Isotopic Studies of Selenium in Environmental Samples.

by

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ABSTRACT

Selenium has aroused much interest in the last decade. It is an essential trace element for man and animals and has been found to be the cofactor in two enzyme systems. The first, glutathionine peroxidase, acts as an anti-oxidant by destroying peroxides which attack cellular membranes. The second, iodothyronine 5'-deiodinase, converts thyroxine to triiodothyronine with the release of iodine. Selenium is obtained in the diet from plant and animal products and the amount present is dependent largely on the Se content and the chemical species present in local soils. Soils low in Se can lead to deficiency problems to the grazing ruminants, and have necessitated the use of Se fertilisers or direct Se injection into the livestock. The disposal of sewage sludge onto agricultural land will increase as new EC legislation comes into force in 1998. This may alleviate Se deficiency in soils or result in toxicity problems. The aims of this project were firstly to develop and validate a method to accurately measure SE in plants, soils and sewage sludges, examine which soil fractions Se was associated with and finally to study the uptake of Se by plants grown on sewage sludge amended soils.

The Se concentration in samples was determined using isotope dilution - mass spectrometry by gas chromatography - mass spectrometry (GC-MS). This was the first application of the determination of Se in environmental samples and the use of a benchtop GC-MS for those analyses. Samples were spiked with ^{76}Se isotope solution. Plants were digested using nitric acid and hydrogen peroxide initially on a heating block and latterly by microwave oven digestion techniques. Soils and sewage sludges were digested using nitric acid and hydrofluoric acid. Selenium in the digests was reduced to Se(IV) and derivatised to 5'-nitropiazselenol. Validation of the methodology was achieved by the use of certified reference materials which gave results within the certified range with a low standard deviation. The Se content of four different freely drained acid Scottish soils under grasslands was in the range 0.5-0.8 $\mu g\ g^{-1}$ air dried soil and three sewage sludge samples contained between 1.1 and 3.5 $\mu g\ g^{-1}$ dry matter.

The ability of the digestion techniques to release organically bound Se as selenomethionine and selenocysteine was found to be around 100% efficient and the use of a microwave oven increased the number of samples which could be processed.

A sequential extraction procedure was used to determine the soil fractions with which Se was associated. The four soils and the sewage sludge examined were all found to have less than 5% of the total Se available for plant uptake.

The uptake of Se by plants grown in soils to which Se had been added as sodium selenate or sodium selenate was studied using both stable and radio isotopes of Se. Results obtained showed that the uptake of Se by plants grown in soils treated with selenite could be related to the soil characteristics, with ferric oxide levels, clay levels and organic matter content being the important factors. The uptake of Se by plants in the soils treated with selenate were less obviously related to soil properties but pH and P levels both appear to be important factors. Good reproducibility was obtained in all cases. Plants grown on sewage sludge amended soils were not found to contain measurable amounts of Se.

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Chapter 1. Introduction

1.1. Background to selenium.

Selenium (Se) was named after the moon goddess Selene by Jons Jacob Berzelius who discovered the element in 1817, in deposits from the lead chambers of the sulphuric acid plant at Gripsholm in Sweden. It has been called the element with the schizophrenic personality (Gissel-Nielsen *et al.*, 1984) due to its ambiguous and versatile behaviour. It is in group VI of the periodic table and has an atomic mass of 78.96 daltons.

There are six stable Se isotopes with masses of 74, 76, 77, 78, 80 and 82 amu. Radioisotopes of Se do not occur naturally but can be generated by neutron activation (eg ^{75}Se and ^{77}Se). Selenium can exist in four oxidation states (-2, 0, +4 and +6). The electrochemical properties of Se have led to its use today in photoelectric cells, photocopiers, light meters and rectifiers. It is also used in the pharmaceutical industry as Se sulphide in anti-dandruff shampoos (Wilber, 1983).

1.2. Geochemistry of selenium.

Selenium is present in the earth's crust in varying amounts depending on the parent rock. Igneous and granite rock generally contain small amounts of Se (<0.2 mg kg^{-1}) with greater amounts occurring in sedimentary rock. Volcanic ashes, tuffs and black shales and are usually rich in Se. This wide variation of Se content along with the amount of weathering which has taken place has resulted in the formation of soils with Se content ranging from very low to toxic (0.03-2.0 mg kg^{-1}). Most natural waters tend to have a low Se concentration (1-10 μg dm^{-3}) (Berrow and Ure, 1989).

1.3. Atmospheric Deposition.

Nriagu and Pacyna (1988) reported that the main source of atmospheric Se comes from fly ashes generated by the combustion of coal and industrial waste of commercial products such as those from the electronics industry. Selenium also enters the atmosphere through volcanic activity. These sources contribute quantities of Se to the soil as do volatile selenide species (Section 1.4).

1.4. Selenium in soil.

The species of Se present in the soil depends mainly on the soil pH and redox conditions (Elrashidi *et al*, 1987) as shown in Figure 1.1. Other influencing factors include the solubility of the Se salts, complexing ability of the soluble and solid ligands, biological interactions and reaction kinetics (McNeal and Balistrieri, 1989). The inorganic species of Se are:-

Selenide (Se^{2-}) which exists in reducing, anaerobic environments as H_2Se and metal selenides which tend to be very insoluble.

Elemental selenium (Se^0) which is stable in reducing environments and insoluble in water.

Selenite (SeO_3^{2-}) which is favoured under mildly oxidising, aerobic conditions in acidic soils. Most selenite salts are less soluble than the corresponding selenate salt and have strong affinities for sorption by iron oxides (Balistrieri and Chao, 1987).

Selenate (SeO_4^{2-}) which is stable in well oxidised aerobic environments in alkaline soils. Selenate salts are very soluble and not strongly adsorbed by soil constituents

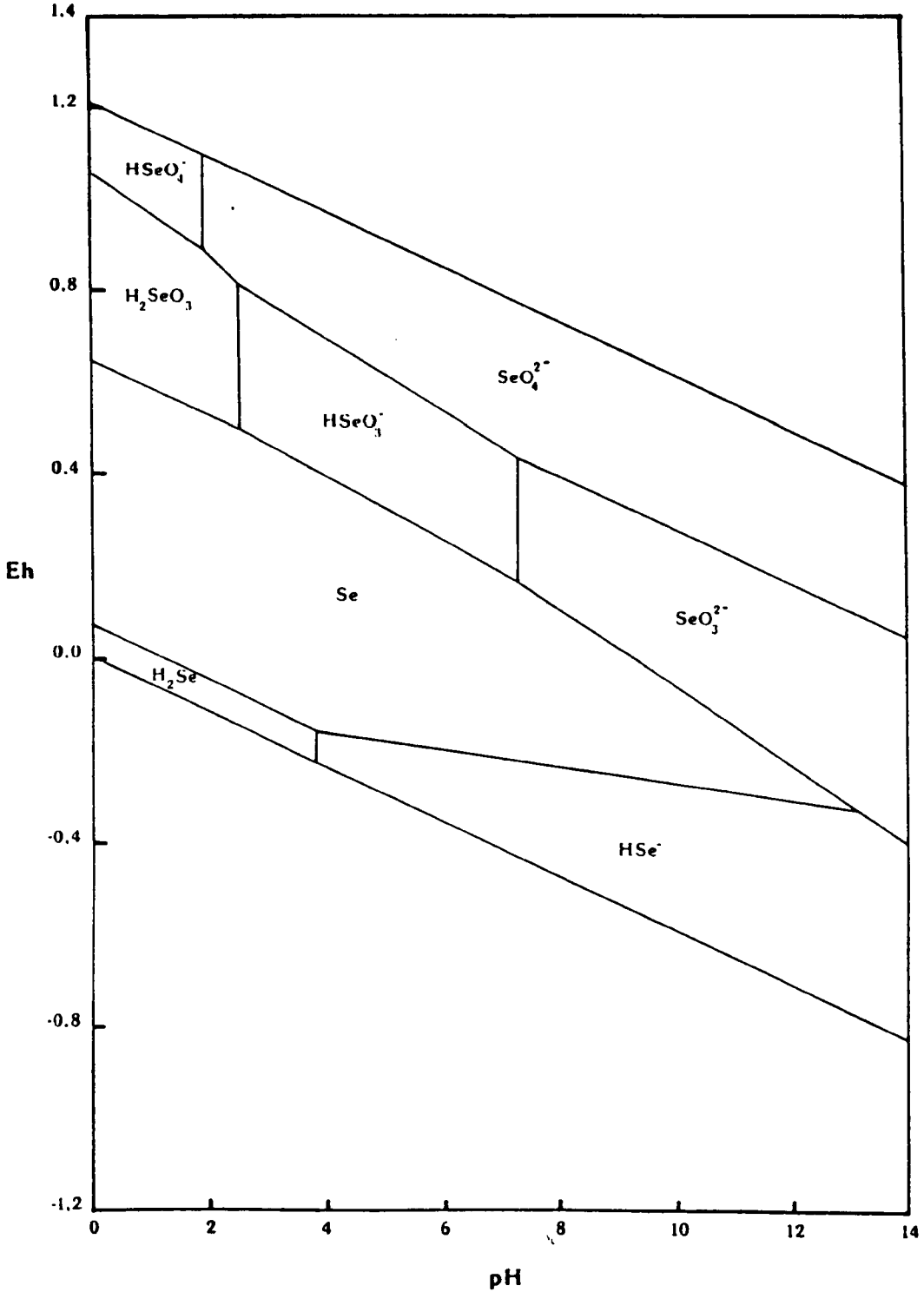


Figure 1.1 Redox potential diagram for Se-H_2O system (from Neal, 1990).

(Merrill *et al*, 1986). This is the most bioavailable form of Se, being readily taken up by plants (Lauchli, 1993). Organic Se species in the soil are predominantly selenomethionine and selenocysteine following the conversion of inorganic Se by microorganisms (Ducros *et al*, 1994; Lauchli, 1993).

The composition of the soil greatly influences the chemical form of Se. Ferric oxides and clays have a strong retention for selenite (Balistrieri and Chao, 1987). On the other hand the presence of manganese oxides do not appear to decrease selenite availability (Balistrieri and Chao, 1990). Organic matter has an even greater binding capacity for selenite than clay minerals. In this case Se is bound in the form of organic complexes or amino acids and proteins, commonly as selenomethionine and selenocysteine (Cary *et al*, 1967). Soil pH influences the form of Se and hence the solubility. Also as pH increases, the adsorption of selenite by clays and mineral oxides decreases (Hamdy and Gissel-Nielsen, 1977). Microbes can absorb available Se and bind it within organic constituents and are also capable of transforming strongly adsorbed selenite to soluble organic Se compounds and to selenate (Shrift, 1967). Microbial activity can produce volatile organic Se compounds, such as dimethylselenide (DMSe) and dimethyldiselenide (DMDSe), that may be lost from the soil system (Francis *et al*, 1974).

Selenium toxic soils are found in California, Wyoming and South Dakota (U.S.A.), Alberta (Canada), Colombia, Venezuela, Huleh Valley (Israel) and Hubei Province (China) (Wilber, 1983, Kubota *et al*, 1967, Winter and Gupta, 1979). These seleniferous soils are found in well-drained, sub-humid areas where selenate and organic Se compounds are the predominant species. One of the best documented cases of Se toxicity is in the San Joaquin valley in California. These Se rich soils have led to water high in Se being carried to the Kesterson Reservoir where it has caused toxicity problems for wildlife, especially birds, and high incidences of embryonic mortality and deformity as well as adult mortality have been recorded (Kilness and Simmons, 1986). Soils deficient in Se are found in many parts of the world including New Zealand, parts of Australia, Scandinavia,

Scotland and a diagonal zone stretching from NE to SW China (Reuter, 1975 ; Wilber, 1983).

1.5. Selenium in plants.

All plants are capable of taking up Se, but plants have been divided into three general groups according to their ability to accumulate Se. Primary Se accumulator plants accumulate large concentrations of Se (up to 4000 mg Se kg^{-1} dry weight). Members of this group include some *Astragalus* and some *Neptunia* species and occur only on seleniferous soils (Marschner, 1986). Secondary accumulator plants accumulate up to a few hundred mg Se kg^{-1} (dry weight) and include members of *Aster*, *Catelleja* and *Grindelia* species. Non-accumulator plants contain around 1 mg Se kg^{-1} dry weight (Lauchli, 1993) even when grown on Se rich soils. Members of the *Cruciferae* family (such as the radish, turnip and cabbage) can concentrate relatively high levels of Se if grown on high Se soils (James *et al*, 1989). These plants have a high sulphur requirement and, since Se and S have similar chemistry, it is proposed that the plant mistakenly takes up Se leading to Se accumulation (Hurd-Karrer, 1935). Since Se is relatively rare, the plants do not appear to have evolved a system to exclude its uptake.

The presence of other ions can influence Se uptake by plants. Sulphate does not affect selenite adsorption but will compete with selenate adsorption (Mikkelson *et al.*, 1988). Phosphate readily displaces selenate ions from absorption sites and competes with selenite for inner surface complexation sites on soil surfaces (Singh and Malholtra, 1976). The addition of phosphate to soil has produced mixed results on Se uptake. Singh and Malholtra (1976) reported the addition of PO_4^{3-} increased Se uptake for *Trifolium alexandra* while Singh (1979) reported a reduction in Se uptake for *Brassica juncea cos*. Levesque (1974) found the addition of PO_4^{3-} decreased Se in lettuce but had no effect on corn. The addition of lime to increases the Se taken up by plants (Gupta *et al*,

1982). This could be because as the pH rises, selenite is replaced by hydroxyl ions on adsorption sites. Although Se is an essential trace element in animals and man (see Section 1.8), its requirement by higher plants has been debated for many years without a conclusion being reached. It should be noted however that the *Astragalus* genus is endemic on seleniferous soils, but these are also normally rich in phosphates and it has not been established whether it is P or Se is the controlling factor (Ernst, 1982). Volatile Se compounds can be lost from plants. Higher plants have been found to emit both DMSe and DMDSe (Lewis, 1976) and can also reabsorb DMSe through their leaves (Zieve and Patterson, 1984). The uptake up of Se by plants is the first step of Se entering the food chain and is an important determinant of the Se status of livestock. A schematic diagram of the Se cycle is shown in Figure 1.2.

Selenium deficiency in soils can cause major problems to livestock production (Section 1.11) but can be rectified by several methods. The direct application of sodium selenate and sodium selenite in solution or mixed with fertiliser has been described by Cary and Allaway (1973) and Cary and Gissel-Nielsen (1973). This method of application has a 3-4 year residual effect and needs to be applied yearly. A study carried out in Finland looked at the effect of Se supplementation to fertilisers on human Se status (Aro *et al*, 1995). Since 1984, Se as sodium selenate has been added to fertilisers at 16 mg kg^{-1} and 6 mg kg^{-1} for cereal and fodder crops, respectively. This was found to raise human Se serum levels by 70% throughout Finland. The amount of Se in these fertilisers was reduced to 6 mg kg^{-1} in 1990 and at this level human Se serum levels remained at double the level prior to supplementation throughout the whole of Finland. This method has also eliminated the need to supplement animal feeds with inorganic Se and was found to increase the Se levels in meat, eggs and milk. Foliar spraying uses much smaller quantities than for soil applications to achieve similar plant concentrations. This could be due to a lesser likelihood of soil fixation, leaching or runoff. Soil amendment has also been achieved by applying Se rich coal fly

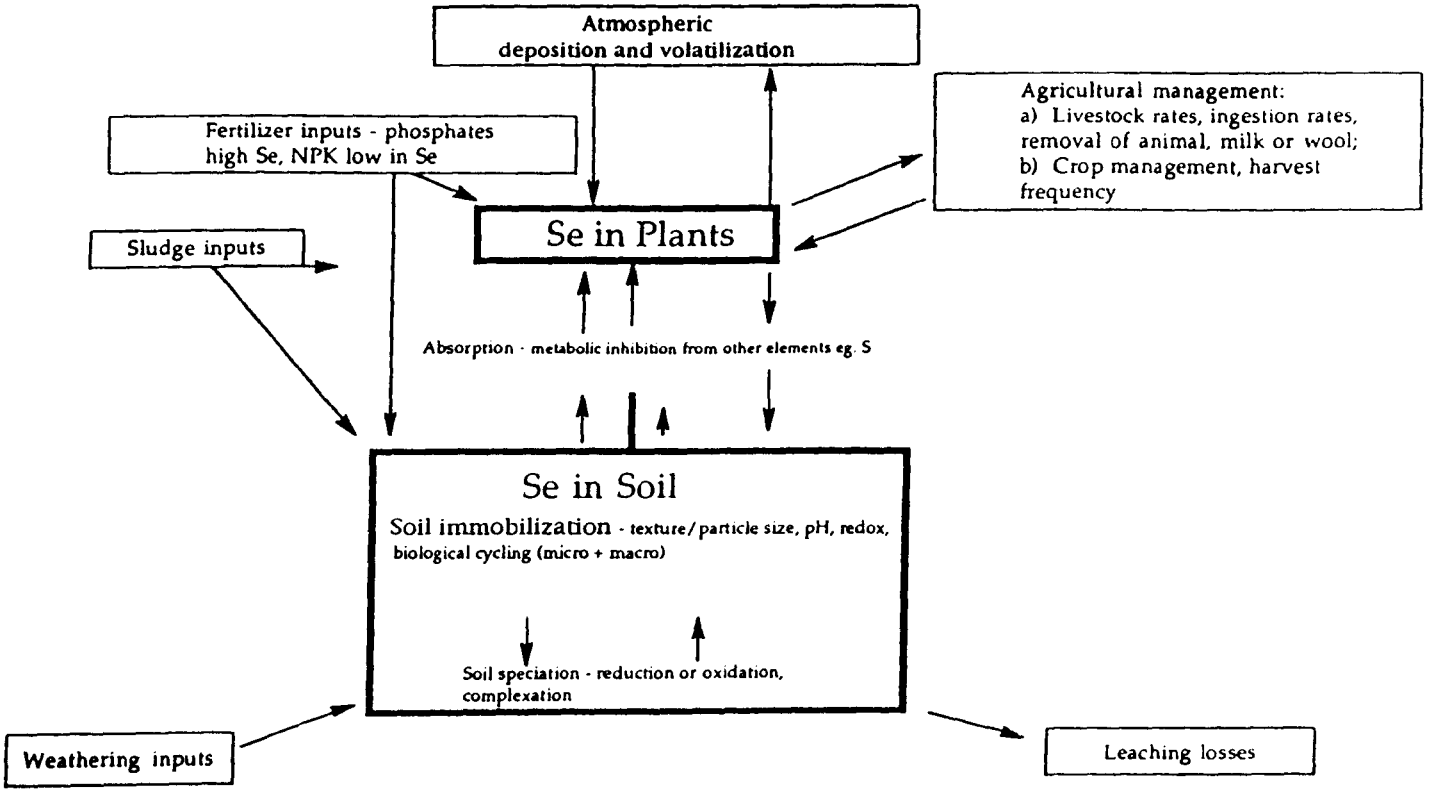


Figure 1.2. Major components in the selenium cycle (from Haygarth, 1994)

ash or Se prills to land. Plants grown on soil amended with coal fly ash became enriched in Se in proportion to the Se content of the added material (Gutenmann *et al*, 1979). Selenium prills have been used on pastures in New Zealand (Watkinson, 1983) to increase the Se status of livestock. These prills are made using specific Se compounds or by using industrial by-products rich in Se. It was found a year later that the prills had caused the concentration of Se in the livestock to rise to a level which would both prevent Se deficiency and provide meat with sufficiently high levels of Se for human consumption (Watkinson, 1983) to ameliorate deficiency. The above methods are commonly used in varying degrees by countries with Se deficient soils. A different method of tackling this problem may emerge in the U.K. in 1998, when the disposal of sewage sludge at sea becomes illegal, and the sludge will be spread on agricultural land (Matthews, 1992). One of the objectives of this project was to examine whether sewage sludge could be used to correct for Se deficiencies in soils, cause toxicity problems in soils or simply have little effect on the Se level present. The policy, in Scotland, for those livestock grazing on Se deficient soils is to supplement their feeds with Se.

1.5.1. Selenium uptake by plants.

The examination of the uptake of Se by plants from soils has had two main objectives:-

(1) The addition of bioavailable Se can increase Se levels in plants and hence overcome Se deficiency problems in livestock and the human population

(2) Se accumulator plants may be useful in removing excess Se from soils and bring about bioremediation of contaminated soils. Early studies often used radiolabelled Se for quantitation of uptake. Peterson and Butler (1962) added

^{75}Se to nutrient solutions and measured uptake by wheat, ryegrass, red clover, white clover and *Neptunia amplexicaulis* (a Se accumulator). No differences in uptake were noticed after a 10 day period but it was observed that the non-accumulator plants contained much of the ^{75}Se in the form of seleno-amino acids compared to *Neptunia amplexicaulis*. The authors recognised that the selenite ion is not absorbed by this Se accumulator plant to the same extent as some other Se forms. The low amount of protein bound ^{75}Se in *Neptunia amplexicaulis* was thought to be due to a mechanism for preferential formation of sulphur amino acids in protein synthesis.

Cary *et al* (1967), using ^{75}Se in neutral soils, estimated that a selenite application rate of Se as a selenized concentrated superphosphate (to give a soil concentration of 0.5 μg Se g^{-1}) to soils growing an alfalfa crop would be sufficient as a feed to protect animals from Se deficiency diseases. Advances in analytical techniques have reduced the need to use radioisotopes of Se and many studies now use hydride generation or graphite furnace atomic absorption spectrometry. Johnsson (1991) found that plants growing in sandy soils had a higher Se uptake from selenite than clay soils. He also observed that the effect of pH on Se uptake decreased as clay and organic matter content increased. Bell *et al* (1992) examined selenate uptake in the presence of the sulphate ion in gypsiferous soils. Using both alfalfa and *Astralagus bisulcatus* (a Se accumulator), the presence of the sulphate ion greatly inhibited selenate uptake by alafalfa. Selenium uptake by *Astralagus bisulcatus* was also significantly affected but to a much lesser extent. This led to the hypothesis that Se accumulator plants are uniquely able to take up selenate even in the face of sulphate competition. Gupta *et al* (1993) calculated that the application of selenate fertiliser at 10 g Se ha^{-1} to soil (pH 6) used for growing cereal grain would protect livestock from deficiency diseases. Using acidic soils, Zhang *et al* (1988) found that yields of Swiss chard were reduced with an application of 1 mg Se kg^{-1} (dry weight) as aqueous selenate but not as selenite.

Bioremediation experiments by Banuelos and Meek (1990), Banuelos *et al* (1992) and Banuelos *et al* (1993) found that wild brown mustard and canola (both *Brassica* species) accumulated high concentrations of Se (50 mg Se kg^{-1} dry weight from soils containing 0.7 mg Se kg^{-1}dry weight). Banuelos *et al*(1991) also found that alfalfa would remove Se from soils amended with Se laden mustard plant tissue (6.0 mg Se kg^{-1} in alfalfa from 5.7 mg Se kg^{-1} in the added mustard addition of 40 g). Parker *et al* (1991) found *Astralagus bisulcatus* and *Astralagus racemosus* (both primary Se accumulators) took up high amounts of Se and could tolerate high salinity.

1.6. Disposal of sewage sludge.

Sewage sludge is the residue which is collected after the contents of the sewage system have been treated. It consists mainly of human wastes but also contains significant amounts of industrial effluents and animal and vegetable waste. Hence sludge may contain many potentially toxic substances including heavy metals, pathogenic bacteria, viruses, protozoa and parasites.

New European Community legislation which prohibits the disposal of sewage sludge at sea from 1998 was announced by the Ministry of Agriculture, Fisheries and Food (1991). The U.K. disposes of over 50% of its sewage sludge into the sea (Towers, 1994), and since the coastline is extensive and easily accessible, this has been both cheap and convenient. Alternative methods must now be considered and these include recycling to land, landfill and incineration.

In Scotland disposal of sewage sludges is the responsibility of the Scottish Environment Protection Agency whereas in England and Wales, privatised water companies are responsible (Matthews, 1992). U.K. sludge production is projected to increase from 1.11 million tonnes of dry solids in 1991 to 2.15 million tonnes in 2006 (ENDS Report, 1993). The method of disposal is chosen on a local basis

using geographical, political and economic factors. Landfill sites at present account for only 15% of sewage sludge in the U.K. (Matthews, 1992). It is unlikely that this will become a principal disposal method due to fewer landfill sites becoming available and increased charges for these sites. Incineration methods have become more efficient and economical but the remaining ash content has to be disposed of to landfill. There is public concern over emissions from incineration sites, and new stringent pollution laws have been introduced (Matthews, 1992). In the future, the main method of disposal of sewage sludges will be to agricultural land.

The presence of high levels of heavy metals in many sewage sludges is a cause for concern. Plant uptake studies, with a range of sewage sludges, have been performed, but little work has examined the effect of adding Se to soils in this form. Waste products from the electronics industry and ore smelting are likely to result in Se in sludge and may be an efficient and cheap method of increasing Se in food for livestock or humans. Alternatively Se toxicity problems may arise. There is clearly a need for work to be carried out to determine the Se levels and chemical species present in these sludges and the availability of these forms to crop and fodder plants.

1.6.1. Code of Practice for Agricultural Use of Sewage Sludge.

This Code (HMSO, 1989) has been published by the Department of the Environment to ensure human, animal and plant health is not put at risk when sewage sludge is used for agricultural purposes. It outlines the benefits of the supply of significant proportions of nitrogen, phosphorus and organic material which will be supplied to the soil. Sludge treatment processes are detailed which will greatly reduce the potential health hazard and sets out the parameters for sludge which require analysis (Table 1.1). These analyses must be carried out every 6 months and the results recorded. Soil analysis must be carried out prior to

application of the sludge and every twentieth year thereafter (of continuous application). The parameters that will be monitored are shown in Table 1.2. In both cases the measurement of Se is not compulsory from the EC Directive 86/278/EEC and has received little attention as a contaminant in sewage sludge.

Table 1.1. Sludge analysis parameters.
(Code of Practice for Agricultural Use of Sewage Sludge).

Parameter	Limit values
Dry matter (%)	Not given
Organic matter (% dry solids)	Not given
pH	Not given
Nitrogen total and ammoniacal (% dry solids)	Not given
Phosphorus total (% dry solids)	Not given
Zinc (mg/kg dry solids)	2500-4000
Copper (mg/kg dry solids)	1000-1750
Nickel (mg/kg dry solids)	300-400
Cadmium (mg/kg dry solids)	20-40
Lead (mg/kg dry solids)	750-1200
Mercury (mg/kg dry solids)	16-25
* Molybdenum (mg/kg dry solids)	3
* Selenium (mg/kg dry solids)	2
* Arsenic (mg/kg dry solids)	2
* Fluoride (mg/kg dry solids)	200

* These parameters are not subject to the provisions of Directive 86/278/EEC and are reference concentrations only.

