TRACE ANALYSIS OF SELENIUM IN BIOLOGICAL SAMPLES USING XRF SPECTROSCOPY

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ABSTRACT

Selenium is both an essential trace element and a toxic element for animals and human beings. It has been established that an extremely narrow concentration range exists between deficient and toxic levels. A knowledge of the concentration of selenium in human tissues and body fluids is quite important for health, and it is necessary to develop analytical methods for the determination of selenium in biological systems.

An X-ray fluorescence (XRF) spectroscopy combined with preconcentration treatment has been developed for the analysis of traces of selenium in biological systems in this work. Tellurium was used as a coprecipitating agent as well as an internal standard because of its similar chemical properties to Se and its absence in most biological systems. The preconcentration treatment consists of two steps: sample decomposition and coprecipitation. A mixture of acids $HNO_3/H_2SO_4/HClO_4$ has been found to be most effective in sample digestions, leading to minimum Se loss while maintaining strong oxidizing conditions. High recovery of selenium (average 98.8%) was obtained from this digestion. Hydrochloric acid and hydrazine dihydrochloride has then been employed in the reduction of the Se in its high oxidation states to form precipitates. The digestion temperature, time and other conditions have been optimized. Experiments have shown that the matrix absorption-enhancement effects are negligible and that the calibration curve obtained without a matrix can be used for

quantitative Se determinations for biological systems.

The precision and accuracy of the method was verified by using standard reference materials of known Se concentration. Seven kinds of NBS and IAEA standard reference materials were examined and excellent agreement was obtained between the measured and the certified values.

The minimum detection limit for most biological systems was 5 ng absolute, corresponding to 10 ng/g for 0.5 g sample and 6 ng/mL for 2 mL human erythrocytes. No interferences were found in this XRF analysis, despite a greater foreign ion concentration than in the Se solution.

In order to verify the validity of using the XRF method, differential pulse cathodic stripping voltammetry (DPCSV) was utilized as an independent technique in the determination of Se in standard reference material and human erythrocyte samples. The DPCSV results correspond closely with XRF/preconcentration determinations.

As an application of this method, a distribution of the Se concentration in human erythrocyte was obtained by performing Se analyses in erythrocyte samples taken from 25 healthy volunteers. The results indicate that about 56% healthy people have the Se concentration between 190 - 210 ppb in erythrocytes.

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Finally, I would like to thank my husband, Mr. H. L. Zhang, for many helpful suggestions and discussions, and especially for being my source of strength in this past year.

ABBREVIATIONS

AAS	atomic absorption spectroscopy
DC	direct current
DAN	diaminonaphthalene
DPCSV	differential pulse cathodic stripping voltammetry
eV	electron volt
FWHM	full width at half maximum
IAEA	International Atomic Energy Agency
IUPAC	International Union of Pure and Applied Chemistry
KeV	kilo electron volt
LLOD	the lower limits of detectability
MCA	multi channel analyzer
NAA	neutron activation analysis
ND66	Nuclear Data, model 66 MCA
NBS	National Bureau of Standards
PIXE	proton induced X-ray emission
ppb	parts per billion
ppm	parts per million
SCE	saturated calomel electrode
Si(Li)	lithium drifted silicon detector
XRF	X-ray fluorescence

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Chapter 1 Importance of Selenium

The element Selenium was discovered early last century (1817)[1] and has been ascertained to be an important element to humans and animals. In the mid 1930's, selenium was recognized as a toxic element responsible for "alkali disease" and blind staggers".[2] Schowarz and Scortt[3] announced their finding of the nutritional requirements of selenium in the late fifties. This is now recognized as one of the most important discoveries in nutrition in the last thirty years.[3] Extensive studies of the role of selenium in human and veterinary health and selenium related decease has continued over the past three decades. The role and importance of selenium in the environment will be discussed from two perspectives, namely, the distribution of selenium, and the role of selenium in human nutrition and health.

1.1 Distribution of Selenium

Se is mainly found in metal sulphide deposits; most commonly it is chemically attached to the metals Cu, Zn, Ag, Hg and Pb. Some Se - minerals, (*e.g.*, Tiemannite and Naumanite) contain up to 24% Se by weight.[4] It is widely

distributed in the environment but in very low concentrations compared to its congener sulphur, the occurrence of which is up to 10^6 times higher.[5] The average crustal abundance of Se is 0.09 µg/g[6] and concentrations in coal and fuel oil range from 0.47 to 8.1 µg/g and 2.4 to 7.5 µg/g, respectively, depending on type and origin of the fossil fuel.[7] In soils where it is present as the element, as selenite and selenate and in the form of minerals, the total levels scatter widely and can range up to 80 µg/g in seleniferous types.[8]

Various factors affect the presence of Se in soils, *e.g.*, its composition, leaching and percolating processes, addition by meteoric or ground water during soil formation in the geochemical cycle.[9] In plants it occurs as selenate and in the form of organic compounds (amino acids). The content varies widely among species. Thus, primary accumulators (species Astragalus) can incorporate several thousand µg/g, where as in locoweed, the concentration is 15000 µg/g.[10] Secondary (species Aster) and non-accumulators (diverse cereals, vegetable and herbage) take up about 200 - 300 µg/g and 0.2 - 20 µg/g, respectively.[8]

Although Se mostly exists in mineralogical rock and soil, the concentration of Se can be very high in some water systems because the Se enters water as soluble selenites and as suspended particles of insoluble and organic forms of the element. A concentration of 9000 ppb Se was detected in a well in Colorado, USA.[11] However, most water systems have very low concentrations of Se due to poor drainage of seleniferous soils. Se concentrations in water increase greatly

when irrigation of seleniferous soils has been carried out. An increase of between 8 and 80 μg/g is observed in the Se content of the Gunnison River in Colorado, due to the collection of irrigation waters from seleniferous soils.[12]

Selenium also exists in air in the form of aerosols and larger particles resulting from windblown dusts, volcanic action, and the combustion of fossil fuels and refinery of nonferrous metals etc. The concentration of Se is in the range of nanograms per cubic meter in most ambient air.[13] Environmentalists believe that of all the contributors of Se to the air, the combustion of fossil fuels contributes the greatest share.

The levels in animal and human tissue[14-18] and food[19-23] show a great variation and can lie anywhere from μg/g to ng/g. As an illustration, some Se levels are presented for environmental samples[24-30] (Table 1.1 and Table 1.2), animal tissues (Table 1.3) and foods (Table 1.4).

The Se in soils can be taken-up by plants which in turn is passed up the food chain to humans and animals. Therefore, it is useful to have a generalized map of the general distribution of Se in crops. Figure 1.1 shows such a regional distribution of Se in food and feed plants in Canada and in the USA.[31] These distributions has raised concerns regarding public health implications for regions with low Se contents.

Table 1.1

Total Selenium contents of some environmental matrices

Matrix	Se Content (µg/g)	Reference
Earth's Crust (average)	0.09	6
Rocks		
igneous rocks	0.05	24
sandstone	0.05	24
limestone	0.08	24
Fossil fuels		
coal	0.47-8.1	7
oil	2.4-7.5	7
Soil		
normal	0.1-0.4	25
dry	0.01-2.0	24
seleniferous	40.0-80.0	8
Marine plants	0.8	26
rye grass	0.01-3.3	7
clover	0.13	25

Table 1.2

Total Selenium Contents of Some Waters

Matrix	Se content (μg/g)	Reference
Sea water		
median	0.09	10
North Pacific	0.06-0.12	27
average	1.0-4.0	7
Lake water		
Arrowhead, Calif. [Se(IV)]	0.08	28
River water	0.085-0.37	29
Rain water		
[Calif. / Se(IV)]	0.052	28
Tap water	0.16	30

Table 1.3

Selenium in Some Human Tissues and Body Fluids

Specimen	Se content (fresh tissue) (μg/g)	Reference
Kidney	0.77-0.109	14
Heart	0.38	15
Liver	0.39-0.54	16
	1.06-2.06[a]	16
Muscle	0.36	17
Skin	0.27	17
Lung	0.19	17
Blood	0.12[b]	18
Urine	0.034-0.1[b]	18

[a] Reported on dry weight, [b] μg/ml

Table 1.4

Selenium Contents in Food

Food	Se content (μg/g)	References
Pork		
kidney	0.2-0.96	19
muscle	0.06	19
ham	0.03	19
Chicken		
muscle	0.1	20
Sea animal average	0.4-0.7	20
fish gills	0.11	21
fish flesh	0.06	21
Whole cow milk	0.003[a]	19
	0.02-0.04	22
Mature	0.02-0.11	23
Skim milk	0.05	20
Dried skim milk powder	0.1-0.25	20
Human milk	0.01-0.17[a]	22
Colostrum	0.05	23
Mature	0.01-0.02	23
Cheese	0.01-0.03	21
Egg white	0.04-0.06	21
Yolk	0.05-0.07	21
Flour, whole meal	0.02	19
White flour	0.01	19
Barley	0.67	20
Fruits, Vegetables	0.01	20
Garlic	0.25	21
Onion	0.02	19
Mushrooms	0.13	21

[a] μg/ml

Figure 1.1

Regional Distribution of Se in Food and Feed Plants in the USA and Canada as Indicated by the Se Contents of Forages and Cereal Crops.[31]

1.2 Se in Human Nutrition and Health

Selenium was recognized as a toxic element in the early 1930's[2] and an essential element for animals in late fifties.[3] However, the role of selenium in human nutrition and health was not well understood until the late seventies.

An important breakthrough resulted from studies of Se in human health in China.[32] These reports indicated a correlation between severe Se deficiency in discrete regions of the People's Republic of China (PRC) and an endemic juvenile cardiomyopathy (*e.g.*, Keshan disease).[33] Keshan disease, a cardiomyopathy, was prevalent in certain areas of PRC for more than a century. This disease is a multifocal myocarditis that occurs primarily in children between the ages of 2 and 10 years. The case-fatality of Keshan disease in PRC was greater than 80% in the 1940's, but has fallen to about 30 % in recent years.

Also, there were observations of Se as a factor in the aetiology of a chondrodystrophic disease (Kaschin-Beck disease) of children in severely Se-deficient parts of PRC.[34] These findings provided the first direct evidence that Se plays in important role in human health.

A map of Se distribution for PRC[35] is shown in Figure 1.2. The mapping of Se in crops is important for the development and understanding of the role of Se in the nutrition of animals and humans. Figure 1.3 shows the distribution of the Keshan disease and Kaschin-back disease in China.[34] Compared to Figure 1.2,

most of these cases occur in the areas with the lowest concentration of Se in food and feed plants. Residents of Keshan disease areas tend to have blood Se levels less than ca. 25 ppb and hair Se concentrations less than 100 ppb.[36] This selenium level is 5 ~ 10 times lower than the normal Se content in countries such as Canada, Australia. This disease has been mostly eliminated after a large scale intervention with selenium supplements.

Magos and Webb[2] published a review on the effect of selenium in different organisms having antagonistic action towards various toxic metals such as arsenic, cadmium, copper, mercury, silver and so on. For several conditions in patients receiving parenteral alimentation, selenium deficiency has been confirmed to have an important role, and alcoholic cardiomyopathy to be aggravated or accelerated by the concomitant occurrence of selenium deficiency.[37]

It is reported that selenium inhibits carcinogenesis by chemical agents such as aminoazo compounds and polycyclic aromatic hydrocarbons.[38] As an explanation for differences in the incidence of cancers in various human populations, variations in dietary selenium intake was suggested.[39] Selenium also plays an important role in the prevention of cardiovascular diseases and myocardical infarction. Inverse epidemiological relationships were observed between human heart disease and environmental or blood selenium.[40]

To summarize, there is a range of selenium intake which is consistent with good health. Deficiency or toxicity effects will occur if the concentration of

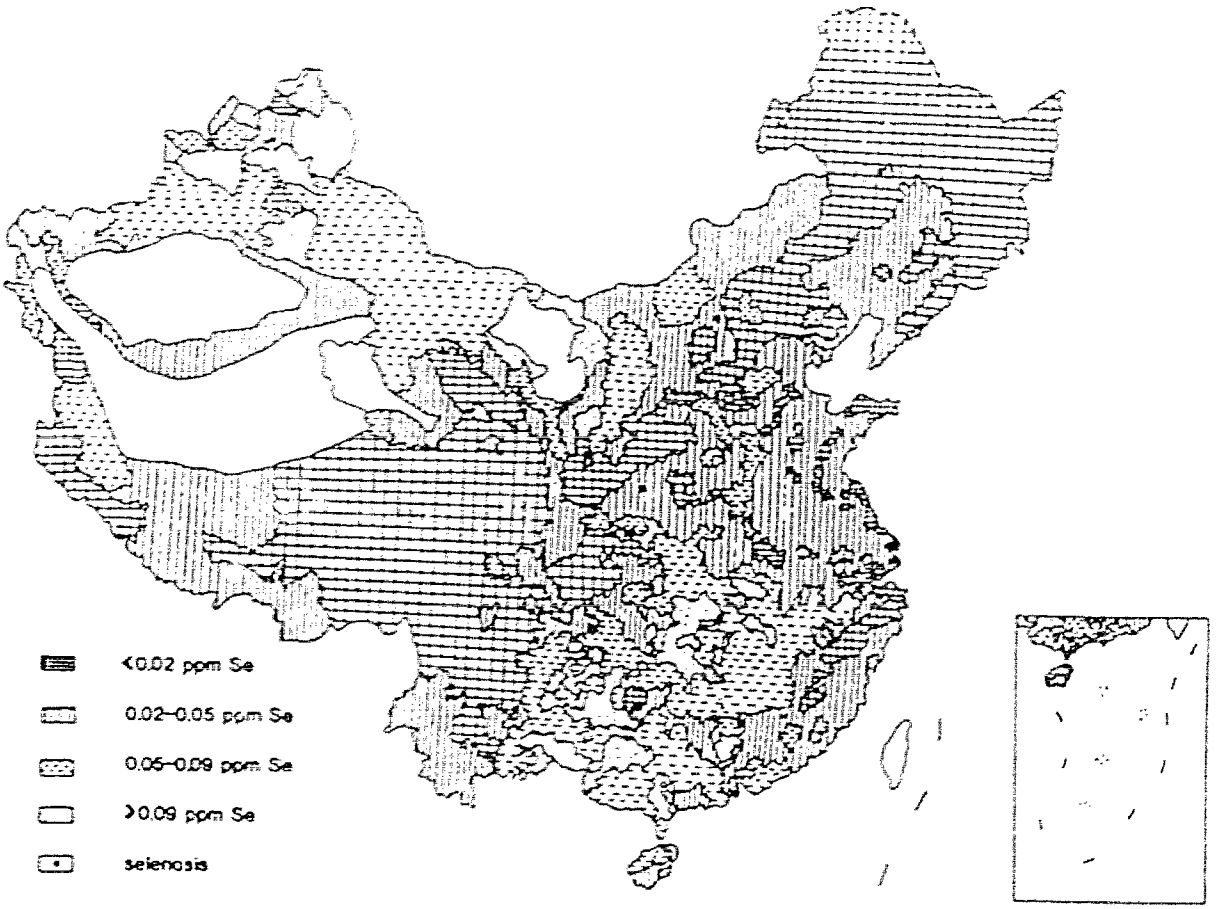


Figure 1.2

Regional Distribution of Se in Food and Feed Plants in the People´s Republic of China.[35]

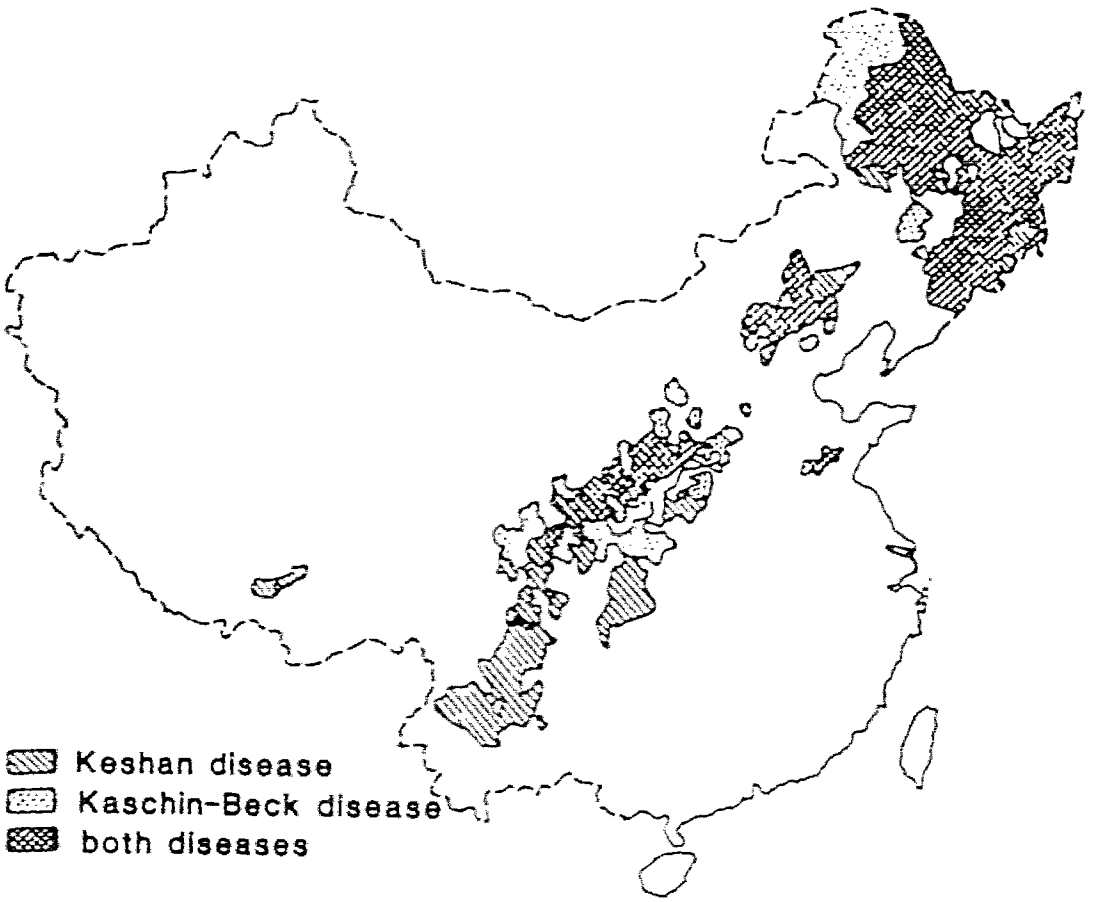


Figure 1.3

Distribution of Keshan Disease and Kaschin-Back Disease in China.[34]

selenium is out of this range. It has been established that an extermely narrow concentration range exists between deficient and toxic levels. The dietary selenium requirement to compensate normal daily losses in humans is 50 - 60 μg/day.[41] Selenium concentrations in the body may vary from 0.034 μg/mL (in urine) to 0.109 μg/g (in kidney).[42] Deficiency symptoms appear at diet dry- matter levels as low as 0.02 - 0.04 ppm, while levels of 2 - 4 ppm or more are toxic.[41] Therefore, a knowledge of the concentration of selenium in human tissues and body fluids is imperative and it is necessary to develop analytical methods for the determination of selenium in biological systems. Further reliable analytical methods are needed to perform required, "on - line" monitoring of Se levels especially to those who are exposed to Se because of their occupations, and for people in the areas with selenium deficient soils.

Chapter 2 Methods for Selenium Determination

The analysis of Se can be accomplished by a variety of techniques but only Some are applicable to biological materials. The following discussion provides a general discussion of the existing methods of Se analyses with emphasis on the strengths or weaknesses of the techniques, particularly when analysing for Se in foods, feeds, and animal tissues.

2.1 Existing Analytical Techniques: Strengths and Weaknesses

Table 2.1 is a brief survey of some analytic methods[43~50] and each of the methods is discussed in the following sections.

Fluorometric method

Among the methods, the fluorometric method using diaminonaphthalene (DAN) has been the most popular one. This method involves oxidizing selenium in samples to Se^{+4}, and reacting with DAN to form benzopiazselenol. The product fluoresces intensely at 520 nm when it is excited at 390 nm. The selenium content is quantified using a fluorometer. The main advantages of the DAN procedure are its good sensitivity (0.002 ppm)[43] and its relatively low cost. Nevertheless, the

Table 2.1

Methods of Analysis of Selenium in Biological Materials

	Detection Limit (ppm)	Sample preparation	Known interferences	References
Fluorometric determination of piazselenol after reaction of Se^{+4} with 2,3-diamino-naphthalene	0.002	Nitric-perchloric acid digestion, or O_2 combustion	Loss of volatized Se in digests	43
Atom-trapping atomic absorption spectrometry	0.1	O_2 combustion	Mineral cations	44
Atomic absorption spectrometry with hydride generation	0.01	Acid digestion; hydride generation	Matrix effects (esp. Cu, As, Sb)	45
Electrothermal atomic absorption spectrometry	0.003	Thermal stabilization with Ni	Matrix effects	46
Neutron activation analysis using ^{75}Se	0.02	-	-	47
X-ray fluorescence spectrometry	0.04	Lyophilization	-	48
Proton-induced X-ray emission analysis	0.01	Lyophilization: pelletization	-	49
Isotope dilution with detection by combined gas-liquid chromatography/mass spectrometry	0.0005	Nitric-phosphoric acid digestion; Chelatioin with 4-nitro-o-phenylenediamine	-	50

method has two potential pitfalls.

The first involves the loss of Se during the acid digestion of samples containing large amounts of organic materials. Adequate acid digestion of selenium in biological materials requires the complete conversion of the native forms of the mineral to Se^{+4} and / or Se^{+6}, and the subsequent reduction of any Se^{+6} formed in the process to Se^{+4} without loss of Se. Inorganic Se can be volatilized to an appreciable extent under the conditions of acidic digestion in the presence of such large amounts of organic materials that charring occurs, especially when sulfuric acid is used as an oxidant.[51] The volatilized Se, probably in the form of H_2Se, can result in significant errors in the analysis of fatty materials, such as egg yolks or adipose tissues. The second potential problem involves interfering fluorescence due to apparent degradation products of DAN itself.[52]

Atomic absorption spectroscopy (AAS)

By far, the most widely used analytical method is AAS with flame, electrothermal and heated quartz cell atomization. In conventional AAS (with flame atomization), a liquid sample is atomized by spraying it into an acetylene burner. Occasionally other flues are used and sometimes an oxidizer such as NO_2 is added to increase flame temperature. The absorbance of the flame is measured at a particular wavelength, then compared against standards.

Conventional atomic absorption spectroscopy (AAS with flame atomization)

is not suitable for the determination of Se in biological samples due to the high limit of detection (0.1 ppm) with that procedure.[44] Variant AAS methods, however, have been developed with sensitivities adequate for biological use. One such method involves hydride generation of sample Se followed by quantitative detection by AAS.[45] This method requires very small sample size (*e.g.*, 0.1 ml of serum). It has adequate sensitivity (0.01 ppm),[45] and the hydride generation step can be automated. However, it suffers from possible interferences due to other elements that can form hydrides as well (*e.g.*, Cu, As, Sb). Of these, Cu causes the most serious interference. Better sensitivity has been obtained using electrothermal AAS. This method avoids the problems associated with wet digestion by employing high temperature oxidation in a graphite furnace. In practice, electrothermal AAS has sensitivity to determine Se levels as low as 0.003 ppm.[46] Use of high temperature (e.g., atomization at 2400^{0}C) reduces interferences due to nonspecific absorption of organic compounds and non-Se salts, but introduces the problem of volatility of Se under such conditions.

Neutron activation analysis (NAA)

A more sophisticated approach is neutron activation analysis. In this technique, samples are bombarded with neutrons in the core of a nuclear reactor. Atoms of elements such as selenium can acquire an extra neutron, and then ultimately decay by gamma emission. The intensity of the decay gamma rays is

proportional to the concentration of selenium in the sample. In some applications NAA has excellent sensitivity, and can be used to analyze solid samples directly.

Neutron activation analysis (NAA) of Se offers the advantages of applicability to small sample size and relative ease in sample preparation. A high sensitivity (0.02 ppm)[47] is obtained by measuring reaction product, ^{75}Se ($t_{½}$ = 120d), but its use necessitates lengthy irradiation times (100 hrs), and long periods of post-irradiation holding (60 days) and counting (2 hrs). Greater economy by increased sample throughput has been achieved, at the expense of sensitivity, through the use of the short-lived product (17.38 sec half-life) ^{77m}Se. This isotope can be produced (5 sec), allowed to decay (15 sec), and counted (25 sec) very quickly in an automated system.[53] Due to the ease of this procedure as well as to its nondestructive nature, some investigators with access to research reactors have found instrumental neutron activation analysis useful for the measurement of Se. Nevertheless, the utility of the "fast" method is limited by its relatively low sensitivity, rendering it unsuitable for accurate quantification of low concentrations of Se in tissues of animals which are chronically deficient of the element. In general, NAA is sensitive enough for direct selenium determination, but requires a nuclear reactor as a neutron source with large start-up costs.[54]

X-ray fluorescence Analysis (XRF)

In XRF analysis, the sample is illuminated with monochromatic X-rays.

Core electrons such as the 1s, 2s, and 2p electrons are knocked out by collisions with incident photons. When other electrons cascade into the newly created vacancies, fluorescent X-rays are emitted. These fluorescent X-rays have energies which are proportional to the atomic number of the emitting atom. The intensity of fluorescent X-rays of a particular energy is a measure of the concentration of a particular element.

One advantage of X-ray fluorescence, as an analytical technique, is the non-destructive sampling. Another advantage is that, unlike the situation in emission spectroscopy, very few lines are obtained for each element, thereby decreasing interferences from background elements. Any element with an atomic number higher than that of magnesium can be readily analyzed.

Unfortunately, the intensity of the few X-ray emission lines of selenium is very low, thus limiting their analytical value. Nevertheless, Olso and Shell[55] developed a procedure for the simultaneous determination of selenium and mercury in organic compounds. Their results were accurate to ±1 ppm in the range of 2-40 ppm for both selenium and mercury. A 600 mg sample was required, but the method is rapid and nondestructive. The average time for each analysis, including sample preparation and X-ray fluorescence analysis, was 45 minutes. Handley[56] used X-ray fluorescence to determine selenium in plant materials containing 7.73-946.0 ppm.