Table 1.2. Soil analysis parameters.

(Code of Practice for Agricultural Use of Sewage Sludge).

Parameter	Limit values
pH	N/A
Nitrogen total and ammoniacal (% dry solids)	N/A
Phosphorus total (% dry solids)	N/A
Zinc (mg/kg dry solids)	150-300
Copper (mg/kg dry solids)	50-140
Nickel (mg/kg dry solids)	30-75
Cadmium (mg/kg dry solids)	1-3
Lead (mg/kg dry solids)	50-100
Mercury (mg/kg dry solids)	1-1.5
* Molybdenum	
* Selenium	
* Arsenic	
* Fluoride	

* These parameters are not subject to the provisions of Directive 86/278/EEC and no reference concentrations were given.

1.6.2. Study of selenium uptake by plants grown on sewage sludge amended soils.

There has been little information about Se uptake by plants grown in soils amended with sewage sludge. In 1988, Logan *et al* looked at Se accumulation in barley, Swiss chard and radish grown in soil to which sewage sludge had been added annually for the previous 10 years (180 Mg ha^{-1} per year at 1.2 mg Se kg^{-1}). The cumulative total was 5.86 +/- 0.1 mg Se kg^{-1} soil at pH 7. There was no measurable Se uptake by the barley and no significant increases in the Se concentrations of either the Swiss chard or the radish. The levels of Se applied were close to the maximum allowable and these results would suggest that there

would be no danger of Se toxicity. The levels are also insufficient to prevent Se deficiencies in livestock. It was also observed that only 13-25% of the Se applied in the sludge could be accounted for in 0-15 cm soil depth and there was no measurable Se (<0.1 mg Se kg^{-1}) in the subsoil to 150 cm. This may have been due to leaching or volatilisation losses. Rutzke *et al* (1993) grew Swiss chard in 4 'composts':-

(1) Sewage sludge (7.2 mg Se kg^{-1}dry weight)
(2) Sewage sludge and yard waste (8.8 mg Se kg^{-1} dry weight)
(3) Sewage sludge, yard waste and municipal solid waste (0.6 mg Se kg^{-1}dry weight)
(4) Yard waste (control) (0.2 mg Se kg^{-1}dry weight)

The pH of the 'composts' ranged from 6.9 to 7.3. The Se concentration (0.09 +/-0.01 mg Se kg^{-1}) in plants grown on sewage sludge was low and insufficient to overcome Se deficiencies in livestock. A higher level (0.17 mg Se kg^{-1}) was found in the plants grown on the sewage sludge/yard waste mixture.

Gutenmann *et al* (1993) applied a liming material which contained a mixture of sewage sludge and cement dust. The cement dust raised the pH of the sludge and contained large amounts of fly ash. The fly ash was rich in Se (12 mg kg^{-1}) which has been shown to be available to plants (Furr *et al*, 1978 and 1979). Swiss chard was grown on soil (pH 5), soil with 5% liming material (pH 6.8) and soil with 10% liming material (pH 7.2). The soil contained 0.33 mg Se kg^{-1} and the liming material 4.7 mg Se kg^{-1}. The uptakes were 0.11 +/- 0.02 and 0.15 +/- 0.03 mg Se kg^{-1}, respectively, for the 2 amended soils. These levels would be sufficient to supply sufficient dietary Se to livestock, however, the concentrations applied were higher than those which would be applied to agricultural land in 'normal' sewage sludges. The studies carried out so far on Se applied in sewage sludge have calculated only the total Se in soils and sludges. No attempt appears to have been made to quantify the Se species present.

1.7. Speciation of selenium in soils.

Quantitation of total Se gives no information about the chemical species or the soil fraction with which it is associated. The oxidation state of Se in soil is of primary importance in determining the availability of Se to crops. The chemical forms and their solubility depends mainly on the redox potential and the pH of the soil (Elrashidi *et al*, 1987). The redox-pH diagram for Se is shown in Figure 1.1. Se can exist in 4 oxidation states all of which are represented in soils, namely Se(IV) (selenite), Se(VI) (selenate), Se (elemental selenium) and Se(II) (selenide and organic selenium). The identification of different chemical forms in the soil is difficult because of the small amounts present and the complex composition of soil. Other factors which influence the Se species present include organic matter content, ferric oxide levels and clay type and content. In acid and neutral soil, Se is usually present as Se (IV) complexes of ferric oxides and oxyhydroxides and has low solubility. Hence in these soils, the Se is largely unavailable to plants (Cary and Allaway, 1969 and Hamdy and Gissel-Nielsen, 1977). In well aerated, alkaline soil, Se(VI) predominates and is usually soluble, mobile and readily available for plant uptake. Clays and mineral oxides have a much greater affinity for Se(IV) than Se(VI) (Gissel-Nielsen, 1973). Amorphous iron oxyhydroxide adsorb more selenite than manganese dioxide and manganese dioxide does not adsorb selenate (Balistrieri and Chao, 1990). Se(IV) is also less soluble than Se(VI) and hence is less available for plant uptake. Geering *et al* (1968) found the solubility of Se(IV) was lowest in the pH range 4-7 and increased with higher or lower pH values. Organic matter has a large capacity to remove Se from solution (Levesque, 1974). Hamdy and Gissel-Nielsen (1977) found that ferric oxides adsorbed more Se(IV) than all the other minerals tested and quickly removed Se(IV) from solution. The authors also found that Se(IV) adsorption by clays reached a maximum at pH 3-5 and that the most important clays for adsorption were kaolinite, vermiculite and montmorillonite, as illustrated in Figure 1.3.

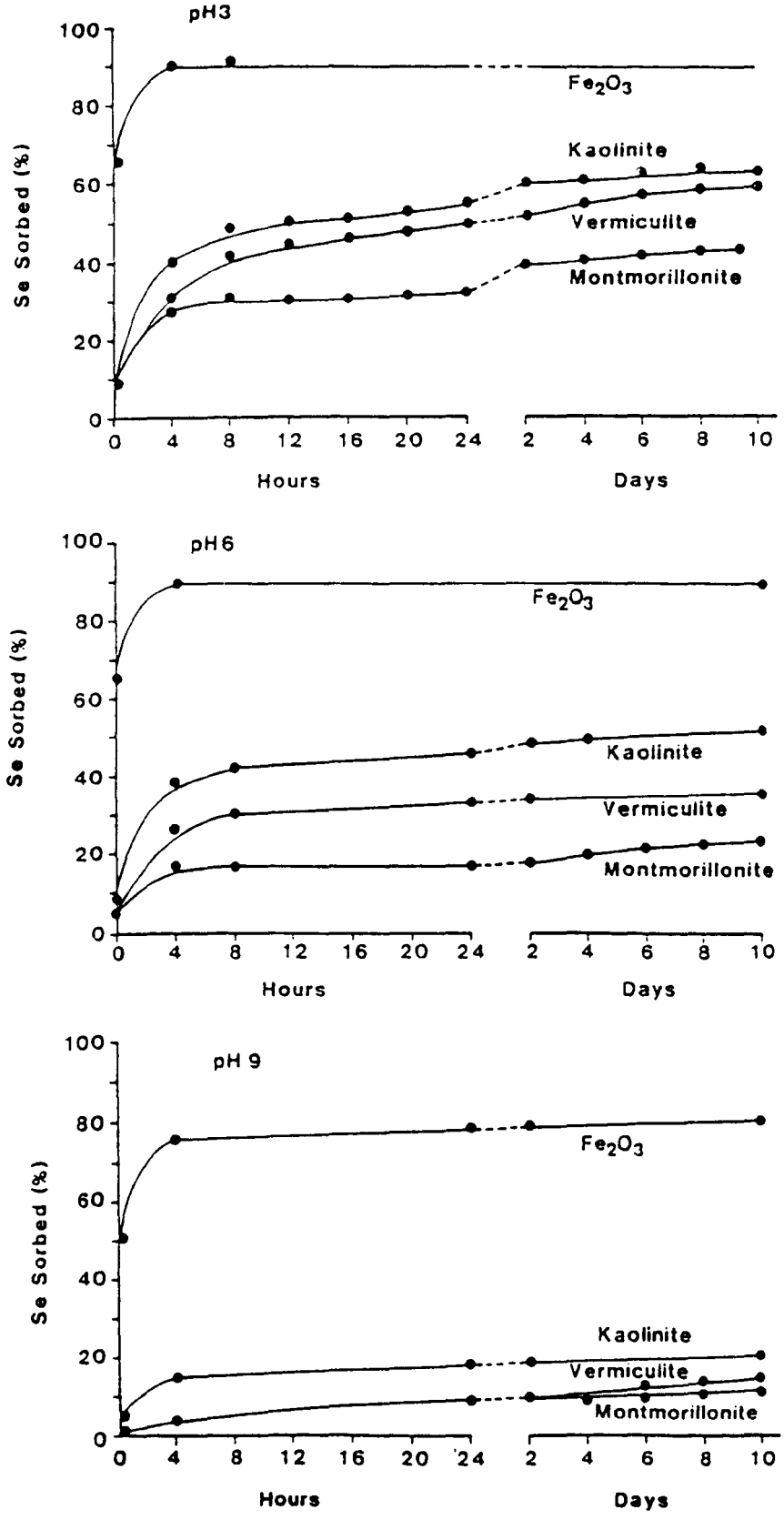


Figure 1.3 Removal of added Se(IV) from solution by clay minerals as Fe_2O_3 as influenced by pH and time (adapted from Hamdy and Gissel-Nielsen 1977).

The liming of soil has been found to increase Se concentration in plants (Cary *et al*, 1967 and Gupta *et al*, 1982). The addition of lime increases the soil pH and reduces the capacity of clays and ferric oxides to adsorb Se (Neal *et al*, 1987). The increased soil pH facilitates the replacement of adsorbed Se(IV) ions by hydroxyl ions and the Se is released into solution (Hamdy and Gissel-Nielsen, 1977). The form of Se will determine its bioavailabilty for plant uptake. However, changes in pH, redox potentials and microbial action can result in a change of the form of Se present. Therefore it is important to be able to assess the amount of Se which can be potentially taken up by plants from both soil and, perhaps more importantly from sewage sludge.

Little work appears has been done on the speciation of Se in soils or sewage sludges in the UK. Speciation of Se in soil or sludge requires extraction or dissolution of the soil, followed by determination of the species using techniques such as ion chromatography or high performance liquid chromatography (Dauchy *et al*, 1994) These analyses determine the selenite, selenate or organoselenium compounds present. Kang *et al*, 1993 achieved quantitative speciation of Chinese soil following extraction by 0.1 M NaOH. They employed humic acid and fulvic acid as extractants and were able to obtain levels of organically bound Se, high molecular weight organic Se and low molecular weight organic Se. Inorganic Se(IV) and Se(VI) levels were also obtained. Calculations of organic Se and Se(VI) were obtained by difference and the NaOH extraction was 83.7% efficient. Karlson and Frakenberger quantified selenite (1986a) and selenate (1986b) in soil using single column ion chromatography. Niss *et al* (1993) determined selenite and selenate in coal fly ash by ion chromatography with conductivity detection after NaOH extraction. Using $CaCl_2$ as an extractant, McGeehan and Naylor (1992) quantified selenate and selenite by suppressed ion chromatography. The determination of soluble Se levels in soils was achieved by Yamada and Hattori (1990) but they found interferences from organic matter requiring digestion with $K_2S_2O_8$. Pitts *et al* (1995) used a fully automated on line system followed by microwave digestion to measure selenite and selenate. Dauchy *et al* (1994)

reviewed current Se speciation methodology, dividing speciation into inorganic and organic groups. The organic Se group was further subdivided into volatiles (GC-FID or GC-AES), selenoamino acids (GC-MS), trimethylselenonium (ion exchange chromatography) and others (ion exchange chromatography). These authors acknowledged that the accurate speciation of Se has many problems and further improvements are needed to obtain accurate information on Se speciation and to correlate this to bioavailability.

An alternative approach is to use sequential dissolution from soils. Chao and Sanzolone (1989) developed a scheme which defined 5 fractions: soluble, ligand exchangeable, acid extractable, oxidative acid dissolution and strong mixed acid digestible. This scheme does not determine individual chemical species but rather indicates the soil phases with which they are associated and the availability of Se for plant uptake. Their overall findings were consistent with the theoretical behaviour of Se. Quantitation of each fraction was by hydride generation atomic absorption spectrometry. The sequential extraction scheme allows rapid estimation of the nutrient or toxicity probabilities of a given soil by a simple 1 step extraction. Criticisms of sequential extractions have been expressed by Gruebel *et al* (1988) and this is discussed more fully in Section 4.10.3.

1.8. The role of selenium in animals and man.

Selenium is known to be the cofactor in two enzyme systems. Se is present as selenocysteine at the catalytic site in glutathionine peroxidase (GSH-Px). This enzyme is responsible for the reduction of lipid hydroperoxides and H_2O_2. During normal oxidative metabolism by the hepatic mixed function oxidases, H_2O_2 and lipid hydroperoxides are generated (Figure 1.4). These are highly reactive intermediates which must be rapidly cleared from the cell to protect the cellular membrane from lipid peroxidation (Rotruck *et al*, 1973). Thus, GSH-Px acting as an antioxidant may be important in the prevention of

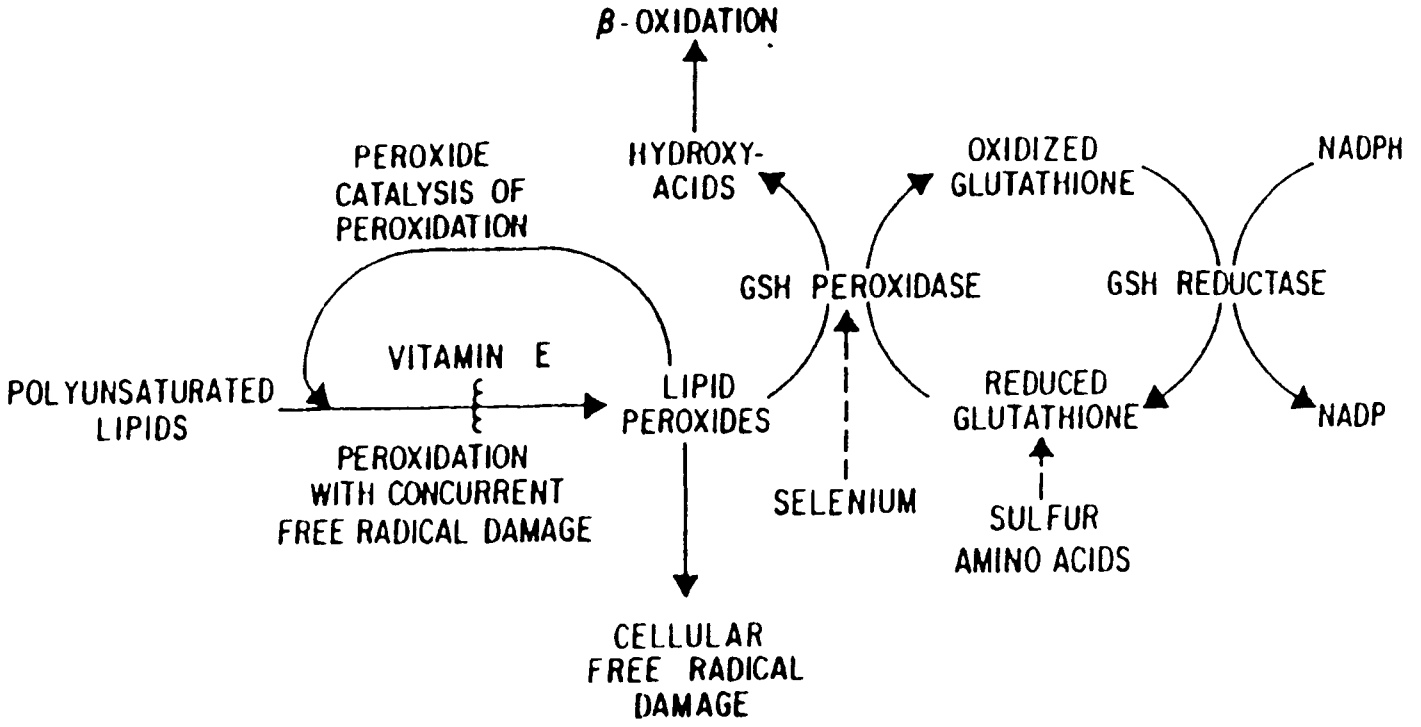


Figure 1.4 The function of selenium in cellular metabolism (from Chow and Tappel, 1974).

cancer (El-Bayoumy, 1991). The role of GSH-Px is complimentary to that of vitamin E. GSH-Px eliminates peroxides which have already formed and vitamin E limits the formation of peroxides (Hoekstra 1974). The second enzyme which requires Se is iodothyronine 5'-deiodinase which converts thyroxin (T_4) to tri-iodothyronine (T_3) with the release of iodine (Behne *et al*, 1992).

1.9. Sources of selenium in the diet.

The study of diet in the U.S.A. showed that meat and fish contained high Se levels (0.1-0.7 μg/g) (Morris and Levander, 1970). The highest Se levels were recorded in oysters, crab and tuna. Offal contain high Se concentrations, as do egg yolks. Cereal grains vary from medium to low status as sources of Se depending upon the parent soil (Arthur, 1972). However, there are worries in the U.K that the drop in imports of Se rich wheat from North America has resulted in lower Se levels in humans (Rayman, 1997). Fruit and vegetables were found to be generally low in Se. In general Se appears to be most concentrated in high protein foods, but the regional influences must be taken into account.

Excretion of Se is primarily *via* urine. Body homeostasis of Se is controlled by the kidney and is excreted primarily as trimethylselenonium (Palmer *et al.*, 1970). Some Se is also excreted in the faeces but Se excreted in this way appears to have resulted from unavailable Se forms (which have not been absorbed in the diet) rather than from endogenous secretions of sloughed cells.

1.10. Effect of selenium deficiency.

Deficiency symptoms are likely to appear in livestock when the Se concentration in the dry matter of the fodder is less than 0.05 mg g^{-1} (Bisbjerg and Gissel-Nielsen, 1969). In cattle, this can result in white muscle disease, infertility and the death of their calves. Pigs and lambs suffer from liver necrosis, muscle

dystrophy and death (Burk, 1978,). To overcome these problems, Se is added to the soil (as discussed in Section 1.4.), the feeds supplemented with Se or by direct injection of Se. The addition of Se to feedstuffs is severely restricted by law in many countries due to the toxicity of this element (Gissel-Nielsen *et al*, 1984). It is usually given to ruminants in the form of "heavy pellets" which contain Se and iron filings. These remain in the fore-stomach and small amounts of Se are released over long periods of time (Oldfield, 1992). In humans, there is no direct evidence of the harmful health effects of a mild Se deficiency. Nevertheless, epidemiological studies carried out in Finland and Denmark suggest that low Se intake (as reflected in low serum Se levels) can increase the risk of cardiovascular disease and cancer (Aro *et al.*, 1995).

Selenium deficiency diseases are endemic to China. Keshan disease occurs in a belt stretching from NE to SW China in a region where the inhabitants diet consists solely of home produced foods grown on Se deficient soils (Yang *et a.l*, 1988). It affects mainly children under the age of fifteen and results in necrotic degeneration of the liver, pancreas and kidneys (Yang *et al.*, 1988,). Kashin-Beck disease occurs in the southwest area of China and affects children in the five to thirteen age group. Initial complaints of tiredness and stiff joints lead to osteoarthritis of the peripheral joints and the spine (Tan *et al.*, 1986). The treatment of both of these diseases with Se supplementation was extremely effective (Chen *et al.*, 1980; Yang *et al.*, 1988).

1.11. Effect of selenium toxicity.

All livestock are susceptible to Se poisoning. Toxicity effects occur in livestock at Se levels above 3 mg g^{-1}dry weight (Bisbjerg and Gissel-Nielsen, 1969). Selenium intoxication has been classified by Rosenfeld and Beath (1964) as acute or chronic.

Acute Se poisoning usually results from the ingestion of excessive amounts of Se accumulator plants. These plants tend to be unpalatable with an unpleasant odour, so this type of poisoning is uncommon. Acute poisoning in aminals results in watery diarrhoea, abdominal pain, prostration and death (James, 1989).

In animals, chronic intoxication is divided into two syndromes, alkali disease and blind staggers. Alkali disease in cattle, horses, pigs and poultry results from eating seleniferous forages such as grasses, over a period of time, in which the Se is bound to protein and therefore relatively insoluble in water (Rosenfeld and Beath, 1964). The first recorded case was probably the observation by Marco Polo in Western China in the thirteenth century that ingestion of a certain poisonous plant by horses resulted in lameness and loss of hooves (Marsden, 1962). Today, alkali disease is characterised by lack of vitality, emaciation, hair loss, hoof changes and lameness (Rosenfeld and Beath, 1964). Blind staggers occur in cattle and possibly sheep and is associated with the consumption of Se accumulator plants over a period of time where the Se is readily extractable in water. It results in necrosis of the functional liver cells and cirrhosis and impactation of the digestive tract (Rosenfeld and Beath, 1957).

Selenium toxicity in humans is rare. Toxicity is normally a result of the ingestion of overly potent Se supplement tablets or inhalation of Se fumes in industrial processes. Symptoms include nausea, vomiting, hair and nail loss and fatigue (Jensen *et al*, 1984).

1.12. Selenium toxicity and carcinostatic activity.

Selenium has been confirmed as a toxic element since the 1930's when its presence in soil was footnoted in a confidential report issued by the United States Bureau of Chemistry and Soil regarding "alkali disease" in livestock (Spallholz,

1995). However, epidemiological studies in the 1970's also suggested that Se possessed anticarcinogenic effects (Rotruck *et al*, 1973).

Hence, the question of the essentiality versus toxicity effects of Se had to be addressed (Hu *et al.* 1982) observed that selenite and selenocysteine could damage rat erythrocyte cell membranes *in vitro*. In 1991, Yan and Spallholz confirmed earlier observations that selenite reacted with GSH-Px to produce superoxide and hydrogen peroxide. Selenocysteine was later shown to react with GSH-Px under different pH conditions and thiol concentrations to also produce superoxide (Spallholz and Whittam, 1992). Selenates and selenoethers were not found to produce superoxides. From these observations, Spallhloz (1994) hypothesised that selenites and selenocysteine will ultimately react to produce superoxides and hydrogen peroxides which are toxic; this Se toxicity becoming acute or chronic when oxidative damage exceeds the capacity of the antioxidant defences. Levander *et al.* (1973) reported that selenite and selenocysteine were reductive catalysts. Reports by Milner *et al.* (1981) using *in vitro* studies found a high correlation between cytotoxicity in tumour cells and loss of GSH-Px with catalytic Se compounds. These catalytic Se compounds were also generally found to be carcinostatic. The likely cytotoxic species is the selenopersulphide anion ($GSSe^-$) which is formed from selenite and endogenous GSH-Px (Levander *et al.*, 1973). *In vivo* studies Young *et al.* (1982) found selenate was reduced to selenite and was equally as toxic. Spallholz (1994) concluded that the production of superoxides and hydrogen peroxide is responsible for the toxicity and carcinostatic activity of Se compounds in vitro. *In vivo*, tumour cells concentrate Se which may explain why carcinostatic activity normally occurs only at dietary levels approaching systematic Se toxicity (Tariq and Priess, 1991).

1.13. Digestion procedures for selenium compounds.

The rate limiting step in any chemical analysis is the sample preparation. Elemental analysis is usually carried out after acid decomposition. This ensures

that the organic forms of Se (selenomethionine and selenocysteine), which are intrinsically bound in protein molecules are released (Ducros *et al.*, 1994). Complete digestion is also necessary for chelate or hydride formation.

Selenium can form relatively volatile compounds and losses can occur at temperatures above 120^0C (Fourie and Peisach, 1973). The use of dry ashing techniques such as the muffle furnace requires the addition of $Mg(NO_3)_2$ or MgO to prevent losses (Siemer and Hagemann, 1975). The main limitation of this procedure is that it is time consuming (48 h) and is prone to contamination. Open wet decomposition is the usual method but is long and tedious and refluxing can lead to Se losses and contamination from external sources.

The classical digestion method for Se in biological samples is wet ashing in a heating block using a HNO_3-$HClO_4$ mixture (Clinton, 1977). This method was used by Ducros and Favier (1992) prior to GC-MS analysis. Complete sample digestion was obtained after 12 h. However, the use of $HClO_4$ which is potentially explosive requires a fume hood designed specifically for handling $HClO_4$ fumes (Reamer and Veillon, 1981). These problems led Reamer and Veillon to investigate other acid mixtures. By using a HNO_3-H_3PO_4-H_2O_2 digestion mixture, complete Se recovery was obtained from biological reference materials. This method was further developed by Dong *et al.*, (1987) for use in plant Se determinations. A manganese salt was incorporated and the accompanying colour change indicated complete oxidation (16 h.). Other digestion methods used include HCl-HNO_3-H_2SO_4 (Quevauviller *et al.*, 1993) and HNO_3-$HClO_4$-H_2SO_4 (Welz *et al.*, 1987). The use of H_2SO_4 in a digestion mixture for Se determination is controversial. Some authors insist the use of sulphuric acid is necessary (Hocquellet and Candillier, 1991) while others deem it detrimental (Quevauviller *et al.*, 1993). Hydrochloric acid is seldom used in digestions of Se containing samples.

Microwave ovens have become a common aid in digestion procedures in the last two decades. Initially, domestic microwave ovens were used but scientifically designed microwave ovens are now commercially available. Microwave digestion carried out in sealed Teflon vessels thus reducing the possibility of contamination and avoiding volatilisation losses and the emission of toxic and corrosive gases. LamLeung *et al.*, (1991) used HNO_3 and a domestic microwave oven to determine Se in fish tissue whereas Ducros *et al* (1994) used HNO_3-H_2O_2 in a commercial microwave for biological samples. A comparison with the traditional HNO_3-$HClO_4$ digestion gave the same mean Se value for reference materials, but better precision was obtained by the microwave digestion method. The microwave digestion time was 45 min compared to a minimum of 20 h for HNO_3-$HClO_4$ digestion. Banuelos and Akohoue (1994) used a HNO_3-H_2O_2-H_2O (2:2:1 v/v) microwave digestion mixture to determine Se in plant samples. It was found necessary to predigest the sample for 4 h and the results were consistent with those obtained by block analysis using the same digestion mixture.

The determination of Se in soil and sewage sludge samples requires an agent such as HF which will dissolve the silicates. Nham and Brodie (1989) investigated 3 acid digestion mixtures: HNO_3-$HClO_4$-HF, HNO_3-H_2O_2-HF and HNO_3-HCl-HF. The samples were analysed by graphite furnace atomic absorption spectrometry (GFAAS) with Zeeman background correction and vapour generation atomic absorption spectrometry (VGAAS). All the mixtures gave similar results using a certified reference material, but the methods which do not use $HClO_4$ are preferred for safety reasons. A rapid microwave method using HF and HNO_3 (Warren *et* al, 1990) has been developed for elemental analysis but has not been used in Se determinations.

1.14. Analytical techniques for selenium determination.

An analytical method for trace element quantitation needs to be able to accurately and precisely determine the levels present over a wide range of concentrations. Traditional methods for Se determination include gravimetry and volumetry. Gravimetric methods are now only used for samples with a high Se content. Volumetric methods are based on oxidation and reduction reactions and are subject to interferences from other oxidants and reductants. Colourimetric and spectrophotometric methods have been largely replaced by the more sensitive fluorometric procedure. This section covers the principles of ID-MS. The commonly used analytical techniques for Se determination including flourometry, chromatography, atomic absorption spectrometry and ICP based methods are discussed. Less commonly used techniques are also briefly examined.

A classification scheme for ranking the quality of a method was proposed in 1976 by the International Federation of Clinical Chemistry and can be applied to Se methodologies (Saris *et al.*, 1976). A definitive method represents the ultimate in quality but is usually sophisticated and common usage is unlikely. The definitive methods for Se include neutron activation analysis (NAA) and mass spectrometry (MS) using isotope dilution which eliminates the use of an external standard (Veillon and Alvarez, 1983). Reference methods are tested against definitive methods or represent the best method available for use under well controlled, routine conditions. For Se, the reference methods, as defined by the Association of Official Analytical Chemists (AOAC), are fluorometry for plants (Williams, 1984a) and foods (Williams, 1984b) and hydride generation atomic absorption spectrophotometry (HGAAS) for foods (Williams, 1984c).

The importance of matrix matched reference materials is also a major consideration and was illustrated by Koh (1987) in his survey of 43 laboratories from 12 countries to determine Se levels in three blood samples. Using aqueous standards, interlaboratory coefficients of variation (CV) were 82% and 143% at

0.5 and 0.2 μmol of Se dm^{-3}. The use of blood standards reduced the CV's to 29%.

1.14.1 Principle of isotope dilution-mass spectrometry (ID-MS).

One way to ensure an analytical method is accurate is to validate the method by the use of certified reference materials (CRM). Another way is to use a definitive method, for example ID-MS.

In ID-MS a small known quantity of an enriched isotope spike solution is added to the sample and mixed completely. The sample and spike solution then undergo the same digestion procedures prior to analyte determination.

The isotope ratio (R) is measured by the mass spectrometer. The two isotopes selected to measure R are the spike isotope and, normally, the isotope with the highest abundance in the sample.

If the sample being analysed contains the elements C, H, N and O, then isotopes of these species will interfere with the isotope ratio measurement. This is called "cross talk" and is corrected for by applying a binomial probability as shown by Pickup and McPherson (1976) and discussed in Section 2.11.

ID-MS offers several advantages. It is precise and accurate, it does not require quantitative recovery and offers high sensitivity with low detection limits (Heumann, 1985). The isotope added is the same element as the sample and hence is assumed to behave in an identical manner. Sample losses which occur during preparation should affect both the sample and the isotope and losses should therefore be in proportion leaving the overall ratio (sample:spike) unchanged.

Disadvantages include the requirement for extensive chemical preparation of the sample and this procedure is a destructive method which is time consuming (Heumann, 1985). Isotope spikes are also expensive and sometimes difficult to obtain.

Spike solutions should be prepared in concentrated acid and diluted. They should be stored in ultracleaned plastic bottles which have been acidified to prevent adsorption effects. The spike solution applied to a sample is normally of a low concentration. This should be prepared freshly each time from the more concentrated stock solution.

The concentration of the spike solution must be be accurately determined. This can be achieved by the use of reverse ID-MS. The precision and accuracy of these spike analyses are generally better than 0.3% (Trettenbach and Heumann, 1985). Reverse ID-MS overcomes the inadequacy of gravimetric analysis and confirms the chemical purity of the spike.

There are limitations associated with ID-MS. The detection of trace amounts of analyte is limited mainly by problems with the blank, precision of the isotope ratio measurement (for maximum precision, the optimum value for the isotope ratio is unity) and by the minimum amount of the analyte which can be isolated and accurately measured (Heumann, 1988).

Several ionisation methods are available for monoelement determination by mass spectrometry including positive and negative thermal ionisation, inductively coupled plasma, field desorption and electron impact. GC-MS with electron impact ionisation was used for the experimental work presented in this thesis. This is the recommended method for volatile organometallic compounds (Nielsen *et al*, 1981) or elements which form stable chelates that can be determined after a GC separation step. This was successfully achieved for Se by Reamer and Veillon (1981).

The mass analyser system can be either a magnetic sector field or a quadrupole system. The quadrupole system was used in this project. The results for the quadrupole are not always as precise but are generally as accurate as those obtained by a high precision magnetic sector field (Heumann *et al*, 1981). Indeed, the limiting factor in precision is not the mass analyser but the chemical treatment and possible inhomogenity of the sample (Heumann *et al*, 1981). The detection system used in the GC-MS was an electron multiplier. This electrical detection system is more accurate than photoplate detection (Heumann, 1988). Quantitation was achieved by the use of the Pickup and McPherson equation (1976) as discussed in Section 2.11.

1.14.2. Fluorometry.

This has been the most widely used method for biological and environmental samples and is still one of the most commonly used techniques. Selenium needs to be present as Se(IV) and a large group of organic reagents can be used as complexing agents, including 3,3- diaminobenzidine, 2,3-diaminonaphthalene and σ-phenylenediamine. Early work was carried out with 3,3-diaminobenzidine (Cousins, 1960) and extensive research on this reagent has been published (Dye *et al.*, 1963). However, for the last thirty years the reagent most commonly used has been 2,3-diaminonaphthalene (DAN) and has also been the subject of many investigations (Parker and Harvey, 1962; Allaway and Cary, 1964 and Cukor *et al*, 1964). The digestion procedure is slow and laborious, using HNO_3-$HClO_4$ acid mix. Reamer and Veillon (1983a) have however developed a procedure for the digestion of biological fluids which eliminates the use of perchloric acid, using a $HNO_3/H_3PO_4/H_2O_2$ mixture instead. Fluorometry utilises the fluorescent complex produced by the reaction of Se (IV) with DAN to give the benzopiazselenol. The complex is extracted into cyclohexane or toluene and after excitation at 360 nm, light is emitted at 520 nm (Watkinson, 1966).

Precise instructions for reagents, apparatus and procedure are available from AOAC (AOAC, 1975). This method has been used to determine Se levels in foods, milk, plants, blood, urine, seawater, sediments and soils (Olson *et al.*, 1973; Raptis *et al.*, 1983). Detection limits range from 10 - 100 ng g^{-1} of sample (Inhat, 1974a and b; Raptis *et al.*, 1983; Watkinson and Brown, 1979). Recoveries of 95-105% have been reported (Wang *et al.*, 1994; Johnson *et al.*, 1976; Watkinson, 1966).

Although fluorometric determination of Se is reasonably simple and one of the most commonly used, problems have been reported. The use of DAN as the derivatising agent limits the useful pH range to 1-2 (Cukor et al, 1964, Bayfield and Romalis, 1985). The digestion of samples by strong acids and the subsequent need for the reduction of Se(VI) to Se (IV) by 6 M HCl means extensive pH adjustment is necessary prior to analysis. However, other authors have argued that the control of pH is unnecessary (Koh and Benson, 1983, Sheehan and Gao, 1990). The benzopiazselenol is sensitive to light and hence the derivatisation and extraction are often performed in darkness (Cukor *et al*, 1964). There are few interfering ions for this method, but Fe can interfere with the formation of the piazselenol (Brown and Watkinson, 1977). This problem can be eliminated by the addition of EDTA (Tamari *et al*, 1986). Interferences by Pd(II) and Sn(IV) have also been reported (Brown and Watkinson, 1977). The automation of the flurometric method has been achieved (Brown and Watkinson, 1977).

Recent work by Johansson et al (1995) used 2,3-diamino-1,4-dibromonaphthalene as the complexing agent. This method offers an analytical procedure to determine Se in small biological samples with the whole analysis is carried out in a single test tube. The acidity constants of Br_2-DAN allow for easy adjustment to the optimum pH for the piazselenol derivatisation and the stability of the reagent means that it can be used in daylight. Initial results indicate that this reagent could advantageously be used for fluorometric determination of Se.

1.14.3. Chromatography.

The chromatographic methods used to determine Se levels can be divided into two groups, namely gas chromatography methods (with electron capture detection or mass spectrometry detection) and high performance liquid chromatography (HPLC) methods.

1.14.3.1. Gas chromatography with electron capture detection (GC-ECD)

Selenium quantification by GC-ECD has been based on the detection of a volatile piazselenol (Figure 2.1). The piazselenol is formed from the reaction between Se as H_2SeO_3 and a substituted σ-phenylenediamine with substituant of Cl, Br, NO_2, F or CF_3. The first piazselenol was synthesised in Hinsberg (1889) using an unsubstituted phenylenediamine. Selenium must be present as Se(IV) after the destruction of the organic matrix of the sample. In order to reduce selenate to selenite, the acid digested samples are boiled with 6 M HCl (Dilli and Sutikno, 1984). Concentrated HCl should not be used as Se can be lost as volatile chlorides (Poole *et al*, 1977). The extraction of the piazselenol is preferred at a low pH (usually <1) (Toei and Shimoishi, 1981). The introduction of a substituant electron withdrawing group into the molecule greatly increases the sensitivity of the method (Toei and Shimoishi, 1981; Poole *et al.*, 1977). Also, this second electron withdrawing group may increase the pH range which facilitates the formation of the piazselenol (Shimoishi, 1977).

The best derivatising agent as regards sensitivity and distribution ratio for use with GC-ECD is 1,2-diamino-3,5-dibromobenzene (Br_2-PDA) (Shimoishi, 1977). The derivatising agent 4-nitro-σ-phenylenediamine (NPD) is an effective commercially available reagent (Young and Christian, 1973). This reagent was investigated by McCarthy *et al* (1981) for biological samples. Initial work found that 2 interfering chromatographic peaks were present after HNO_3 digestions. The

addition of EDTA and hydroxylamine sulphate in the presence of urea was found to eliminate these peaks occurring in the analysis of Se in standards and biological materials. The detection limit was found to be 0.5 pg with a recovery of 95-105%. McCarthy *et al* found that this method gave good agreement with certified reference materials (CRM) and that around 60 samples could be analysed over 12 h. The Se-NPD complex has also been used in GC-ECD to determine Se levels in soils, waters and sludges with a detection limit of around 0.2 ng/g (Cappon and Smith, 1978). Limitations of the Se-NPD method however include poor chromatographic properties arising from on-column decomposition of the derivative and of various reaction products. This decomposition results in severe "gumming up" of the GC column and detector, even after clean up procedures have been applied (Cappon and Smith, 1978).

At present Br_2-PDA (now commercially available) is the most commonly used ligand (Johansson and Olin, 1992). This ligand has been studied for several reasons. It is available in a highly pure form and the derivatised piazselenol has a relatively high boiling point which gives the longer retention time which was important for water samples which eluted interfering peaks at shorter retention times. Also, the protonation constants of Br_2-PDA make it easy to adjust the pH to optimum values for the formation of the piazselenol. Previous work (Shimoishi, 1977) had experienced problems with interfering chromatographic peaks with packed columns. This resulted in the use of clean up procedures but these were not totally effective (Shimoishi and Toei, 1978). The introduction of capillary columns, which have higher efficiency, has eliminated interferences even without clean up techniques and the piazselenol is well separated from co-extractants. A clean up step could be included to prolong the lifetime of both column and detector. A detection limit of a few ng dm^{-3} of Se in water was obtained by this method.

Although GC is most commonly used with ECD detection, other methods of detection include flame photometry (Hancock *et al.*, 1980), thermal

conductivity (Akiba *et al.*, 1975), flame ionisation (Schwedt and Russell, 1973), and atomic absorption spectrometry (Jiang *et al.*, 1982; Wolf and LaCroix, 1988).

1.14.3.2 Gas chromatography-mass spectrometry (GC-MS).

The use of stable isotope - dilution mass spectrometry for quantitation of Se in biological samples was demonstrated by Reamer and Veillon (1981b). Reamer and Veillon utilised the reaction between selenite and 4-nitro- σ-phenylene-diamine (NPD) in an acidic solution to form the piazselenol (Se-NPD). The Se-NPD was isolated and measured by stable isotope dilution gas chromatography-mass spectrometry. This method has the advantages of freedom from matrix effects and does not require quantitative recovery (Aggarwal *et al*, 1992). The samples were spiked with ^{82}Se isotope solution, digested using HNO_3, H_3PO_4 and H_2O_2 as described by Reamer and Veillon (1981a). After digestion, concentrated HCl was added and the samples boiled for 5 min in order to reduce any selenate to selenite. The samples were derivatised and the Se-NPD was extracted into toluene prior to analysis. The method was validated by the analysis of three standard reference materials. Tracer studies showed a 90% recovery of Se-NPD. Precise and accurate results for the determination of Se were obtained which showed good agreement with values obtained by the established perchloric acid digestion / DAN fluorometric method.

Further work by Reamer and Veillon (1983b) extended this single isotope dilution method to a double isotope dilution method. This employed one stable isotope as the internal standard and the other isotope as the tracer. The method was found to be specific, sensitive (detection limit = 50 pg), precise, accurate and rapid. An important application of this method is in human metabolic studies where the use of radio-isotopes is inappropriate.

The stable isotope dilution GC/MS method using Se-NPD was further developed by Ducros and Favier (1992) who analysed biological samples, using a capillary column rather than the packed column used by Reamer and Veillon (1983b). The capillary column increases the efficiency of the chromatographic separation and the speed of analysis. It also decreases the "bleeding" time of the column. Precise and accurate results were obtained using a certified reference material (bovine liver 1577a, NIST) and a limit of detection of 2.8 ng cm^{-3} was reported from three times the standard deviation of the analytical blank (deionised water).

Different derivatising agents for the formation of the piazselenol complex have been investigated. Wolf *et al*, 1988 evaluated 4-trifluoromethyl-σ-phenylenediamine (TFMPD) for quantitation of Se in biological materials by both GC-AAS and GC/MS. Recovery problems were experienced with GC-AAS and led to the conclusion that further study and modifications were required to prevent Se losses which occurred during derivatisation. However, using CRM (bovine serum RM-8419 and mixed diet RM-8431, NBS), good agreement was obtained by ID-MS. Results showed Se-TFMPD had better sensitivity (detection limit 0.3 ng Se per injection) than Se-NPD and gave sharper peaks for peak height isotope ratio measurements. The higher volatility of Se-TFMPD and its increased thermal stability also resulted in cleaner chromatography and fewer problems with "gummed up" columns and dirty sources. Lower column temperatures used also meant the GC/MS could be used more efficiently with less down-time.

Aggarwal *et al* (1992) investigated the derivatising agents 4-nitro-σ-phenylenediamine (NPD), 3,5-dibromo-σ-phenylenediamine(DBPD) and 4-trifluoromethyl-σ-phenylenediamine (TFMPD). Using CRM (urine 2670, NIST) these derivatising agents were examined in terms of accuracy, precision and memory effect. No significant differences were observed in terms of accuracy and precision, but the authors expressed some difficulties in preparing the piazselenol using TFMPD for urine samples. The piazselenol formed was not stable for more

than a day when stored at room temperature, but was stable for at least a week when stored at -70^{0}C.

Using a capillary column, Se-NPD showed a longer retention time than Se-DBPD and Se-TFMPD showed the shortest retention time. Se-TFMPD also exhibited the least GC/MS memory effect. However, in deciding upon which derivatising agent is to be used, it should perhaps be noted that 4-nitro-σ-phenylenediamine is commercially available, whilst the two others require to be synthesised.

The availability of GC/MS equipment in laboratories has increased greatly in recent times and it has been proposed that stable isotope dilution should become the method of choice for routine Se analyses (Lewis, 1988) on the grounds that it is accurate, has good reproducibility and the methodology is straightforward.

1.14.3.3 High performance liquid chromatography (HPLC).

HPLC is a versatile technique for Se analysis as it can be used with a variety of detectors. The most commonly used detectors for Se analysis (as Se-DAN derivative) include spectrophotometric (Hoffman and McConnell, 1987), fluorometric (Handelman *et al.*, 1989), atomic absorption spectrometry (Blais *et al.*, 1991) and radio-chemical (Baldew *et al.*, 1989). Initial studies used spectrophotometry, but later, the use of fluorometry with Se-DAN derivative was favoured (Vezina and Bleau, 1988). Synthesis of Se-DAN and other Se aromatic diamines is laborious and analysis can be problematic due to the presence of interfering peaks from the DAN solutions themselves.

1.14.4. Atomic absorption spectrometry (AAS).

The AAS methods which have been used for the quantitation of Se include flame AAS, graphite furnace AAS (GFAAS) and hydride generation AAS (HGAAS). Flame AAS does not have the required sensitivity (LOD = 1 mg g^{-1}) for most Se determinations. GFAAS is more sensitive than HGAAS (LOD = 50 pg compared to 100 pg respectively) and samples for HGAAS need to be tenfold larger (Welz *et al*, 1983). Samples analysed by GFAAS do not require pre-digestion (unless in solid form) and analysis time is 3 to 4 min per sample (Ringstad and Thelle, 1986). It is a relatively expensive as the lifetime the graphite tubes is short due to the high atomisation temperature required (Brown *et al*, 1982). HGAAS requires sample digestion and has an analysis time of 1 to 2 min per sample (Ringstad and Thelle, 1986). Verlinden *et al* (1981) recommend HGAAS for routine analysis and a sample throughput of 100 to 150 duplicate analysis within a working week. Both techniques suffer from problems of poor precision, interferences and losses due to volatilisation.

1.14.4.1 GFAAS

Selenium can exist in several oxidation states which results in difficulties in determination by GFAAS. The different oxidation states can exhibit different thermal stabilities depending on the matrix modifier used (MacPherson *et al*, 1988). Many different elements have been employed as matrix modifiers. Nickel and Ag have been found to raise ashing temperatures to 1050 and 1250^{0}C respectively without losing Se (Saeed *et al*, 1979). Garcia-Olalla *et al* (1991) studied the effects of various single and mixed metal chlorides, sulphates and nitrates. Among the metals studied (Pd, Hg, Cd, Ni, Cu, Mg and Ag), the best enhancement of the Se atomic absorption signal was obtained by the mixed pair Hg-Pd chloride. The use of a Pd modifier produces a Se peak height signal at least twice that produced by Ni modification (Pohl *et al*, 1987).

Interferences are problematic with this technique (Saeed and Thomassen, 1981). Deuterium background correction and Zeeman effect background correction eliminate many interferences and allows Se determination in many types of biological samples. GFAAS with Zeeman background correction and a L'Vov platform is thought to be as accurate a method as ID-MS (Reamer and Veillon, 1981b), but ID-MS is twice as precise as GFAAS (Lewis *et al.*, 1986). The use of an electrodeless discharge lamp also increased the sensitivity of this technique. GFAAS has been used to determine Se levels in water, food, urine, plasma and geological samples.

1.14.4.2 HGAAS.

HGAAS requires that the sample be digested prior to analysis. Indeed many of the problems encountered by HGAAS are as a result of improper sample decomposition (Inhat *et al.*, 1986). After digestion Se is often present as selenate and this needs to be converted to selenite for hydride generation. This step is achieved by the use of $NaBH_4$ and HCl. At this stage interferences from ions such as Cu(II), Fe(III) and Co(III) can be overcome by the use of a masking agent (e.g: quinidin-8-ol or thiourea). A limiting factor in HGAAS is that foam formation occurs after the tetraborate addition which reduces the applicable sample volume to 20 µl (Ringstad and Thelle, 1986). In order to overcome this, complete sample mineralisation is essential but this increases the complexity of the digestion procedure and may lead to sample losses and contamination. Haygarth *et al.* (1993) found that the use of a flow injection HGAAS system had superior sensitivity compared to the single sample system. HGAAS has been used to determine Se levels in water, soil, ores, blood, food and environmental materials (Raptis *et al.*, 1983).

1.14.5. Inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS).

The determination of Se by ICP-MS was found to be problematic due to insufficient ion beam intensity for the available sample sizes and the relatively large ion beam backgrounds due to the argon plasma interfering ions ($^{38}Ar^{40}Ar^{+}$, $^{40}Ar_2^{+}$) which have mass/charge ratios that overlap with Se isotopes (Ting *et al.*, 1989). Later work by Buckley *et al.* (1992) overcame these problems by using a continuous flow hydride generation technique in conjunction with isotope dilution ICP-MS. A detection limit of 1.3 ng g^{-1} in the sample was observed for routine analysis and the method was verified by the use of CRM. The purchase price of an ICP-MS ranges from £150,000-200,000 and the operating costs are very high due to the large amounts of Ar used in its operation (Bersier *et al.*, 1994). ICP-MS is most cost effective when it is used in multi-element determinations and is not routinely available in most analytical laboratories. An experienced operator is also required.

ICP-AES is an expensive instrument both to purchase and to run. Again an experienced operator is required and it is best suited to multi-element analysis. However, it has about the same sensitivity as flame AAS (1 mg g^{-1}) for Se (Bersier *et al*, 1994) and therefore is not often used for Se determination.

1.14.6. Other analytical techniques.

1.14.6.1. Neutron Activation Analysis (NAA).

NAA is a highly sensitive and highly specific analytical technique. Little sample pretreatment is required and either the short-lived ^{77}Se (half-life = 17.5 s)

or the long-lived ^{75}Se isotope (half-life = 120 days) can be used in analysis. This method has been used to determine Se levels in blood, plasma, urine, food, water, hair and geological materials (Raptis *et al*, 1983). Limits of detection are in the order of 4 to 10 ng with relative standard deviations of less than 10% (Lewis and Veillon, 1989). Disadvantages include availability of a neutron source, high costs, long analysis time and the disposal of radio-active samples.

1.14.6.2. Negative ion thermal ionisation mass spectrometry (TIMS) of selenium.

This technique uses a hot metal filament to form negative Se ions. A linear amount of an artificially enriched Se isotope (^{82}Se) is added to the sample prior to digestion and Se converted to SeH_2, which is absorbed in conc. HNO_3. Evaporation of the solution is followed by mixing the residue with silica gel suspension and this is placed on the evaporation filament of the TIMS for the determination of the $^{80}Se/^{82}Se$ m/z ratio value. Negative ion TIMS has been used to determine Se levels in food, water and soil samples with a limit of detection of 6 ng g^{-1} (Heumann and Wachsmann, 1989). However, the instrumentation is not a commonly available technique, is very expensive and is capable of analysing only small numbers of samples per day.

1.14.6.3. Electroanalytical methods.

Electroanalytical methods include potentiometry (Setzik and Umland, 1978), ac-polarography (Alam *et al.*, 1976) and anodic stripping voltammetry (Posey and Andrews, 1981). These methods are not in common use for Se determination and the commercial availability of highly automated electroanalytical instrumentation is limited. The techniques also suffer interferences from Cu and Fe, thus requiring the use of a separation step such as liquid chromatography. Losses occurring from incomplete mineralisation will not be accounted for by this method.

1.14.6.4. Nuclear techniques.

Nuclear techniques include proton induced X-ray emission (PIXE) (Hyvonen-Dabek *et al*, 1984) and direct energy-dispersion X-ray fluorescence spectrometry (Pella and Dobbyn, 1988). Both techniques require complex and expensive equipment and suffer from poor sensitivity.

1.14.7. Summary.

The definitive methods for Se determination are NAA and ID-MS. Hydride generation atomic absorption spectrometry has been commonly used for large sample batch analysis. The availability of GC-MS equipment may now see ID-MS becoming both the routine as well as the definitive analytical methodology.

1.15. Aims of research.

The determination of Se concentrations in environmental samples has been problematic due to the volatile nature of Se and interferences from other ions present. Isotope dilution-mass spectrometry gives accurate results and complete quantitative recovery is not essential. The effects of applying sewage sludge containing Se to agricultural land has received little attention. The aim of this work work was to develop a method for Se determination in plants, soil and sewage sludges by ID-MS. After optimisation of the methodology, the aim was to use a sequential extraction procedure followed by ID-MS to examine which soil fractions Se was associated with. The last stages of the project was the application of the methodolgy to study the uptake of Se by plants grown in soils amended with sewage sludge.

Chapter 2. Development of methodolgy to measure selenium by ID-MS.

2.1. Introduction.

This section describes the development of the methodology for the quantitation of Se by ID-MS. The relative abundances of the ions in the molecular ion region (M^+) of the selenium nitrophenylenediamine (Se-NPD) were determined for both naturally occurring Se and the ^{76}Se isotope solution. The chemical purity and the concentration of the isotope solution were confirmed by reverse isotope dilution. Detection limits and the percentage yield of Se-NPD were established. The method was validated for accuracy by the use of certified reference materials (CRM). Reagents and procedures which were used throughout experimental work are common to both this and later Sections.

2.2. Instrumentation.

A gas chromatograph (GC) (Model 5890) coupled to a 5791A quadrupole mass spectrometer (Hewlett Packard, Cheshire, England) was used. A capillary DB-1 GC column (Jones Chromatography, Mid Glamorgan, U.K.) of 25 m length, 0.2 mm internal diameter and 0.33 μm film thickness was connected to the ion source. The carrier gas was He at a flow rate of 50 cm^3 min^{-1}. The injections were in splitless mode and performed manually. The initial temperature was 120^0C, rising to 200^0C at a rate of 5^0C min^{-1}. The injector and transfer line temperatures were 250 and 280^0C respectively, with a purge gas flow rate of 20 cm^3 min^{-1}. All injections were in splitless mode and performed manually. These conditions were adapted from those used by Ducros and Favier (1992). The mass spectrometer was operated in electron impact (EI) mode with an electron energy of 70 eV and was programmed using MS Chemstation 1989/90 software (Hewlett Packard, Cheshire, England). The electron multiplier voltage (EMV) was set by

the Quicktune performed at the start of each day. This was in the range of 1850-2000V each time and a change in EMV was found to have no effect on quantitation results.