A recent modification to XRF has been the use of protons to induce X-ray

emission (PIXE).[57] PIXE is still in the development stages, but detection limits of 0.01 ppm have been achieved.[49] PIXE has several problems including a short half thickness for protons, and the need for equipment capable of generating beams of protons with energies on the order of Mev.

Isotope dilution mass spectrometry

The procedure for determining Se by double isotope dilution involves the use of two stable isotopes of Se as tracer (^{76}Se) and internal standard (^{82}Se).[50] Samples spiked with a known quantity of the internal standard are digested in nitric-phosphoric acid, undigested lipids are removed with chloroform, and hydrochloric acid is used to reduce any Se^{+6} to Se^{+4}. Selenite is reacted with 4-nitro-o-phenylenediamine to from 5-nitropiazselenol, and the nitropiazselenonium ion cluster is detected by combined gas-liquid chromatography/mass spectrometry. The native Se in the sample is calculated from the measured isotope ratios, using the ^{80}Se naturally present in the sample. Reamer and Veillon[50] have carefully developed this technique and have reported a sensitivity of less than 0.001 ppm.[50] Their method employs a rapid digestion, which avoids several problems associated with the use of perchloric acid, and is capable of fully oxidizing the often problematic trimethylselenonium.[50] It thus appears to be suitable for biological measurements and there have been applications in biological systems.[58]

A IUPAC interlaboratory (12 sites) comparison of widely used methods for

the determination of Se in clinical materials[59] found statistically significant difference among the mean concentrations reported for Se in lyophilized human serum. The samples were analyzed by either (a) acid-digestion/DAN-fluorometry, (b) electrothermal AAS, (c) acid-digestion/hydride generation AAS, or (d) acid-digestion/isotope dilution mass spectromery, with slightly higher values reported by the first procedure. The four methods compared very favourably for the analysis of pooled lyophilized urine samples. However, only the fluorometric method showed homogeneity of variance among laboratories.

The DAN fluorometric procedure remains one of the most widely used method for the analysis of Se in biological materials due to its high sensitivity and reliability. Despite the tedious nitric-perchloric acid digestion which it entails, the operating costs of the method are not great because the only instrumentation required is a good quality fluorometer. Therefore, this procedure has been the method of choice for many biomedical laboratories. Newer methods, including electrothermal AAS, AAS with hydride generation, PIXE, "fast" INAA, and isotope dilution with gas-liquid chromatography/mass spectrometry, offer good options for biological investigations, but they generally require large amounts of background development with large start-up costs. As these methods are improved, they will be employed more extensively in future analysis of Se.

2.2 Goals of This Thesis

We have been interested in developing an alternate experimental procedure to quantify Se in tissues and body fluids. The availability and advantages of the XRF system could make it very suitable as a technique of choice for Se determination in biological specimens. XRF is basically a multi - element technique and is one of a few instrumental techniques which can analyze solid samples directly. XRF can be performed rapidly, accurately and reliably. Cost for such analyses can be modest. The XRF analysis usually does not require any special sample preparation. This keeps the overall technique simple and more reliable because each step in a sample preparation procedure has the potential to introduce errors.

Conventional XRF spectroscopy usually has a relatively poor sensitivity for Se and suffers from the interference of matrix absorption - enhancement effects.[60] Direct determination of selenium in biological samples by XRF is not possible[61] because the typical selenium concentration in a biological sample is well below the detection limit of XRF for all modes of sample excitation. It was, therefore, necessary to develop a preconcentration procedure to bring selenium concentrations up to the detectable limits of this technique. This long objective can be sub-divided into a series of discrete goals.

1. Development of a rapid and simple chemical preconcentration technique

to quantitatively extract selenium from solutions, using tellurium as a coprecipitating agent as well as an internal standard. An efficient preconcentration technique consists of two steps: optimization of sample decomposition, followed by a coprecipitation procedure to obtain a thin sample.

2. Identification and optimization of conditions to improve the sensitivity of the new approach.
3. Identification of spectator ions which may interfere with quantitative selenium determination.
4. Assessment of sources of error in the XRF analysis, and develop methods to minimize the error.
5. Expand the application of the new approach for routine selenium analysis of a large number of biological samples.
6. In order to verify the validity of using this XRF method, an electrochemical technique, namely differential pulse cathodic stripping voltammetry (DPCSV), was utilized as an independent technique in the determination of Se in standard reference material and human erythrocyte samples.

Chapter 3 Principles of Experimental Techniques

A. X-ray Fluorescence Analysis: Overview

Energy dispersive X-ray fluorescence induced by photons (XRF) provides a simple and sensitive method for multi-element analysis of elements with atomic number Z greater than 12. This technique presents the following general characteristics:

(1) It is non destructive and non radioactive

(2) It is a surface analysis, as information comes from a very thin layer of the specimen.

(3) It allows qualitative and quantitative multi-element analysis in a measuring time of 10^2 - 10^3 s with real time information.

(4) It presents a wide dynamic range (from trace analyses for samples of less than 0.1 ppm (w/w) to analyses of major elements with percent concentrations up to 100%).

When very thin samples are analyzed, on the order of 5-100 mg cm^{-2} (the value depends on the energy of incident and energing radiation), the method gains additional advantages. Firstly, the X-ray intensity of a given element is a linear function of mass per unit area of the element. Secondly, interelement effects

become negligible. Thirdly, the ratio of fluorescence X-ray to background is increased.

Assuming the use of thin samples, typical sensitivity values for XRF-analysis are in the range 1-100 ng cm^{-2}, in a measuring time of 10^2 s.[62]

XRF analysis, has become an invaluable instrumental method to obtain both qualitative and quantitative information on many different types of samples. XRF analysis has applications in many areas such as clinical chemistry, archaeology, criminology, mining, medicine, etc.

3.1 X-ray Fluorescence (XRF) - Basic Principle

Figure 3.1 shows an idealized, simplified diagram of the electron configuration of an atom. The electrons around the nucleus are in different shells. These are designated as the "K" (up to 2 electrons), the "L"(up 8 electrons) and then the "M" (up to 18 electrons) shells.

If the atom is bombarded by an incoming radiation (photon or charged particle) that has an energy greater than E_M (see Figure 3.1), electrons in the M shell will be forced from their regular orbit creating vacancies in this shell. Similar situations would occur in the L and K shells, if the incoming photon has an energy greater than E_L or E_K, respectively.

The electron vacancies thus created are quickly filled by higher energy

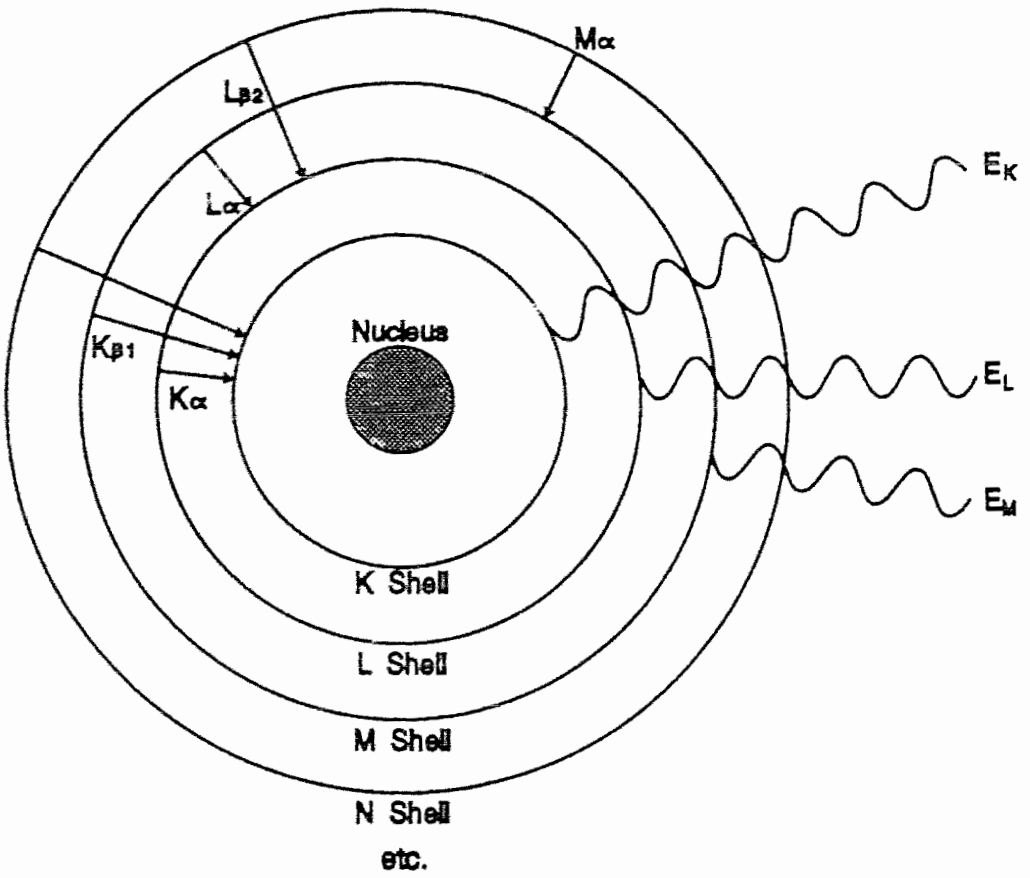


Figure 3.1

Electron transitions giving rise to the major emission lines. The moving particle or photon must have energy greater than the binding energy of the respective electron orbit.

electrons from the outer shells, thus maintaining equilibrium of the atom. Electrons from the outer shells possess more energy than the energy necessary to enter an inner orbit. This excess energy may be released as the emission of electromagnetic radiation, namely, photon as one option. Because the energy released is in the X-ray range, i.e.: 1 KeV to 100 KeV, the phenomenon is known as "X-ray fluorescence".

Given appropriate excitation conditions, vacancies in the inner electron orbits can lead either to the emission of X-rays characteristic of the element (fluorescence) or to the emission of Auger electrons. These competing processes vary as a function of the atomic number, Z, of the element, with the fluorescence process dominating at higher values of Z.

By definition, fluorescence that results from electron transitions to fill vacancies in the K shell of an atom are called "K lines". Fluorescence from electron transitions to fill L shell vacancies are called "L lines" etc..

Because of the regularity in atomic structure of all the elements, differences in electron orbit energies for the various elements are also regular. In fact, there is a linear relationship between the XRF line energies and the atomic numbers of the elements. Since the binding energies of the electrons are known, it is possible to theoretically calculate the XRF energy lines for all the elements; this information is readily available to the operator or analyst. Additionally, XRF line energies are closely spaced, forming 2 lines of known relative intensities for K series, 5 lines

for the L series etc.. Thus X-ray fluorescence analysis is a powerful and convenient tool for identifying elements in a sample with a Z number greater than 12, and is unmatched by any other analytical technique for speed, comprehensiveness, accuracy and cost.

Figure 3.2 shows a schematic diagram of an XRF system. The sample is irradiated by a photon or an X-ray source and characteristic X-rays are emitted from each element present in the sample. X-rays are then detected and converted to electric pulses, which are then amplified and analyzed by means of a pulse-height analyzer. From the energy of the emitted X-ray, elements present in the sample can be identified. Elements can be quantified from the X-ray intensity (the X-ray intensity depends upon the concentration of that element in the sample).

3.2 Quantitative Analysis with XRF and Interference Effects

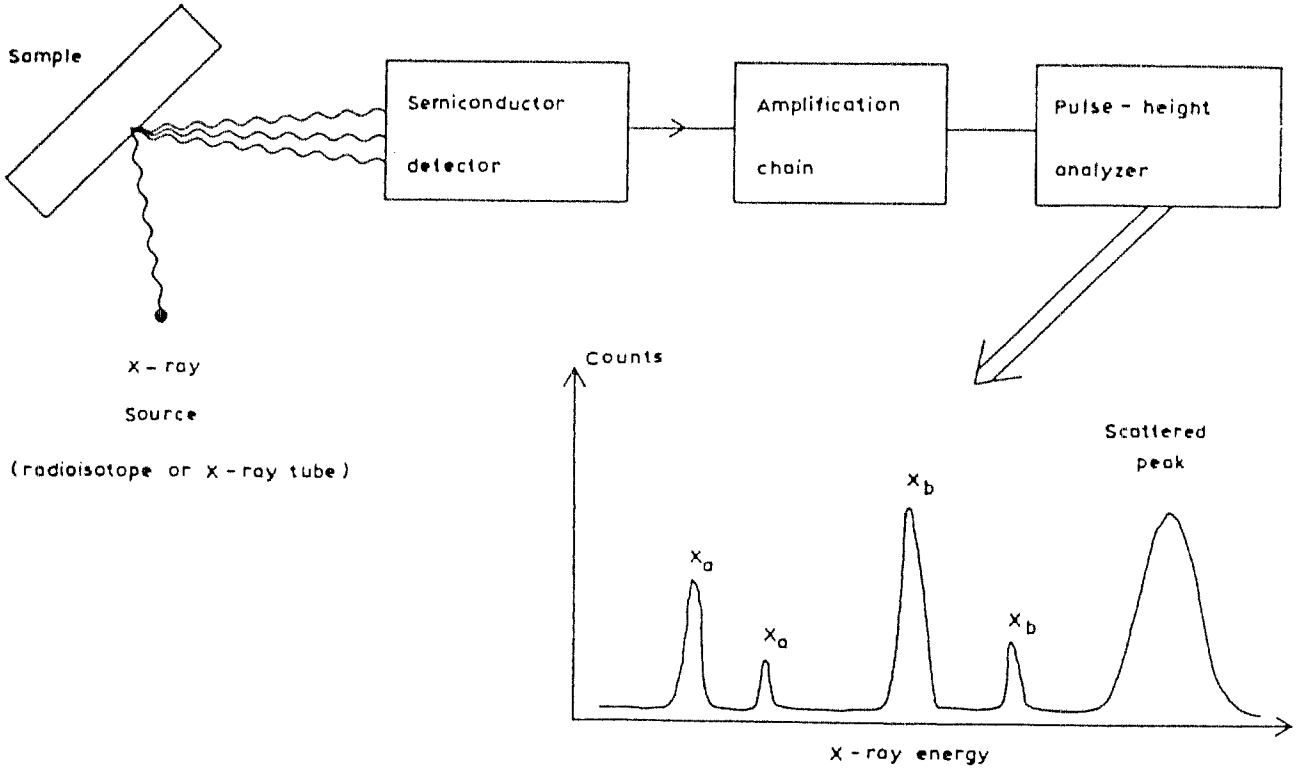
In general, the intensity I_x of emitted X-rays of a particular line (e.g., Kα) from a trace element with concentration C_x present in a sample is given by:

$$I_x = I_0 PGAC_x \tag{3.1}$$

where I_0 is the intensity of the incident radiation and G is the detection efficiency of system (including geometry of the source-detector configuration and intrinsic detection efficiency for the X-ray of interest); P is the total cross-section for the

Figure 3.2

A sample irradiated by an external X-ray source emits characteristic X-rays of elements a and b. These are detected by a semiconductor, the relative pulses are then amplified and analysed. A typical spectrum is then obtained, containing the X-ray lines (two lines Kα and Kβ for each element) and a peak due to elastic and inelastic scattering of incident radiation by sample.



production of a particular line for the element considered and A is the self-absorption factor, depending on the sample matrix. Normally, parameters such as P, G and A are difficult to estimate, especially for a thick sample. However, if a thin sample is used, the interelemental effects and absorption losses can be minimized. For a thin sample, equation (3.1) simplifies to:

$$I_x = I_0 P G W_x \tag{3.2}$$

where W_x is the weight (or number of atoms) of element x in the sample. This assumes that the sample is completely irradiated by the beam, i.e. the beam area is large than the sample area. Equation (3.2) is valid only if the absorption of the incident and emitted beam by the sample matrix is negligible: i.e.,

$$I_x > 0.9\ I_0 \tag{3.3}$$

which translates into the thickness of the sample, m_x (expressed in mg/cm^2).

$$m_x \leq 0.1 / (\mu(E_0) + \mu(E_i)) \tag{3.4}$$

The terms $\mu(E_0)$ and $\mu(E_i)$ are the mass absorption coefficients (cm^2/mg) of the sample matrix materials for incident (E_0) and emitted (E_i) X-ray energies.

Even with this simplification, the absolute magnitude of the various terms in equation (3.2) are difficult to estimate.

One way to circumvent this problem is to perform measurements relative to

some internal standard. Using equation (3.2) for the internal standard element, element x and taking the ratio, one obtains:

$$I_x/I_s = (I_0P_xG_x/I_0P_sG_sW_s) * W_x = K_xW_x \qquad (3.5)$$

where I_x is the intensity of emitted X-rays of interest and I_s is the intensity of the particular internal standard element line. The calibration constant K_x (relative system response constant for element x) can be determined for each element from a series of measurements on thin standard samples with known W_x. This information can then be used to quantitatively analyze for the corresponding elements in an unknown sample.

Theoretically, X-ray fluorescence is inherently quantitative in nature, since the yield of X-ray quanta is proportional to the number of atoms actually present in the specimen. A complication arises, however, due to interaction of X-rays. This phenomenon is referred to as "matrix effects". Matrix effects are the result of two basic processes: the "absorption" of X-rays by the matrix and the subsequent "enhancement" or production of additional X-rays by certain elements in the matrix.

The external (incoming) photon that excites the atom to produce XRF is absorbed by all elements in the sample until it reaches a depth in the material where it has been totally absorbed. During this time, X-rays that are characteristic of the different absorbing elements (XRF) are produced. In the process of reaching the X-ray detector, however, these X-rays are again absorbed by all the elements

in the specimen. This is the absorption phenomenon and is the predominant cause of matrix effects.

The use of thin samples greatly reduces this effect. A sample is defined as infinitely thin when the intensity of the emitted fluorescent X-rays is reduced by less than 10% on leaving the sample (equation 3.3). For selenium in biological samples, this corresponds to a thickness of about 1 mg/cm^2 (calculated from equation 3.4 and assuming that the bulk matrix is carbon).

If some of the X-ray fluorescence produced is energetic enough to excite other elements in the specimen, "secondary fluorescence" will also be produced. This condition is normally called "enhancement".

3.3 The XRF System at SFU: Selection of Operational Parameters

The optimal condition for XRF analysis of thin samples is obtained when monoenergetic radiation of adequate intensity is employed as incident radiation, with energy above the binding energy of the element or elements to be analyzed. Therefore, X-ray sources which emit preferably one or very few lines in the range 5-150 KeV can be usefully employed.

In general, one approach is to employ an X-ray tube with variable acceleration potential with changeable secondary targets, so that the energy of the excitation radiation can be changed (Figure 3.3).[62] The SFU system is a photon

exaltation secondary target system of this type. In this approach the primary radiation emitted by the X-ray tube, mainly a continuous X-ray background (called Bremsstrahlung) produced from interaction of electrons in the anode, is converted to a secondary quasi-monoenergetic radiation through the excitation of and successive X-ray emission of an appropriate monoelemental secondary target.

Two XRF spectrum of 100 μg Fe are shown in Figure 3.4.[62] In the top spectrum, the Fe was excited by Bremsstrahlung radiation from the X-ray tube; in the spectrum at the bottom, the Fe was irradiated with the secondary radiation emitted by a zinc target. Both spectra were collected using essentially identical conditions (*e.g.*, collection time, primary beam current, etc.). The advantage of the latter solution is apparent.

The choice of an appropriate secondary target is predicated upon the need to have the energy of the exciting photon radiation only slightly greater than the absorption-edge energy, K_{ab}, of the elements of interest. For the analysis of biological specimens a Mo target was used since its Kα radiation (17.5 keV) is above the K_{ab} energy of selenium (12.65 keV). The highest number of vacancies occurs as the energy of the K_{ab} approaches the excitation energy.[60] As a general rule, the most efficient secondary target is usually about 5 to 10 atomic numbers higher than the element to be determined.

What are the optimum kV and mA of X-ray tube settings for a sample determination? The following equation governs the choice of kV and mA:

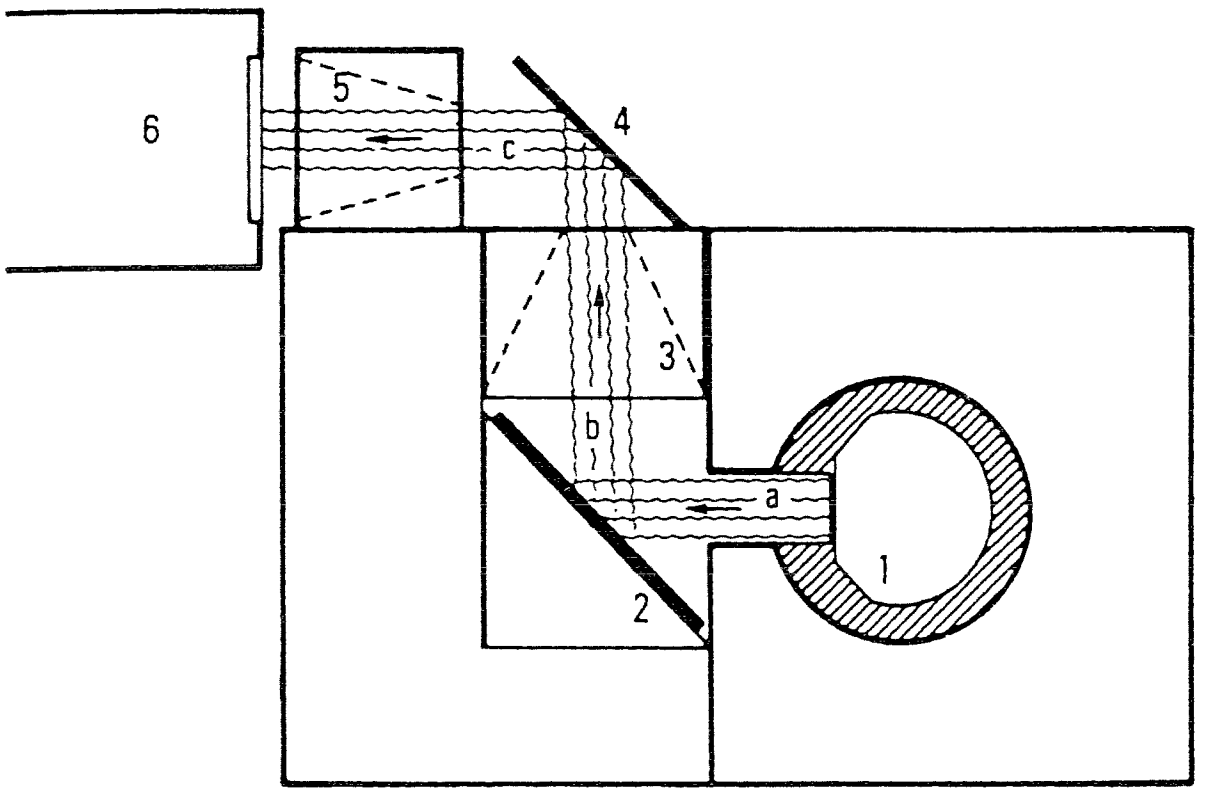


Figure 3.3

Schematic set-up of the apparatus employed for converting primary Bremsstrahlung radiation from the tube to secondary monoenergetic radiation. 1. = X-ray tube; 2 = Secondary target; 3 = conic collimator; 4 = sample; 5 = conic collimator; 6 = detector; a = primary Bremsstrahlung radiation; b = secondary radiation; c = X-rays emitted by the sample.[62]

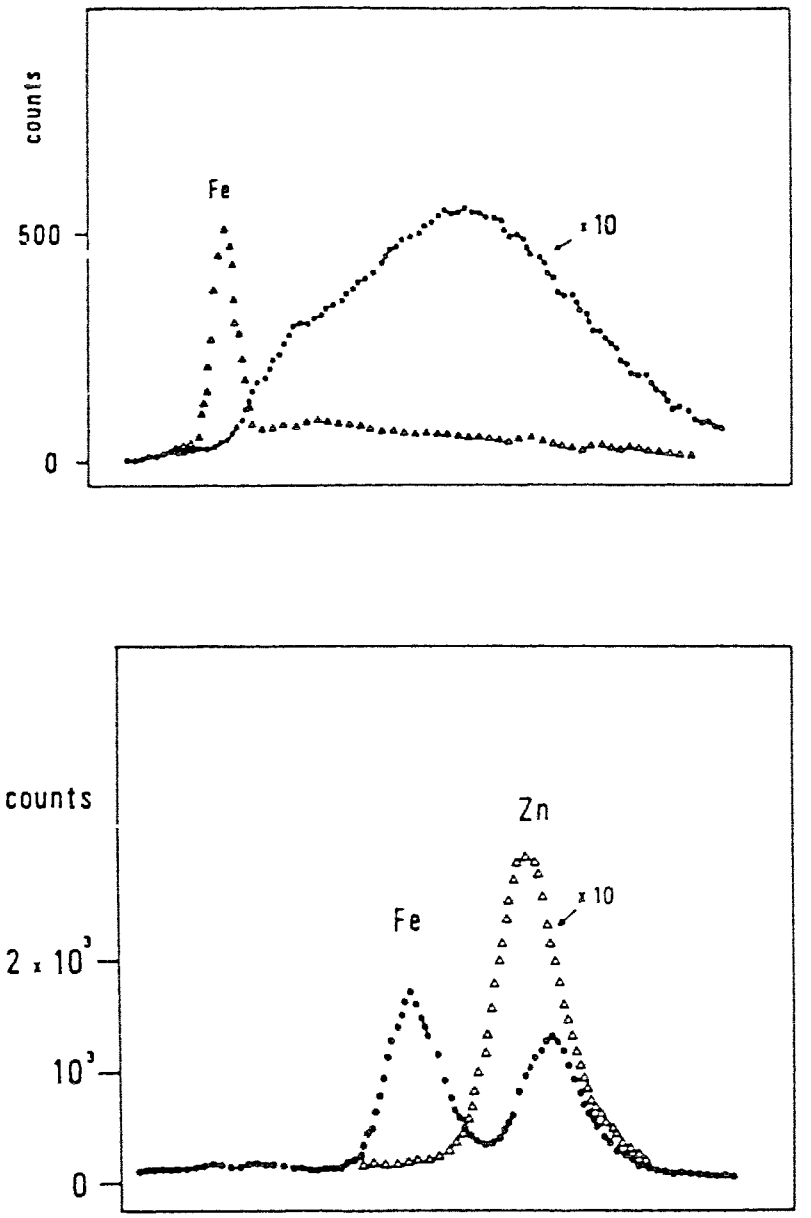


Figure 3.4

XRF - spectrum of 100 µg iron (▵) excited by the primary Bremsstrahlung radiation emitter by the X-ray tube (•) (upper spectrum) compared with the XRF-spectrum of the iron sample (•) excited by a secondary Zn-target (▵) (lower curve).[62]

$$I_0 \alpha (kV)^n mA \text{ (within certain limits)} \quad (3.6)$$

where: I_0 is the intensity from the source, and n is greater than 1 but usually less than 3. Note that to obtain the greatest I_0, maximum kV and mA should be used. Of course, there are some practical limitations. First, there is a maximum kW (kVA) rating on the X-ray generator and X-ray tube to consider, and secondly the use of high kV and mA settings will probably cause the detector/electronics circuitry to be swamped with counts and system dead time will therefore become excessive.