2.3. Materials and reagents.

Reagents were of 'AristaR' grade: HNO_3 (18.5 M), HCl (12 M), HF (28.9 M) (Merck, England), ammonium hydroxide (14.8 M), chloroform and H_2O_2 (30 volume) (Fisons, England). Standards were prepared from 1000 mg dm^{-3} Se atomic absorption standard (AAS) solution (Fisons, England). Water with a resistivity of 18 $M\Omega cm^{-1}$ and obtained from an Elgastat UHQ II pure water system (Elga, England).

The derivatising agent was 4-nitro-1,2-phenylenediamine (Merck, Germany). A solution was prepared by dissolving 100 mg in 25 cm^3 of 0.1 M HCl. The solution was purified by extraction with cyclohexane (Fisons, England) (3 x 25 cm^3). The solution was then centrifuged to remove small amounts of particulate matter and the supernatant transferred to a plastic container and stored in the dark (foil wrapped) at 4^0C. A fresh solution of the derivatising agent was prepared every 2 weeks (or as required).

An indicator solution consisting of 20 mM EDTA (disodium salt), 10 mg dm^{-3} bromocresol purple (Fisons, England) and 7 M ammonium hydroxide was prepared.

2.3.1. Cleaning of containers and silanisation of GC insert.

Glassware and plasticware were cleaned by soaking for 16 h in reagent grade 4 M HNO_3 and then for 24 h in 18 $M\Omega cm^{-1}$ water, dried at 50^0C and finally

wrapped in cling film for storage. The glass GC insert was silanised by sequential rinsing with methanol, then acetone and finally methanol. After drying it was covered (0.5 h) with dimethylchlorosilane (2%) in dichloroethane (Fisons, England) and drained. After air drying the insert was returned to the GC/MS.

2.3.2. Preparation of ^{76}Se isotope solution.

Elemental ^{76}Se was obtained (19.8 mg, certified isotope enrichment = 98.5% +/-0.2) (Europa Scientific, Cheshire, England).
Preparation and calculation of concentration was obtained by the following procedure:

Total weight of bottle + ^{76}Se = 3.6516 g
Total weight of bottle (Se removed) = 3.6345 g
Weight of bottle after wash + HNO_3 = 3.6318 g
Hence weight of ^{76}Se isotope = 19.8 mg
The sample was heated at 80^0C for 4 h in an oven to dissolve the Se in the concentrated HNO_3. After 4 h a few pieces of undissolved material remained. The solution was filtered through silanised glass wool into a 200 cm^3 Azlon bottle and approximately 100 cm^3 deionised water added.
Weight of bottle + top + label = 16.6549 g
Weight of bottle + top + ^{76}Se + HNO_3 + water = 125.6101 g
Hence concentration of ^{76}Se solution = 181.73 $\mu g\ g^{-1}$

The stock solution was stored at 4^0C. The exact concentration and isotopic composition of this solution was determined by GC/MS: see Sections 2.11 and 2.12.

2.4. Derivatisation of standards.

The method used was that of Ducros and Favier (1992). The Se AAS solution (0.5 cm^3) was placed in a pyrex tube and 200 µl of 6 M HCl added. The tube was placed in a water bath (95^0C) and heated for 5 min. This step was repeated until 1 cm^3 of HCl had been added. This ensured all the Se was present as Se(IV). The solution was cooled and 2 cm^3 of the indicator solution added. The solution was again heated in the water bath (95^0C) until a pale yellow colour appeared (on cooling the purple colour sometimes reappeared necessitating further heating). The addition of the indicator solution ensured the sample was of the correct pH prior to derivatisation. After cooling, 5 cm^3 of 0.1 M HCl was added, the tube covered and stored in darkness at room temperature overnight.

The following day 0.5 cm^3 of the derivatising agent was added and the tube heated at 40^0C for 30 min. After cooling 5 cm^3 of $CHCl_3$ was added and the tube shaken on a vortex mixer for 5 min to extract the piazselenol complex (Se-NPD) (Figure 2.1). The organic phase was transferred to a round bottomed flask and the $CHCl_3$ evaporated using a rotary film evaporator. The residue was redissolved in 200 µl of $CHCl_3$ and transferred to 1 cm^3 Eppendorf tubes where the $CHCl_3$ again evaporated. Prior to analysis the residue was redissolved in 100 µl of $CHCl_3$.

2.5. Preparation of ^{76}Se isotope solution for determination of isotope abundance.

The ^{76}Se isotope solution (0.5 cm^3) was placed in a pyrex tube and derivatised as before. The isotope sample was stored in an Eppendorf tube ready for analysis.

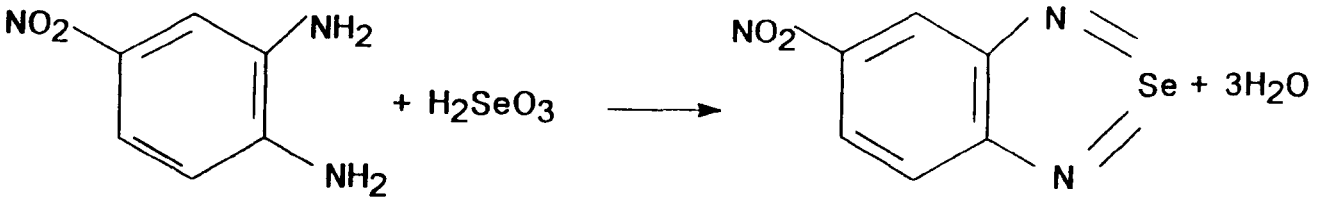


Figure 2.1. Derivatisation of selenium to selenium nitrophenylenediamine (Se-NPD).

2.6. GC/MS programmes for analysis.

2.6.1. Programme set up for mass spectrum of Se-NPD.

The GC/MS was set up as described in Section 2.2. The SCAN mode was selected for a mass range of m/z 20-250. The solvent delay was set for 12 min and 1 μl of the Se-NPD in $CHCl_3$ (Section 2.4) was injected. The mass spectrum of Se-NPD obtained is shown in Figure 2.2.

2.6.2. Setting up in selected ion monitoring (SIM) mode.

Peak centering was performed for the base peak m/z 229 and was found to be m/z 228.9. The GC/MS was programmed in SIM mode to examine the mass at m/z 229 and 225 only. This also results in an increase in sensivity compared to SCAN mode. The SIM cycle time was 4.1 cycles s^{-1}.

2.7. Time window for calculation of m/z 229/225 ratio.

A time window of 420 ms was found to account for each of the m/z 229 and 225 chromatograms and give the same start and stop time for both peaks during integration. The start of each peak was better defined than the end and 420 ms ensured the complete end of the tail was included. Although the length of the time window was kept constant, the time the window opened was dependent on the retention time of the peak to ensure the whole peak appeared in the window.

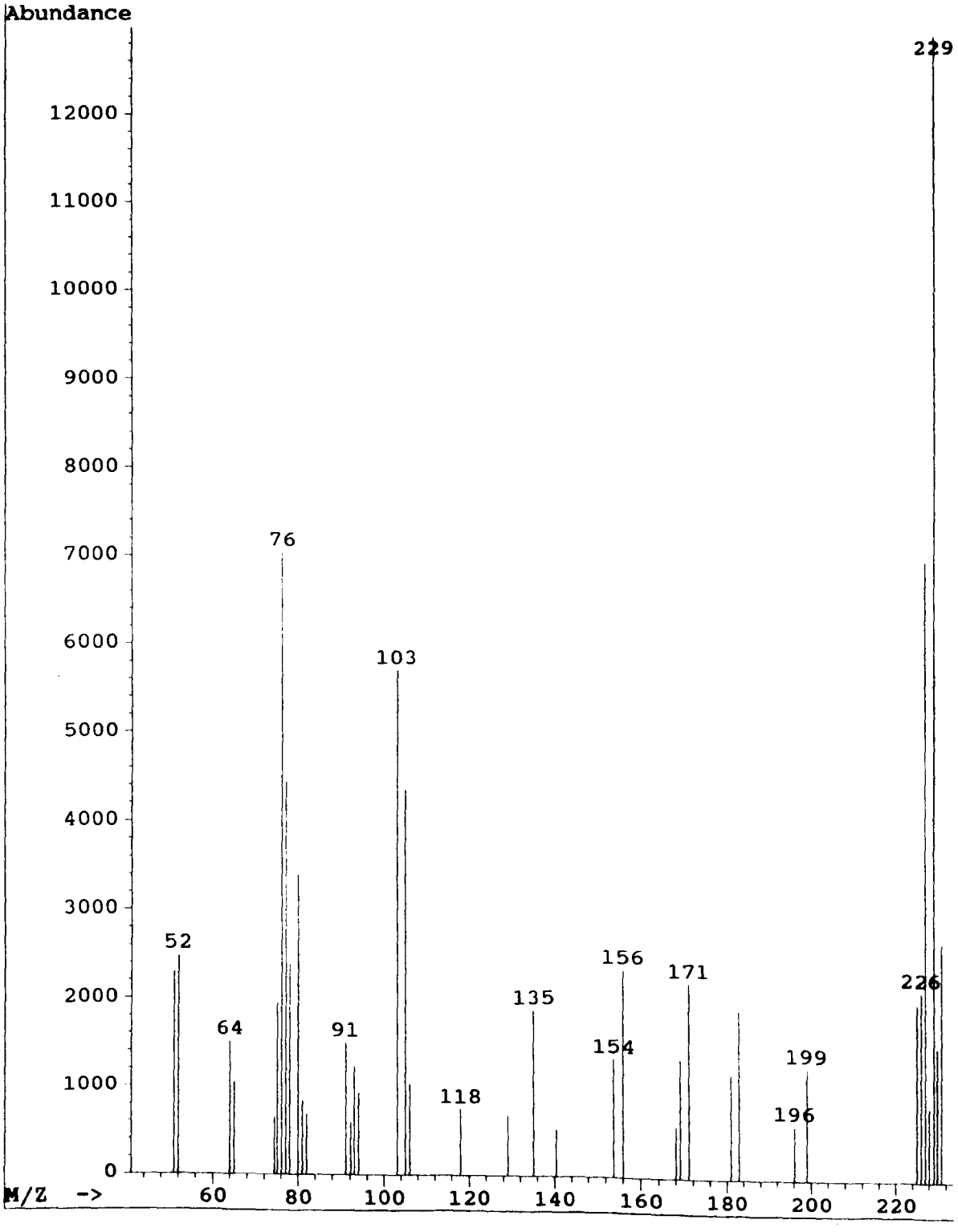


Figure 2.2. Mass spectrum of selenium standard as Se-NPD.

2.8. Integration of the mass chromatograms.

The software of MS Chemstation has 3 integration packages. The calculation of the m/z 229/225 ratio was determined from 10 replicate samples of the standard. The theoretical m/z 229/225 ratio of Se-NPD based on the natural isotope abundances of Se and ^{2}H, ^{13}C, ^{15}N, ^{17}O and ^{18}O is 5.5365 (De Bievre and Barnes, 1985) . The 3 integration packages are described as follows:

2.8.1. RTEint.

The RTEint integrator is a fast integrator designed to handle simple chromatograms such as those found in target compound identification or extracted ion chromatograms. The RTEint parameters are shown in Table 2.1. The default parameters are shown below:-

Threshold = 5%
Max^{m} number of peaks = 100
Bunch = 1
Start = 0.200%
Stop = 0.00%
Baseline reset = 5
Valley = 100

These parameters gave m/z 229/225 ratios of:
mean = 4.99, standard deviation (s.d) = 0.29, % coefficient of variation (C.V.) = 5.7%.

Changing the baseline reset to 200, 500 and -500, and valley to 50 gave no change in results. A threshold value of <5% and changing stars and stop values also did not influence results.

Table 2.1. RTEint integration parameters

Parameter	Range	Meaning
Area threshold	0 to 100 Relative	Peaks with areas less than the value entered are not reported
Maximum # of peaks	1 to 100	Limits number of peaks reported
Bunch	1 to 9 and -1 to -9	Number of points used in peak detection calculation. Negative values give additional smoothing
Detector threshold -start	0 to 100%	Determines how much the slope sensitivity calculation must exceed the absolute data to trigger a peak start. (0.2 optimal for most cases)
Detector threshold -stop	0 to 100%	Determines the peak end
Baseline reset	-500 to 500	Determines number of scans that must separate 2 peaks for the peak start/stop points to define the baseline
Valley	1 to 100%	Determines whether baseline drop (100%) or tangent skim (1%) is used in drawing baseline

The setting of valley to 1 gave m/z 229/225 ratios of:

mean = 5.39, s.d = 0.23, C.V. = 4.3%

Keeping the valley as 1, the bunch value was changed as follows;

(a) bunch = -1 gave 229/225 ratios of mean = 5.36, s.d = 0.20, C.V. = 3.8%

(b) bunch = 3 gave 229/225 ratios of mean = 5.59, s.d = 0.23, C.V. = 4.0%

(c) bunch = -3 gave 229/225 ratios of mean = 5.54, s.d = 0.20, C.V. = 3.7%

From these results the optimum parameters for this package were:-

Threshold = 5%

Max^{m} number of peaks = 100

Bunch = -3

Start = 0.200%

Stop = 0.00%

Baseline reset = 5

Valley = 1

2.8.2. Autointegration.

Autointegrate determines the optimum values for the initial threshold and peak width parameters, sets the initial area reject to zero, and then automatically integrates each peak in the chromatogram. Ten replicate injections gave 229/225 ratios of mean = 5.53, s.d = 0.12, C.V. = 2.2%. The autointegrate package generaly gave much better precision, often better than 2.2%.

2.8.3. Integration.

If autointegration does not integrate the required peaks, for example a small peak (abundance < 800), then the integration command allows a lower threshold setting to be selected and used.

2.8.4. Integration package selection.

It was decided to use the Autointegrate package for this project. Allowing the software to select the optimum parameters gave both the theoretical isotope ratio and excellent reproducibility.

After the selection of the integration package, which gave optimal results, 10 replicate injections were carried out to determine the m/z 229/225 ratio. This procedure was also used to determine the isotopic composition of Se as Se-NPD as discussed in Section 2.11.

2.9. Comparison of total ion current (TIC) abundance vs m/z 229/225 ratio.

The injection of replicate samples of Se-NPD resulted in a large variance in the spread of TIC abundance values which led to worries that 229/225 m/z ratio may be related to TIC abundance. In Section 2.8 ten replicate injections were used to determine the m/z 229/225 ratio. The TIC abundance from each injection was recorded and plotted against the 229/225 ratio. This is shown in Figure 2.3 where it can be seen that there is a large spread of TIC abundances despite all the injections being of the same volume from the same solution and using identical GC/MS conditions. However, the total TIC abundance appears to have no correlation with the m/z 229/225 ratio. Good precision (C.V. <3%) was also observed within the m/z 229/225 ratio range of values.

2.10. Determination of yield of Se-NPD.

The Se standard solution (0.5 g x 3) was derivatised as in Section 2.3. After the $CHCl_3$ was evaporated off, the Se-NPD complex was digested in 4 cm^3 HNO_3 and 400 µl H_2O_2 on a hot plate for 2 h. The digest was diluted by a factor

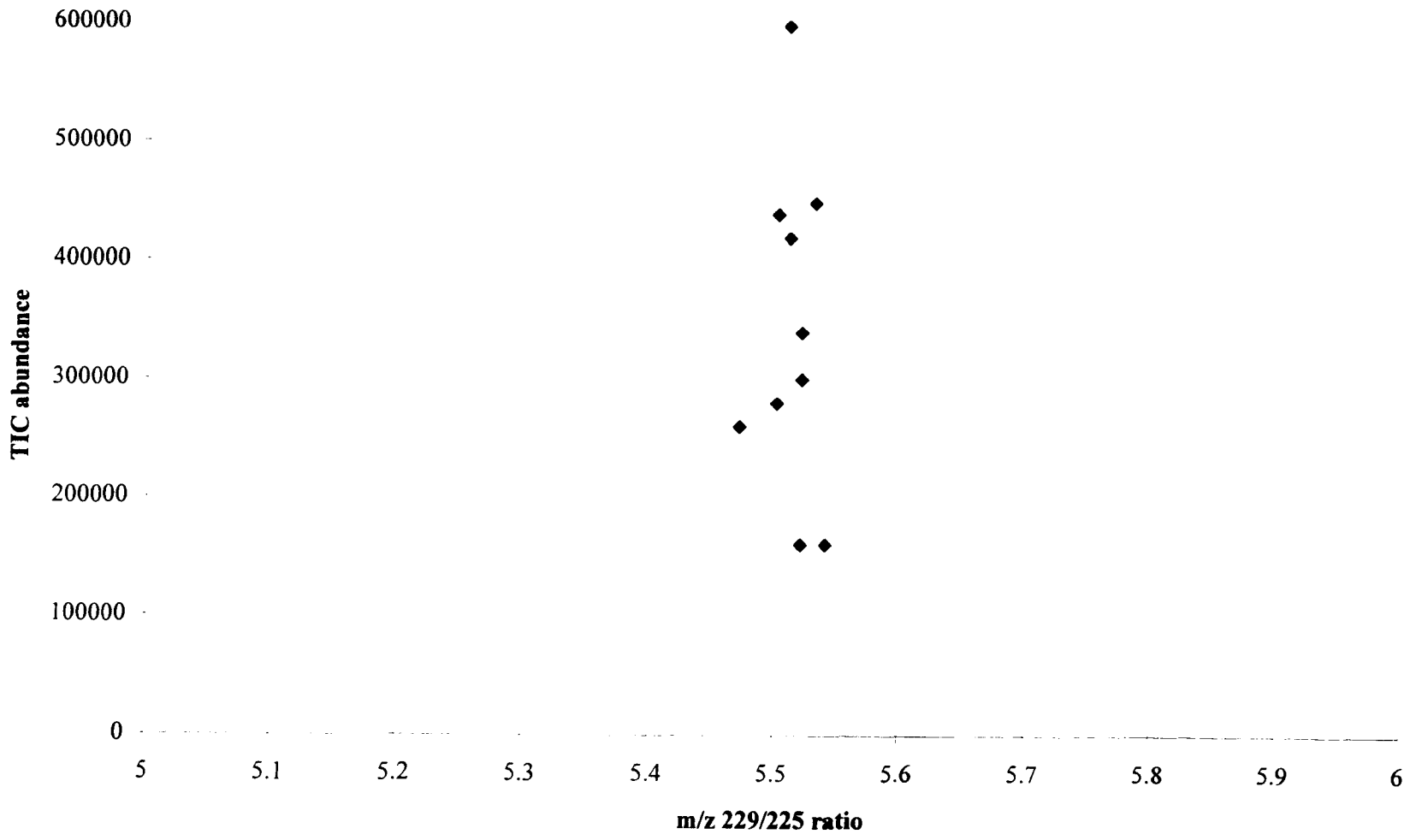


Figure 2.3. Plot of TIC abundance versus m/z 229/225 ratio.

of 5000 with 18 $M\Omega cm^{-1}$ water. Standards of 10, 25 and 40 μg/l were prepared. A calibration graph was obtained on a Perkin Elmer model 1100B atomic absorption spectrophotometer equipped with an autosampler. The samples were analysed in triplicate and the concentration determined from the graph. A Se hollow cathode lamp was used for determination of Se at a wavelength of 196.1 nm and a slit of 2 nm. The temperature programme used was as follows:-

(a) temperature 120^0C, ramp 10 s, hold 10s
(b) temperature 900^0C, ramp 5 s, hold 10 s
(c) temperature 2200^0C, ramp 1 s, hold 8 s
(d) temperature 2600^0C, ramp 1 s, hold 5 s

From Table 2.2 it can be seen that the mean recovery of Se was 93.3% after 1 extraction and 94.4% after 2 extractions.

Table 2.2. Recovery of selenium

Sample	Original Se content ($\mu g\ cm^{-3}$)	Se recovered ($\mu g\ cm^{-3}$) after 1 extraction	Recovery (%)	Se recovered ($\mu g\ cm^{-3}$) after 2 extractions	Recovery (%)
1	103.3	90.0	90.5	94.2	90.1
2	102.8	101.5	98.7	102.5	99.7
3	102.9	93.5	90.8	96.1	93.4

2.11. Determination of the isotopic composition of natural selenium.

The Se AAS standard (0.5 cm^3) was derivatised as in Section 2.3. The GC/MS was centred on the most abundant ion (Section 2.6.2) and ions were selected in SIM mode (after peak centering) at the following m/z; 222.9, 223.9, 224.9, 225.9, 226.9, 227.9, 228.9, 229.9, 230.9, 231.9, 232.9. These ions were selected to cover the molecular ion (M^+) region of all the major

isotopic species of Se-NPD. The results for these ions allow the calculation of the relative abundances of the 6 Se isotopes. The derivatised standard (Section 2.3) was injected (no. of replicates = 10) and the abundances recorded shown in Table 2.3. The relative abundances of the 6 Se isotopes as Se-NPD are illustrated in Figure 2.4. A statistical analysis of the data is shown in Table 2.4.

The percentages of Se at m/z 223, 225, 226, 227, 229 and 231 correspond, in the main, to the Se isotopes ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se and ^{82}Se but isotopes of C, H, N and O cause a significant proportion of these signals to 'cross-talk' at neighbouring m/z values. This is not a problem for quantitation work as all Se being measured is as Se-NPD and these contributions can be compensated for so that the true abundances of each isotope of Se in the sample can be calculated. The corrections are obtained by assuming a decreasing percentage of the isotope in question (M^+) is in the peaks directly above it (M^++1, M^++2, M^++3). This calculated percentage must be removed from the mean percentage abundances before the value for the base isotope present in the M^++ 1 peak can be calculated. For Se the following peak spread (Table 2.5) is present:-

Table 2.5. Peak Spread for Se-NPD (The underlined numbers are the Se-NPD peaks corresponding to the Se isotope shown).

Se isotope	m/z of Se-NPD peaks									
74	<u>223</u>	225	226	227						
76		<u>225</u>	226	227	228					
77			<u>226</u>	227	228	229				
78				<u>227</u>	228	229	230			
80						<u>229</u>	230	231	232	
82								<u>231</u>	232	233	234

	m/z										
	223	224	225	226	227	228	229	230	231	232	233
Sample											
1	91229	11264	1009284	897033	2688102	202715	5518398	407119	988338	74677	0
2	100751	12457	1106897	985955	2947472	222548	6084149	454222	1093987	81257	0
3	130869	14895	1395013	1245317	3720782	279403	7702986	568368	1383508	101955	0
4	115000	13980	1288986	1148907	3429125	257548	7112327	528741	1278067	95189	0
5	48596	0	519799	462600	1381108	108349	2882055	217803	519209	37880	0
6	50635	0	557017	497349	1470756	112355	3073663	229762	553790	40990	0
7	241238	25854	2646491	2376320	7021753	525072	14577258	1079516	2607443	193599	16429
8	170149	19448	2123945	1898256	5644493	422844	11752901	866789	2093168	154406	13343
9	167118	18361	1926195	1721774	5142880	381996	10611107	778616	1903758	139709	11867
10	225759	25492	2103827	1877856	5617377	415565	11569991	847855	2053853	151021	13431
	% 223	%224	%225	%226	%227	%228	%229	%230	%231	%232	%233
	0.7674	0.09475	8.489826	7.545601	22.61159	1.705184	46.41928	3.424576	8.313634	0.628163	0
	0.7697	0.095166	8.456247	7.532299	22.5175	1.700177	46.48045	3.470073	8.35762	0.620771	0
	0.7911	0.090038	8.432599	7.527714	22.49145	1.68894	46.56315	3.435681	8.363054	0.616299	0
	0.7532	0.091565	8.442474	7.524999	22.45975	1.686863	46.58362	3.463096	8.370958	0.62346	0
	0.7867	0	8.414529	7.488589	22.35744	1.753958	46.65483	3.525804	8.404978	0.613203	0
	0.7688	0	8.457185	7.551246	22.33048	1.705885	46.6674	3.488475	8.408189	0.622351	0
	0.7705	0.082572	8.45228	7.589416	22.42585	1.676958	46.55639	3.447724	8.327569	0.61831	0.05247
	0.6763	0.077298	8.441839	7.544815	22.43462	1.680637	46.71312	3.445143	8.319513	0.613703	0.053033
	0.7329	0.080519	8.446971	7.550521	22.55315	1.675173	46.53304	3.414476	8.348578	0.612668	0.052041
	0.9066	0.102369	8.448417	7.540976	22.55791	1.6688	46.46205	3.404763	8.247734	0.606461	0.053935
Mean	0.7723	0.0714	8.4482	7.5396	22.474	1.6943	46.5633	3.452	8.3462	0.6175	0.0211
S.D	0.0575	0.0384	0.0193	0.0254	0.0899	0.0246	0.0948	0.0364	0.0473	0.0064	0.0273
%C.V	7.44	43	0.23	0.34	0.4	1.45	0.2	1.05	0.57	1.01	0

Table 2.3. Ion abundances at m/z values across molecular ion region of Se as Se-NPD.

	%223	%224	%225	%226	%227	%228	%229	%230	%231	%232	%233
Mean	0.77232	0.071428	8.448237	7.539618	22.47397	1.694258	46.56333	3.451981	8.346183	0.617539	0.021148
Standard Error	0.018189	0.012139	0.006098	0.008036	0.02842	0.00777	0.029981	0.011523	0.014961	0.00201	0.008635
Median	0.76925	0.086305	8.447694	7.542896	22.4756	1.687901	46.55977	3.446433	8.353099	0.617305	0
Mode	#N/A	0	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	0
Standard Deviation	0.05752	0.038387	0.019284	0.025411	0.089871	0.024572	0.094807	0.036439	0.047312	0.006356	0.027306
Sample Variance	0.003309	0.001474	0.000372	0.000646	0.008077	0.000604	0.008988	0.001328	0.002238	4.04E-05	0.000746
Kurtosis	3.892205	1.078298	2.536056	2.482873	-0.76767	3.694177	-0.89539	0.5113	1.013725	-0.21977	-2.27323
Skewness	1.078013	-1.61126	0.593678	-0.09615	-0.17812	1.724093	0.103721	0.789058	-0.72248	-0.03389	0.485214
Range	0.2303	0.102369	0.075297	0.100827	0.281115	0.085158	0.293841	0.121041	0.160455	0.021702	0.053935
Minimum	0.6763	0	8.414529	7.488589	22.33048	1.6688	46.41928	3.404763	8.247734	0.606461	0
Maximum	0.9066	0.102369	8.489826	7.589416	22.61159	1.753958	46.71312	3.525804	8.408189	0.628163	0.053935
Sum	7.7232	0.714276	84.48237	75.39618	224.7397	16.94258	465.6333	34.51981	83.46183	6.175388	0.211479
Count	10	10	10	10	10	10	10	10	10	10	10
Confidence Level	0.03565	0.023792	0.011952	0.01575	0.055702	0.015229	0.058761	0.022585	0.029324	0.00394	0.016924

Table 2.4. Statistical analysis of relative isotope intensities in molecular ion region of Se-NPD.

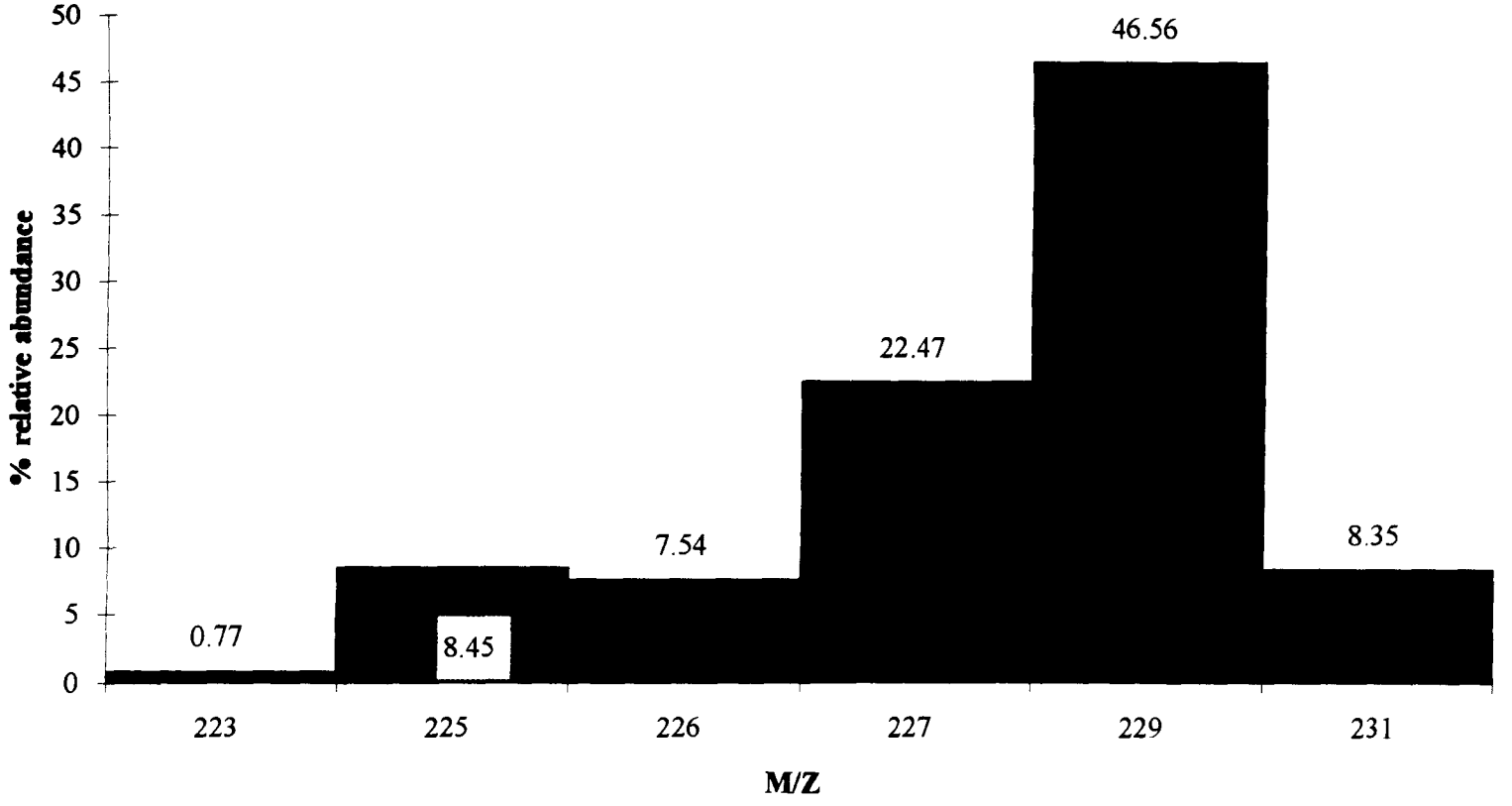


Figure 2.4. Relative abundances of m/z ratios of major ions in molecular ion region of Se-NPD.

As an example the peak at 224 contains ^{74}Se as a base isotope but due to interferences from C, N, O and H the peaks at m/z 225, 226 and 227 also contain some ^{74}Se. These "cross talk" values must be subtracted from each of the mean values. Four assumptions are made in this calculation, namely: the relative ion intensities of the major ions in the M^+ cluster for $C_6N_3O_2H_3$ are M^+, M^++1, M^++2 and M^++3 are 91.81, 7.25, 0.82 and 0.03 respectively. Hence, the equations for ^{74}Se and ^{76}Se are:-

^{74}Se = %223 + (%223 x 7.25/91.81) + (%223 x0.82/91.81) + (%223 x 0.03/91.81).

^{76}Se = [%225 - (%223 x 0.82/91.81)] + [(%225 - (%223 x 0.82/91.81)) x 7.25/91.81] + [(%225 - (%223 x 0.82/91.81)) x 0.82/91.81] + [(%225 - (%223 x 0.82/91.81)) x 0.03/91.81].

The resultant calculations are shown in Table 2.6. This calculation will be used again in section 2.12 as a check on the isotopic purity of the ^{76}Se isotope purchased.

Table 2.6. Abundances of isotopes of natural selenium

Se isotope	Evaluated limits of isotope (%)[a]	Calculated (%) abundance for Se-NPD
74	0.908-0.897	0.8404
76	9.052-8.982	9.1860
77	7.630-7.590	7.4794
78	23.550-23.487	23.7841
80	49.718-49.655	50.4565
82	9.209-9.141	8.6319

[a]Values from De Bievre and Barnes (1985)

2.12. Determination of the isotopic composition of ^{76}Se isotope solution.

This was carried out as in Section 2.11 for natural abundance Se and the mass spectrum obtained (Figure 2.5). The abundances are shown in Table 2.7 with the statistical analysis in Table 2.8. After correction for contributions from other isotopic species, the relative abundances of the Se isotopes are shown in Table 2.9. Figure 2.6 shows the relative abundances of the 6 isotopes of Se of the spike solution as Se-NPD. The isotopic purity (Table 2.9) was calculated as before and the Se in the isotope is > 99% ^{76}Se.

Table 2.9. Abundances of isotopes of enriched selenium

Se isotope	Calculated (%) Abundance for ^{76}Se-NPD
74	1.1617
76	99.3950
77	-0.4034
78	-0.1685
80	0.1171
82	-0.0010

2.13. Calculation of the concentration of selenium in the spike solution by reverse ID-MS.

The chemical purity of the ^{76}Se isotope solution is an important criterion for ID-MS; the presence of impurities would lead to errors in determining the concentration of the spike solution if this were done by gravimetric methods alone. To confirm the chemical purity and calculate the concentration of the spike solution, reverse ID-MS was used. In this case the Se AAS standard was used as the 'spike'. The greatest accuracy occurs in a 1:1 ratio mixture for ID-MS (Heumann, 1988). By assuming the concentration of the isotope solution was

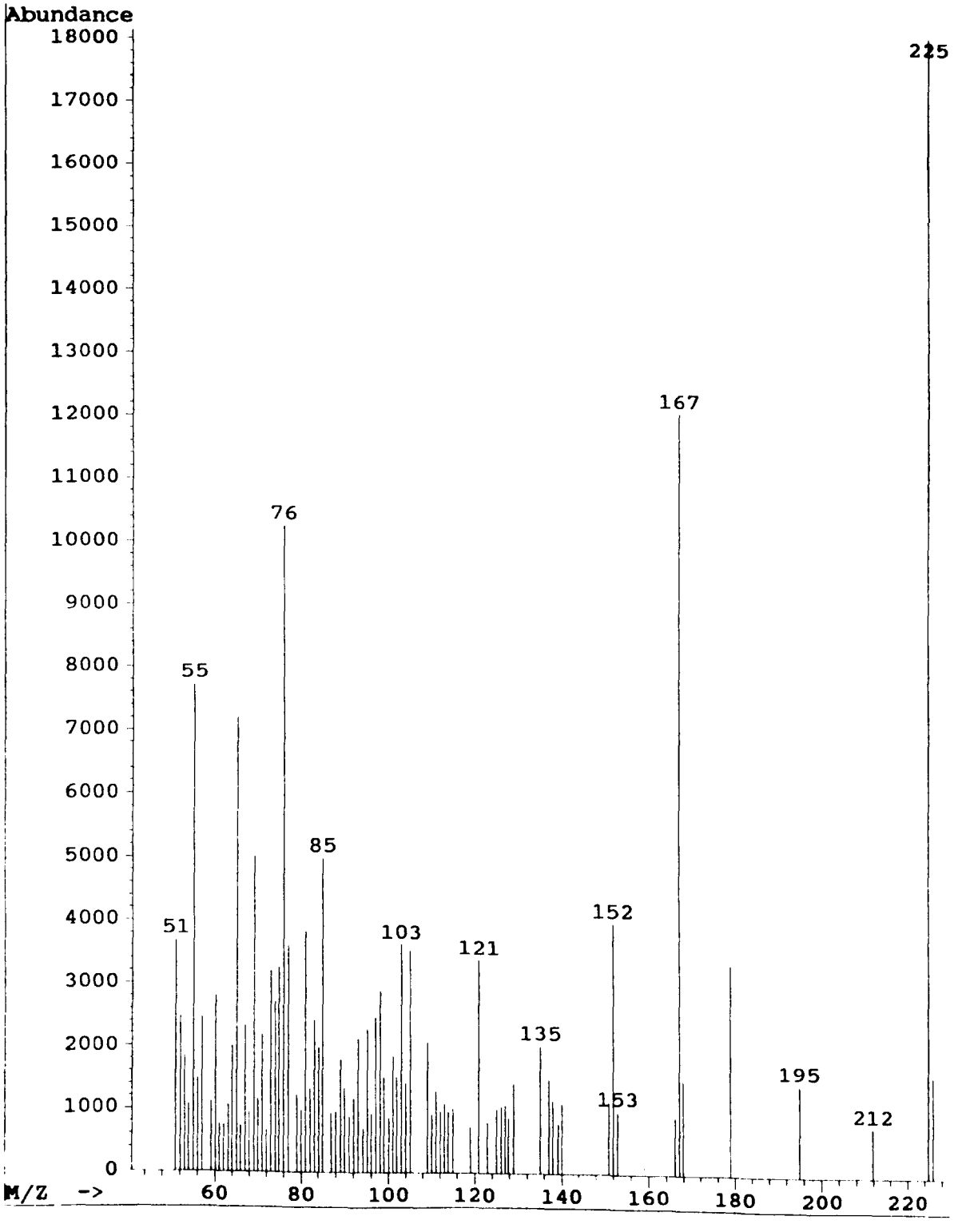


Figure 2.5. Mass spectrum of ^{76}Se isotope solution as Se-NPD.

	m/z										
	223	224	225	226	227	228	229	230	231	232	233
Sample											
1	98012	11835	7878122	598040	0	0	0	0	0	0	0
2	128515	16249	10668165	802063	75848	0	13888	0	0	0	0
3	81549	11165	6785791	511541	47038	0	9681	0	0	0	0
4	88646	12184	7764036	578166	54514	0	0	0	0	0	0
5	94505	11966	8266909	616094	57278	0	10233	0	0	0	0
6	132534	16475	11488147	860705	78882	0	11313	0	0	0	0
7	101248	7773	8522690	635926	59418	0	10206	0	0	0	0
8	180669	22527	15571544	1158044	105896	0	16516	0	0	0	0
9	141197	17626	12278771	917816	84761	0	13797	0	0	0	0
10	187314	24020	17055011	1277145	114910	0	16498	0	0	0	0
	%223	%224	%225	%226	%227	%228	%229	%230	%231	%232	%233
	1.141532	0.137841	91.75534	6.965285	0	0	0	0	0	0	0
	1.097975	0.138824	91.14407	6.85247	0.648012	0	0.118653	0	0	0	0
	1.095093	0.149931	91.12401	6.869305	0.631657	0	0.130003	0	0	0	0
	1.043195	0.143383	91.36798	6.803917	0.641526	0	0	0	0	0	0
	1.043449	0.132119	91.27661	6.802418	0.632418	0	0.112985	0	0	0	0
	1.052855	0.130878	91.26228	6.837474	0.626642	0	0.089871	0	0	0	0
	1.088679	0.08358	91.64103	6.837854	0.638898	0	0.109741	0	0	0	0
	1.059319	0.132083	91.30088	6.789978	0.620902	0	0.096839	0	0	0	0
	1.049482	0.13101	91.26505	6.821898	0.630007	0	0.10255	0	0	0	0
	1.003026	0.128622	91.32586	6.838833	0.615318	0	0.088343	0	0	0	0
Mean	1.067	0.1308	91.3463	6.8392	0.5683	0	0.1061	0	0	0	0
S.D	0.0385	0.018	0.1734	0.0507	0.1999	0	0.0465	0	0	0	0
%C.V.	3.61	13.76	0.19	0.74	284.29		43.83	0	0	0	0

Table 2.7. Ion abundances at m/z values across molecular ion region of Se isotope solution as Se-NPD.

	%223	%224	%225	%226	%227	%228	%229	%230	%231	%232	%233
Mean	1.067027	0.130794	91.3463	6.83922	0.568284	0	0.106113	0	0	0	0
Standard Error	0.012195	0.005684	0.054844	0.016028	0.063213	0	0.014706	0	0	0	0
Median	1.056087	0.132101	91.27637	6.829686	0.630832	0	0.099694	0	0	0	0
Mode	#N/A	#N/A	#N/A	#N/A	#N/A	0	0	0	0	0	0
Standard Deviation	0.038564	0.017974	0.173433	0.050683	0.199896	0	0.046505	0	0	0	0
Sample Variance	0.001487	0.000323	0.030079	0.002569	0.039959	0	0.002163	0	0	0	0
Kurtosis	0.5939	6.693636	5.640022	4.37394	9.939109	#DIV/0!	0.805288	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Skewness	0.418972	-2.33479	2.060436	1.910762	-3.14913	#DIV/0!	-1.44585	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Range	0.138506	0.066684	0.631331	0.175307	0.648012	0	0.130003	0	0	0	0
Minimum	1.003026	0.083247	91.12401	6.789978	0	0	0	0	0	0	0
Maximum	1.141532	0.149931	91.75534	6.965285	0.648012	0	0.130003	0	0	0	0
Sum	10.67027	1.307937	913.0982	68.3922	5.682835	0	0.848547	0	0	0	0
Count	10	10	10	10	10	10	10	10	10	10	10
Confidence Level(95.(	0.023902	0.01114	0.107493	0.031413	0.123895	#NUM!	0.028824	#NUM!	#NUM!	#NUM!	#NUM!

Table 2.8. Statistical analysis of relative isotope intensities in molecular ion region of ^{76}Se-NPD.

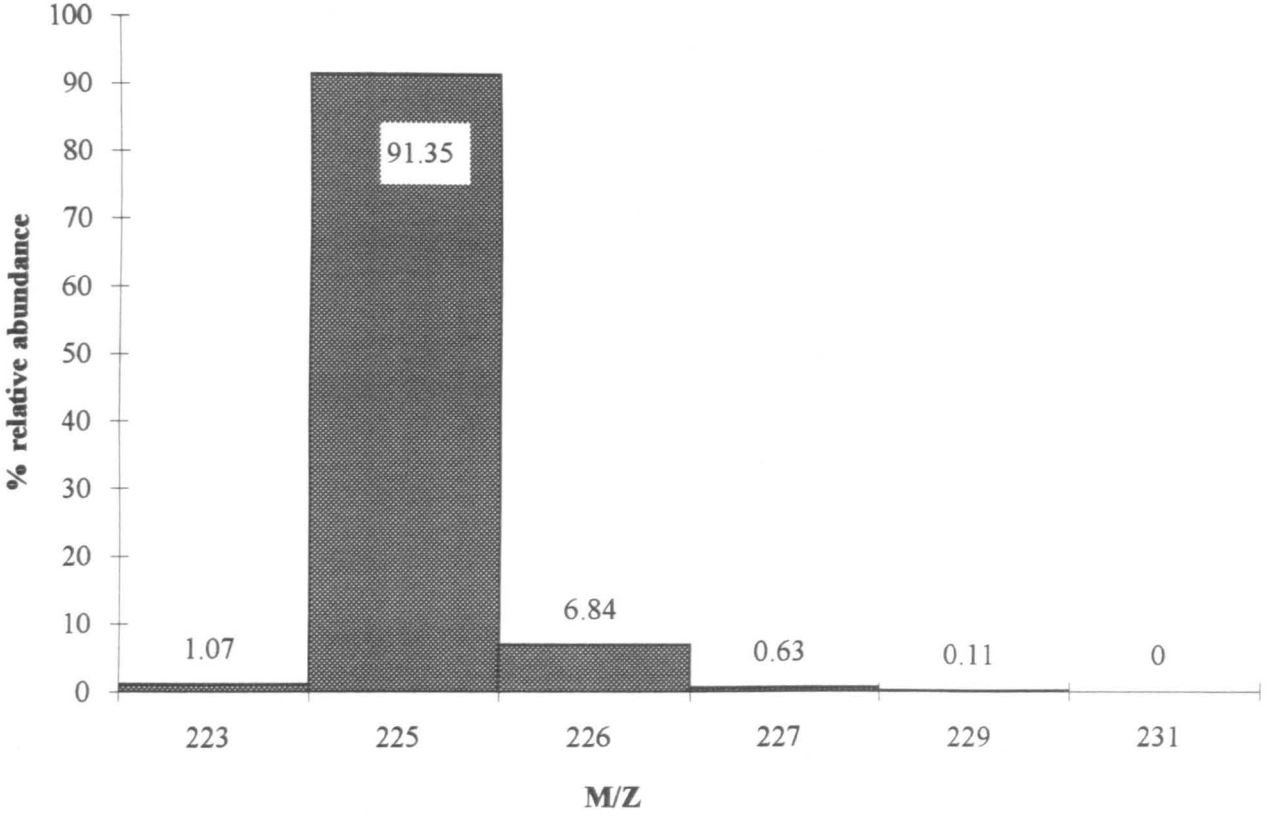


Figure 2.6. Relative abundances of m/z ratios of major ions in molecular ion region of ^{76}Se-NPD.

approximately 181.7 μg g^{-1} (Section 2.2) and the concentration of the standard solution was 1000 μg g^{-1}, the following mixtures were prepared (Table 2.10) to give m/z 229/225 ratios close to unity.

Table 2.10. Weights of standard and isotope solution for reverse ID-MS (*Expected weight assuming 100% chemical purity).

Sample	Standard Se (g) →	Weight Se (μg)	^{76}Se solution (g)→	Weight Se (μg)*
1	0.2049	204.9	0.5070	92.1
2	0.2045	204.5	0.5097	92.6
3	0.2051	205.1	0.5113	92.9
4	0.2049	204.9	0.5146	93.5
5	0.2071	207.1	0.5142	93.4

The solutions were derivatised as in Section 2.3. The GC/MS was focused on the most abundant ion and 3 standards run to ensure the m/z 229/225 ratio was correct. The GC/MS was programmed in SIM to determine the m/z 229/225 ratio of the 5 solutions. Each solution was analysed 5 times and the mean ratios are shown in Table 2.11.

Table 2.11. Mean m/z 229/225 ratios obtained for reverse ID-MS

Solution	Mean m/z 229/225	Standard deviation	C.V. (%)
1	0.9062	0.0023	0.25
2	0.8947	0.0013	0.15
3	0.8959	0.0021	0.24
4	0.9000	0.0031	0.35
5	0.9064	0.0020	0.22

The concentration of the isotope solution was calculated using the Pickup and McPherson equation:-

$$x = y(RQ_l - Q_k)/(P_k - RP_l)$$

where x=amount of Se in the sample in moles (in this case only this is the spike solution), y=amount of Se added in moles (in this case only this is the Se AAS solution), R=observed m/z 229/225 in the mole mixture ratio P_k, P_l, Q_k and Q_l are the relative abundances of the isotopes of natural Se and the spike solution at m/z 229 and 225 respectively. Hence the equation can be rewritten as:

$$x = y(91.35R - 0.11)/(46.56 - 8.45R).$$

The mean concentration of the isotope solution was found to be184.77 $\mu g\ g^{-1}$ (s.d = 4.007, C.V. = 2.1%). The concentration obtained by using gravimetric methods of ^{76}Se was 181.73 $\mu g\ g^{-1}$ (Section 2.3.3). Using the reverse isotope dilution data the stock solution was diluted accurately (by weight) for experimental work to give a second stock solution with a Se concentration of 18.507 $\mu g\ g^{-1}$.

2.14. Practical calibration data.

A set of calibration mixtures was prepared from Se AAS standard and the isotope solution weights shown in Table 2.12.

The solutions were derivatised and analysed on the GC/MS as before. The ratios obtained are shown in Table 2.13.

Table 2.12. Weight of standard and isotope solution for practical calibration graph (*Concentration = 184.77 μg g^{-1}).

Sample	Standard Se (g) →	Weight Se (μg)	^{76}Se isotope solution (g) →	Weight Se (μg)*	Mole ratio (unlabelled/labelled)
1	0.2077	207.7	2.0544	379.6	0.54
2	0.2063	206.3	1.5424	285.0	0.72
3	0.2068	206.8	1.0263	189.6	1.09
4	0.2063	206.3	0.4127	76.3	2.70
5	0.2064	206.4	0.2607	48.2	4.28

Table 2.13. m/z 229/225 ratios obtained for practical calibration graph

Sample	Mean m/z 229/225 ratio	S.D	C.V. (%)	Expected m/z 229/225 ratio*
1	0.2500	0.0008	0.33	0.2591
2	0.3272	0.0005	0.15	0.3373
3	0.4831	0.0022	0.45	0.4924
4	1.0744	0.0033	0.30	1.0778
5	1.5494	0.0066	0.43	1.5319

*Based on the chemical (100%) and isotopic purity of the ^{76}Se spike and AAS standard.

A practical calibration graph of isotope ratio versus mole ratio was prepared. The practical and theoretical calibration curves are shown in Figure 2.7. These are a quadratic functions (Rsq = 1 using curvefit in SPSS for windows). The curves show excellent agreement between theoretical and measured m/z 229/225 ratios across an acceptable working range of ratios (i.e: around unity range = 0.2 to1.5 where maximum precision is achieved).

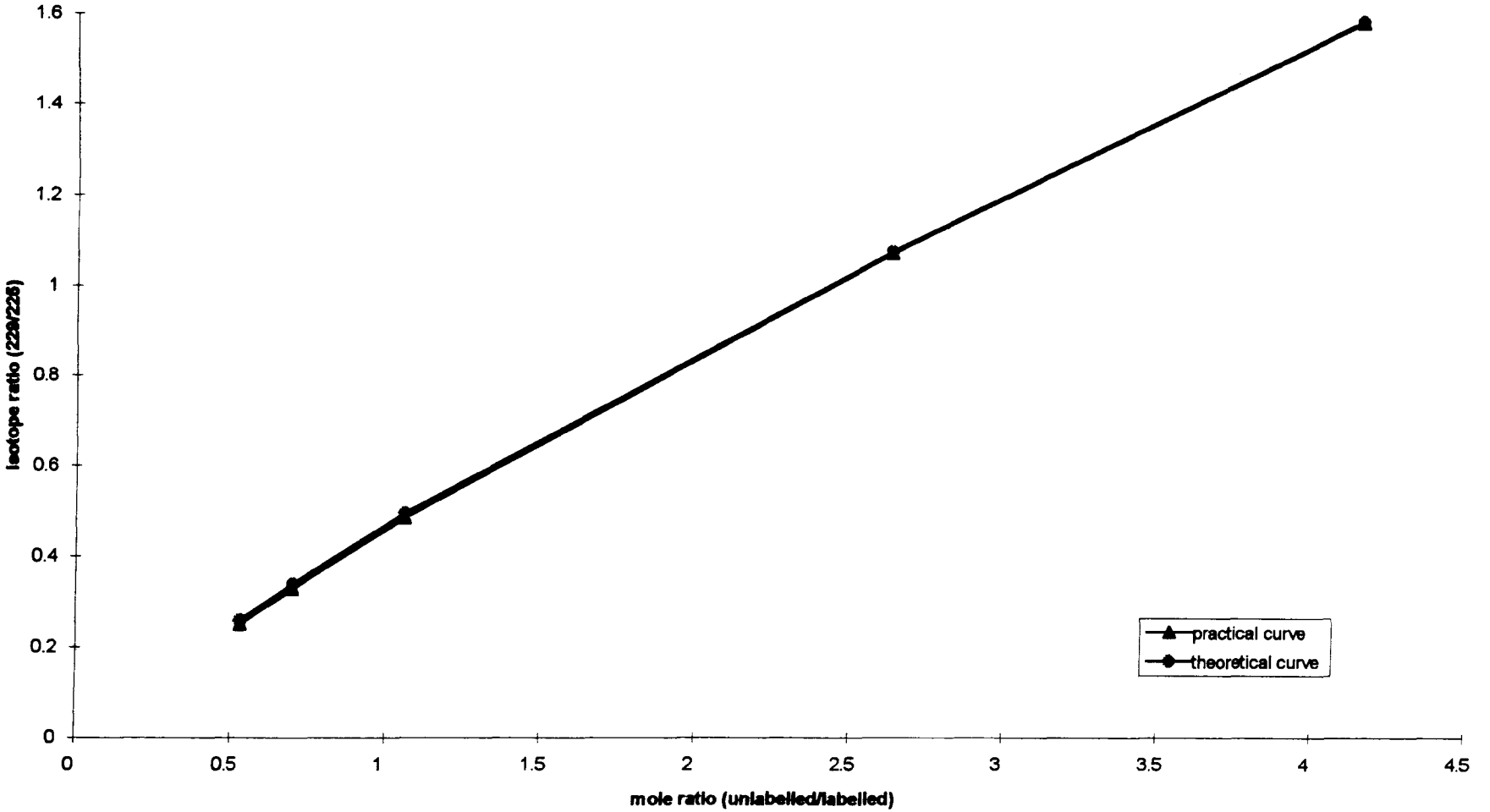


Figure 2.7. Calibration curves using enriched and standard selenium in various mole ratios against isotope ratios.