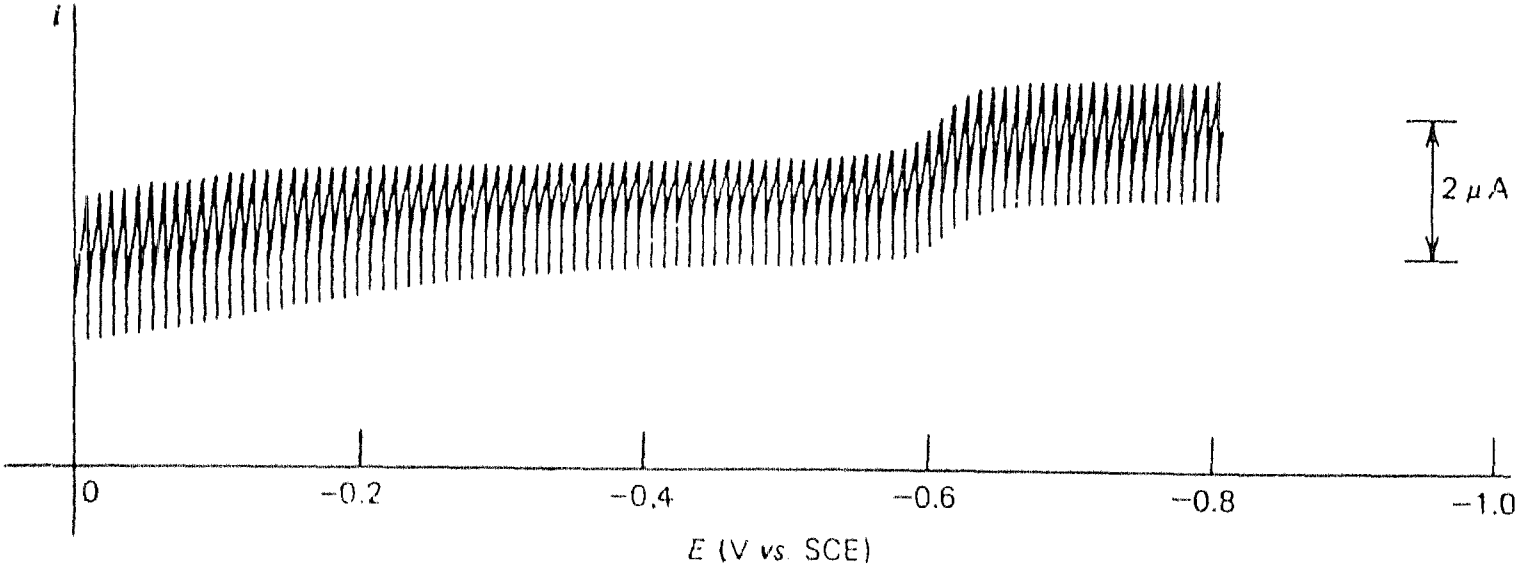
As a general rule, a KV setting roughly equal to about 2 times the energy of the characteristic line of the secondary target is used, and a mA setting sufficient to achieve a dead time of about 5% is used. In this study, the XRF spectrometer was used with operational parameters of 40kV and 15mA. For quantitative work, it is imperative that the samples be analyzed at the same KV and mA settings as when the system is calibrated.

B. Differential Pulse Cathodic Stripping Voltammetry

Differential Pulse Cathodic Stripping Voltammetry is a modified polarographic measurement. The conventional polarography is constructed with a dropping mercury electrode and an electrochemical cell. Polarographic data are

Figure 3.5

The Conventional Polarogram (Polarograms of 10^{-4} M Cd^{2+} in 0.1 M HCl).[63]



obtained by measuring current as a function of the potential applied to an electrolytic cell containing analytical samples. A plot of the data gives current vs voltage curves. If an electrochemical reaction occurs, a sharp increase of current is observed; this is called the polarographic wave (Figure 3.5).[93] The current wave provides both the qualitative information (from the potential of the current wave) and the quantitative information (from the height of the current wave).

However, this conventional polarography has very limited sensitivity; usually the optimum concentration range lies between $10^{-2} \sim 10^{-4}$ M. Also, the use of a potential more positive than 0.4 V versus the SCE is not accessible because of the ready oxidation of mercury. Several modifications of the conventional polarographic procedure overcome these limitations to various degrees.

In differential pulsed polarography, a DC potential, which is increased linearly with time, and an additional DC pulse of 10 ~ 100 mV at regular intervals of about 60 ms is applied to the polarographic cell (as shown in Figure 3.6).[93] The pulse terminates with detachment of the mercury drop from the electrode. To synchronize the pulse with the drop, the latter is detached by an appropriately timed mechanical movement of the electrode. As depicted in Figure 3.7[93] two currents are measured, one just before DC pulse and one near the end of the pulse. The difference in current, per pulse is recorded as a function of the linearly increasing voltage. A differential curve gives a peak as shown in Figure 3.8.[93] The peak current is directly proportional to the concentration of the analyzed element. The

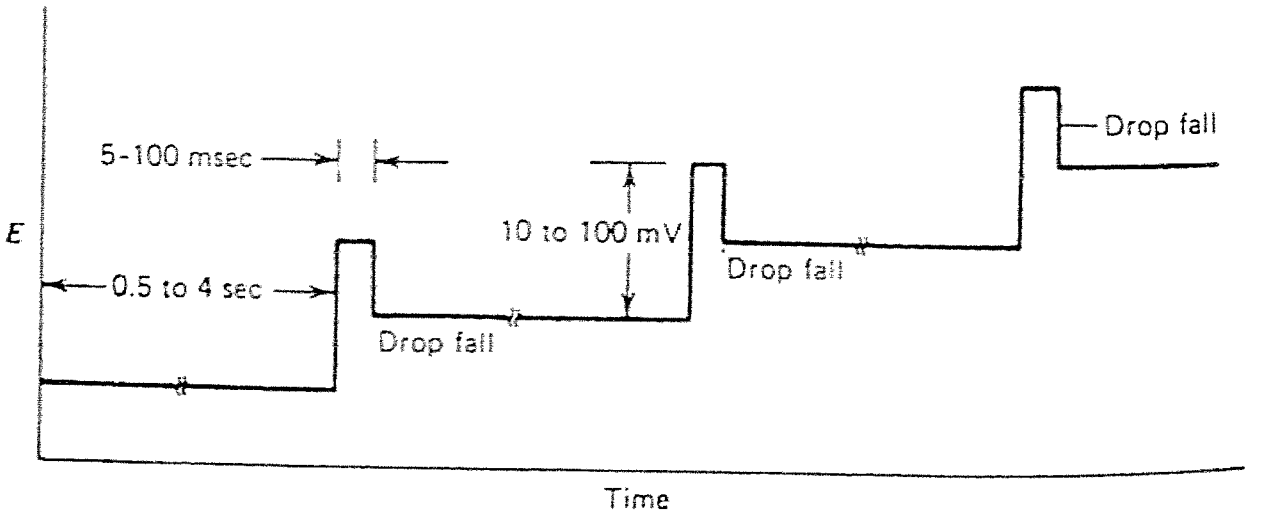


Figure 3.6

Potential Program for Several Drops in a Differential Pulse Polarographic Experiment.[63]

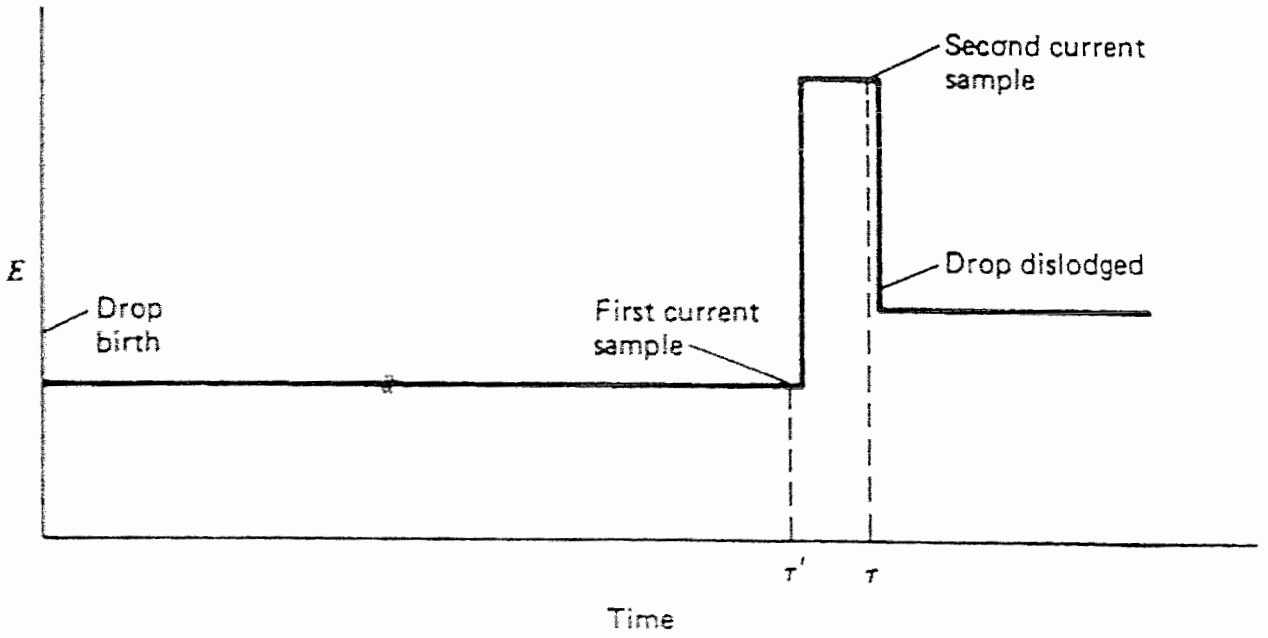
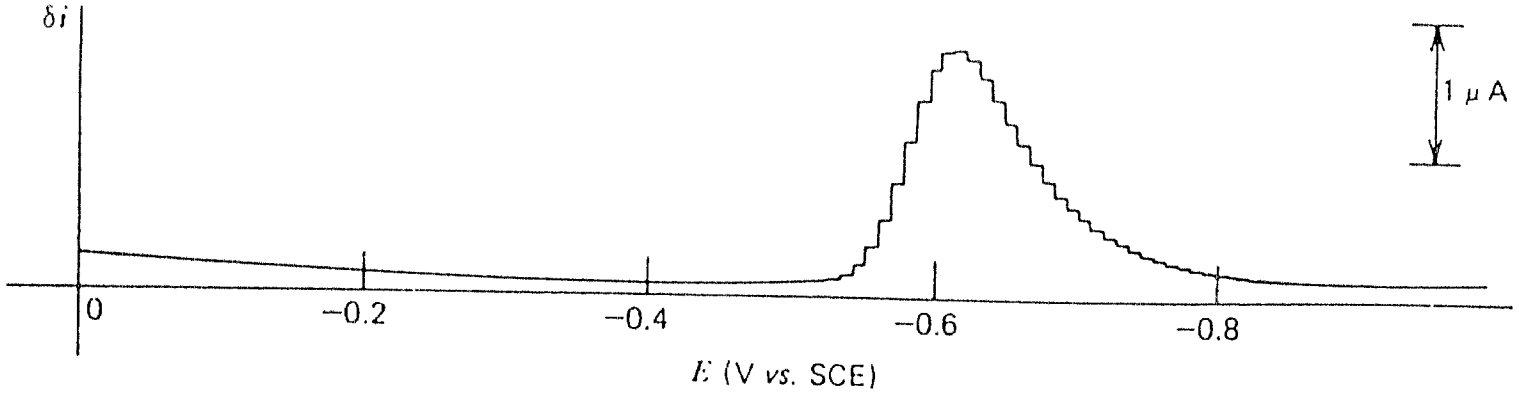


Figure 3.7

Events for a Single Drop of a Differential Pulse Polarographic Experiment.[63]

Figure 3.8

The differential Pulse Mode, ΔE = -50 mV (Polarograms of 10^{-4} M Cd^{2+} in 0.1 M HCl).[63]



differential pulse polarography increases the sensitivity of the polarographic method by about 3 orders of magnitude, to 10^{-8} M.

The sensitivity of the differential pulse method is further enhanced by combining the differential pulse technique and a stripping method. In the stripping procedures, the solution is first collected by electro - deposition at a mercury electrode; it is then redissolved (stripped) from the electrode to produce a more concentrated solution than originally existed. This preconcentrated solution around the mercury electrode is then measured by differential pulse polarography. The whole process is called differential pulse stripping voltammetry. If the stripping is a reduction reaction, it is known as differential pulse cathodic stripping voltammetry (DPCSV). The detection limits can be as low as 10^{-9} M.

In this work, the principle of the differential pulse cathodic stripping voltammetry utilized a bulk electrolysis step to preconcentrate selenium from solution into the small volume of a mercury electrode (a hanging mercury drop). After this electro-deposition step , the selenium was redissolved (stripped) from the electrode using a scan of cathodic potential (in the form of differential pulse voltammetry). The measured voltammetric response (peak current) is proportional to the bulk concentration of selenium. During the preconcentration step at a potential of -0.2 V (*vs* Ag/AgCl reference electrode), the following reactions occur:

$$Se^{4-} + 2H^{+} + 6e^{-} \rightarrow H_2Se \qquad (3.7)$$

$$H_2Se + Hg \rightarrow HgSe + 2H^+ + 2e^- \quad (3.8)$$

The preconcentrated Se is then stripped (redissolved) at -0.58 V as:

$$HgSe + 2H^+ + 2e^- \rightarrow H_2Se + Hg \quad (3.9)$$

According to the theory of the method,[64] the peak potential is characteristic of Se and peak current is proportional to the concentration of Se in solution.

Chapter 4 Experimental

4.1 Analysis Using XRF

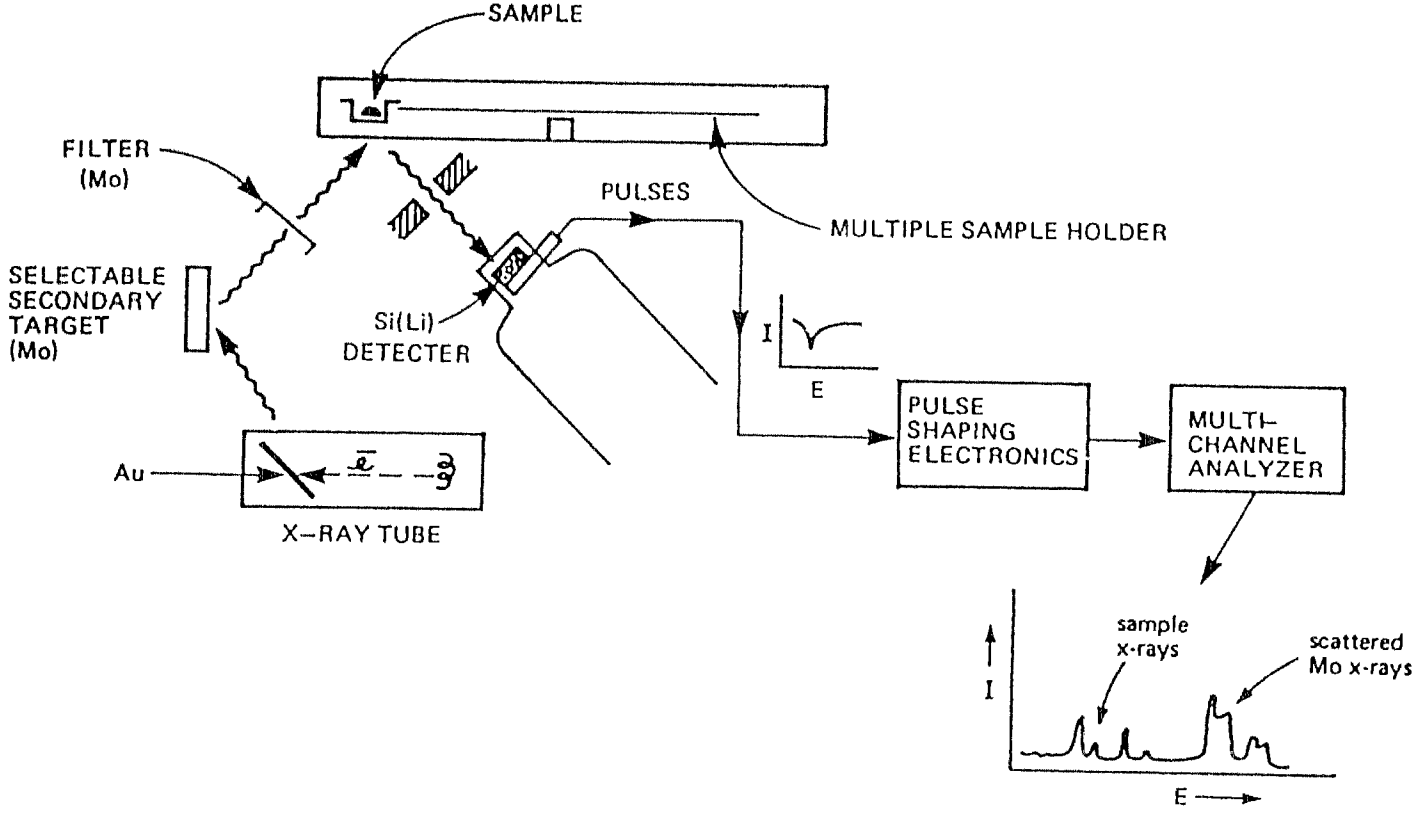
4.1.1 XRF - Instrumentation and Measurements

The X-ray fluorescence (XRF) facility used in this work as discussed earlier is a secondary target, photon excitation, energy-dispersive system. Figure 4.1 shows a schematic representation of this system. An X-ray tube (SIEMENS Model 1162023V 4004), equipped with a gold anode, was employed to generate the primary source Bremsstrahlung photon radiation which in turn irradiated a selectable secondary target, (molybdenum was used in this study). X-rays from this secondary target (including M_o Kα and Kβ lines) was filtered by a thin layer of Mo before radiating the sample.

The samples rested on a AAWP, millipore filter paper with a diameter of 25mm and a pore size of 0.8 μm. The deposited area of the sample on the millipore filter was 78.78 mm^2. The fluorescence from the sample was detected using a Kevex Si(Li) semiconductor detector (measured FWHM 180 eV for 5.9 keV) which produces a current pulse for each photon received. Pulses were amplified, shaped, and digitized by standard electronic equipment (originally developed for nuclear spectroscopy) including a Kevex Model 2002 Pre-amplifier

Figure 4.1

Schematic Diagram of the XRF Spectrometer

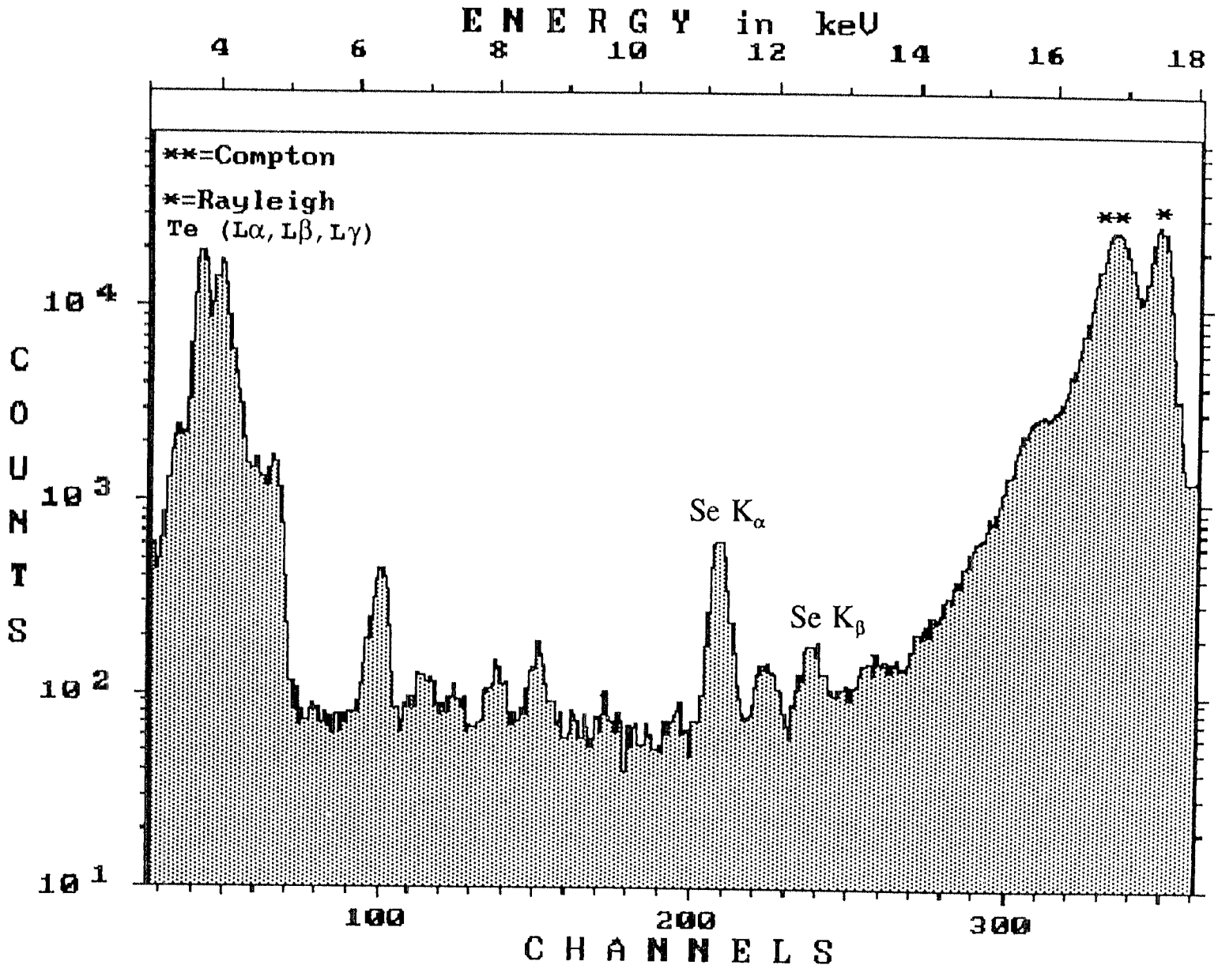


and a Kevex Model 4500 amplifier (see Figures 3.2, 4.1). A multichannel analyzer (NUCLEAR DATA INC. Model 66) sorted the signals into a frequency histogram. Irradiation time was 1000 seconds with an electron current in the X-ray tube of 15 mA. The intensities of the selenium Kα line (11.2Kev) and the combined tellurium Lα Lβ Lγ lines (3.77Kev, 4.30Kev and 4.57Kev, respectively) were extracted from the observed spectrum using an internal program of the ND66 analyzer (see Figure 4.2). The net area of the peak was taken to be the total counts minus the background counts. The background counts are the summation of all counts below an projected line drawn from the left limit channel of the peak to the right limit channel of the peak. The desired accumulated counts in the present study was a net peak of 2500 counts. Standard counting error then was 50 and relative fractional counting error was 2%.

4.1.2 XRF - Materials

Reagents. All reagents and acids were analytical grade. Glass-distilled, deionized water was used for all the experiments. Stock solutions containing 1.0 mg/mL of selenium in 1N HCl were prepared with elemental selenium (Aldrich Chemical Company, Inc.) and the internal standard tellurium (1.0 mg/mL in 1N HCl) was prepared from Na_2TeO_3 (Aldrich Chemical Company, Inc.). All stock solutions were stored in plastic bottles. Dilutions from these stock solutions were made prior to the experiments to prevent any losses of these elements, possibly by adsorption and volatilization. Reducing agent was freshly prepared by dissolving

Fig. 4.2 X-ray Spectrum From Inorganic Sample (300 ng Se, 600 µg Te).



hydrazine dihydrochloride (Aldrich Chemical Company, Inc.) in water to a final concentration of 10% m/m. All glassware and plastic bottles were cleaned with detergent and acetone, rinsed and soaked overnight in a 5 M nitric acid bath, rinsed with distilled water, and air-dried prior to use.

Samples. Standard reference materials such as National Bureau of Standards (NBS) Oyster tissue (SRM 1566), Citrus leaves (SRM 1572), Rice flour (SRM 1568), Bovine lever (SRM 1577a), International Atomic Energy Agency (IAEA) Animal blood (A-2), Animal muscle (H-4) and Fish homogenate (MA-A-2) were employed. Biological samples with Se such as human serum and erythrocytes (from Royal Columbia Hospital in New Westminster) and food samples (from the supermarket, *e.g.*, fruits, vegetables, fish, meat, liver etc) were also available for analysis. Sample weights in this work were generally in the range of 0.2-0.5 g for solid samples and about 1.0 mL for liquid samples.

Digestion apparatus. Destruction of biological samples was conducted in 100 ml Pyrex long neck (6 cm long and 4.2 cm diameter) flasks. Temperature-controlled heat plates were used to heat the samples to desired temperatures. A glass air-condenser (> 30 cm long) was fitted to the flasks to ensure reflux of acid vapour.

Filtration Apparatus. The filtration apparatus (Figure 4.3) consisted of a Pyrex filter holder, a reservoir, a flat 10 mm diameter circular sintered glass and metal clamps. The collection disc was a 25 mm diameter Millipore filter (AAWP,

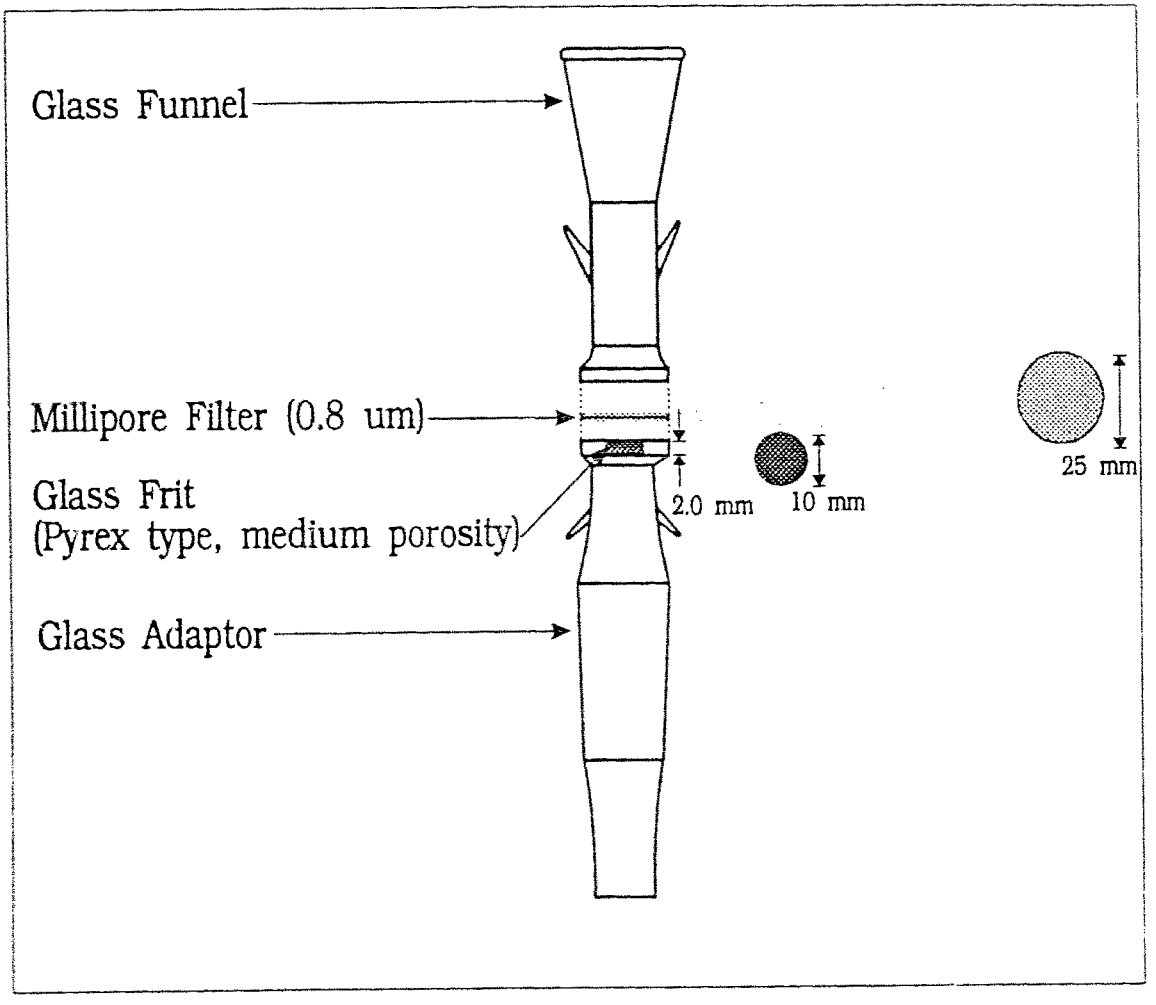


Figure 4.3

Filtration Apparatus

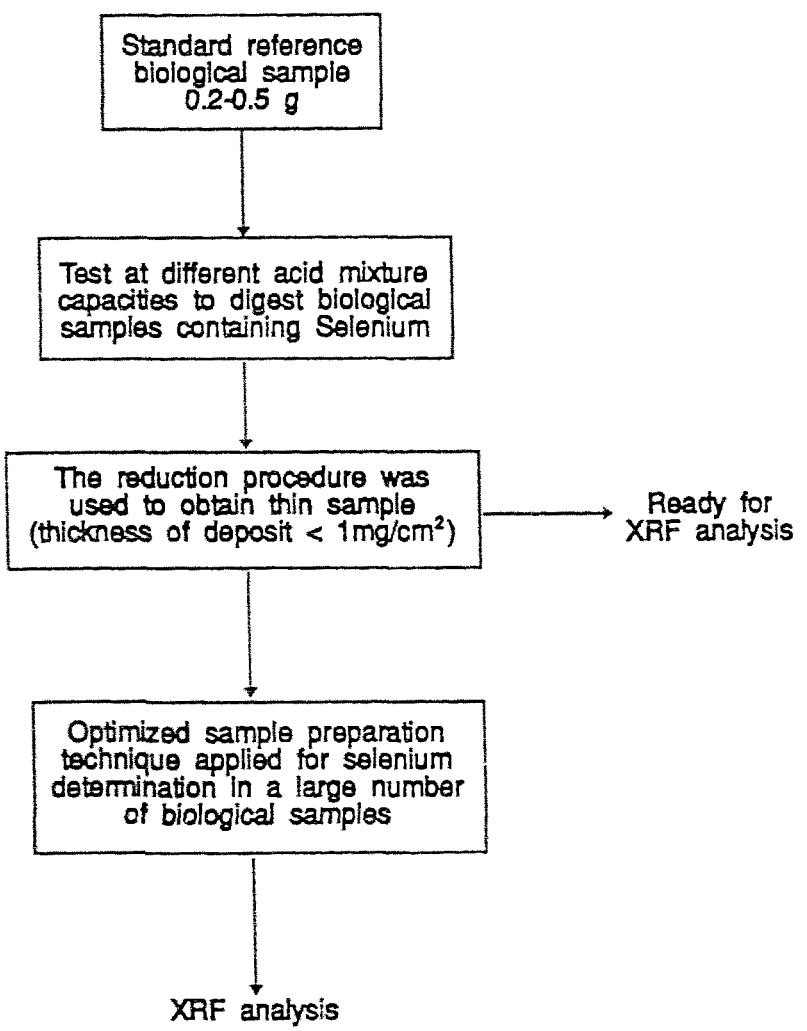
0.8μm).

4.1.3 XRF - Biological Sample Preparation Approach

The general experimental procedure to be followed to develop the optimum acid mixtures is illustrated in flow diagram (Figure 4.4). Rseults of these tests and rationale discussion in Chap.5.

Figure 4.4

Flow Diagram for the Biological Sample Preparation Approach



4.1.3a Test of Different Acid Mixtures for Wet Digestion

600 µg amounts of Te from the stock solution (1.0 mg/mL in 1N HCl) were added to five biological standard reference samples as an internal standard; four kinds of digestion mixture acids were then tested for each sample. In order to avoid losses of volatile selenium compounds (*e.g.*, in the forms of $SeOCl_2$ or $SeOBr_2$) during digestion, a condenser was routinely used.

a) Sulphuric acid and hydrogen peroxide Sulphuric acid 5.0 ml (96%) was added to the sample in a Pyrex long neck flask (100 ml). The resulting mixture was heated to approximately 100 °C for 15 min, then cooled to room temperature where upon 2mL of hydrogen peroxide was subsequently added. The mixture was then heated for 20 min at ≤ 100 °C, and was subsequently cooled in an ice bath for 10 min. The foregoing hydrogen peroxide treatment was repeated three times in order to oxidize all forms of selenium to Se(VI).

b) Nitric and perchloric acids Concentrated nitric acid (10 ml) and a few glass beads (to prevent bumping) were added to the sample in a Pyrex long neck flask (100 ml) and heated to 75 °C. The mixture was left to stand overnight at this temperature. Further heating at 125 °C for 6 hours was applied to reduce the volume of the sample solution to 3 ~ 5 mL. After addition of 2 mL of concentrated perchloric acid (72%), the temperature was increased to 170 °C. Upon the appearance of perchloric acid fume, the digestion was carried out for 30 min. The heating was halted when the liquid volume was reduced to approximately

1 mL.

c) Nitric, perchloric and sulphuric acids The sample was allowed to stand overnight with 10 mL of concentrated nitric acid. At this stage, all of the protein matrix dissolved to give a yellow solution, with only the fat matrix floating on the surface of the solution. After addition of 2 mL of concentrated sulphuric acid and 2 mL of 60% perchloric acid, the mixture was gradually heated to 140 °C and was maintained at this temperature for 30 min. The sample was kept at 170-180 °C until all of the perchloric acid was removed and dense white fumes of sulphur trioxide appeared. The addition hydrogen peroxide and the heating process were repeated twice again after which the heating was allowed to continue 5 min at 180°C after the evolution of white fumes.

d) Nitric, phosphoric acids and hydrogen peroxide Concentrated (65%) nitric acid (5 ml) and 85% phosphoric acid (1 ml) was added to the sample and left overnight at room temperature. After this predigestion, the sample was heated at 50 °C for 1/2 h after which the temperature was kept at 145-150 °C for 4~12h depending on the digestibility of the organic matter. When the brown NO_2 fume disappeared, the heating was halted. After cooling the sample to room temperature, hydroperoxide (2 mL of 50% H_2O_2) was added. The samples were allowed to react for 10 min after which 1 drop of 2.5 g/L Mn as $MnSO_4$ was added. The flask was returned to the hot plate and heated at 145 °C for ~4 hours until the volume was reduced to ~1-1.5 mL. and the colour of the digest turned purple. If purple

coloration did not appear, a further 1-2 mL of nitric acid-hydrogen peroxide mixture (3:1, v/v) added to the flask and to the digestion continued.

4.1.3b The Reduction Procedure Used to Obtain Thin Samples (< 1mg/cm^2)

As will be discussed later, an optimal reduction procedure was developed experimentally as part of this study. The final procedure used is given below.

A two-step reduction was carried out using hydrochloric acid (HCl) and hydrazine dihydrochloride ($N_2H_4 \cdot 2HCl$). The digested sample was diluted with water and 30% hydrochloric acid to produce a 5 M HCl solution. The mixture was boiled gently (100 °C) for 20 min to reduce all selenate to selenite. Selenite was further reduced to elementary selenium by adding 10% aqueous hydrazine dihydrochloride (2 mL) and a saturated solution of SO_2 (4 mL). After 15 min, the sample was filtered on millipore filter paper (AAWP, 0.8µm). The resulting fine grey precipitate was washed with 100 °C water and hot ethanol (95%) to dissolve the white precipitate of hydrazynium chloride. The sample was then ready for XRF analysis under thin target conditions, with the thickness of the deposit being less than 1 mg/cm^2.

4.1.3c Optimized Sample Preparation Technique Applied for Selenium Determination in Biological Materials

The Optimized sample preparation technique used in the study is shown in flow diagram (Figure 4.5).

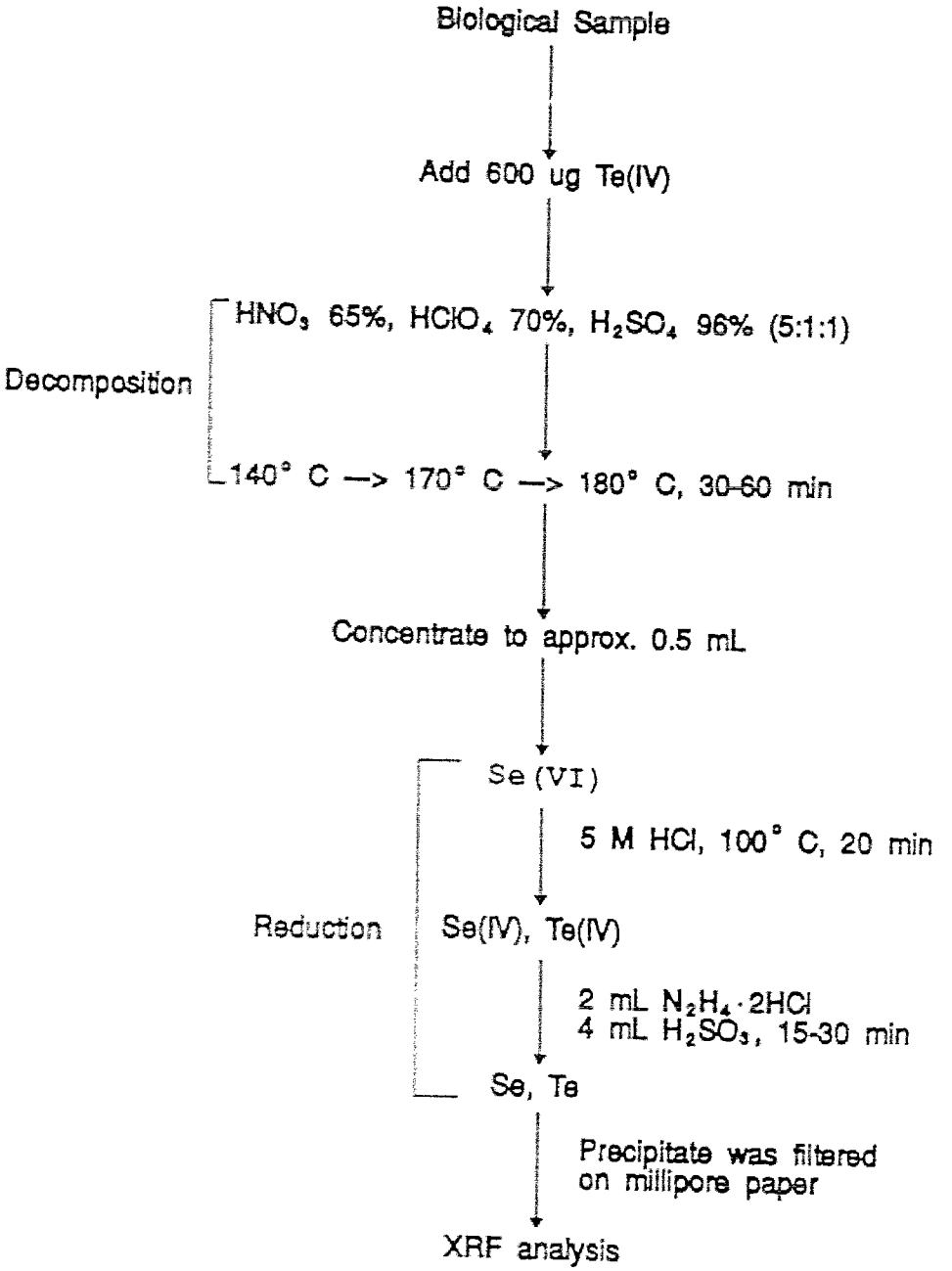


Figure 4.5

Flow Diagram for the Trace Analysis of Selenium

1. Tissue samples. Individual rainbow trout fish (15 individuals, 90 % male) were obtained from the Westcreek Trout Farm in Vancouver. The fish were kept for a period of two weeks in an aquarium, then sacrificed and weighed. Muscle, liver and gill tissues were recovered. Each gill sample was a combination from five organisms due to the very low weight of the organs.

A known amounts (fresh weights) of the tissue was placed in a 100 mL Pyrex glass flask and concentrated nitric acid (10 mL) was added, the sample was allowed to stand at room temperature overnight. At this stage the protein tissues were dissolved to yield a yellow solution with the fat tissue floating on top of the solution. Concentrated sulphuric acid and 60% perchloric acid (2 mL each) were added and the sample gently heated at 140 °C. At this stage the nitric acid was evaporated. When the volume of the digest was reduced to 4-5 mL, the temperature was increased to 170-180 °C. At this temperature, boiling of nitric acid was vigorous and care was necessary to prevent the solution from escaping from the flask. If the solution turned yellow, 1 ~ 2 mL of nitric acid was added after cooling the solution for about 1 minute. The solution was boiled again and more nitric acid was added until the solution became colourless. The sample was boiled until white fumes of perchloric acid appeared to ensure the absence of charring.

2. Food samples. The selection of samples was based on the popularity of the food. The popularity was based on information received from various government agencies and directors of supermarkets serving Western Canada,

particularly in the region of British Columbia. The samples analyzed in this work were considered to be particularly representative of the food products consumed in Western Canada. The sample size was usually determined by two factors: (1) water content (wt %) and (2) fat, starch, or sugar content. Generally, 5-10 g of products containing >50% water and low in fat, starch, or sugar (*e.g.*, fruits, vegetables, fish, meat, liver) were used; food products weighing 3-5 g that contain 10-50% water; and 1-3 g for products containing <10% water (*e.g.*, flour, cereals, dried foods) were employed. For products high in fat or sugar (*e.g.*, cheese, butter oils, sirrups, jams), sample sizes were limited to 1-2 g.

Having placed appropriately sized samples (weighed to an accuracy of 0.001 g) and 3 glass beads into a 100 mL Pyrex glass flask, 30 mL of HNO_3-$HClO_4$ (5:1, v/v) was added. This sample was digested overnight at room temperature. The temperature was gradually increased to achieve a steady vigorous boil with care taken to prevent loss by foaming or bumping. When the volume was reduced by approximately one-half, 10 mL of HNO_3-H_2SO_4 (1:1, v/v) solution was added at room temperature. The flask was further heated at 170-180 °C during $HClO_4$ oxidation, a process which was characterized by vigorous surface darkening. Charring was avoided by cautious addition of HNO_3 in 1 mL portions (HNO_3 had to be added to maintain oxidizing conditions). Failure to closely observe the $HClO_4$ oxidation stage of this digestion would result in possible loss of elements sought, or even explosion. High fat, sugar, or carbohydrate products required more HNO_3

to maintain oxidizing conditions. When digestion was applied to any such product, a conservatively small sample (1-2 g at most) was initially utilized and digestion was cautiously allowed to proceed. Upon increasing the temperature to 140°-170°-180°C, the solution rapidly turned an intense yellow-green. Heating was continued until the solution became clear and colourless, and dense white fumes of SO_3 appeared. The samples were kept at this temperature for another 5 min, then final digest was colourless and the volume of solution was reduced to 2 mL.

3. Serum and erythrocyte samples. Serum and erythrocyte samples were taken from 25 healthy volunteers. 1.0 mL of serum or 1.0 mL of erythrocytes and 2.5 mL of nitric acid were placed in long-necked Pyrex glass flasks, heated slowly to 140 ^{0}C on a hot plate and kept at this temperature for 30 min. After cooling to room temperature, sulphuric and perchloric acid (0.5 mL each) were added. The temperature was slowly raised to 160 °C, 170 °C, 180 °C, and held at each of these temperatures for 15 min before the next increase. The final temperature of 180 ^{0}C was held for at least 20 min so that most of the perchloric acid was removed and the volume of the digestion solution was reduced to approximately 0.5 mL.

In order to determine selenium content after sample digestion, the reduction technique (see 4.1.3b) was carried out to obtain a thin sample.

4.2 Analysis Using Differential Pulse Cathodic Stripping Voltammetry (DPCSV)

4.2.1 Reagents and Instrumentation

All acids including electrolytes were analytical grade. Distilled, deionized water was used for all sample and solution preparations. Ar (99.999%) was employed as purging gas. The stock Se solution was selenium atomic absorption standard solution (Aldrich, 1030 μg/ml of Se in 1% HNO_3). The required standard solutions were freshly prepared by diluting the stock solution with electrolyte-containing solution.

A microprocessor-based polarographic analyzer/stripping voltammeter (PAR Model 264, GE and G Princeton Applied Research), equipped with a PAR Model 303A static mercury drop electrode was used to obtain all stripping voltammograms. A Hewlett-Packard Model 7046A X-Y recorder was used. The electrode compartment consisted of a hanging mercury drop, Ag/AgCl (in saturated KCl, all the potential is relative to Ag/AgCl) and a Pt wire were used as its working, reference, and counter electrodes, respectively (Figure 4.6).

Sample solution (10 ml) was transferred to the polarographic cell following 4 minutes of degassing. The selenium (VI) was measured at room temperature under the following conditions:

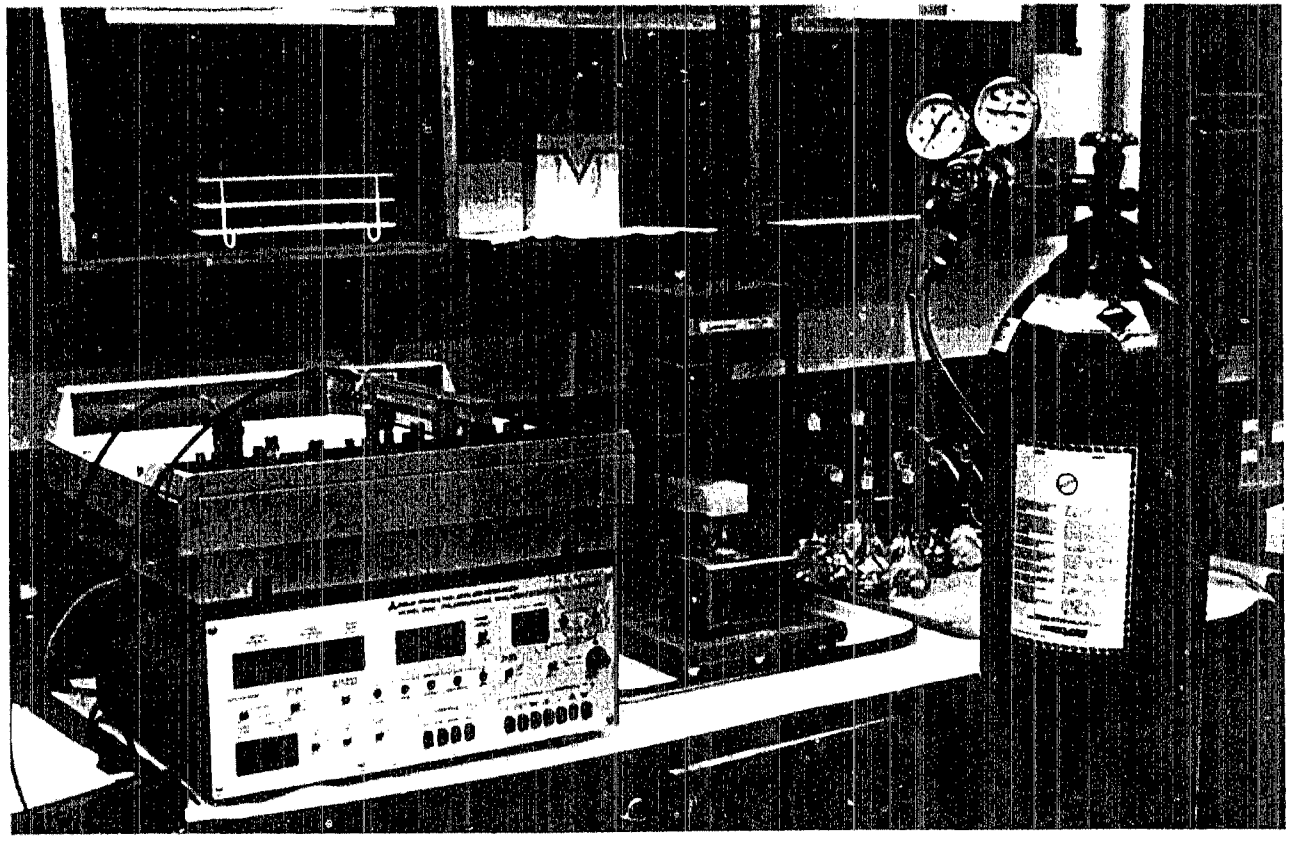
initial potential	-0.20 V
final potential	-0.90 V
purge time	240 sec.
scan rate	5 mV
deposition time	120 sec.
equilibrium time	10 sec.
pules height	0.05 V

4.2.2 Sample Digestion

Samples of erythrocytes were digested using methods similar to those employed in XRF analysis. Erythrocytes (0.5 mL) were transferred into a long-necked flask and 65% HNO_3 (10 mL), and 72% $HClO_4$ (4 mL) were added. The flask was heated slowly to 135 °C. After 30 minutes at 135 °C, the temperature was gradually raised to 140, 160, 170, and 210 °C, and the sample was held at each of these temperatures for 15 minutes. The HNO_3 and $HClO_4$ were evaporated during the digestion and approximately 0.5 mL remained in the flask. The resulting Se(VI) was reduced to Se(IV) by heating the sample with HCl (1.0 ml HCl,36.5% added) at 100 °C for 20 minutes. The sample solution was then diluted to 50.00 mL with water.

Figure 4.6

Differential Pulse Cathodic Stripping Voltammetry (DPCSV) Equipment.



Chapter 5 Results and Discussion: Development of Biological Sample Preparation Method

Direct excitation XRF usually has limited sensitivity: the detection limit for Se is generally in the range of 2-40 ppm.[55] In order to bring the concentration of trace elements to a detectable level, a preconcentration step is required. An efficient preconcentration treatment consists of two steps: sample decomposition, followed by a coprecipitation procedure to obtain a thin sample (<1 mg cm^{-2}). The most important considerations for preconcentration treatment for biological materials are: 1) the choice of an acid mixture that avoids loss of Se and maintains strong oxidation conditions; 2) the choice of a digestion temperature and a time to fully decompose the organic structure without loss of Se; 3) and the design of the reduction procedure. The sample was treated with different strong oxidizing acids at different ratios and different temperatures depending on the types of biological samples. The biological sample preparation technique will be discussed in two aspects: optimization of the acid mixture to digest selenium matrices, and optimization of the reduction step.

5.1 Optimization of the Acid Mixture to Digest Biological Samples Containing Selenium

Although the chemical nature of selenium in biological materials is not entirely known,[32] some organic selenium compounds have already been identified in plant and animal tissues. For example, selenoamino acids such as selenocysteine and selenomethionine have biological activity, and the trimethylselenonium ion is an important urinary excretory product.[32] These bioselenium samples proved to be a way to check the validity of the various methods of determination of selenium in biological materials and their capacity to digest some organic selenium matrices.