2.15. Memory effect.

A memory effect was observed to occur when samples were run after standards. For example, prior to each of the samples being run for the practical calibration graph, a standard was run. The m/z 229/225 ratio obtained for the first sample injected after the Se standard solution was run is shown in Table 2.14. The next column shows the mean m/z 229/225 value from 5 different sample injections. The first value obtained was as a result of memory effect from the previous standard and was not included in the calculation of the mean m/z 229/225 ratio. This problem was eliminated by thorough rinsing of the syringe with $CHCl_3$ after each injection. A blank run of $CHCl_3$ prior to sample injection confirmed the memory effect was no longer present.

Table 2.14. "Actual" data is a result of 1st injection of sample after standard. "Expected" data refers to the value achieved after the 3rd injection when the memory effect was no longer present.

Sample	Actual m/z 229/225 after standard run	Expected m/z 229/225
1	0.8369	0.2500
2	0.8957	0.3272
3	0.9852	0.4831
4	1.557	1.0744
5	2.3951	1.5494

2.16. Detection limits.

The Se AAS standard (1 cm^3) was derivatised and analysed on the GC/MS as before and by injecting progressively lower amounts on column sample dilution was achieved. It was analysed in quadruplicate and the mean result recorded. This was continued until a statisically different m/z 229/225 ratio was obtained. This

established a practical detection limit. The results are shown in Table 2.15. This shows that with injection sizes of > 10 ng natural abundance selenium, it is possible to obtain m/z 229/225 values that are not significantly different ($p<0.1$) from theoretical. With injection sizes of < 10 ng Se the ratio became increasingly inaccurate and imprecise. In all subsequent experiments attempts were made to ensure that > 10 ng Se were available for each SIM analysis.

Table 2.15. Practical detection limits for quantitation of selenium.

Sample size (ng) Se loaded onto column*	Mean m/z 229/225 ratio (n = 4)	C.V. (%)	P difference
100	5.59	2.3	<0.1
50	5.59	2.3	<0.1
20	5.53	2.1	<0.1
10	5.61	2.2	<0.1
5	4.32	5.2	
1	3.59	6.2	

* Injection volume = 2 µl

2.17. Validation of methodology.

In order to ensure the method gave accurate results, certified reference materials (CRMs) of plants and soils were analysed. There are no sewage sludge CRMs which are certified for Se.

2.17.1. Plants.

For plant material, the CRM used was cabbage leaves (C85-04, Laboratory of the Government Chemist, Middlesex, England). Cabbage was

selected because it is one of the few plant CRMs with certified Se levels. It is also a member of the *Cruciferae* family which are known Se accumulators (James *et al.*, 1989). Also it was another member of this family, the radish, which was used in pot experiments in this project at a later date (Chapter 5).

The CRM cabbage leaves were digested using a heating block at 150^{0}C. The CRM (5.0 g) was dried (McGaw, 1996), weighed, spiked with 0.1 g of the isotope solution (concentration = 18.51 μg g^{-1}) and the sample digested with 28 cm^3 18.5 M HNO_3, 4 cm^3 H_3PO_4 and 8 cm^3 H_2O_2. This was heated for 10 h or until a clear digest was obtained. This method was adapted from that of Dong *et al*, (1987). Digestion methods are discussed more fully in Chapter 3.

2.17.2. Soils.

The soil CRM was Chinese soil (C74-05, Laboratory of the Government Chemist). The method used for digestion was a modification of that used by Nham and Brodie (1989). The CRM soil (0.5 g) was placed in a 50 cm^3 Teflon bomb (Savillex, Minnesota, U.S.A.) and spiked with 0.1 g of the isotope solution as above. Nitric acid (18.5 M, 2 cm^3) was added and the mixture placed in an 800 W microwave oven. The sample was heated on low power for bursts of 0.5, 1, 1.5, 2 and 3 min. In between, the samples were cooled and vented. This eliminated organic material from the sample. Hydrofluoric acid (28.9 M, 10 cm^3) was added and the lid replaced and hand tightened. The sample was heated on a hot plate at 180^{0}C for 6 h and cooled for 16 h. The top was then removed and the HF evaporated at 120^{0}C for approximately 6 h. After cooling, 10 cm^3 of 18.5 M HNO_3 was added and evaporated at 120^{0}C for about 6 h. The residue was redissolved in 4 cm^3 of 3 M HNO_3.

2.17.3. Determination of selenium levels in plant and soil CRM.

These were derivatised as described in Section 2.4 and stored in Eppendorf tubes. Prior to analysis, the residues were redissolved in 25 µl of $CHCl_3$. The parameters in the GC-MS were set as before and the m/z 229/225 ratio obtained. The Se content of the cabbage leaves was determined from 3 replicate samples analysed 3 times each to give a mean of means value (+/-95% confidence interval) of 0.091+/-0.007 µg g^{-1} (certified value=0.083+/-0.008 µg g^{-1}) (Table 2.16). The soil CRM was analysed in the same manner to give a mean of means value of 1.67+/-0.04 µg g^{-1} (certified value=1.56+/-0.12 µg g^{-1}) (Table 2.16).

Table 2.16. Selenium levels in CRM cabbage leaves and soil (* n = number of replicates).

CRM	Mean Se concentration (µg g^{-1}) (n=3) *	C.V.(%)
Cabbage leaves a	0.087	5.5
Cabbage leaves b	0.094	2.2
Cabbage leaves c	0.092	<0.05
Soil a	1.67	1.5
Soil b	1.65	2.4
Soil c	1.68	0.3

For both CRM the random errors for our measurements are lower than the uncertainty values given for the certified materials and the mean Se concentrations lie within their 95% confidence intervals. In both cases the mean values are <10% higher than the certified concentration.

2.18 Discussion

The quantification of Se at low concentrations has in the past been problematic due to volatilisation losses and interference from other matrix ions in the sample. The concept of the ID-MS method originated with Reamer and Veillon (1981b) for biological materials and was further developed by Ducros and Favier (1992), also for biological samples. In this Chapter the method has been used on a standard benchtop GC/MS with a basic software package. The optimum integration method and time window have been identified and it has been shown that the m/z 229/225 ratio is independent of column loading with samples > 10 ng Se. The method gave good recovery (mean = 93.3%) after a single extraction.

The isotopic composition of natural Se (both as 'free' Se and as Se-NPD) was determined. The calculation of isotopic purity of the spike solution allowed verification that the spike solution was very close to the certified value of ^{76}Se (98.5 +/- 0.2%). The isotopic purity was determined to be >99% ^{76}Se. The chemical purity of the spike was obtained by reverse ID-MS and the concentration (184.77 $\mu g\ g^{-1}$) was found to be similar to that calculated by gravimetric methods assuming 100% purity.

The m/z 229/225 ratio was obtained for a range of molar ratios using a mixture of the Se AAS standard and the spike solution. Excellent agreement was observed between the obtained and the expected values and problems observed concerning a memory effect were overcome.

The practical detection limit was found to be 10 ng Se per 2 µl injection. The extraction into $CHCl_3$ is a preconcentration step and hence the detection limit of 10 ng can be referred back to the original sample weight to give the actual detection limit for each sample. As the m/z 229/225 ratio approaches unity in ID-MS experiments it may be possible to obtain good data with lower sample sizes.

The method was validated by the use of plant and soil CRM and the data showed no significant differences ($p<0.05$) between our data and the certified values.

2.19 Conclusion

This study has shown that isotope dilution - mass spectrometry using a benchtop instrument can give accurate and precise data in the quantitation of Se in standard and isotope solutions at a relatively low cost.

Chapter 3. Comparison of digestion procedures.

3.1. Introduction.

Sample preparation is the critical step in any chemical analysis. This is particularly necessary in the analysis of samples where Se is the element of interest. Unless complete sample digestion has occurred, then total Se concentrations cannot be accurately determined. There are also problems associated with sample digestion prior to analysis. Selenium forms relatively volatile compounds and losses can occur at temperatures above 120^{0}C (Fourie and Peisach, 1973). The method of analysis also influences the choice of digestion method. Determination of Se concentrations in this project were by ID-MS following the formation of the piazselenol. The formation of this chelate is dependent on Se being converted to Se(VI), which is then reduced to Se(IV) by heating with 6 M HCl.

3.2. Categorisation of digestion methods.

Digestion methods can be divided into 2 main groups:-

(1) Dry decomposition
(2) Wet decomposition.

The use of dry decomposition techniques such as dry ashing in muffle furnaces leads to volatile Se losses unless $Mg(NO_3)_2$ or MgO additives are added to the sample (Siemer and Hagemann, 1975). This method also suffers from possible sample contamination and is time consuming (48 h.).

Wet decomposition can be achieved in open or closed systems. In open systems, volatile Se losses can be minimised by using reflux condensers fitted to the digestion tubes. However, for complete digestion, the reagents used must

have a high oxidising potential. Various acid digestion mixtures have been reviewed by Raptis *et al.*, 1983, who concluded that in many cases $HClO_4$ was necessary to retain Se in solution. Perchloric acid is potentially explosive and its use requires a specifically designed fume hood and great care to be taken during handling. However, despite these problems, it has been the usual method of biological sample preparation for Se determination (Raptis *et al.*, 1983).

Wet decomposition in closed systems eliminate volatile Se losses and sample contamination. This method has become more commonly used with the increased availability of microwave oven techniques.

3.3. Digestion methods for soil and sewage sludge samples.

In order to quantify Se in soil and sewage sludge samples, the digestion procedure must be able to break down the silicon lattice network and hence free the Se associated with this phase. This procedure involves the use of HF which is a strong oxidisng agent. Studies carried out by Nham and Brodie (1989) investigated 3 digestion mixtures:-

(1) HNO_3-$HClO_4$-HF

(2) HNO_3-H_2O_2-HF

(3) HNO_3-HCl-HF.

The test sample used was the CRM Coal Fly Ash (1633A) and Nham and Brodie concluded that all 3 digestion methods gave similar results which fell within the certified values for Se concentration. Hence, the use of $HClO_4$ was not essential to achieve total sample digestion. Indeed, the use of a HNO_3-HF digestion mixture was found to be sufficient for complete digestion and was used

to determine Se concentrations in the soil CRM used for validation of the methodology (Section 2.17.2).

3.4. Digestion methods for plant material.

3.4.1. Wet digestion in digestion block (open vessel).

The digestion mixtures applied to biological samples for Se determination must be able to release the Se which is intrinsically bound in proteins as selenomethionine and selenocysteine. Although $HClO_4$ is an excellent oxidising acid for biological samples, its explosive nature has led to the investigation of other acid digestion mixtures. In 1981, Reamer and Veillon found that a digestion mixture of H_3PO_4-HNO_3-H_2O_2 gave results with the limits for Se concentrations in CRM (wheat flour, bovine liver and rice flour). The authors concluded that the digestion mixture was safe, efficient and relatively rapid. Reamer and Veillon (1983) applied this digestion mixture to serum, plasma and urine CRM samples that were predigested overnight and compared the results to those obtained using a $HClO_4$ mixtures. The analytical techniques used were fluorometry and isotope dilution GC-MS. Excellent agreement was obtained between the 2 digestion mixtures and the authors concluded that the phosphoric acid mixture could be used, eliminating the need to use $HClO_4$. Schreiber and Linder (1979) used an acid mixture of H_2SO_4-HNO_3-H_2O_2 for Se determination in plant samples. Again, the samples were left overnight to pre-digest.

3.4.2. Wet digestion in microwave oven (closed vessel).

Microwave technology has led to an increase in closed system wet digestions. Microwave ovens allow increases in temperature and pressure to be exerted on the sample, and hence reduces digestion time. Banuelos and Akohoue (1994) used a HNO_3-H_2O_2-H_2O (2:2:1 v/v) digestion mixture in Teflon bombs to

determine Se concentrations in plant samples. A commercial scientific microwave oven was used that allowed the internal vessel pressure to be controlled. Samples were predigested for 4 h and a comparison of results with samples digested in a digestion block showed good agreement.

3.4.3. Wet digestion in microwave oven (open vessel).

Ducros and Favier (1994) used a HNO_3-H_2O_2 digestion mixture in an open vessel system with heating under reflux in a microwave oven. No pre-digestion step was included. The Se concentrations in plasma and CRM bovine liver samples were compared to those obtained using the traditional HNO_3-$HClO_4$ open wet digestion system. Both methods were found to give results close to the certified values and there were no significant differences between the means ($p=0.05$) for any of the samples.

3.5. Selection of digestion method.

The digestion method chosen initially for plant material samples was H_3PO_4-HNO_3-H_2O_2 by open wet ashing as used by Reamer and Veillon (1981a). The use of CRM cabbage leaves showed that this mixture gave accurate results. However, the isotope dilution method for Se concentration determination involves the addition of inorganic ^{76}Se isotope solution (as selenate) to the sample prior to analysis. It was considered that it was possible that organo-selenium in the form of selenomethionine and selenocysteine may not be completely mineralised and converted to Se(VI) by this procedure. Hence it was decided to investigate 2 objectives:-

(1) The ability of the digestion mixture to liberate the Se intrinsically bound in proteins as selenomethionine and selenocysteine.

(2) To compare the effectiveness of microwave and open wet ashing procedures for mineralising organo-selenium.

Hence it was decided to digest selenomethionine and selenocysteine samples spiked with ^{76}Se isotope solution using the following digestion methods

(1) H_3PO_4-HNO_3-H_2O_2 (open wet ashing) as descibed by Reamer and Veillon (1981)

(2) H_2SO_4-HNO_3-H_2O_2 (open wet ashing) as descibed by Screiber and Linder (1979)

(3) HNO_3-H_2O_2 (microwave closed wet ashing) as described by Ducros and Favier (1994) and Banuelos and Akohoue (1994)

(4) H_3PO_4-HNO_3-H_2O_2 (microwave closed wet ashing) using the same v/v ratios as in (1).

Method (3) in the literature used a commercial microwave oven which could control pressure within the digestion vessel. This was not possible with the domestic microwave oven used in our procedure. Also, while Banuelos and Akohoue (1994) used a relatively high power system (600 W), Ducros and Favier used a maximium power of only 60 W. Preliminary work showed a power of 90 W for 25 min produced the brown NO_2 fumes associated with the oxidation of organic material and it was decided to follow a low power programme for method (3) and use a higher power programme for method (4).

In order to monitor the progress of the digestion, it was decided to remove 1 cm^3 of the sample for analysis at appropriate time intervals, convert them to the Se-NPD derivative and determine their m/z 229/225 ratios.

3.6. Reagents and materials.

All reagents used for digestion mixtures were of AnalaR grade and as described in Section 2.3. Seleno-L-methionine and seleno-DL-cysteine (Sigma Chemicals, St. Louis, USA) solutions were prepared as follows:-

(1) Selenomethionine: 0.0106 g was dissolved in 18 MΩ H_2O and made up to 25 cm^3 to give a Se concentration of 171 $\mu g\ g^{-1}$.

(2) Selenocysteine: 0.0102 g was dissolved in 18 MΩ H_2O and made up to 25 cm^3 to give a Se concentration of 193 $\mu g\ g^{-1}$.

These were stored below -6^{0}C. The ^{76}Se isotope solution used was of concentration 184.7 $\mu g\ g^{-1}$ (Section 2.13).

3.7. Digestion methods.

3.7.1. Open wet digestion.

(1) H_3PO_4-HNO_3-H_2O_2.

A schematic diagram of the digestion is shown in Figure 3.1. A 625 µl aliquot of each sample was placed in the digestion tube with 250 µl of ^{76}Se isotope solution. The digestion mixture (7 cm^3 HNO_3 + 1 cm^3 H_3PO_4) was added and the samples left overnight in the Buchi 426 digestion block (Buchi, Switzerland) at room temp to pre-digest. Three samples of each of the selenomethionine and selenocysteine were used for the analysis to be carried out in triplicate.

The following day the digestion unit was pre-heated and the samples heated at 50^{0}C (0.5 h). A 1 cm^3 subsample was taken from each tube. After

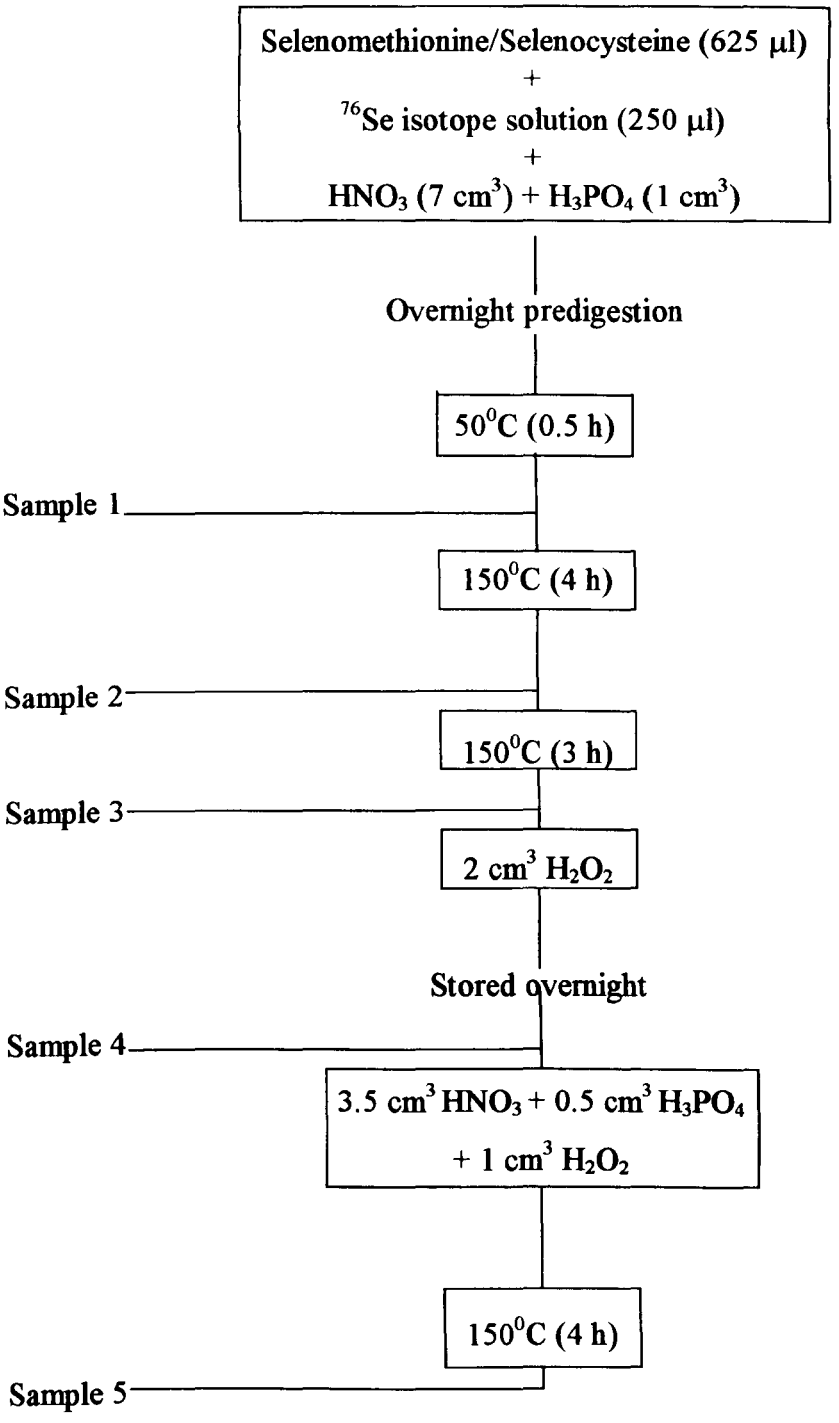


Figure 3.1. Schematic diagram of digestion procedure H_3PO_4-HNO_3-H_2O_2.

heating at 150^{0}C (4 h), another 1 cm^3 subsample was taken. Heating continued at this temp until there were no further brown fumes visible in the digestion tubes (3 h) and another sub-sample was taken. The tubes were cooled and 2 cm^3 H_2O_2 was added in 0.2 cm^3 increments. The samples were left overnight and the following day a further sub-sample was taken. A further 3.5 cm^3, 0.5 cm^3 and 1 cm^3 of HNO_3, H_3PO_4 and H_2O_2 respectively were added to ensure sufficient liquid was present in the digestion tubes and v/v ratio remained constant. Finally the samples were heated at 150^{0}C (4 h) and the final sub-sample obtained. The sub-samples were derivatised to the piazselenol complex as described in Section 2.4 and stored in Eppendorf tubes prior to analysis.

(2) HNO_3-H_2SO_4-H_2O_2.

The selenomethionine and selenocysteine samples (625 µl) were 'spiked' with the ^{76}Se isotope solution (250 µl). An acid mixture of HNO_3-H_2SO_4 (10 cm^3) in a 4:1 ratio was added and the samples left overnight to predigest (Figure 3.2). After heating at 120^{0}C (1 h), the first subsample was taken. Heating continued at 250^{0}C (1 h) and a second subsample was obtained. The tubes were cooled before 3 cm^3 H_2O_2 was added in 0.5 cm^3 increments at 2 min intervals to allow the samples to react. The tubes were heated again at 160^{0}C (1.5 h) then, the temp was raised to 250^{0}C (1 h). After each heating stage, a further sub-sample was taken. The sub-samples were derivatised and stored as before.

3.7.2. Closed wet digestion.

3.7.2.1. Instrumentation.

A domestic microwave oven (Panasonic NN 5402/52) rated at 800 W was used. The power levels were warm (90 W), simmer (240 W), defrost (270 W), low (420 W), medium (560 W) and high (800 W).

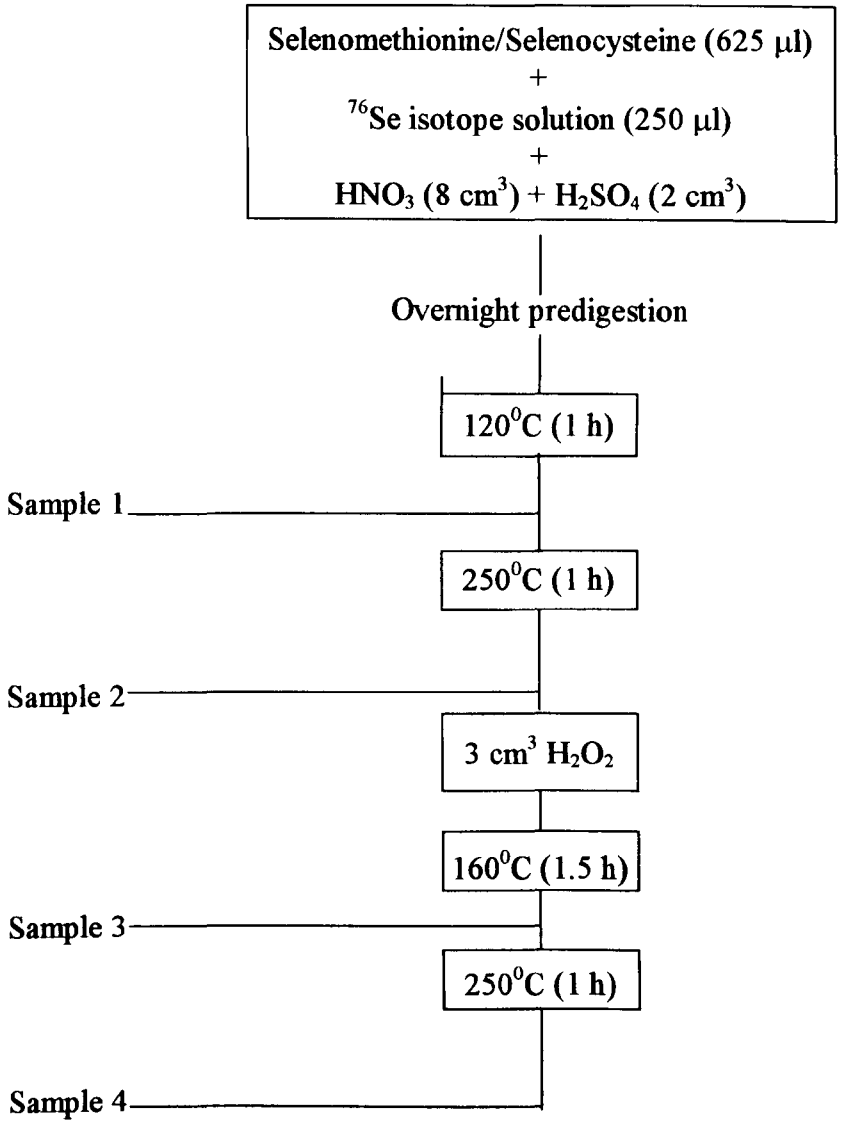


Figure 3.2. Schematic diagram of digestion procedure H_2SO_4-HNO_3-H_2O_2.

3.7.2.2. Digestion methods.

(1) HNO_3-H_2O_2.

The selenomethionine and selenocysteine samples (625 µl) and the ^{76}Se isotope solution (250 µl) were added to 50 cm^3 Teflon bombs (Savillex, USA) containing 6 cm^3 HNO_3 and left overnight to pre-digest (Figure 3.3) with the lids loosely fitted. The next day the lids on the bombs were tightened, the bombs heated at 90 W (25 min) and after cooling, the first sub-sample was taken. The samples were vented, 1 cm^3 H_2O_2 was added and the samples were heated at 90 W (6 min). After cooling and venting, a second sub-sample was obtained. A further 7 cm^3 H_2O_2 was added and after heating at 90 W (8 min) a final sub-sample was removed. The sub-samples were derivatised and stored as previously described (Section 2.4).

(2) H_3PO_4-HNO_3-H_2O_2

The samples were predigested in 50 cm^3 Teflon bombs containing HNO_3 (7 cm^3) and H_3PO_4 (1 cm^3) as shown (Figure 3.4). The samples were heated at 420 W (5 min), cooled and vented. A sub-sample was taken and H_2O_2 (2 cm^3) added. The samples were heated for a further 3 min, cooled and a final sub-sample taken. The sub-samples were derivatised and stored as before.

3.8. Analysis of sub-samples.

The Se-NPD derivatised subsamples were redissolved in CH_3Cl (25 µl). The m/z 229/225 ratios in the subsamples were determined by ID-MS as previously described (Section 2.4.). The parameters for the GC-MS were as described in Section 2.2 and the m/z 229/225 ratios were obtained using Autointegration (Section 2.8.2).