Wet digestion is fast and is much less troubled with volatilization loss, because of the lower temperature as compared to the dry ashing technique. The major disadvantage is the possibility of contamination from the large excess of reagents employed. Generally, wet decompositions for the complete destruction of organic matter require agents with high oxidation potential. In many cases, HNO_3 and even a mixture of HNO_3 and H_2SO_4 are insufficient. Not only is this acid mixture unable to digest tissues with high fat content, but charring of the sample is possible. Therefore, a host of mixtures and oxidizing agents at various temperatures have been investigated, to completely mineralize the sample without loss of Se.

In this work, wet digestion methods were employed for the XRF analysis of

selenium in five biological standard reference materials (described in chap.4 - 4.1.3a). Samples were treated with concentrated mineral acids and/or strong oxidizing agents in solution. Oxidizing conditions are maintained throughout the procedure. Usually, the mixture was heated to 100-200 ^{0}C to accelerate the digestion process.

Four different combinations of acid mixtures described in chap.4 were used in this work:

I. in H_2SO_4/H_2O_2 ratio 5:1 ;

II. in $HNO_3/HClO_4$ ratio 5:1 ;

III. in $HNO_3/H_2SO_4/HClO_4$ ratio 5:1:1 ; and

IV. in $HNO_3/H_3PO_4/H_2O_2$ ratio 5:1:1 ;

The recoveries of Se using different mixtures in wet digestion techniques are given in Table 5.1, and each of the procedures are discussed in the follow sections.

Procedure I.

Compared to other procedures, relatively low recoveries of selenium were achieved by sulphuric acid and hydrogen peroxide mixture. Samples were routinely dehydrated and charred by sulphuric acid. Charring of the sample is an indication of the reduction, although Agemian and Thomson[65] reported that complete loss of selenium occurred when samples were charred. Using this mixture, satisfactory results were obtained for NBS 1577 and IAEA A-13. When volatile selenium

Table 5.1

Comparison of Some Wet Decomposition Procedures

Matrix	Parameter	Procedure			
		I	II	III	IV
Rice flour NBS 1568	R, % n S, %	90 3 5	95 3 11	102 5 5	101 3 9
Citrus leaves NBS 1572	R, % n S, %	87 3 13	104 3 13	97 3 14	95 4 12
Bovin liver NBS 1577a	R, % n S, %	98 4 7	101 3 4	101 4 6	96 3 10
Fish homogenate IAEA MA-A-2	R, % n S, %	71 3 13	93 3 10	96 3 11	95 3 13
Animal blood IAEA A-13	R, % n S, %	104 3 8	100 4 8	95 3 9	102 4 12

I. in H_2SO_3/H_2O_2; II. in $HNO_3/HClO_4$; III. in $HNO_3/H_2SO_4/HClO_4$;

IV. in $HNO_3/H_3PO_4/H_2O_2$;

R = Selenium Recovery; n = Number of Analysis;

S = Relative Standard Deviation

compounds were absent, the charring of the sample did not lead to a loss in selenium. The recoveries of selenium after using this procedure were generally low and its use for this purpose should be avoided.

Procedure II and III

Mixtures such as nitric-perchloric and nitric-perchloric-sulphuric acids have were found to be satisfactory in this work; the average recovery of Se was 98.6% and 98.2%, respectively. The high recoveries of Se in these mixtures can be explained by the following:

It is well known that selenium in high oxidation states is less volatile. The loss of selenium during sample decomposition can be avoided by maintaining the solution under oxidizing conditions. The presence of perchloric acid was also important in decomposing organoselenium compounds completely and facilitating oxidation of the highly resistant fatty materials. These conditions were achieved by introducing perchloric acid to the digestion mixture.

In order to avoid loss of volatile selenium compounds, the temperature was increased slowly during the initial stage of digestion to avoid excessive boiling and bumping. The duration of digestion in the perchloric acid mixture was at least 30 min. When the digestion was completed, the volume of the solution was reduced to 1 mL. In this way the selenium containing organic material was found to be completely decomposed.

As discussed earlier, the role of perchloric acid was to prevent selenium losses by maintaining oxidation condition throughout digestion. The use of concentrated perchloric acid in wet digestion procedures required several precautions. The high reactivity of $HClO_4$ could cause violent explosions if the acid were mishandled. Perchloric acid is an oxidizing agent only when both hot and concentrated. The cold, concentrated (70%) acid (although a strong acid) is not an oxidizing agent and will not even oxidize iodide to iodine or iron (II) to iron (III). In other words, care must be taken to follow the basic rules[66] of safe handling:

1. Never bring undigested organic matter directly into contact with hot, concentrated $HClO_4$; a fire or explosion may result.
2. Always predigest organic matter with HNO_3 first to deplete the easily oxidized compounds.
3. If it is hot, concentrated $HClO_4$ is spilled, dilute immediately with large quantities of water. This dilutes and cools the acid and effectively "turns off" its oxidizing power.
4. Never boil $HClO_4$ to dryness.

Nitric acid mainly prevents the samples from charring at high temperature. It is important because charring is a characteristic of nonoxidizing or reducing conditions in which selenium compounds are apparently converted to selenides (probably hydrogen selenide) which are lost by volatilization.[65]

Additional use of sulphuric acid does not seem to result in a better recovery of organic selenium, but it does reduce the time of decomposition with perchloric acid. Sulphuric acid was excluded if samples contained a large amount of calcium because of the possibility of coprecipitation of analyzed element with $CaSO_4$. The precipitation of other cations (e.g. Pb^{2+}, Ag^{+}, Ba^{2+} with sulfate; Ag^{+}, Pb^{2+} with chloride) limited the selection of suitable digestion acids.

Further, attention must be made to the slow oxidation procedure of selenite to selenate in boiling perchloric acid, because under rapid heating conditions, virtually complete losses were evident.

The mixture of acids most commonly used was 5:1:1 (v/v) of HNO_3, H_2SO_4 and $HClO_4$ which completely dissolved organic material in all kinds of biological materials.

Procedure IV

The high reactivity of $HClO_4$ could cause violent explosions if the acid were mishandled. A mixture of phosphoric acid, nitric acid, and hydrogen peroxide has been proposed as an alternative to the use of the nitric/sulphuric/perchloric acid mixture to digest biological materials. The purpose of this study was to test the applicability of this digestion method for the determination of Se in standard reference materials. Selenium contents of standard reference materials were determined following acid digestion with $H_3PO_4/HNO_3/H_2O_2$ (Table I). The values

for all standard reference materials ranged from 95% to 102% of their certified values. The results reported here indicate the $H_3PO_4/HNO_3/H_2O_2$ mixture to be a satisfactory alternative to the use of $HNO_3/HClO_4/H_2SO_4$ for digesting biological samples for Se determination.

The oxidation status of the digesting solution was monitored by solution color up on the addition of a small amount of manganese (Mn) which served as an indicator of completion of digestion. Due to the formation of permanganate (MnO_4^-) ion, a purple coloration of the digesting solution appeared when the oxidation was complete. The addition of Mn also identified samples that had not received sufficient oxidant at the end of the digestion to avoid Se loss from charring of samples.[67] The mechanism of MnO_4^- formation has been explained by Allen Dong et.al.[68] During the initial stage of the digestion, the color of the solution was yellow-brown due to the presence of NO_2 gas. After the addition of H_2O_2 and heating to evaporate excess HNO_3, the phosphate in the digest was condensed to form pyrophosphate. Pyrophosphate reacted with H_2O_2 or phosphate hydroperoxidate to form peroxophosphate; this in turn reacted with colourless Mn(II) to form purple MnO_4^-. The purple color of MnO_4^- usually formed when the volume of the digest was reduced to ~1.5 mL. However, depending on the properties of samples, *e.g.*, when digesting a Se standard, the purple color appeared when the volume was reduced to ~4 mL.

However, some digesting solutions of difficult-to-digest samples did not

show the purple color even when the volume of the digest was reduced to 1 mL because of insufficient oxidant. Also, samples with high lipid content had lipids floating on top of the digest. For both situations, the digestion was terminated before the volume reached 1 mL with yellow-brown color. The remaining dissolved organic materials or lipids were removed with chloroform.[69] For complete digestion of organic material, additional HNO_3+H_2O_2 was added before the volume of the digest reached 1 mL. Upon the addition of HNO_3+H_2O_2 to the digest, the purple color changed back to colourless due to the initial destruction of MnO_4^- by free H_2O_2. The destruction of H_2O_2 by Mn was diminished in the presence of phosphate.[70]

With prolonged heating, it is possible to evaporate, decompose, or consume the remaining oxidants in the digest. The change in color from the purple MnO_4^- to colourless Mn(II) readily identifies reducing conditions in the digest that occurs prior to the formation of charred particles. Thus continued heating can result in Se loss from precipitation or volatilization.

Experimental evidence indicated that reducing conditions could occur even prior to the formation of black charred particles in the digest. Thus it is important to identify and avoid this "precharring" stage during H_3PO_4/HNO_3/H_2O_2 digestion. $MnSO_4$ was added for this purpose as a redox indicator. The addition of Mn(II) to the digest did not interfere with the XRF determination of Se; this was in agreement with the findings of S. E. Raptis et.al.[71]

Careful control of digestion temperature was critical for a successful decomposition. Decomposition times varied from one hour to a whole day depending on the nature of acid mixture used.

A nitric acid, phosphoric acid and hydrogen peroxide mixture provided an efficient means of sample digestion with high recovery, possibly without Se loss. However, the process was long, tedious and not suitable for fats. The mixture of nitric, perchloric and sulphuric acids was therefore, deemed most suitable for the routine analysis of large number of biological samples.

5.2 Design and Optimization of the Reduction Procedure

The reduction of Se(VI) is based on a two-step reduction using hydrochloric acid and hydrazine dihydrochloride. Selenium was co-precipitated with tellurium (coprecipitant), and then deposited on a millipore filter.

5.2.1 Conversion of Se(VI) to Se(IV)

In geological,[72] biological[73] and soil extract[74] samples, some Se is present in the hexavalent state. It can also be oxidized to the highest oxidation state during decomposition, depending on the oxidation potential of the reagents. For example, approximately 15 and 50% of the total Se was oxidized to selenate in mixtures consisting of $HNO_3/H_2SO_4/V_2O_5$ and $HNO_3/HClO_4$, respectively.[73]

Reductants that are normally applied are, *e.g.*, H_2O_2,[75] KBr,[76] KI-$SnCl_2$,[77]

Zn powder,[78] $TiCl_3$-Mg.[79] The efficacy of KI is, however, often impugned.[80] The reductant HCl was employed in this work. The efficiency of the reaction depends, however, on many factors, e.g. concentration of the HCl (Figure 5.1),[81] reduction time (Figure 5.2),[81] and temperature of the solution.[81] The optimal conditions in our experiment involved use of a 5 M hydrochloric acid solution and boiling gently (100 °C) for 20 min.

5.2.2 Precipitation of selenium using tellurium as the coprecipitant

Trace amounts of Se present in biological samples does not permit direct precipitation of the element. Therefore the selenium was coprecipitated with added carrier Te. The use of Te in this work has two noteworthy advantages in the biological applications of XRF. First, it is an ideal coprecipitant for Se because its chemical properties are similar to Se. Even in extremely small quantities, Se can be coprecipitated with Te to make the analysis possible. Secondly, it has been reported that Te does not naturally occur in most biological systems.[82] Tellurium is, therefore, ideally suitable as an internal standard which is important in compensating for matrix absorption-enhancement effects and long-term instrument drift (see Chapter 6). The quantities of added Te used are usually in the range of 200-1000μg. We chose 600μg Te as a good compromise, taking into account the final counting rate.

The mixture of hydrazine dihydrochloride and H_2SO_3 was used to reduce the selenites and tellurites to selenium and tellurium, respectively. Figure 5.3 gives the

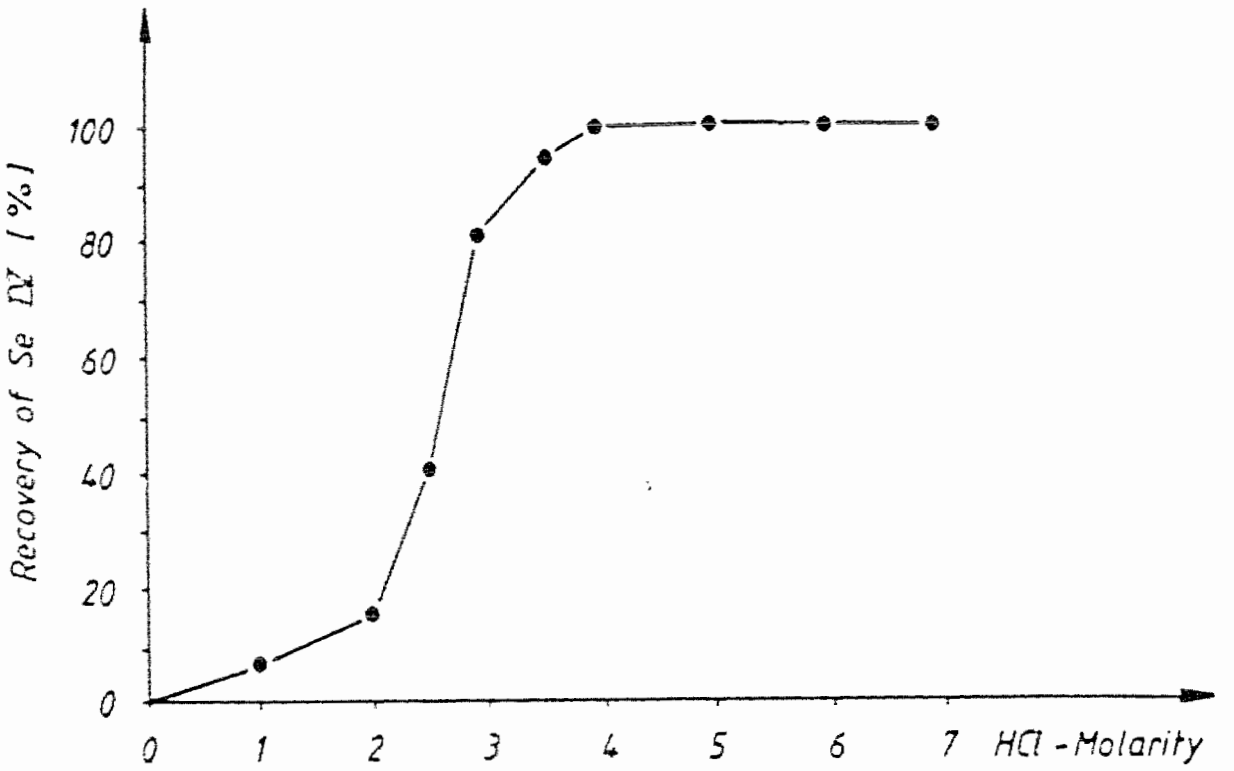


Figure 5.1

Dependence of the Recovery of Se(IV) on the Concentration of HCl in the Reduction of Se(VI) to Se(IV).[81]

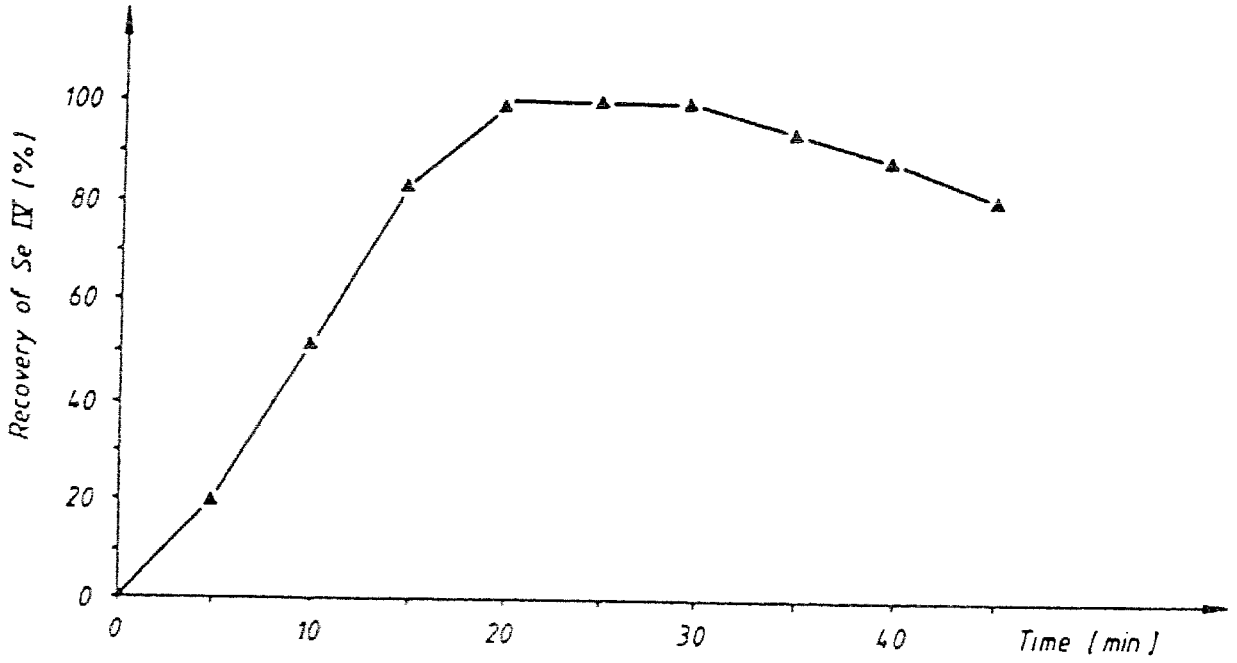


Figure 5.2

Dependence of the Recovery of Se(IV) on the Reaction Time in the Reduction of Se(VI) to Se(IV) with 5 N HCl at 100 °C.[81]

dependence of the recovery on the hydrazine dihydrochloride concentration. Quantitative reduction was achieved only if the hydrazine dihydrochloride concentration was greater than 1%. The use of strong oxidizing acids (HNO_3, $HClO_4$, H_2SO_4) in the decomposition step requires an operation at the upper end of the curve to ensure a sufficient excess of hydrazine dihydrochloride (a final concentration of about 10%).

Figure 5.4 shows that a period of at least 10 min was required for crystallization (precipitation). After 2 h, the dissolution of Se began. The decrease of the curve demonstrates that the time for the reaction prior to filtration can not be too long otherwise in completing the reduction, precipitation may occur. The dissolution is also volume-dependent but the precipitation is quantitative for solution volumes less than 40 mL (Table 5.2). To avoid lengthy filtration times, the optimal sample volume was between 10 and 15 mL. Quantitative precipitation was achieved between 10ng and 200μg Se. The filtration time was varied between 3 and 15 min and had no influence on the recovery or the homogeneity of the deposit. It has been reported that the millipore filters that were used have a blank value of 0.4 ng of Se,[83] which is well below the detection limit of the present method. To summarize, we optimized the procedure of various reagents used for the wet digestion of different materials and reduction of Se to its elemental form with Te as coprecipitant. A biological sample preparation method was developed which includes an optimization of a wet digestion and reductio procedure.

Figure 5.3 Recovery of Selenium as a Function of $N_2H_4 \cdot 2HCl$ Content (Sample: 1 μg of Se, 15 - min reaction time, 5 - min filtration time).

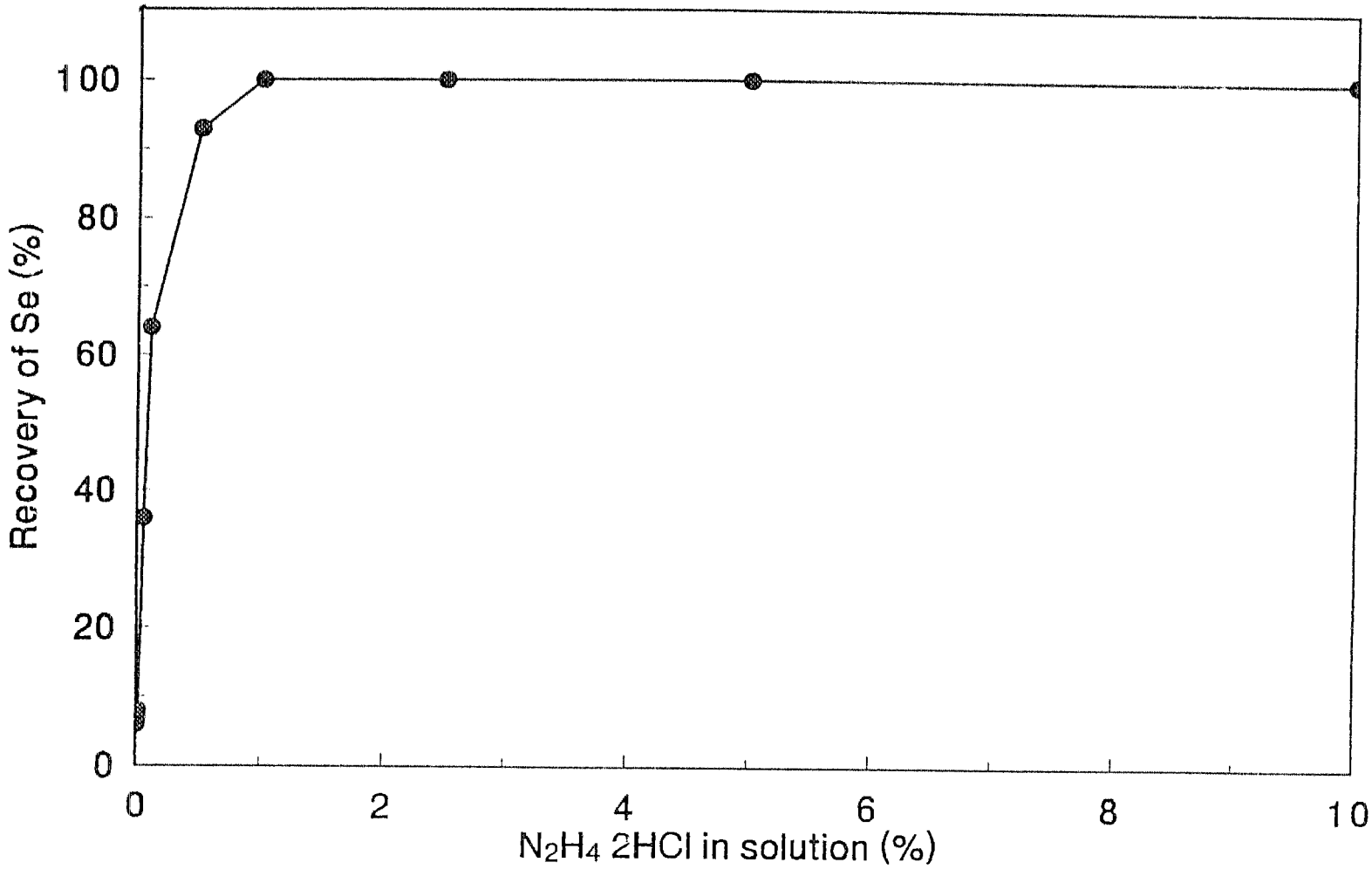


Figure 5.4 Recovery of Selenium as a Function of Reaction Time (Sample: 1 μg of Se, 10% $N_2H_4 \cdot 2HCl$, 5- min filtration time).

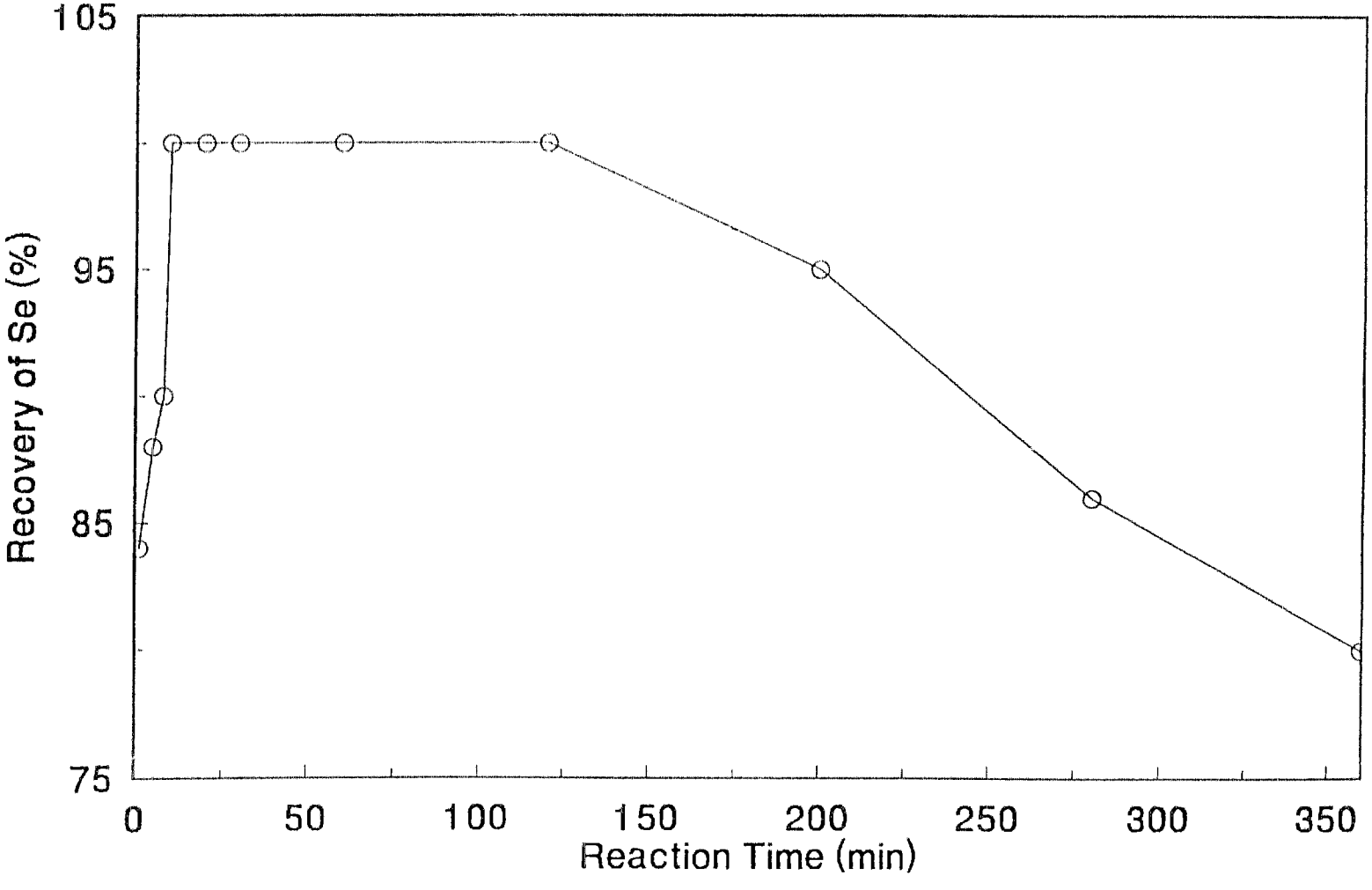


Table 5.2

Dependence of Recovery on Sample Volume

Volume of sample (mL)	Recovery on the filter[a], (%, N=5)
0.5	99.0 ± 2.0
1.0	98.7 ± 1.9
2.0	98.5 ± 1.8
5.0	98.1 ± 1.6
10	98.3 ± 1.7
20	98.4 ± 1.5
30	98.0 ± 1.9
40	97.1 ± 1.6
50	93.7 ± 2.0
60	93.0 ± 1.9
80	92.5 ± 2.0
100	90.0 ± 2.0

[a] 200 ng of Se

Chapter 6 Results and Discussion: Quantitative Se Analysis

6.1 Method of Quantitative Analysis

X-ray spectrometric analysis is an analytical technique in which intensity data are converted to concentration units by the use of calibration curves or mathematical relationships derived from the measurements of standards. In this study, "internal standardization" was used for quantitative analysis (quantitative addition to all specimens of an internal-standard element having excitation, absorption, and enhancement characteristics similar to those of the analyze in a matrix). The internal standard compensates for absorption-enhancement effects and instrumental drift for all types of specimens. The calibration function involves the intensity ratio of the analyze to internal-standard lines, as shown in equation (3.5) (described in chap. 3 - 3.2).

$$I_x/I_s = (I_0P_xG_x/I_0P_sG_sW_s) * W_x = K_xW_x \qquad (3.5)$$

For the quantitative analysis of Se, tellurium is an ideal coprecipitant and is also used as the internal standard for purposes of normalization.

when the weight of tellurium is a known quantity and kept constant throughout the analysis, a linear relation between the ratio I_{Se}/I_{Te} and the weight of selenium is obtained as:

$$I_{Se}/I_{Te} = K_{Se}\ W_{Se} \qquad (6.1)$$

Line intensities are measured from the selenium Kα line and tellurium L lines for all samples and standards. A calibration curve of intensity ratio versus selenium quantity may be established from a series of standards.

6.1.1. Calibration Curves of Standard Se (IV) Solutions

Solutions of known concentrations of Se were used to determine the constant K_{Se} (system response constant for selenium). The calibration was carried out by analyzing the X-ray fluorescence spectra for standards which were precipitated from solutions with known amounts of selenium using the precipitant $N_2H_4.2HCl$ and the coprecipitant Te (600μg) in the absence of an organic matrix. The analytical conditions used were the same as those for the samples. Each Se standard level was analyzed three times and least squares analysis[54] was carried out as well. Se Kα peak area was normalized to the Te L peak areas; normalized peak areas were then plotted against the known weight of selenium to yield calibration curves. Figure 6.1a shows a calibration curve covering the Se (as H_2SeO_3) quantity range from 0 - 700 ng for trace amounts of Se analysis. The linear relationship between

Figure 6.1a Precipitation Efficiency of Filter Samples (without organic matrix).

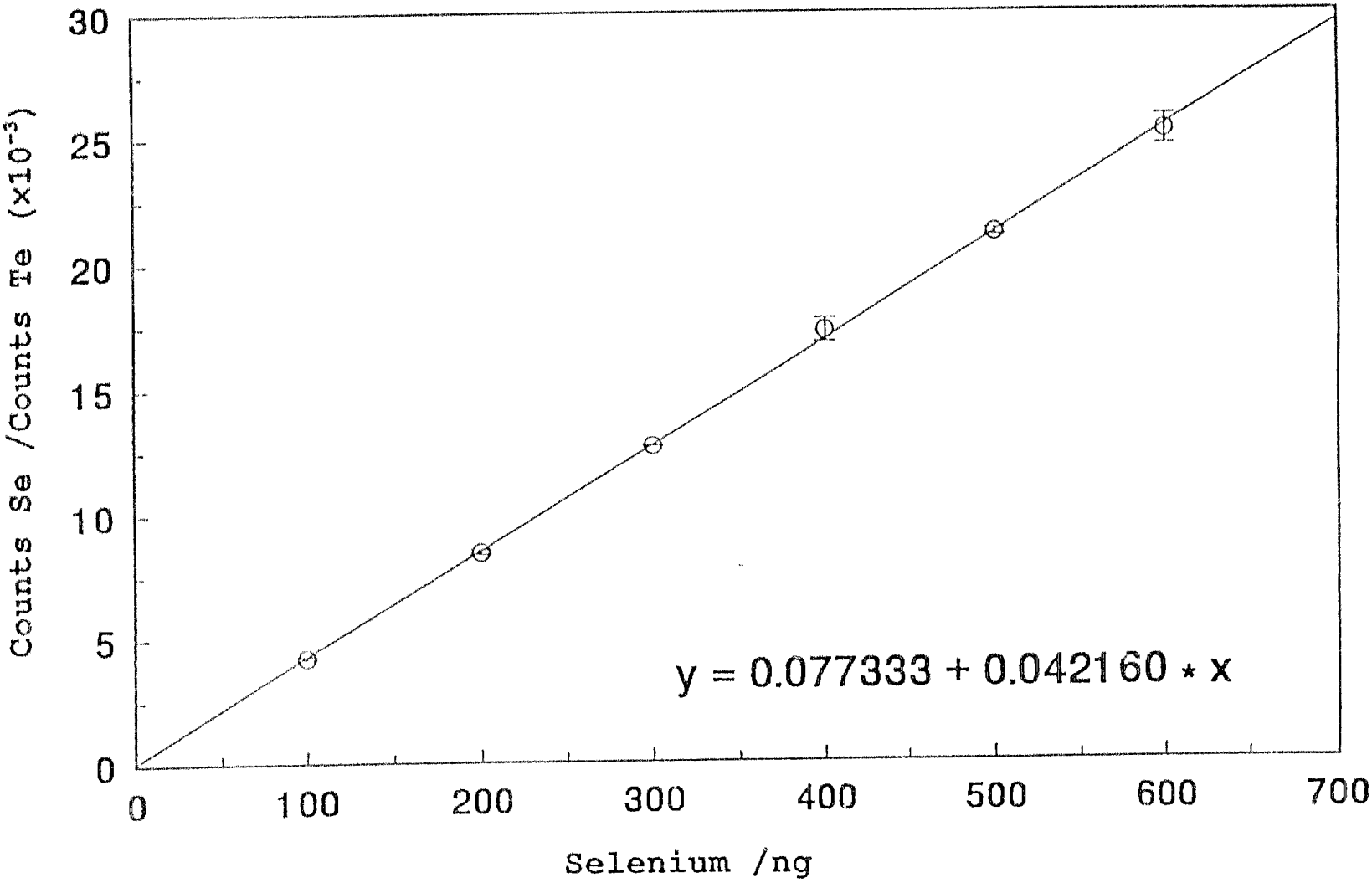
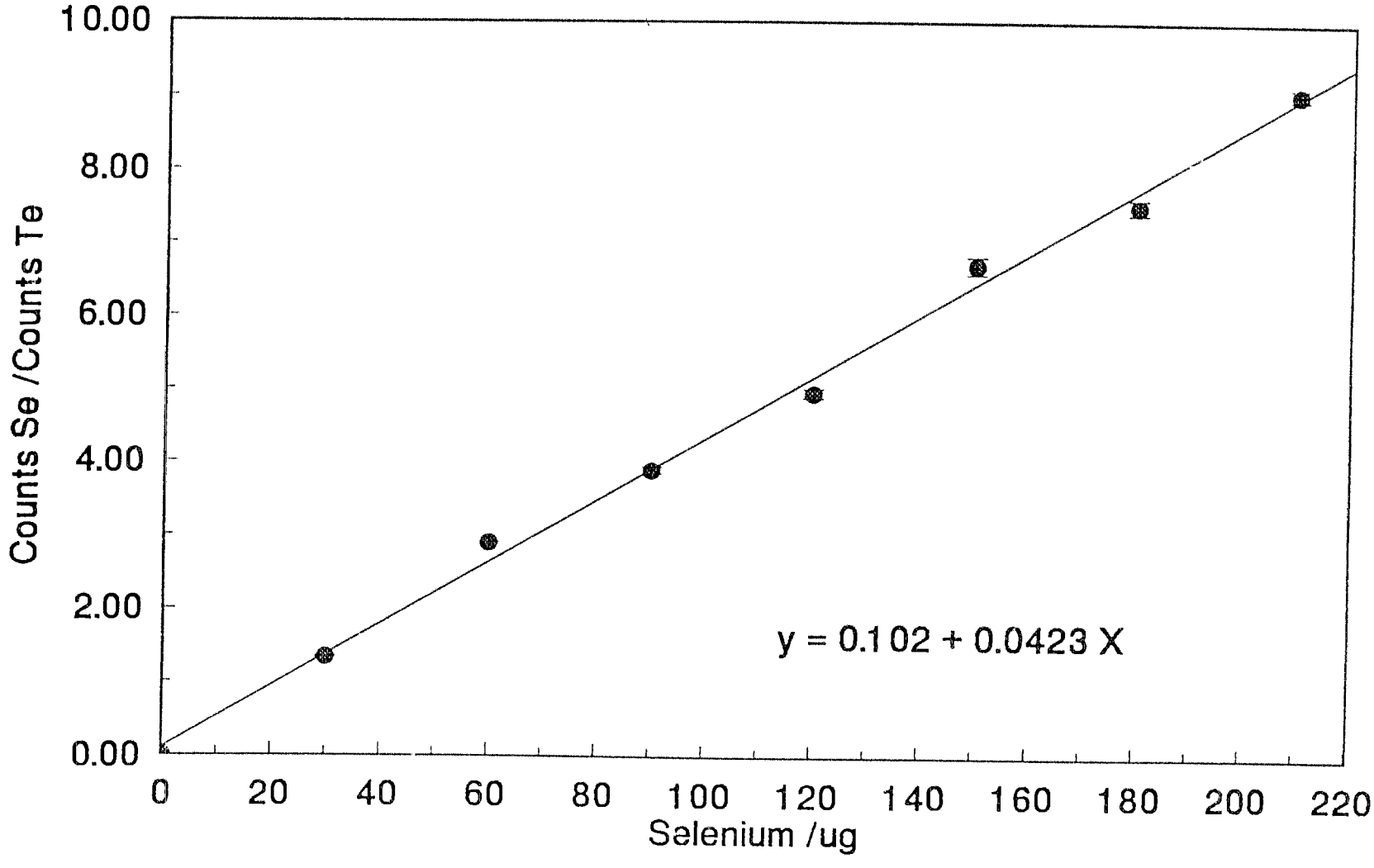


Figure 6.1b Precipitation Efficiency of Filter Samples (Without Organic Matrix).



the intensity ratio and Se quantity can be extended to more than 210 µg as shown Figure 6.1b. The excellent linear relationship between the count ratio and the quantity of Se demonstrates a consistent precipitation efficiency for a large range of Se quantities.

Alternatively, a calibration constant K_{Se} may be derived from a series of standard analyses. Thereafter, K_{Se} can be used to calculate selenium concentration in unknown samples from the intensity ratio measured. An average calibration constant of $0.423 * 10^{-4}$ ng^{-1} for selenium in standards was calculated from Figure 6.1a and 6.1b. The weight of selenium in an unknown sample was calculated using the following equation:

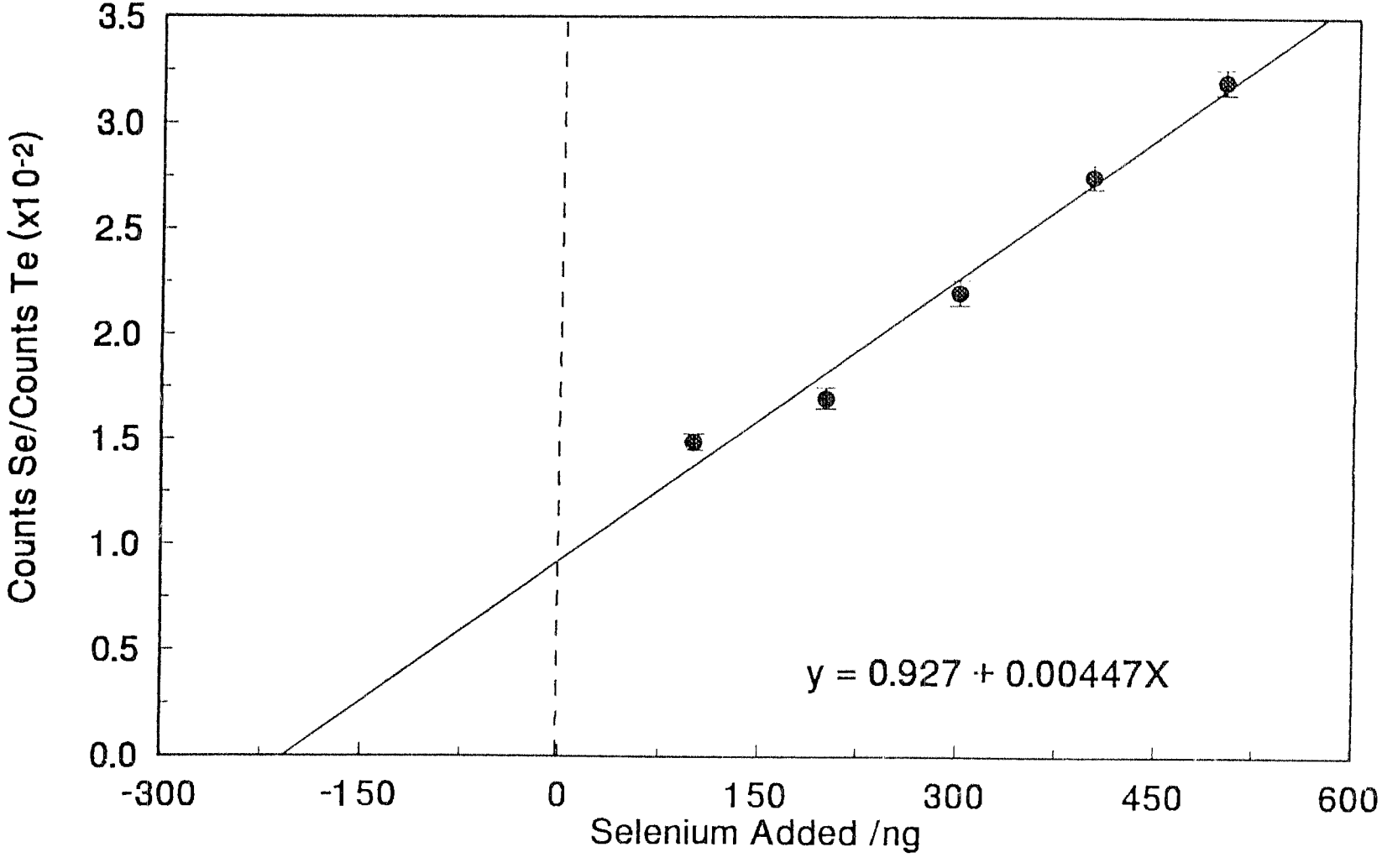
$$W_{Se} = A_N / K_{Se} \qquad (6.2)$$

where W_{Se} is the weight of selenium in the sample (µg); A_N is the normalized selenium area, and K_{Se} is the calibration constant. The concentration can be determined from the ratio W_{Se}/W_s (ppm), where W_s (g) is the weight of sample.

6.1.2 Matrix Effects Studies

Matrix effects were evaluated by standard addition. Two kinds of calibration curves were obtained (one is shown in Figure 6.2). Samples prepared from 1 mL of erythrocytes were predigested with nitric acid and spiked with 100, 200, 300, 400 and 500 ng of Se (as H_2SeO_3). The result of this analysis is shown in Figure 6.2. A quantity of 195 ng Se in 1 mL erythrocyte sample was obtained from the

Figure 6.2 Precipitation Efficiency of Filter Samples (With Organic Matrix 1 ml Erythrocytes).



standard addition method by extrapolating the curve to the X intercept. Analysis of this sample by the standard calibration curve described in section 6.1.1 yielded a quantity of 200 ng. The difference between the two procedures is only 2.5%; this is well within the experimental error (±15%). Therefore, the matrix of erythrocytes does not significantly interfere with the analysis of Se. Figure 6.3 shows another calibration curve which was obtained at the nanogram level of Se in a 100 mg matrix (Oyster tissue NBS1566). The linear relationship demonstrates that equation (6.1) is applicable to the biological samples. From Figure 6.3, the selenium content of the sample was determined (X-intercept) to be 208 ± 11 ng. This is in good agreement with the certified value 210 ± 50 ng.

It is important to note that the slope (K_{Se}) of the calibration curves for the biological sample (with matrix) is almost identical to those obtained without a matrix, within experimental error (±15%) (Table 6.1). This indicates that the matrix absorption-enhancement effects are negligible and that the calibration curve obtained without the matrix can be used for quantitative determinations of Se in biological systems.

6.2. The Lower Limit of Detectability

In the development of a new trace element analysis technique, one of the most important parameters for comparison to other established techniques is the

Figure 6.3 Precipitation Efficiency Of Filter Samples (With Organic Matrix 100 mg Oyster Tissue).

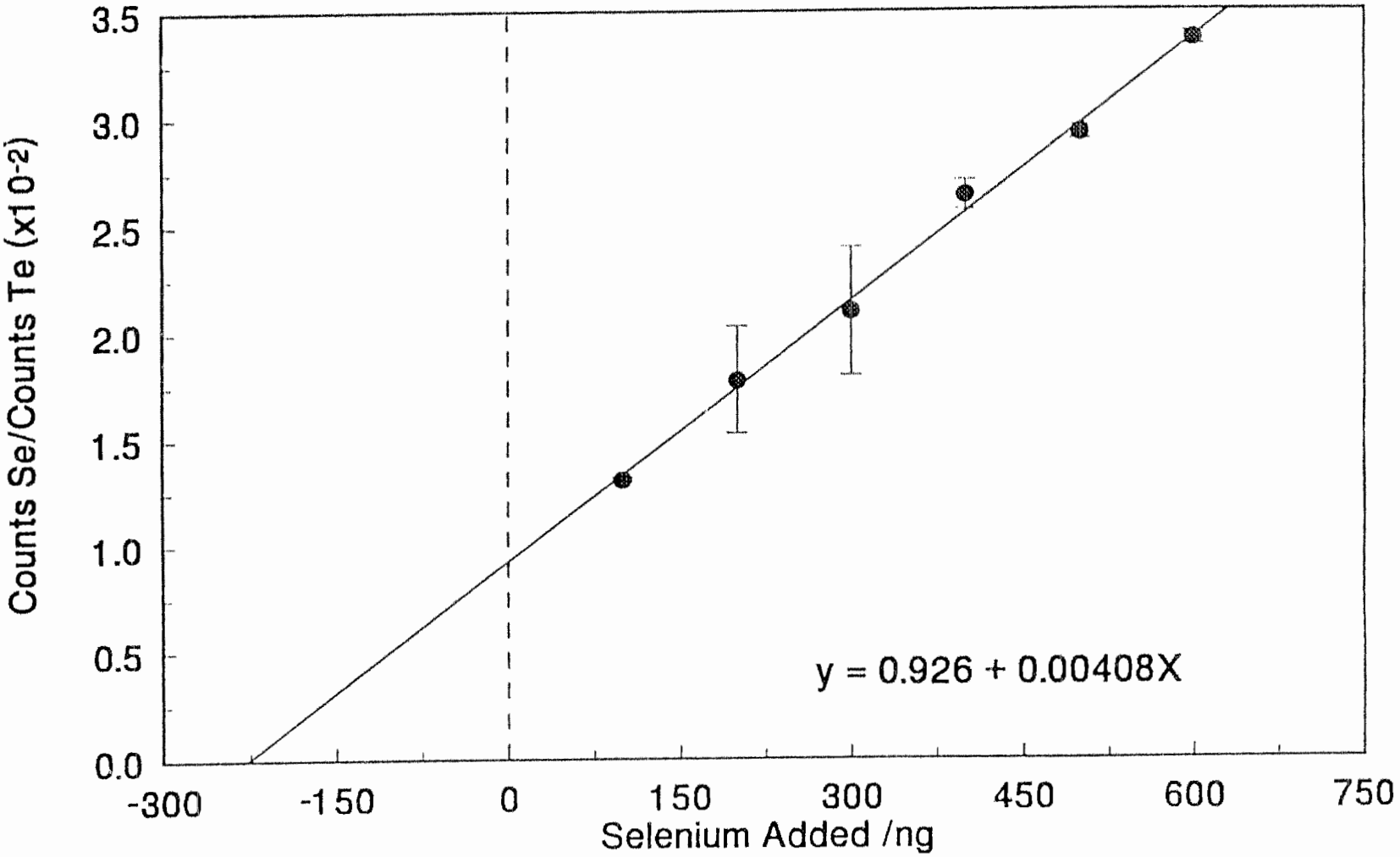


Table 6.1

Comparison of the Slopes of the Calibration Curves

Sample	Calibration curve 1 (Figure 6.1)	Calibration curve 2 (Figure 6.2)	Calibration curve 3 (Figure 6.3)
Slope	$(0.422 \pm 0.012) \times 10^{-4}$	$(0.427 \pm 0.017) \times 10^{-4}$	$(0.428 \pm 0.022) \times 10^{-4}$
Relative variance of the slope	2.8 %	3.9 %	5.1 %
Original selenium	____	(203 ± 8) ng	(210 ± 11) ng
Certified value	____	____	(210 ± 50) ng

evaluation of the lower limit of detectability (LLOD).

The LLOD can be defined in several ways. A generally accepted definition is the amount of analyze that gives a net peak intensity equal to 3 times the standard counting error of the background intensity.[60] Since the counting error is approximately the square root of the number of counts in a peak, the LLOD is simply:

$$\text{LLOD} = (3\sqrt{N_b})\ \frac{C}{N_c} \qquad (6.3)$$

where C is the amount of analyze which gives a total of N_c counts in the spectral peak; N_b is the total counts in the background below the spectral peak.

Direct determination of selenium in biological samples by X-ray emission spectroscopy is not possible due to the limited sensitivity of all modes of sample excitation. Figure 6.4 shows an X-ray spectrum obtained by direct determination of selenium in a biological sample (fish homogenate, IAEA MA-A-2 SRM). The signal for Se is almost immersed in the background due to the limited sensitivity. However, the LLOD is greatly improved for the same sample as that of Figure 6.4 under identical determination conditions after Se preconcentration (see Figure 6.5). Therefore, for selenium trace analysis, a preconcentration step preceding the determination by XRF brings selenium up to detectable levels. Clearly, Figures 6.4 and 6.5 indicate the different levels of the background originating from Rayleigh and Compton scattering; the ratio of fluorescence X-ray to background is

Fig. 6.4 X-ray Spectrum From Fish Homogenate, IAEA MA-A-2 SRM.