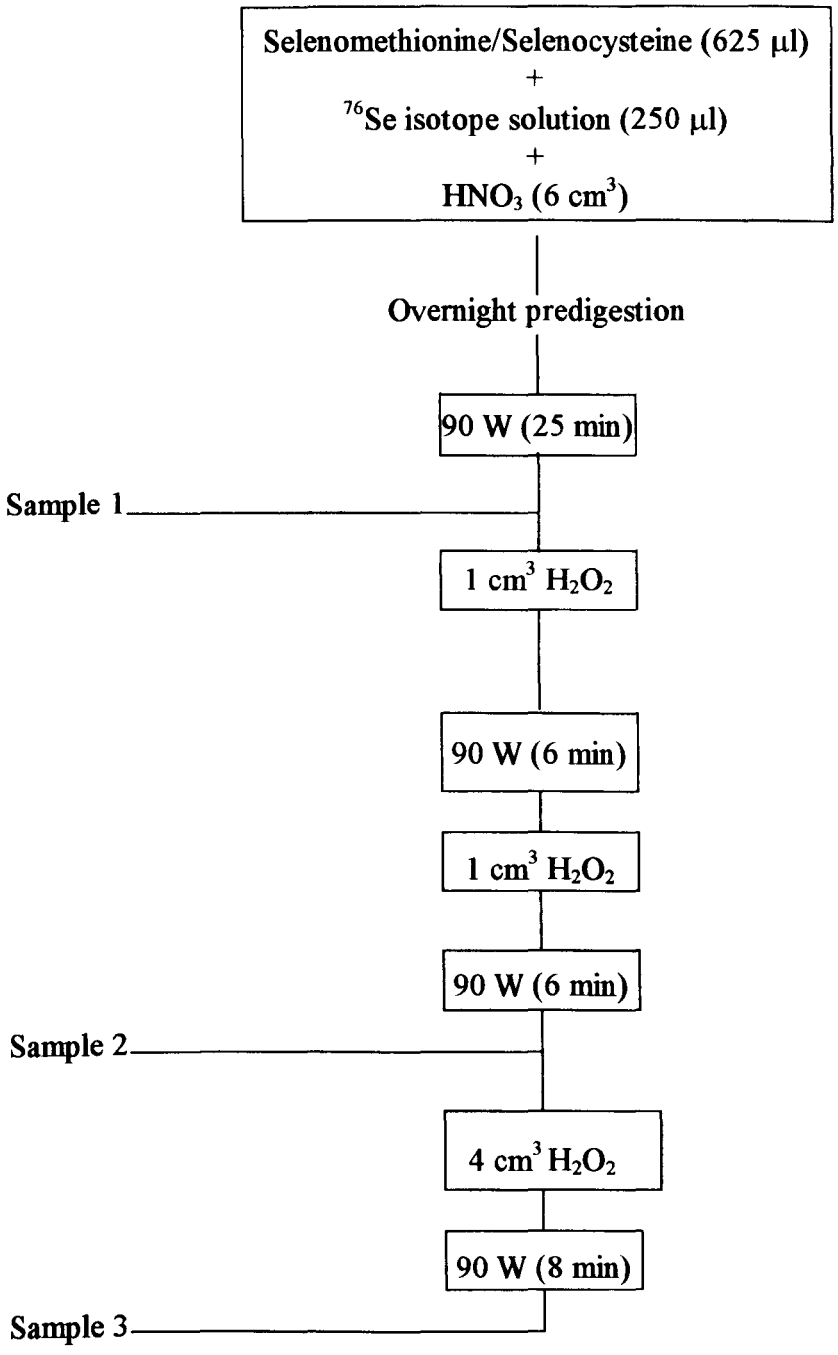


Figure 3.3. Schematic diagram of digestion procedure HNO_3-H_2O_2.

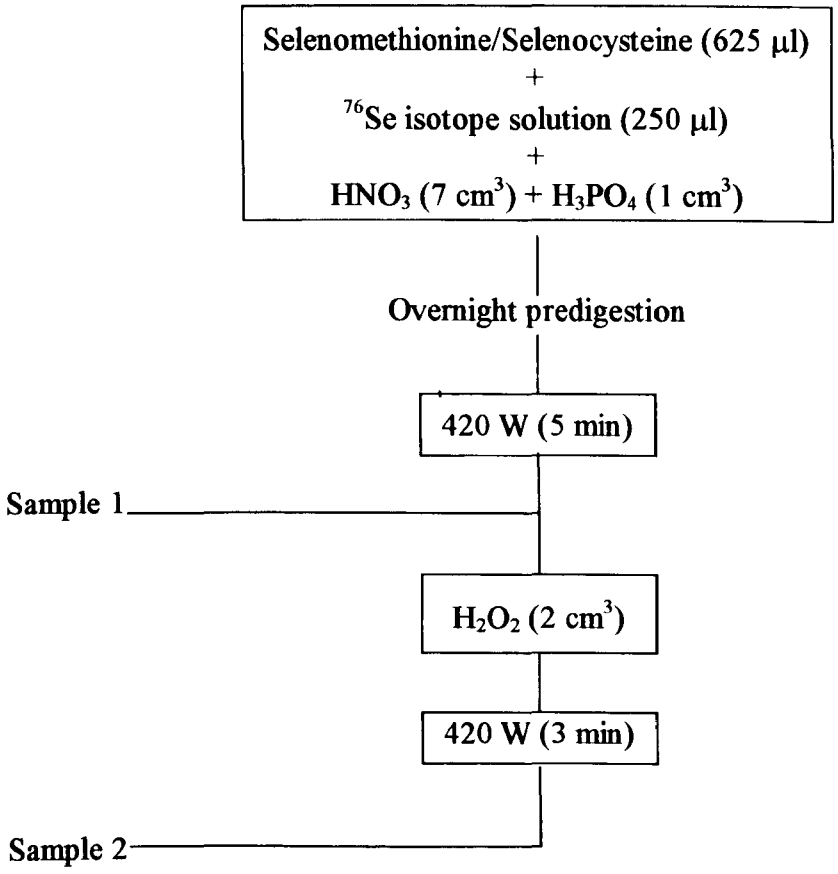


Figure 3.4. Schematic diagram of digestion procedure H_3PO_4-HNO_3-H_2O_2.

3.9. Results.

Initially 100 mg (approximately) of selenomethionine and selenocysteine were made up to 25 cm^3. The amount of Se in these solutions was calculated at 171 and 193 μg/g respectively. After derivatisation, the expected amounts of Se which would give m/z values 229 and 225 were calculated as follows:-

(1) Selenomethionine

0.0106 g in 25 cm^3 gives a Se concentration of 171 μg g^{-1}.
625 μl contains 106.9 μg Se (1.35 μmoles)
After derivatisation, m/z 229 of Se-NPD (46.74%) = 50 μg Se (0.63 μmoles).
After derivatisation, m/z/225 of Se-NPD Se (8.33%) = 8.9 μg Se (0.11 μmoles).

(2) Selenocysteine

0.0106 g in 25 cm^3 gives a Se concentration of 193 μg/g.
625 μl contains 120.6 μg Se (1.53 μmoles).
After derivatisation, m/z 229 of Se-NPD (46.74%) = 56.4 μg Se (0.71 μmoles).
After derivatisation, m/z/225 of Se-NPD Se (8.33%) = 10.0 μg Se (0.13 μmoles).

(3) ^{76}Se isotope solution

Se concentration 184.77 μg/g.
250 μl contains 46.2 μg Se (0.61 μmoles).
After derivatisation, m/z 229 of Se-NPD (0.11%) = 0.05 μg Se (0.0007 μmoles).
After derivatisation, m/z 225 of Se-NPD (91.27%) = 42.2 μg Se (0.55 μmoles).

Hence the expected m/z ratio 229/225 was calculated:-

Selenomethionine m/z 229/225 = 0.95

Selenocysteine m/z 229/225 = 1.05

These ratios represent 100% recovery of Se and therefore 100% mineralisation of the organoselenium compounds.

3.9.1. Open wet digestion.

(1) H_3PO_4-HNO_3-H_2O_2.

The recovery of Se from selenomethionine and selenocysteine at each stage of the digestion with H_3PO_4-HNO_3-H_2O_2 are shown in Tables 3.1 and 3.2 respectively. These show a recovery of 96% and 78% of Se from the seleno-methionine and selenocysteine solutions.

The digestion of selenomethionine showed little Se was released after prediestion and heating at 50^{0}C (14%). Increasing the temp to 150^{0}C and maintaining this temp for 4 h released 65% of Se, with 88% released after a further 3 h at this temperature. The addition of H_2O_2 and leaving the digest overnight allowed 92% Se to be released. A final heating burst of 4 h at 150^{0}C completed the digestion and gave a final Se recovery of 96% (Figure 3.5).

The digestion of selenocysteine followed an entirely different pathway. Gentle heating (50^{0}C) after predigestion gave a Se recovery of 81%. Increasing the temp and adding H_2O_2 had no further effect with a measured final recovery of 78% (Figure 3.5).

(2) H_2SO_4-HNO_3-H_2O_2.

The recovery of Se from selenomethionine and selenocysteine at each stage of the digestion with H_3PO_4-HNO_3-H_2O_2 are shown in Tables 3.3 and 3.4 respectively. The final recoveries of Se were 91% and 93% for selenomethionine and selenocysteine respectively. The initial recovery Se from selenomethionine

was low (31%) despite heating at 250^{0}C. It was not until H_2O_2 was added and the digest heated at 160^{0}C for 1 h that a recovery of 88% was obtained. Further heating at 250^{0}C gave a final recovery of 91% (Figure 3.6).

The greatest amount of Se from selenocysteine was recovered after heating at 120^{0}C for 1 h (91%). Recovery changed little during the remainder of the digestion to give a final 93% Se (Figure 3.6).

Table 3.1. Recovery of Se from selenomethionine using H_3PO_4-HNO_3-H_2O_2 in open wet ashing apparatus.

Subsample	Mean Se conc ($\mu g\ 625\ \mu l^{-1}$)	C.V. (%)	Recovery[a] (%)
1	15.18	7.7	14.2
2	69.98	7.8	65.5
3	94.31	1.9	88.2
4	98.27	4.9	91.9
5	102.5	4.4	95.9

a=based on 625 µl containing 106.9 µg Se

Table 3.2. Recovery of Se from selenocysteine using H_3PO_4-HNO_3-H_2O_2 in open wet ashing apparatus.

Subsample	Mean Se conc ($\mu g\ 625\ \mu l^{-1}$)	C.V. (%)	Recovery[a] (%)
1	98.48	3.5	81.7
2	96.88	3.8	80.4
3	95.58	4.5	79.3
4	90.09	3.9	79.7
5	94.48	3.9	78.4

a=based on 625 µl containing 120.5 µg Se

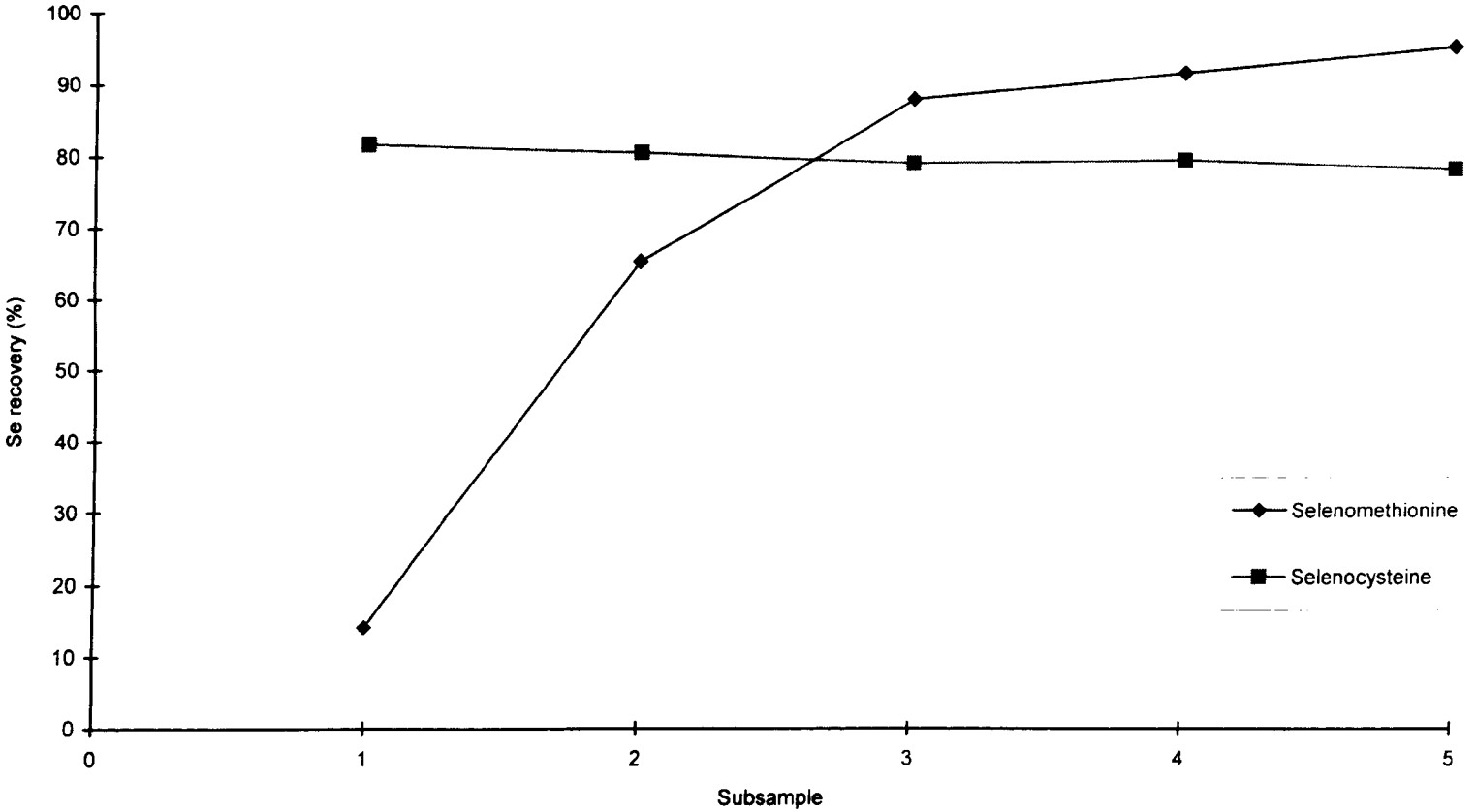


Figure 3.5. Recovery of selenium by open wet ashing using H_3PO_4-HNO_3-H_2O_2.

Table 3.3. Recovery of Se from selenomethionine using H_2SO_4-HNO_3-H_2O_2 in open wet ashing apparatus.

Subsample	Mean Se conc (μg/625 μl)	C.V. (%)	Recovery[a] (%)
1	29.17	3.9	27.3
2	33.53	6.8	31.4
3	94.28	0.05	88.4
4	97.45	2.0	91.3

a=based on 625 μl containing 106.9 μg Se

Table 3.4. Recovery of Se from selenocysteine using H_2SO_4-HNO_3-H_2O_2 in open wet ashing apparatus.

Subsample	Mean Se conc (μg/625 μl)	C.V. (%)	Recovery[a] (%)
1	110.34	5.2	91.5
2	112.77	2.0	93.6
3	109.99	0.04	91.3
4	112.04	1.7	92.9

a=based on 625 μl containing 120.5 μg Se

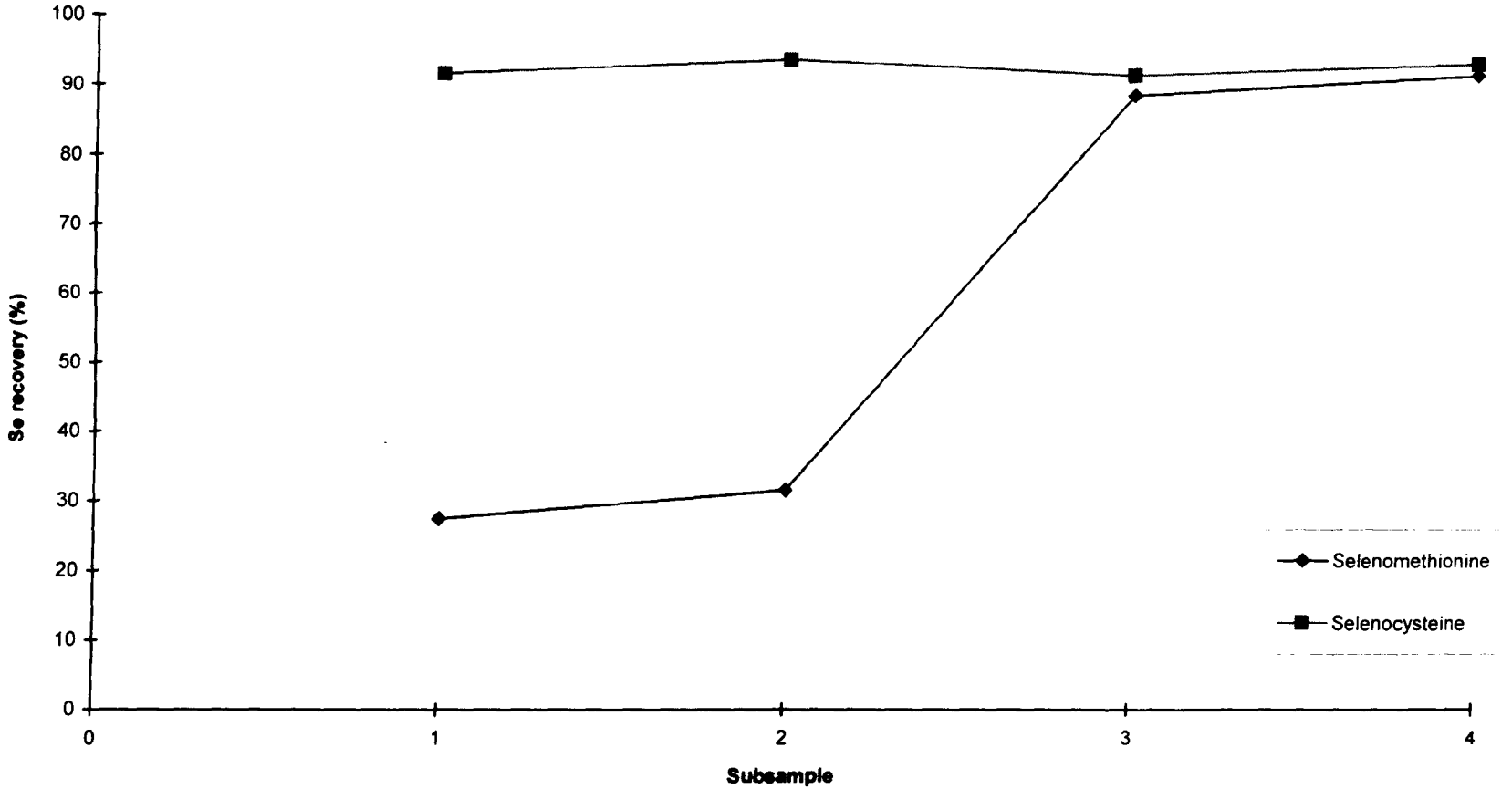


Figure 3.6. Recovery of selenium by open wet ashing using H_2SO_4-HNO_3-H_2O_2.

3.9.2. Closed wet digestion using microwave oven techniques.

(1) HNO_3-H_2O_2.

The use of HNO_3-H_2O_2 digestion mixture at low power (90 W) gave recoveries of Se of 86% and 89% respectively from selenomethionine and selenocysteine (Tables 3.5 and 3.6). The use of HNO_3 alone gave a low Se recovery from selenomethionine (21%) and H_2O_2 was required for digestion. However, Se was recovered from selenocysteine (89%) after heating for 25 min (90 W) with HNO_3. Again, further digestion made little difference to total recovery figures (Figure 3.7).

(2) H_3PO_4- HNO_3-H_2O_2.

This digestion mixed yielded Se recoveries of 92% and 91% from selenomethionine and selenocysteine (Tables 3.7 and 3.8). Heating at 420 W power for 5 min gave a Se recovery of 83 % for selenomethionine which increased to 92 % with the addition of H_2O_2 and heating for 3 min (420 W). The Se from selenocysteine again was recovered after stage 1 (91%) with the addition of H_2O_2 having made little difference to the final recovery (Figure 3.8).

3.10. Discussion.

The objectives of this section was to alleviate any doubts about the digestion procedure being able to release organically bound Se (as selenomethionine and selenocysteine) and to find a digestion method which was not time intensive. The amounts of selenomethionine and selenocysteine used were chosen to obtain an m/z 229/225 ratio close to unity in order to give optimum accuracy.

Table 3.5. Recovery of Se from selenomethionine using HNO_3-H_2O_2 digest in closed wet ashing apparatus.

Subsample	mean 229/225 m/z ratio(n=3)	Mean Se conc (μg/625 μl)	C.V. (%)	Recovery[a] (%)
1	0.223	21.97	10.9	21.2
2	0.779	85.29	3.7	82.4
3	0.805	88.61	1.2	85.6

a=based on 625 μl containing 106.9 μg Se

Table 3.6. Recovery of Se from selenocysteine using HNO_3-H_2O_2 in closed wet ashing apparatus.

Subsample	mean 229/225 m/z ratio(n=3)	Mean Se conc (μg/625 μl)	C.V. (%)	Recovery[a] (%)
1	0.949	107.58	1.2	89.3
2	0.962	109.55	0.07	90.9
3	0.947	107.44	1.1	89.1

a=based on 625 μl containing 120.5 μg Se

Table 3.7. Recovery of Se from selenomethionine using H_3PO_4-HNO_3-H_2O_2 in closed wet ashing apparatus.

Subsample	mean 229/225 m/z ratio(n=3)	Mean Se conc (μg/625 μl)	C.V. (%)	Recovery[a] (%)
1	0.810	89.19	3.2	83.6
2	0.882	98.60	1.1	92.4

a=based on 625 μl containing 106.9 μg Se

Table 3.8. Recovery of Se from selenocysteine using H_3PO_4-HNO_3-H_2O_2 in closed wet ashing apparatus.

Subsample	mean 229/225 m/z ratio(n=3)	Mean Se conc (μg/625 μl)	C.V. (%)	Recovery[a] (%)
1	0.963	109.62	1.6	90.9
2	0.965	109.99	1.5	91.2

a=based on 625 μl containing 120.5 μg Se

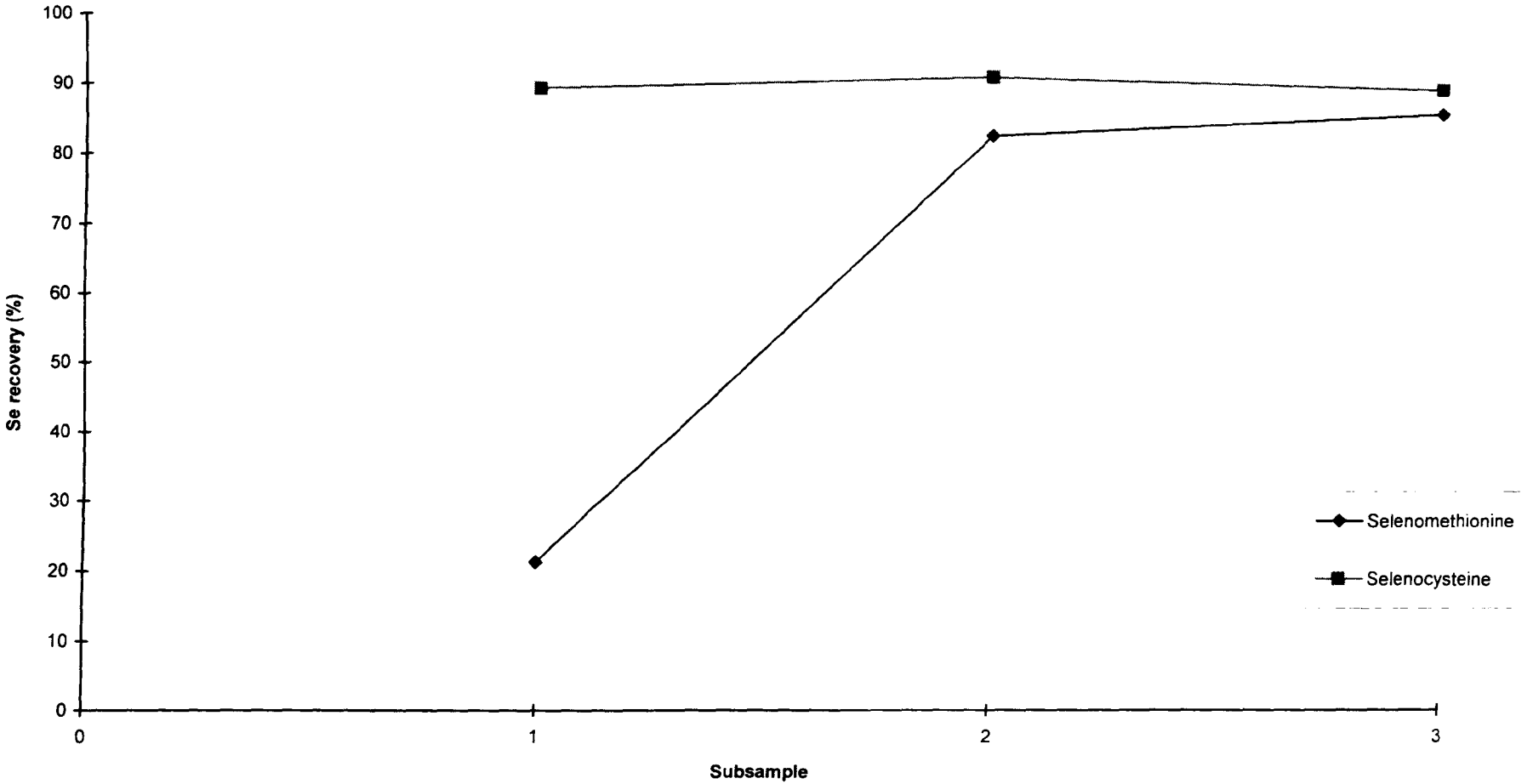


Figure 3.7. Recovery of selenium by closed wet ashing using HNO_3-H_2O_2.