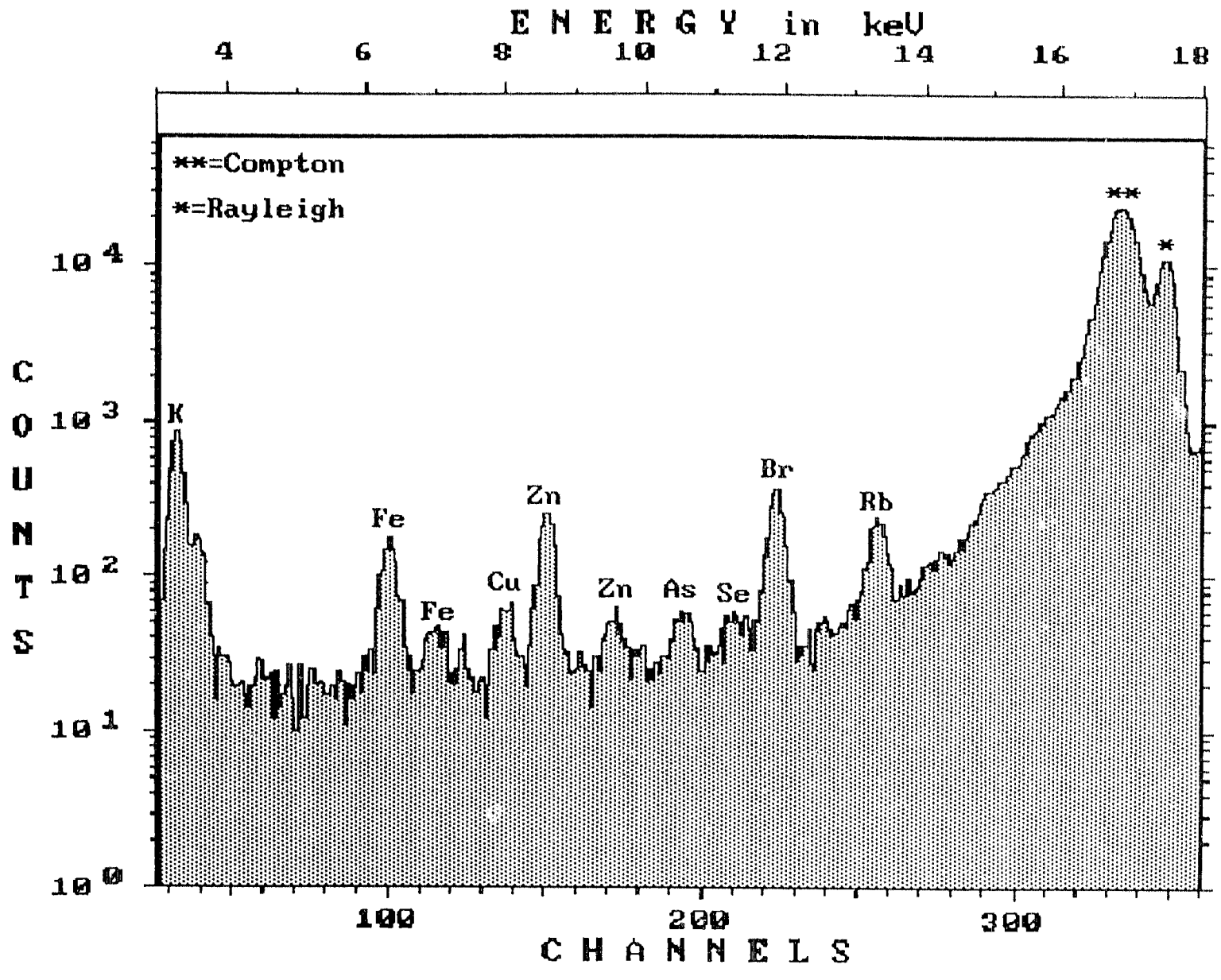
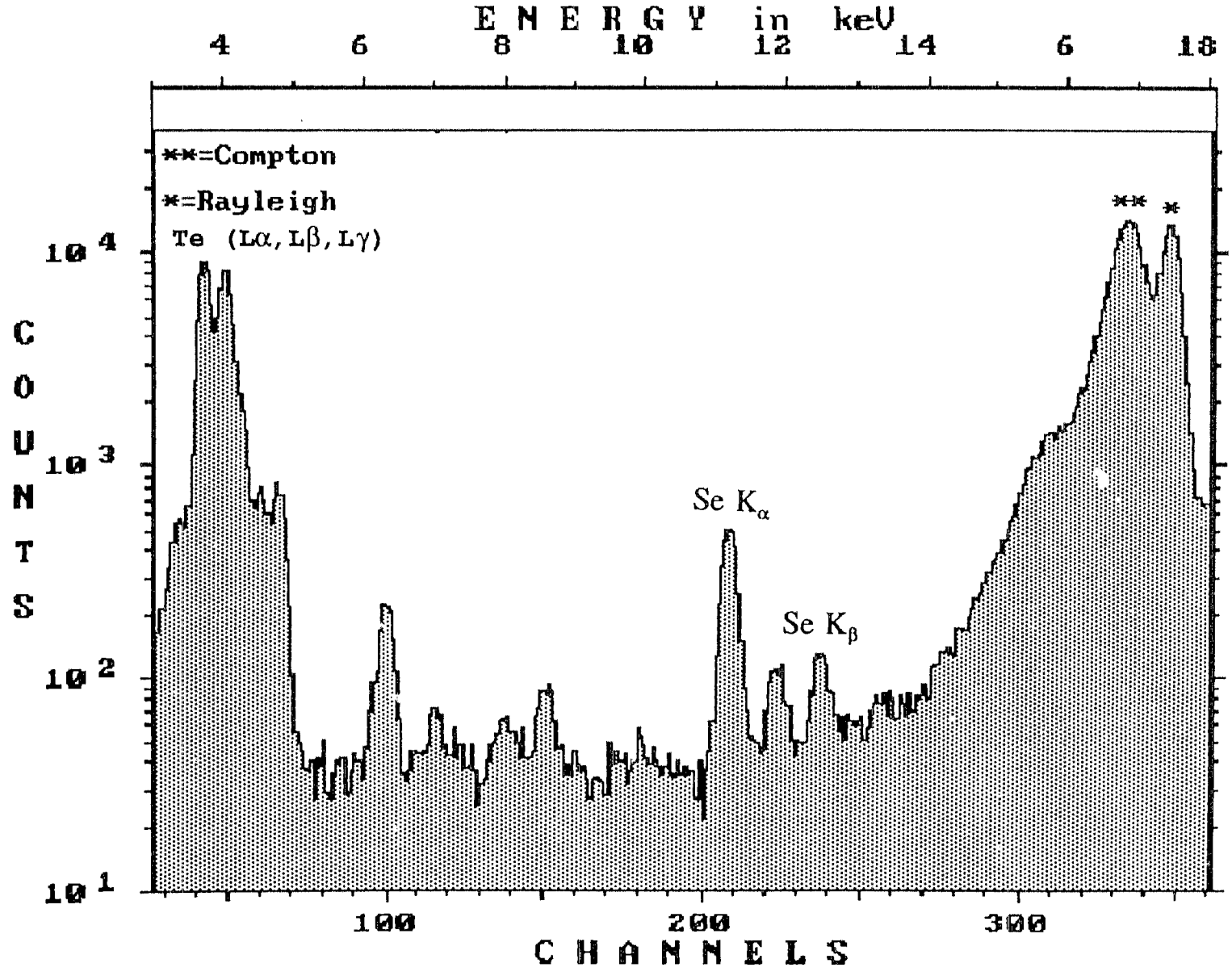


Fig. 6.5 X-ray Spectrum From Fish Homogentate, IAEA MA-A-2 SRM, After Preconcentration of Se.



increased because of the preconcentration treatment for thin samples. However, the detection limit of the technique is also highly dependent on the spectrometer and is influenced by the X-ray tube power, detector efficiency, geometry, sample volumes and count time. Table 6.2 lists the detection limit of the technique for several standard reference samples and their uncertainties. The "theoretical detection limit" in Table 6.2 is calculated as being 3 times the height of the background, near the selenium Kα peak. The lower limit of detectability (LLOD) is 5 ng absolute, corresponding to a 0.5g sample that has a Se concentration of 10 ng/g. Measurements as low as 6ppb can be determined in human erythrocytes for a 2000 second counting time. This limit can be lowered by using longer counting times or larger volumes of erythrocytes.

In general, the LLOD of the technique studied in this work is limited by the detection limit of the spectrometer. Thus, an obvious way to improve the detection limit is to decrease the XRF detection limit. There are several methods of lowering the detection limit of the XRF spectrometer. One method is to bombard the sample with a more intense beam of X-rays. The X-ray tube in the SFU XRF spectrometer is not operated above 1000 watts in order to prolong its life. However, many modern X-ray tubes are designed to operate at five to ten times this power. For a fixed counting time, both the background and the selenium peaks should be 5-10 times higher. The detection limit is proportional to the square root of the background intensity, while the selenium concentration is proportional to the

selenium X-ray intensity. Therefore, the detection limit should be improved by 2.2 to 3.2 times. Another method to lower the spectrometer detection limit is to use a larger, more efficient detector. Detectors for measuring X-rays are manufactured in a variety of sizes having a range of collection efficiencies. The detector used in this study has a surface area of only 30 mm^2, and intercepted approximately 0.23% of the X-rays (depends on source to detector distance) emitted by the sample. Modern detectors have a surface area at least ten times larger, so they can collect ten times more X-rays. A better detector should increase the size of the selenium peak by about ten times and improve the detection limit by a further 3.2 times.

Table 6.2

Detection Limit of the Technique for Several Standard Reference Samples

Sample	Sample weight (g)	Detection limit		Uncertainty, (%)
		(ppb)	(ng)	
Oyster tissue NBS 1566	0.5	77	39	2.62
Animal blood IAEA A-2	0.5	28	14	2.83
Orchard Leaves NBS 1571	1	16	16	3.18
Animal muscle IAEA H-4	0.5	10	5	1.80
Bovine lever NBS 1577a	0.5	28	14	2.38
Rice flour NBS 1568	1	18	18	2.92
Fish homogenate IAEA MA-A-2	0.5	30	15	1.36

Chapter 7 Results and Discussion: Errors in X-ray Spectrometric Analysis

7.1 Nature of Error

The error in a measurement in an analytical result is the difference between the measured value and the "true" value. However, since the true value must itself be determined by measurement, it might seem impossible to evaluate the error. Strictly speaking, this may be true, but in practice, error can be evaluated satisfactorily in terms of precision and accuracy, which, although often used synonymously, are quite different.

7.1.1 Precision

The precision of a measurement or analysis is the degree of agreement among replicate determinations made under conditions as nearly identical as possible. Quantitatively, precision P_i is the difference between the individual measurement m_i or analysis and the mean m of a large number or set of independent replicate measurements or analyses, usually expressed relative to the mean as a percent, that is,

$$P_i = [(m_i - m)/m] \times 100 \qquad (7.1)$$

Thus the greater ("better") the precision, the smaller is the numerical value of P_i. The mean m is regarded as the "best known" value, that is, the value most likely to be true. Precision can be evaluated experimentally or calculated by summation of individual contributing errors [Equation (7.1)].

To evaluate the precision of the XRF technique, replicate analyses of the same sample were performed, keeping all conditions constant. The precision of the XRF technique for twenty analyses (n=20) was determined to be 4.5% for samples containing 500 ng of Se.

7.1.2 Accuracy

The accuracy of a measurement or analysis is the degree of agreement with the "true", accepted, or most reliably known value. Quantitatively, accuracy, a_i, is the difference between the individual measurement, m_i, of analysis and the true value, t, usually expressed relative to the true value as a percent. That is,

$$a_i = [(m_i - t)/t] \times 100 \qquad (7.2)$$

Thus again, the greater ("better") the accuracy, the smaller is its numerical value.

The accuracy of the method can be established by conducting (a) the recovery studies of inorganic Se(IV) added to different biological samples and (b) determination of Se in standard reference materials. Both methods were carried out in this work to estimate the accuracy of the present technique. The absolute amount of Se recovered is not measured, but the initial concentration of Se can be

calculated from the K_{Se} and the ratio I_{Se}/I_{Te}.

The recovery of selenium added to blood samples, plasma, whole blood and erythrocytes is shown in Table 7.1. The mean recovery for all types of sample was 100.6% with a range of 93 to 108%. Table 7.2 shows recoveries of Se for 12 different types of food products (vegetables, meat, and grain). The average recovery was 97.5% (range 92-105%). These studies indicate the high accuracy of the present technique.

The results of Se determinations in standard reference materials are listed in Table 7.3. Seven kinds of NBS and IAEA standard reference materials were used. Method precision and accuracy are also presented in Table 7.3. For a variety of samples, precision ranged from 4.3 to 11.5% for Se, depending on the level of concentration in the sample and the LLOD of the instrumental method used. Accuracy of the method was estimated by comparing mean levels of analyze found, with certified values. The average recovery was 98.8% (range 92.4-101.5%). A good agreement was achieved within experimental error. Both recovery study and determinations of standard reference materials demonstrate that the method has acceptable accuracy.

7.2 Sources of Error

The errors affecting an X-ray spectrometric analysis may be classified as random and systematic. Random and systematic errors limit the precision and accuracy, respectively.

Table 7.1

Selenium Recovered from Different Types of Blood Samples

Sample	Concentration of Selenium ($\mu g \cdot L^{-1}$)				Recovery (%)
	Initial	Added	Found	Recovered	
Blood A	180	50	228	48	96
Blood B	209	50	263	54	108
Erythrocyte A	200	50	250	50	100
Plasma A	86	50	135	49	98
Blood A	180	100	288	108	108
Blood B	209	100	309	100	100
Erythrocyte A	200	100	303	103	103
Erythrocyte B	210	100	308	98	98
Plasma B	95	100	195	100	100
Blood A	180	150	320	140	93
Blood B	209	150	359	150	100
Plasma B	95	150	255	160	107
Plasma C	100	150	245	145	97

Table 7.2

Selenium Recovered from Food Samples

Sample*	Concentration of Selenium ($\mu g \cdot L^{-1}$)				Recovery (%)
	Initial	Added	Found	Recovered	
Pork kidney	0.88	0.60	1.47	0.59	98
Pork muscle	0.055	0.60	0.675	0.62	103
Chicken muscle	0.12	0.60	0.69	0.57	95
Egg white	0.045	0.60	0.595	0.55	92
Garlic	0.23	0.60	0.84	0.61	102
Onion	0.018	0.60	0.618	0.60	100
Mushroom	0.126	0.60	0.706	0.58	97
Flour (white)	0.014	0.60	00.574	0.56	93
Skim milk powder	0.21	0.60	0.84	0.63	105
Fish gills	2.10	0.60	2.64	0.54	90
Fish liver	1.73	0.60	2.29	0.56	93
Fish muscle	1.45	0.60	2.06	0.61	102

* All Samples are 1.00 g in weight.

Table 7.3

Analysis of Standard Reference Materials

Standard reference material	n	x ± S µg/g	Sr (%)	95% confid. intev.	Recomd. conc. µg/g*	Recovery %
Oyster tissue NBS 1566	6	2.10±0.09	4.3	2.01-2.19	2.1 (1.5-2.6)	100
Animal blood IAEA A-2	6	0.599±0.065	10.9	0.531-0.667	0.59 (0.45-0.73)	101.5
Orchard leaves NBS 1571	5	0.080±0.004	2.5	0.075-0.085	0.08 (0.07-0.09)	100
Animal muscle IAEA H-4	5	0.265±0.017	6.4	0.244-0.286	0.28 (0.25-0.32)	94.6
Bovine liver NBS 1577a	4	0.715±0.032	1.8	0.664-0.766	0.71 (0.64-0.78)	100.7
Rice flour NBS 1568	3	0.41±0.02	2.4	0.360-0.460	0.4 (0.35-0.45)	102.5
Fish homogenate IAEA MA-A-2	3	1.57±0.18	11.5	1.123-2.017	1.7 (1.4-2.0)	92.4

n = Number of analyses
s = Standard deviation
x = Average Se concentration
S_r = Relative standard deviation *(95% confidence interval)

7.2.1 Random errors

Random error consists of small differences in successive values of a measurement made repetitively with great care by the same competent person under conditions as nearly constant as possible. The magnitude of random errors can be evaluated, and the errors can be minimized, but not eliminated. The random errors besetting X-ray spectrometric analyses may be contributed from the following sources:

1. Statistical counting error. This error constitutes the best possible attainable precision and depends only on the total accumulated counts. The counting error always represents the maximum precision attainable in an X-ray spectrometric analysis. In X-ray emission spectography the possibility of predicting and controlling the best precision attainable as long as the analytical-line intensity is established (that is, limited) by counting.

In X-ray spectrometric analysis, intensities are given in "counts" per unit time - that is, X-ray photons per unit area per unit time (the unit area is usually the useful area of the detector). For a single measurement of N counts at the net area of peak, the counting error for accumulated counts is given by

$$\sigma_N = \sqrt{N} \tag{7.3}$$

$$\varepsilon_N = \sigma_N / N = \sqrt{N} / N = 1 / \sqrt{N} \tag{7.4}$$

where σ_N is the standard counting error and ε_N is relative fractional counting error.

Table 7.4 gives values of σ, 2σ, 3σ and $\varepsilon_{68.3\%}$, $\varepsilon_{95.4\%}$, $\varepsilon_{99.7\%}$ (the subscript of ε is the confidence level) for $N = 10^2$-10^7 counts. From Table 7.4, it is evident that standard counting error decreases as N increases, and in principle, may be made as small as required if a sufficiently long counting time is permissible. However, accumulation of a large N at low intensity requires a very long counting time; this may be disadvantageous for several reasons: the analysis time is increased, and extremely high stability is required in the X-ray tube potential and current, and in the electronic detector and readout components. The desired accumulated counts in the present study was a net peak area of 2500 counts. Standard counting error was 50 and relative fractional counting error was 2%.

2. Instrumental errors. These errors consist of short-term and long-term variation, instability, and drift in instrumental components, conditions, and parameters including the following: (a) X-ray tube potential (kV) and current (mA), (b) intensity and distribution of the primary X-ray beam (caused by changes in dimension and position of internal components of the tube), (c) coincidence (dead-time) losses in the detector and electronic circuitry, (d) shift and distortion of pulse-height distributions (only when pulse-height analysis in used), (e) electronic circuitry.

Long-term instability usually has several times the magnitude (as high as 10% in a year) of short-term instability. There are two approaches to reduction of error due to instrumental drift. One is to reduce the instability itself: efficient

Table 7.4

Standard and Relative Counting Error

Number of Counts N	σ,[a] Counts/s	$\varepsilon_{68.3}$,[b] %	2σ,[a] Counts/s	$\varepsilon_{95.4}$,[b] %	3σ,[a] Counts/s	$\varepsilon_{99.7}$,[b] %
100	10	10.0	20	20.0	30	30.0
200	14	7.1	28	14.1	42	21.2
500	22	4.5	44	9.0	66	13.4
1000	32	3.2	64	6.3	96	9.5
2000	45	2.2	90	4.5	135	6.7
5000	71	1.4	142	2.8	213	4.2
$1x10^4$	100	1.0	200	2.0	300	3.0
$2x10^4$	141	0.70	282	1.4	423	2.1
$5x10^4$	224	0.45	448	0.90	672	1.3
$1x10^5$	316	0.32	632	0.63	948	0.95
$2x10^5$	447	0.22	894	0.45	1341	0.67
$5x10^5$	707	0.14	1414	0.28	2121	0.42
$1x10^6$	1000	0.10	2000	0.20	3000	0.30
$2x10^6$	1414	0.07	2828	0.14	4242	0.21
$5x10^6$	2236	0.05	4472	0.09	6708	0.13
$1x10^7$	3162	0.03	6324	0.06	9486	0.10

[a] $\sigma = \sqrt{N}$

[b] the subscript of ε is the confidence level

line stabilization (±0.05% or less) and adequate warm up time (at least 30 min) reduce instrument instability. The other is to choose a counting strategy that reduces the effect of any remaining instability on the intensity measurement: in this study. Having reduced instability as much as possible, the operator can select a counting strategy to minimize the effects of the residual instability on the intensity measurement. These effects are minimized in "internal standardization" techniques (Chapter 6), and are substantially eliminated when the two intensities (Se, Te) are measured simultaneously.

3. Operational errors (manipulative or resetting errors). These errors consist of slight nonreproducibility in settings of instrumental conditions, principally the following: (a) X-ray tube potential (kV); (b) X-ray tube current (mA); (c) amplifier gain; (d) pulse-height analyzer baseline and window and (e) detector-tube potential.

Operational error is substantially dependent on care of the operator. It is a problem only when settings must be changed during an analysis. The error is minimized by always approaching a setting from the same direction and using an internal standard. Operational errors are essentially absent in modern semiautomatic and automatic instruments.

4. Error in estimation of concentration from the calibration curve.

The concentration of selenium in unknown samples was calculated using the equation: $W_{Se} = A_N/K_{Se}$. Calculation from four calibration curves (Figure 6.1a,

6.1b, 6.2, 6.3) of the relative variance of the calibration constant, K_{Se}, is 3.93%.

The precision of an X-ray spectrometric analysis is obtained by combining the variances for the individual sources of error, so that the precision may be represented by

$$\sigma_{T1}^2 = \sigma_N^2 + \sigma_I^2 + \sigma_O^2 + \sigma_C^2 \qquad (7.5)$$

where the subscripts represent, in order, total, counting, instrumental, operational, and calibration curve errors, respectively. For selenium analysis, the total coefficients of variation (σ_{T1}) ranged from 4.4 to 11.5%.

7.2.2 Systematic Errors

Systematic errors are those that can be avoided, or at least evaluated and corrected. They may be constant. In that case, systematic errors account for the deviation or bias of the experimental result from the true value. They may fluctuate about a mean value, which contributes to the precision. The systematic errors inherent in X-ray spectrometric analyses are contributed by the following sources:

1. Specimen errors.

These errors arise in the specimen itself. However, specimen errors do not include sampling errors, that is, errors arising from failure of the submitted sample to be representative of the bulk of the material to be analyzed. Specimen errors consist of many components such as: (a) absorption-enhancement ("matrix") effects.

(b) position effects, including variations in specimen plane, take off angle, position, orientation, and flatness (warp, ripple, wrinkle, etc.); position effects include the specimen-insertion effect, (c) physical attributes, including thickness; heterogeneity of composition; heterogeneity of density; surface texture; particle size etc.

In matrix effects studies (chapter 6), we chose Te as an "internal - standard". It compensates for absorption-enhancement effects. The relative variance of the matrix effects was 2.5%. Other kinds of specimen errors were estimated; the relative variance was about 5%.[60] Total specimen errors were estimated to be 6%.

2. Spectral-Line Interference.

Spectral-line interference, refers to what a nearby spectral line can contribute to the intensity measurement of an analyze line.

Arsenic, bromine and lead give an X-ray emission line very close to selenium. Potassium, calcium and titanium give an X-ray emission line very close to tellurium. Table 7.5[60] lists energies (KeV) of Kα, Kβ, Lα, Lβ for these elements. When these interference elements are present in large amounts in the sample, spectral line interference may constitute a major source of error. But generally, there are very low concentrations of arsenic, bromine and lead in blood and most of the food samples.[2] Titanium is absence in most biological matrices.[3] As will be discussed later, in our experimental study, reduction of Se is very selective, and most interfering species (including Ca^{2+}, K^{+}) did not precipitate along with the Se (7.3). Spectral line interference is estimated at about 3.2%.

The accuracy of an X-ray spectrometric analysis may be represented by

$$\sigma_{T2}^2 = \sigma_{S1}^2 + \sigma_{S2}^2 \qquad (7.6)$$

where the subscripts represent, in order, total, specimen and spectral-line interference errors. For selenium analysis the total coefficients of variation (σ_{T2}) ranged from 6.8 to 10%

The total relative errors of X-ray spectrometric analyses (8 to 15%) are summarized as:

$$\sigma_T^2 = \sigma_{T1}^2 + \sigma_{T2}^2 \qquad (7.7)$$

7.3 Interferences

To study interference effects in XRF measurements, two sets of samples containing 1 μg of Se and 600 μg Te each were prepared: one sample contained thirteen potential interferents (see Table 7.6) including Co^{2+}, Ni^{2+}, Cr^{3+}, Cd^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}; the other was a control. The results are listed in Table 7.6. The total excess in Se was more than 12000-fold, about 5-10 times more than that which can reasonably be expected in biological samples. The intensity ratio Se(Kα)/Te(Lα) for the sample containing the added ions was 3.2% lower relative to the control. However, this was within the experimental error of ±15%. No interferences were

Table 7.5[60]

The X-Ray Energy for Some Elements Measured in This Separation

Se	11.220 (Kα1)	12.494 (Kβ1)	----	----	----
As	10.542 (Kα1)	11.724 (Kβ1)	----	----	----
Pb	10.550 (Lα1)	12.612 (Kβ1)	----	----	----
Te	3.758 (Lα2)	3.769 (Lα1)	4.029 (Lβ1)	4.301 (Lβ2)	4.570 (Lγ1)
K	3.310 (Kα2)	3.313 (Kα1)	3.589 (Kβ1)	----	----
Ca	3.688 (Kα2)	3.691 (Kα1)	4.012 (Kβ1)	----	----
Ti	4.504 (Kα2)	4.510 (Kα1)	4.931 (Kβ1)	----	----

found in the chemical preparation step or in the X-ray fluorescence measurements. This is as expected since the reduction of Se is very selective and most interferents did not precipitate along with the Se.

Due to the simplicity of X-ray spectra, spectral-line interference is relatively infrequent. However, when it does occur, there are many ways to deal with it. First, prevention or reduction of excitation of the interfering line by operating the X-ray tube below the interferant excitation potential, selection of the X-ray tube target, or monochromatic excitation. Secondly, selection of the detector for maximum efficiency for the analyze line or minimum efficiency for the interfering line. Other methods to minimize spectra-line interference such as pulse-height selection, mathematical correction, including unfolding (stripping) of overlapped lines etc.

Table 7.6

Amount of Concomitant Elements Used in Interference Experiment

Ion	Excess over Se weight ratio to Se	Corresponding conc. in a 0.5 g sample ug/g
Co^{2+}, Ni^{2+}, Pb^{2+}, Ag^{+}, Cr^{3+}	50	100 (each)
Cd^{2+}	100	200
Mg^{2+}, Ba^{2+}	200	400
Zn^{2+}	250	5000
Fe^{3+}, Cu^{2+}, Ca^{2+}	500	1000
K^{+}, Na^{+}	5000	10000
Total	12050	24100

Chapter 8 Results and Discussion: Selenium in biological samples

8.1 Distribution of Se in Human Serum and Erythrocytes

The role of Se in human nutrition remained unclear for almost two decades after the recognition of the nutritional requirement for the element in experimental animals. It is important to assess the nutritional status of Se in resident populations on the basis of tissue concentrations of Se because some human diseases are associated with severe nutritional Se deficiency. Se concentrations are measured in human tissues most readily through biological specimens (i.e., whole blood, plasma, serum, erythrocytes, urine and hair). These results show a strong geographic variation in Se status, which correlates with the geographic variation in the Se contents of food supplies. From such information, we may be able to determine a correlation between tissue Se levels and the average Se intake of a given population.

For Se elemental Se levels in humans, blood and hair are normally taken as indicator samples.[85] Selenium determination in serum has come to be considered a better indicator than selenium determination in plasma or whole blood.[86] Se in serum is remarkably stable over a long period of time, provided that no major

changes in lifestyle occur.

In this work, selenium concentrations were measured both in serum (20 donors) and in erythrocytes (20 donors) from 25 healthy men. The results are given in Table 8.1. We have established a mean serum selenium level for healthy people at about 84 ng/mL and a mean erythrocyte selenium level of 200 ng/mL. They are in agreement with the literature value (for serum, erythrocytes 82ng/mL[87,88] and 200 ng/mL,[89,90] respectively). Figures 8.1 and 8.2 show the distribution of the concentrations of selenium in serum and erythrocytes respectively. As for the distribution of the concentrations of selenium in serum samples, about 30% of healthy people have Se concentrations between 70 and 90 ng/mL; for the distribution of the Se concentration in human erythrocyte samples, about 56% of healthy people have Se concentrations between 190-210 ng/mL.

8.2 Routine Analysis of A Large Number of Food Samples

Because of the importance of selenium as an essential trace element for human and animal nutrition alike, accurate analyses of Se in biological materials and food samples are necessary. Additionally, there is also the need to define more precisely the physiological role of Se and the dietary intake in Canada. The analytical procedure employed in this work by XRF combined with chemical preconcentration, gave reliable and accurate results. The reliability and accuracy

were validated by those results obtained for NBS and IAEA standard materials. In addition, this method is rapid since the analysis of 10 to 40 samples requires 1 day of chemical preparation and 1 day of photon irradiation. The present work shows that it may also be employed for the precise measurement of Se levels down to the order of nanograms per gram of food sample. The above described method was used for the determination of selenium in different kinds of food samples. The results of the analyses are presented in Table 8.2.

8.3 Verification of Results

The XRF method employed in this work has been verified by an independent analytical method. For the determination of selenium in biological systems at the trace to ultratrace levels (which are often encountered), only a few methods (as discussed in chapter 2) provide the necessary reproducibility and sensitivity required. Among electrochemical techniques, differential pulse cathodic stripping voltammetry (DPCSV) is an inexpensive, accurate and convenient approach. It was therefore chosen as the independent technique for comparison with the XRF method employed in this work.

8.3.1 DPCSV Calibration Curve, Detection Limit and Interference

Figure 8.3 shows a series of differential pulse polaragrams for the reduction of Se at different concentrations. Sharp current peaks at -0.58 V (distinguishable from the background) are depicted. The peak currents increase with the increase in Se

concentration. A calibration curve as shown in Figure 8.4, is obtained so that the analysis could be carried out under the same conditions as for the erythrocyte samples. The linear working range of detection for selenium is 0.1-12 ppb and the detection limit is 0.1 ppb for erythrocyte samples. The concentrations of Se in unknown erythrocyte samples were calculated from this calibration curve.

It has been noticed that the sample digestion is a crucial step in the determination of Se in biological samples. In order to confirm the absence of Se loss during the digestion procedure, a recovery experiment was carried out. A known amount of Se was added to ten erythrocyte samples and the Se was digested under the identical conditions as the actual samples. The remaining Se was then measured. The results are shown in Table 8.3. An average recovery of 95.2% was obtained, indicating the Se loss during the digestion procedure was minimum.

The precision and accuracy of the DPCSV method was further confirmed by the determination of Se in six types of NBS and IAEA standard reference materials. The results are shown in Table 8.4. The precision of the method ranged from 5 to 20% for Se. Accuracy of the method was estimated by comparing mean levels of analyze found with the certified values. The average recovery was 96%.

Elements such as Cu(II), Cr(III), Cd(II) and Pb(II) produce interference. For example, Cu(II) may form an intermetallic compound with selenium as suggested by reaction[64]

$$H_2SeO_3 + 4H^+ + Cu^{2+} + Hg + 6e^- \rightleftharpoons Cu(Hg)Se + 3H_2O \qquad (8.1)$$

The formation of such compounds could account for a negative shift of the peak potential (E_p) in addition to modification of the peak height (i_p). No significant interference to the analysis was found at the concentration of the interfering elements up to 12 ppm.

8.3.2 Analysis of erythrocyte samples

Selenium concentrations in human erythrocytes which were used as samples in the XRF analysis were determined using the DPCSV method. The results from both DPCSV and XRF are illustrated in Table 8.5. The average concentration of Se in human erythrocytes as determined by DPCSV is 199 ppb. This result confirms the results of the XRF analysis (200 ppb). They are in excellent agreement within experimental error. The XRF method is verified by the electrochemical technique.

An attempt was made to find the form in which Se exists. The erythrocyte sample was extracted with cyclohexane from aqueous solution. The Se in organic form was extracted using the hexane, and the inorganic form remained in the aqueous solution. Table 8.6 shows the results of the concentrations of Se in organic and inorganic solvents after extraction from the erythrocyte sample. This result shows that half of total Se exists in the inorganic form in human erythrocytes.

8.4 Comparison

Both XRF and DPCSV have been shown to be adequate techniques for the study

of selenium concentration levels in biological materials. However, there are some differences between the two methods. The three primary differences are:

First (in comparison with DPCSV technique), the new XRF method is less sensitive, but more accurate with much better precision.

Second, the organic matrix effects are negligible and a single calibration curve can be constructed without an organic matrix and used to determine selenium in a wide range of biological samples for the XRF method. However, in DPCSV determinations, organic matrix effects are not negligible. For different organic matrices, different calibration curves must be made.

Finally, the linear working range for selenium is 0.1-12 ppb for the DPCSV technique but this range can be extended to 210 μg on the filter in XRF determination. In addition, the great reliability and the relative simplicity make the XRF technique a competitive technique among currently used methods for the determination of selenium.

Table 8.1

Selenium Concentrations in Human Serum and Erythrocytes as Determined by XRF

Sample	Se concentration in serum (ng/mL)	Se concentration in erythrocytes (ng/mL)
1	30±4	140±21
2	67±10	190±28
3	109±16	227±34
4	102±15	223±30
5	56±8	167±25
6	89±13	208±30
7	71±10	202±30
8	64±9	197±29
9	77±11	206±30
10	114±17	232±34
11	84±12	207±30
12	120±18	263±39
13	49±7	144±20
14	60±9	189±28
15	96±14	210±30
16	71±10	203±30
17	91±13	209±31
18	76±11	200±30
19	58±8	179±26
20	82±12	204±30
21	109±16	-
22	71±10	-
23	138±20	-
24	101±15	-
25	133±19	-
26	96±14	-
27	40±6	-
Average	84	200

Table 8.2

Selenium Content in Food Samples as Determined by XRF

Food samples	Se content (ug/g)	Reference (ug/g)	Reference number
Pork kidney	0.880±0.132	0.20-0.96	19
Pork muscle	0.055±0.008	0.06	19
Chicken muscle	0.120±0.018	0.10	20
Egg white	0.045±0.008	0.04-0.06	21
Garlic	0.230±0.035	0.25	21
Onion	0.018±0.003	0.02	19
Mushrooms	0.126±0.019	0.13	21
Flour (white)	0.014±0.002	0.01	19
Skim milk powder	0.210±0.032	0.10-0.25	20
Fish gills	2.100±0.315	2.1±0.6	91
Fish liver	1.730±0.259	1.9±0.5	91
Fish muscle	1.450±0.218	1.6±0.5	91

Table 8.3

Recovery Efficiency of Acid Digestion Method for Selenium

Sample number	Se(IV) added (ng)	Se(IV) found* (ng)	Recovery (%)
Erythrocytes #1	51.5	51.0±2.0	99
Erythrocytes #2	51.5	47.5±1.9	92
Erythrocytes #3	51.5	53.0±2.1	103
Erythrocytes #4	51.5	46.0±1.8	89
Erythrocytes #5	51.5	50.0±2.1	97
Erythrocytes #6	51.5	52.5±2.1	102
Erythrocytes #7	51.5	48.9±1.9	95
Erythrocytes #8	51.5	46.9±1.9	91
Erythrocytes #9	51.5	46.4±1.9	90
Erythrocytes #10	51.5	48.4±2.0	94

*Errors are standard Deviation

Table 8.4

Concentration of Se Found in Selected Biological Standard Reference Materials

Ref. material	Number of detns	DPCSV Value (ppm)[a]	Certified value (ppm)[a]	Recovery (%)
Animal blood IAEA A-2	4	0.576±0.115	0.59±0.14	97.6
Animal muscle IAEA H-4	3	0.259±0.018	0.28±0.03	92.5
Fish homogenate IAEA MA-A-2	3	1.650±0.149	1.7±0.3	97.1
Orchard leaves NBS 1571	3	0.078±0.005	0.08±0.01	97.5
Rice flour NBS 1568	3	0.385±0.019	0.4±0.05	96.3
Bovine liver NBS 1577a	4	0.679±0.054	0.71±0.07	95.6

[a] Errors are Standard Deviation

Table 8.5

Concentration of Se in Human Erythrocytes
as Determined by XRF and DPCSV

Sample	XRF (ng/mL)	DPCSV (ng/mL)
1	140±21	145±29
2	190±28	191±38
3	227±34	228±45
4	223±30	221±44
5	167±25	170±34
6	208±30	207±40
7	202±30	204±40
8	197±29	196±39
9	206±30	207±41
10	232±34	227±45
11	207±30	207±41
12	263±39	255±51
13	144±20	148±29
14	189±28	175±35
15	210±30	211±42
16	203±30	204±40
17	209±31	202±40
18	200±30	199±39
19	179±26	186±37
20	204±30	205±41
Average	200	199

Table 8.6

Concentration of Se in Organic and Inorganic Solvents after Extraction from Erythrocyte Samples

Samples	Organic (ppb)	Inorganic (ppb)	Total (ppb)
No. 1	110	95	205
No. 2	112	95	207
No. 3	113	93	206
Average	112±2	94±1	206±2
Percentage	54%	45.6%	100%

Figure 8.1 Distribution of Se Concentration in Human Serum.

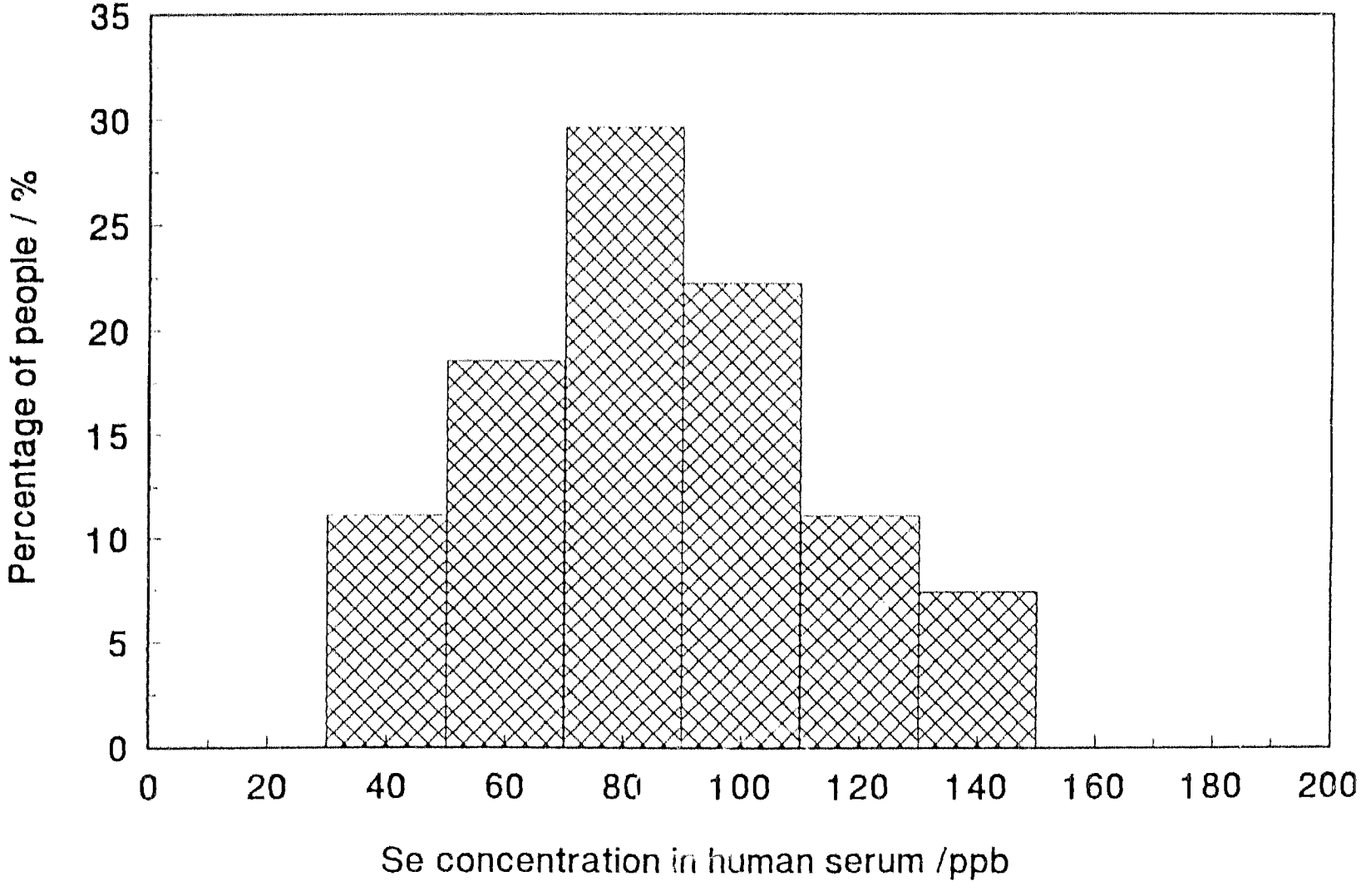
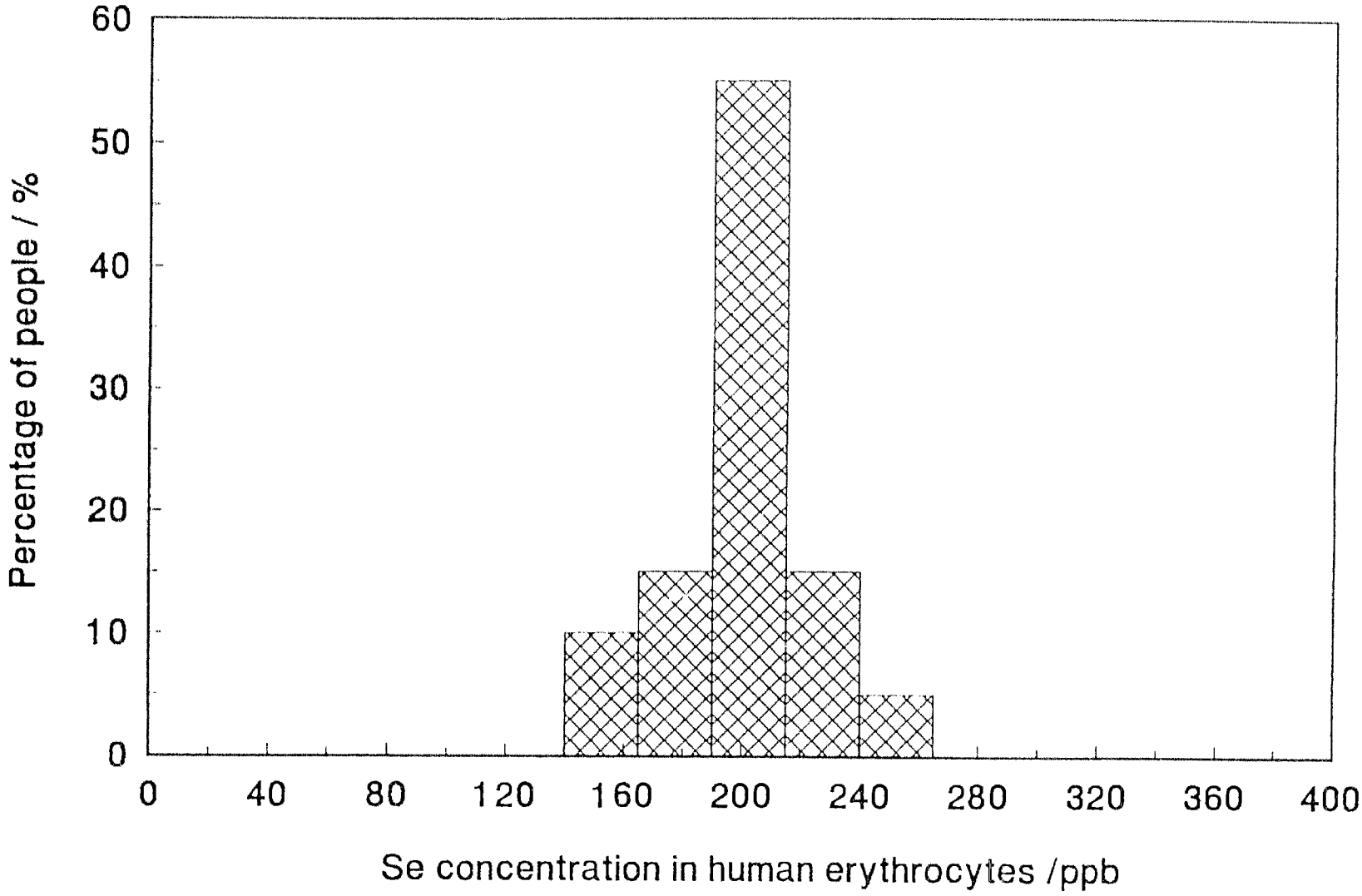


Figure 8.2 Distribution of Se Concentration in Human Erythrocytes.



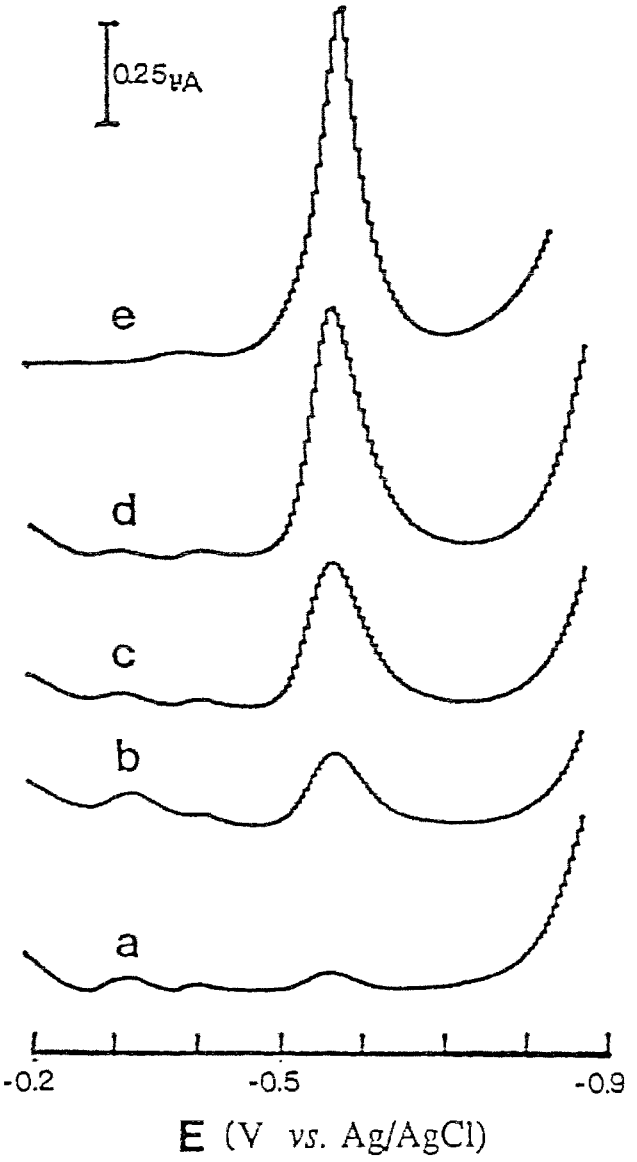
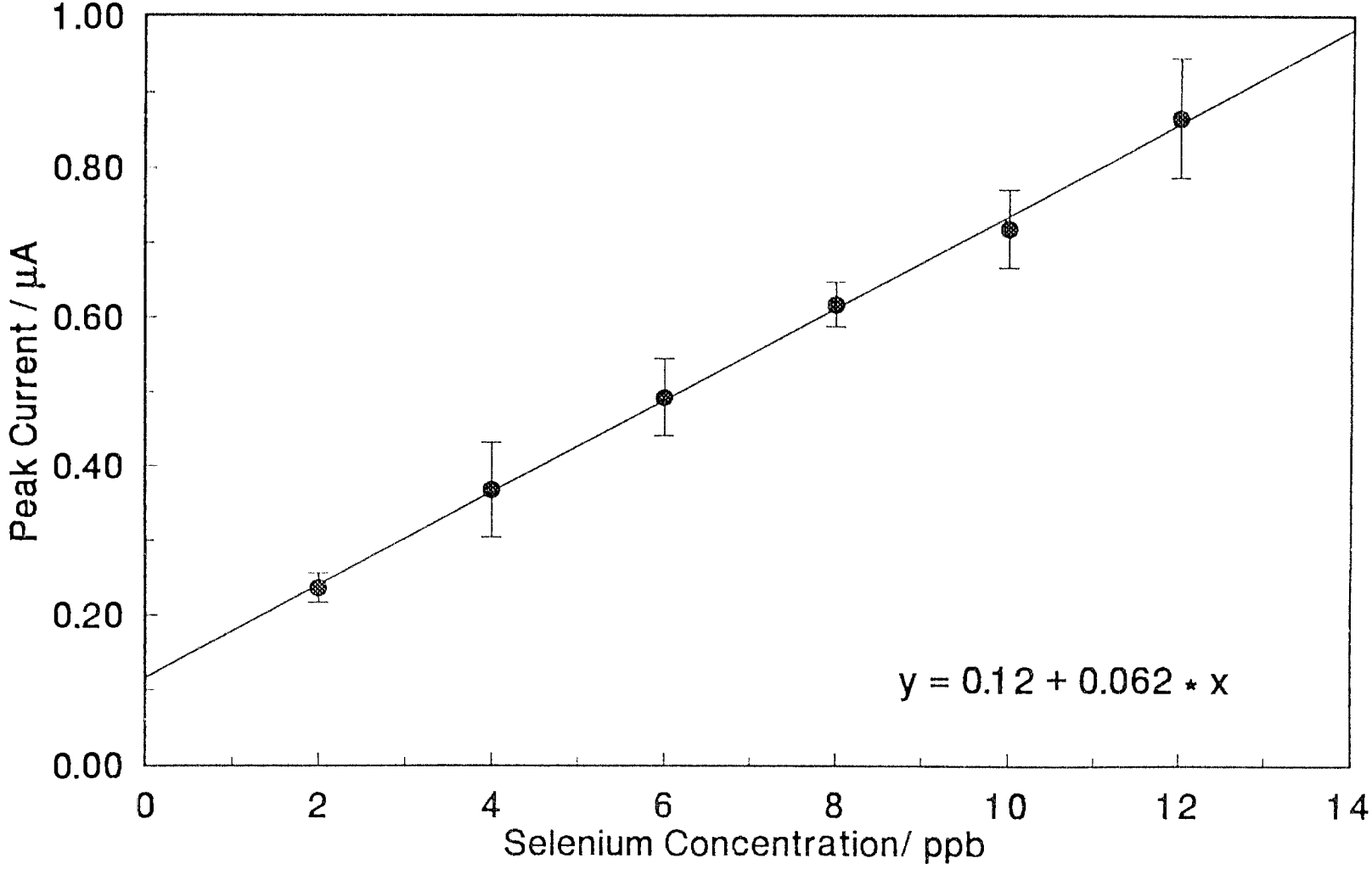


Figure 8.3

Relationship between Se Content and Peak Current: (a) Blank and (b), (c), (d), (e) Are Peak Currents for Se Concentrations of 2, 4, 6, 8, ng ml^{-1}, Respectively. Deposition time 120 s and Deposition Potential -0.2 V vs. Ag/AgCl.

Figure 8.4 Calibration Curve for Selenium Obtained by Differential Pulse Cathodic Stripping Voltammetry.



Chapter 9 Conclusions

9.1 Conclusions

It has been shown that accurate concentrations of selenium in biological samples can be obtained by XRF analysis when coupled with an effective preconcentration technique as developed in this study. The experimental approach was verified using NBS and IAEA standard materials; no significant deviations from the certified values could be detected. Random errors (range 4.3 to 11.5%) and systematic errors (range 6.8 to 10%) were determined.

Experimental evidence has shown that no potential interferents were found in the XRF analysis. This is as expected since the reduction of Se is very selective and most interferents did not precipitate along with the Se. The concentration of Se in human erythrocytes were measured and the results were confirmed by an independent electrochemical method. In comparison with the DPCSV technique, the new method is less sensitive, but more accurate with much better precision. An attempt was made to apply this method to the routine analysis of biological samples.

An effective preconcentration technique is described that allows for a determination of selenium at the ppb level in biological samples. A detection limit for the XRF approach was determined to be 5 ng absolute, corresponding to 10 ng/g for a 0.5 g sample and the linear range extends up through 210 μg Se. The approach involves first the

decomposition of the organic matrix and then the precipitation of selenium using tellurium both as the coprecipitant and as the internal standard, to obtain thin specimens (< 1mg/cm^2).

The wet digestion procedure and the subsequent reduction of selenium to its elemental form with tellurium as coprecipitant have been optimized. The use of perchloric acid under certain conditions, and sulphuric acid in conjunction with nitric acid, leads to an excellent recovery of the selenium in biological materials. The average recovery of selenium obtained with the use of optimized analytical procedure was about 98.8%.

In summary, the XRF approach used in this work presents the following advantages:

(1) The X-ray intensity of a given element is a linear function of mass per unit area of the element because a thin target is used.

(2) Matrix adsorption-enhancement effects are negligible and, therefore, a single calibration curve can be constructed without an organic matrix and be used to determine selenium in a wide range of biological samples.

(3) The ratio of fluorescence X-ray to background is increased because a thin target is used.

(4) Direct determination of selenium in biological sample is possible, through the use of a preconcentration procedure to bring selenium concentrations up to detectable levels.

(5) The need for complex analytical chemistry skills is eliminated.

9.2 Suggestions For Future Work

Knowledge of the speciation of environmentally and biomedically relevant elements, i.e., their distribution in different chemical forms, is important because bio-availability and toxicity both depend critically on the chemical form (since different forms have different assimilability).[1] Generally, the free (hydrated) metal ion is the form most toxic to aquatic life. Strongly complexed metal, or metal associated with colloidal particles, is much less toxic.[2] For speciation determination at ultratrace concentration levels, very sensitive analysis is required and the whole analytical procedure must be designed to keep contamination to an absolute minimum. Also, any preliminary separation step and the analytical measurement must, wherever possible, avoid altering the equilibria between the various chemical species in the sample.

For multiple-oxidation-state elements such as selenium, the existence of different chemical forms necessitates further analysis of particulate associations. Selenium commonly exists in four formal oxidation states: -II, 0, IV, and VI. In natural waters the principle dissolved selenium species are Se(IV) and Se(VI), which exist as selenite and selenate ions, respectively.[3,4] Within particulate material, Se may be found in any of four oxidation states. Since the biological uptake and toxicity of selenium are controlled by its chemical form,[5,6] an evaluation of this chemical speciation in particulate matter is needed. Furthermore, many processes that affect the selenium cycle in natural waters can be elucidated with particulate chemical speciation data. Future research can quantitatively reveal the chemical speciation of selenium in particulate materials.

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