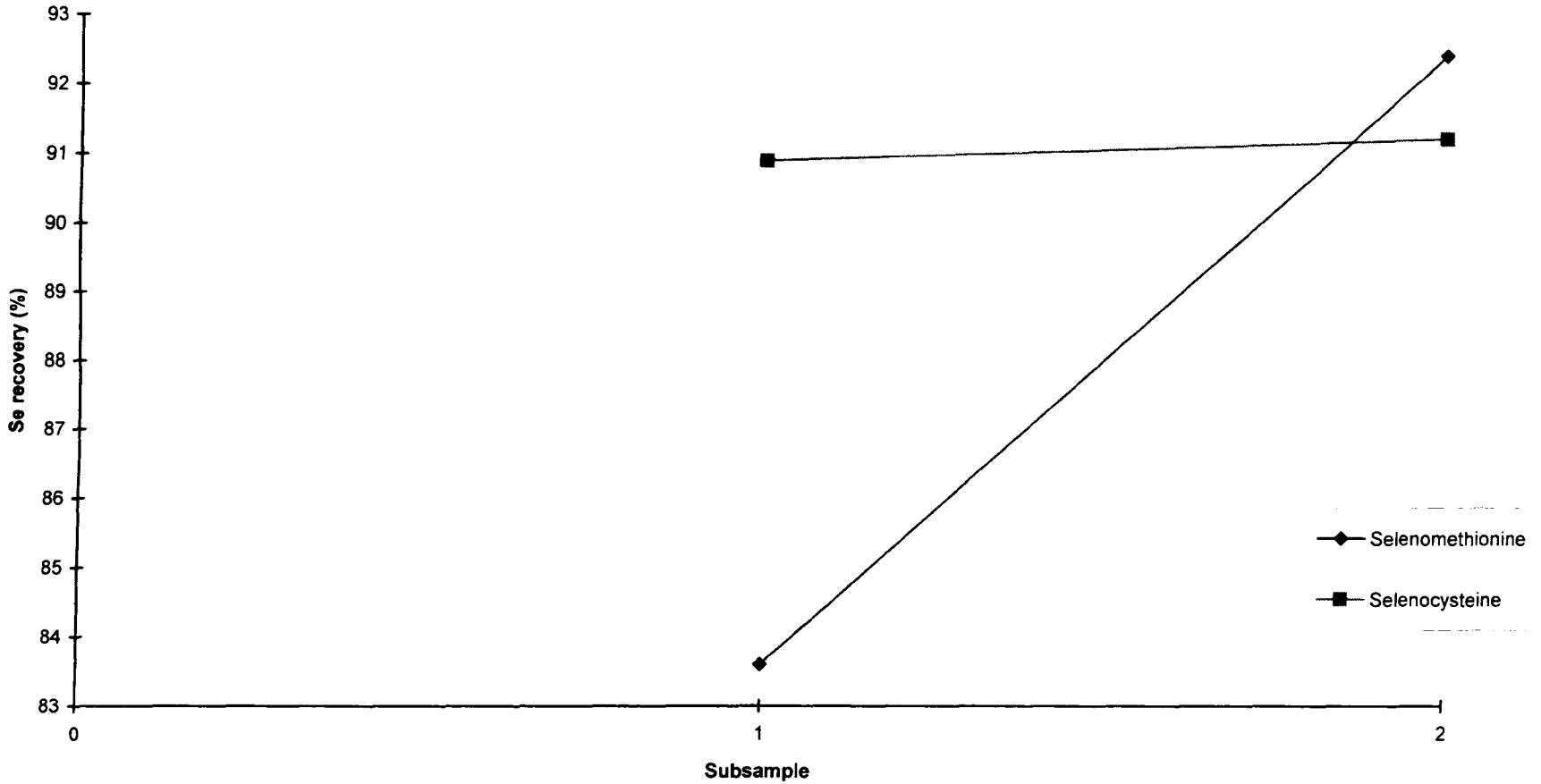


Figure 3.8. Recovery of selenium by closed wet ashing using H_3PO_4-HNO_3-H_2O_2.

3.10.1. Recovery of selenium from selenomethionine.

Selenium intrinsically bound as selenomethionine required the addition of H_2O_2 and further heating before it could be released. The H_3PO_4-HNO_3- H_2O_2 open wet ashing digestion gave the greatest recovery (96.1%). This was the method used to determine Se concentrations in CRM plant samples (Section 2.16). Application of this mixture as a microwave oven digestion mixture also gave a good recovery (91.3%) (Table 3.9).

3.10.2. Recovery of selenium from selenocysteine.

The recovery of Se from selenocysteine did not require strongly oxidising conditions. Overnight predigestion followed by low temp heating was sufficient to release the Se present. Indeed, further digestion may lead to small Se losses, possibly as volatile species. Highest Se recoveries were by open wet ashing using H_2SO_4-HNO_3- H_2O_2 (92.9%) and closed microwave digestion using H_3PO_4-HNO_3- H_2O_2 (91.3%) (Table 3.9).

3.10.3. Factors affecting recovery of selenium.

Although Se recoveries were good, 100% recovery was not achieved. Possible errors may have resulted from the hydroscopic properties of the selenomethionine and selenocysteine samples or the presence of impurities in the samples. To examine these possibilities, firstly the samples were weighed, placed in beakers, covered with perforated parafilm and left in a dessicator containing KOH and P_2O_5. After 12 h they were reweighed. No weight difference was recorded, hence it was assumed neither sample was hydroscopic. An carbon and nitrogen analysis was carried out (by MLURI analytical staff) which showed only

90% of the expected C value was present (Table 3.10). Taking the impurity of the samples into account, Table 3.11 shows the adjusted recovery figures.

Table 3.9. Final recoveries of Se from selenomethionine and selenocysteine.

Digestion method	Se recovery from selenomethionine (%)	Se recovery from selenocysteine (%)
H_3PO_4-HNO_3-H_2O_2	95.9	78.4
H_2SO_4-HNO_3-H_2O_2	91.3	92.9
HNO_3-H_2O_2 (microwave)	85.6	89.1
H_3PO_4-HNO_3-H_2O_2 (microwave)	92.4	91.3

Table 3.10. Elemental analysis of selenomethionine and selenocysteine.

Se compound	Theoretical Nitrogen (%)	Actual Nitrogen (%)	Theoretical Carbon (%)	Actual Carbon (%)
Selenomethionine	7.14	7.11	30.8	27.5
Selenocysteine	8.38	8.07	21.5	19.3

Table 3.11. Adjusted recoveries of selenium from selenomethionine and selenocysteine.

Digestion method	Se recovery from selenomethionine (%)	Se recovery from selenocysteine (%)
H_3PO_4-HNO_3-H_2O_2	106.5	87.1
H_2SO_4-HNO_3-H_2O_2	101.4	103.2
HNO_3-H_2O_2 (microwave)	95.1	99.0
H_3PO_4-HNO_3-H_2O_2 (microwave)	102.7	101.4

3.11. Conclusion.

The use of H_3PO_4-HNO_3-H_2O_2 digestion mixture in a closed wet ashing microwave oven procedure has been shown to release around 100% of organically bound Se. This may have been greater as some doubts remain over the chemical purity of the samples used. Although this digestion mixture was originally used to determine Se concentrations in CRM plant material using open wet ashing techniques and satisfactory results were obtained, it was decided for future analysis to use the microwave oven closed system. This has the added benefit of shorter digestion times as well as greater Se recoveries.

Chapter 4. Speciation of selenium

4.1. Introduction.

Quantification of the total Se in soils or sewage sludge gives no information about the chemical species or the soil fraction with which it is associated and, therefore, provides little information about the availability to plants. The chemical forms of Se and their solubility depend mainly on the redox potential and the pH of the soil (Elrashidi et al., 1987). Selenium can exist in four oxidation states all of which are represented in soils, namely: Se(IV) (selenite), Se(VI) (selenate), Se (elemental selenium) and Se(II) (selenide and organic Se). Other factors which influence the species of Se present and its bioavailability include organic matter content, ferric oxide levels and clay type and content. These factors are more fully discussed in Section 1.7. Hence, prior to speciation studies, these soil parameters were examined.

Total Se concentration was calculated for 4 Scottish soils and 3 British sewage sludges. In order to assess bioavailability to plants of Se, a speciation scheme was needed. One approach was soil dissolution followed by chromatography (Section 1.7) to speciate selenite, selenate or organoselenium compounds. However, rather than determining individual chemical species, a sequential extraction procedure was used to assess the availability of Se for plant uptake. The 4 soils and the sewage sludge with the highest Se concentration were used and the procedure identified 5 fractions: anion exchangeable, ligand exchangeable, acid extractable, oxidisable and residual Se. This method assesses how much Se is likely to be readily available, potentially available or unavailable rather than quantifying the chemical species present. Previous work by Chao and Sanzolone (1989) using sequential extraction and HGAAS found that the amount of Se determined for each fraction was consistent with the theoretical expected form of Se associated with a particular soil environment. It is hoped that this

method will be able to predict the effects of Se uptake by plants in relation to overcoming Se deficiency or toxicity problems prior to sewage sludge application to agricultural land.

4.2. Reagents.

Reagents were of analytical grade: hydrous calcium chloride, acetic acid (17.5 M), ammonium oxalate, sodium hexametaphosphate, sodium carbonate, ammonium hydroxide (14.8 M), nitric acid (18.5 M), hydrochloric acid (12 M), hydrofluoric acid (28.9 M), potassium chloride, potassium dihydrogen phosphate, potassium chlorate (Merck, UK) and silica gel (0.125-0.250 mm) (Fisons, UK). Elemental ^{76}Se (Europa Scientific, Cheshire, UK) was dissolved in nitric acid and diluted as described in Section 2.3.2 to provide a 'spike' solution for addition to each extract.

4.3. Soils.

Freely drained soils of the Countesswells, Insch, Strichen and Tarves Associations (Soil survey of Scotland, 1982) from North East Scotland (Figure 4.1.) were chosen for this study. Their location and parent material are shown in Table 4.1. These soils were selected because such soil types meet the suitability criterion for sewage sludge application (Towers, 1994). Soil was taken to a depth of 25 cm^3 and sieved in a 2 mm sieve to remove stones and visible roots and air dried at 30^0C. Each soil was homogenised and sub-sampled by coning and quartering. For sequential extraction of Se the soils were ground to pass a 150 μm sieve.

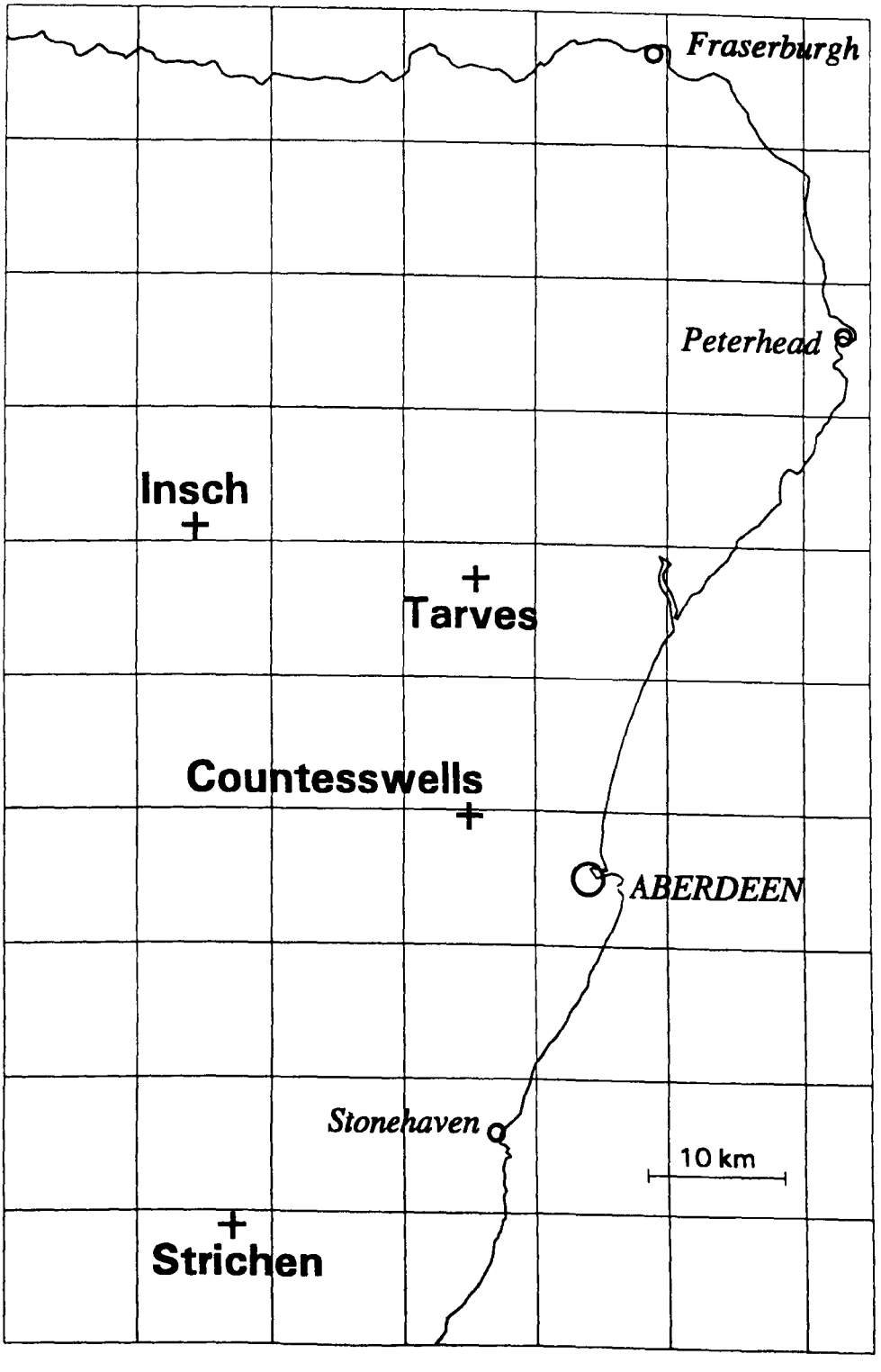


Figure 4.1. Map of North East Scotland showing the location of soil Associations sampled.

Table 4.1. Parent rock and location of 4 soils from the North East of Scotland.

Soil Association[a]	Parent Material	National Grid Reference
Countesswells	Drifts derived from granite and granitic rocks	NJ 850096
Insch	Drifts derived from gabbros and igneous rocks	NJ 643312
Strichen	Drifts derived from arenaceous schists and strongly weathered argillaceous schists of the Dalradian Series	NO 671789
Tarves	Drifts derived from intermediate rocks or mixed acid and basic rocks, both metamorphic and igneous	NJ 855274

a=Soil Survey of Scotland, (1982)

4.3.1. Characterisation of soils.

4.3.1.1. Physical and chemical properties.

The physical and chemical properties determined for the soils are shown in Tables 4.2-4.5. All analyses were carried out in triplicate and the mean obtained.

(A) Organic content.

The organic content (loss on ignition) was determined from soil subsamples which had been dried (150^{0}C) for 1h and weighed. The samples were transferred to a muffle furnace (450^{0}C) for 12 h and re-weighed.

(B) pH.

The soil pH (calcium chloride solution) was determined from 15 g soil in 30 cm^3 $CaCl_2.H_2O$ (0.01 M). This was left to equilibrate for 20 min. A soil standard was made up with $CaCl_2.H_2O$. The pH meter (Orion, USA) was calibrated with pH 4 and pH 7 buffers. The pH was determined for each soil, and the soil standard pH verified between soil analyses.

The soil pH (water) was determined as above using 15 g soil and 30 cm^3 de-ionised water.

(C) Acetic acid extraction.

Soil (2.5 g) was shaken (2 h) with 100 cm^3 0.43 M acetic acid (2.5%), filtered into bottles and analysed by inductively coupled plasma-optical emission spectrometry (ICP-OES) to determine of Ca, Na, K, Mg and P levels.

(D) Oxalate extraction.

Soil (2.5 g) was shaken with acid ammonium oxalate (McKeague and Day, 1966). The extracts were filtered and the Fe, Mn, Al and Si contents determined by ICP-OES.

(E) Clay analysis.

Clay fractions (< 2 μm) for X-ray diffraction (XRD) were determined with a Siemens (Germany) D5000 2 kW X-ray diffractometer using cobalt kα radiation with a secondary monochromator counter. Slides (air dried) were prepared by dispersing the soil in water, centrifuging and coating the slide with soil (< 2 μm) from the filtered solution. This deposited around 30 mg soil onto the slide.

(F) Particle size distribution.

Particle size distribution in the soils was determined hydrometrically following dispersion in 2.3 mM sodium hexametaphosphate containing 3.0 mM sodium carbonate (Day, 1965).

Table 4.2. Properties of Countesswells Association soil

Loss on ignition (%)	11.9
pH (H_2O)	5.6
($CaCl_2$)	5.0
British standard textural class (BSTC)[a]	
Sand (%)	76.4
Silt (%)	15.4
Clay (%)	8.2
Ammonium oxalate extractable elements (g kg^{-1})	
Fe	10.2
Mn	0.24
Al	7.4
Si	0.39
Acetic acid extractable elements (mg g^{-1})	
Ca	2076
Na	124
K	284
Mg	132
P	128
Dominant clay minerals[b]	Illite, Plagioclase, Kaolinite

[a]Sand 2000-20, silt 20-2 and clay < 2 μm

[b]Comparison of data for dominant clays in the 4 North East of Scotland soils in shown in Figure 4.1.

Table 4.3. Properties of Insch Association soil

Loss on ignition (%)	13.3
pH (H_2O)	5.9
($CaCl_2$)	5.3
British standard textural class (BSTC)[a]	
Sand (%)	60.2
Silt (%)	27.2
Clay (%)	12.6
Ammonium oxalate extractable elements (g kg^{-1})	
Fe	28.8
Mn	0.97
Al	5.5
Si	0.42
Acetic acid extractable elements (mg g^{-1})	
Ca	3208
Na	168
K	212
Mg	104
P	60
Dominant clay minerals[b]	Vermiculite, Plagioclase, Kaolinite

[a]Sand 2000-20, silt 20-2 and clay < 2 μm

[b]Comparison of data for dominant clays in the 4 North East of Scotland soils in shown in Figure 4.1.

Table 4.4. Properties of Strichen Association soil

Loss on ignition (%)	8.5
pH (H_2O)	5.2
($CaCl_2$)	4.6
British standard textural class (BSTC)[a]	
Sand (%)	63.3
Silt (%)	24.4
Clay (%)	12.2
Ammonium oxalate extractable elements (g kg^{-1})	
Fe	9.6
Mn	0.6
Al	13.5
Si	3.8
Acetic acid extractable elements (mg g^{-1})	
Ca	1172
Na	148
K	92
Mg	112
P	88
Dominant clay minerals[b]	Illite, Kaolinite, Vermiculite

[a]Sand 2000-20, silt 20-2 and clay < 2 μm

[b]Comparison of data for dominant clays in the 4 North East of Scotland soils in shown in Figure 4.1.

Table 4.5. Properties of Tarves Association soil

Loss on ignition (%)	10.5
pH (H_2O)	5.0
($CaCl_2$)	4.6
British standard textural class (BSTC)[a]	
Sand (%)	58.9
Silt (%)	24.7
Clay (%)	16.4
Ammonium oxalate extractable elements (g kg^{-1})	
Fe	9.1
Mn	0.5
Al	5.6
Si	0.77
Acetic acid extractable elements (mg g^{-1})	
Ca	1556
Na	172
K	208
Mg	108
P	80
Dominant clay minerals[b]	Vermiculite, Kaolinite, Illite

[a]Sand 2000-20, silt 20-2 and clay < 2 μm

[b]Comparison of data for dominant clays in the 4 North East of Scotland soils in shown in Figure 4.1.

4.3.1.2. Determination of total selenium in soil.

The soil was air dried and 0.5 g transferred to a 50 cm^3 Teflon bomb (Savillex, Minnesota, USA) containing 2 cm^3 18.5 M HNO_3 and 0.1 g of ^{76}Se isotope solution (concentration =18.615 $\mu g\ g^{-1}$). Organic material was eliminated by heating in a microwave oven (800W) for bursts of 1,2 and 3 min. The sample was cooled and vented between heating bursts. After digestion, 10 cm^3 HF (28.9 M) was added and the lid was tightened. The sample was heated on a hot plate at 180^{0}C for 6 h and cooled for 16 h. The lid was removed and the HF evaporated by heating at 120^{0}C for about 6 h. After cooling, 10 cm^3 of 18.5 M HNO_3 was added and evaporated by heating at 120^{0}C for about 6 h. The residue was redissolved in 4 cm^3 of 3M HNO_3 and the Se derivatised to Se-NPD to determine the Se concentration (Table 4.6) by ID-MS (as described in Section 2.4).

4.4 Sewage sludges.

Sewage sludge samples were taken from 3 locations in the UK, namely London, Birmingham and Selkirk (Scottish Border region). The first 2 samples were anaerobically digested. These sludges were analysed for total Se using the same method as described for total soil Se (Section 4.3.1.2). The results (Table 4.6) identified a sludge with a high Se concentration which would be used for speciation and pot plant experiments. To eliminate pathogens, these sludges were autoclaved three times (120^{0}C), freeze dried, sieved (2 mm) and homogenised by coning and quartering. For sequential extraction of Se the sludges were ground to pass a 150 μm sieve. Prior to application to agricultural land, the sludges will undergo treatment to reduce pathogens present and it is acknowledged that this may influence the Se species present.

Table 4.6. Selenium levels in selected soils and sewage sludges.

Sample	Mean Se ($\mu g\ g^{-1}$) (n=3)	C.V. (%)
Countesswells	0.637	4.8
Insch	0.761	1.6
Strichen	0.710	2.5
Tarves	0.546	4.5
London	3.554	1.8
Birmingham	1.156	1.8
Selkirk	3.137	2.9

4.4.1. Characterisation of sewage sludges.

The pH, organic matter content, acetic acid extractable Ca, Mg and K, and ammonium oxalate extractable Fe, Mn and Al were obtained in the same manner as for the soils (Table 4.7.).

4.5. Speciation of selenium in soils and sewage sludge by sequential extraction.

The sequential extraction scheme used separated the total Se into fractions according to their ease of extraction. The extraction sequence, mechanisms involved and an indication of Se availability is summarised in Table 4.8. For fractions 1-4, the extract was stored in polythene bottles containing 2 cm^3 HNO_3 (18.5 M) had been added.

Table 4.7. Physical properties of London, Birmingham and Selkirk sewage sludges.

Sewage sludge	Loss on ignition(%)	pH (H_2O)	pH ($CaCl_2$)	Ammonium oxalate extractable elements (g kg^{-1})			Acetic acid extractable elements(mg g^{-1})		
				Fe	Mn	Al	Ca	Mg	K
London	23.4	7.0	6.7	19.4	0.4	19.1	1208	40	8
Birmingham	60.7	7.3	6.9	23.6	0.6	46.2	1630	92	20
Selkirk	56.9	5.1	5.0	0.9	0.2	0.8	260	32	12

Table 4.8. Summary of sequential extraction procedure.

Extractant	Fraction	Se form extracted	Mechanism of extraction	Availability to plant
0.25 M KCl	1	Soluble, non-specifically adsorbed selenate	Anion exchange	Highly available
0.1 M KH_2PO_4	2	Exchangeable, specifically adsorbed selenite	Ligand exchange	Available
4 M HCl	3	Se associated with mineral oxides, some hydrolysed organic matter	Acid dissolution, acid hydrolysis	Potentially available
$KClO_3$+c.HCl	4	Se from sulphides, organic matter	Oxidative acid dissolution	Unavailable
HF+c.HNO_3	5	Se associated with silicon lattice network	Destruction of lattice network	Highly unavailable

Fraction 1. Soluble Se.

The ground soil (2 g) was placed in a centrifuge tube (100 cm^3) with 0.5 g of silica gel (150-250 μm) added and mixed using a vortex device. The silica gel increases the efficiency of extraction by dispersing the particles and prevents caking of the particles on the bottom of the centrifuge tube after centrifugation. Following the addition of 50 cm^3 0.25 M KCl, the tube was shaken (30 mins). The mixture was centrifuged (33,000 x g) at 0^0C for 10 mins or until the supernatant was clear. The supernatant was decanted and stored.

Fraction 2. Ligand exchangeable Se.

The residue from Fraction 1 was mixed with 50 cm^3 0.1 M KH_2PO_4 and shaken for 30 mins. The mixture was centrifuged and the supernatant decanted off.

Fraction 3. Acid extractable Se.

The residue from Fraction 2 was mixed with 4 M HCl (50 cm^3) and heated with occasional stirring in a water bath (95^{0}C) for 45 mins. After cooling, the mixture was centrifuged and the supernatant decanted off.

Fraction 4. Oxidisable Se.

The residue from Fraction 3 was mixed with 0.5 g of $KClO_3$ and 10 cm^3 of 12 M HCl was slowly added. The mixture was left for 45 min and stirred occasionally. The sample was centrifuged and the supernatant decanted off.

The extract derived for each Fraction (1-4) was stored in poltethene bottles containing 2 cm^3 of 18.5 M HNO_3.

Fraction 5. Residual Se.

The residue from Fraction 4 was air dried, weighed. 0.5 g transferred to a 50 cm^3 Teflon bomb (Savillex, Minnesota, USA) and treated by the same procdeure as described for the determination of total Se (Section 4.3.1.2).

Prior to analysis Fractions 1-4 were each transferred to pyrex beakers containing 0.1g of ^{76}Se isotope solution(concentration =18.615 $\mu g\ g^{-1}$). These were heated on a hot plate (100^{0}C) and evaporated to 15 cm^3. Heating with HNO_3 added prior to storage ensured that all the Se present was in the form of Se(VI).

4.6. Determination of selenium levels in soils and sewage sludge.

The samples for fractions 1-5 and for total Se were derivatised to Se-NPD and the Se levels determined by ID-MS (Section 2.4).

4.7. Statistical analysis.

Extractions of soils and the sewage sludge were carried out in triplicate. In order to assess the effect of soil type on Se concentrations within each fraction, a one way analysis of variance was performed using 'Genstat 5' (1990).

4.8. Results.

4.8.1. Sequential extraction of selenium in soils.

The concentrations of soluble Se (Fraction 1) were low (9-11 ng g^{-1}) (Table 4.9.) and not significantly different between the soils ($p<0.05$). The concentrations of ligand exchangeable (Fraction 2) Se were not significantly different ($p<0.05$) between Countesswells, Insch and Strichen Associations soils (21-23 ng g^{-1}), but there was a significant decrease for Tarves soil Association (15 ng g^{-1}). The acid extractable fractions were significantly different between the 4 soils. The Insch Association contained the least Se (63 ng g^{-1}) and the Strichen Association the most Se (128 ng g^{-1}). Oxidative acid extraction released between 138 and 162 ng g^{-1} Se for the Countesswells, Insch and Strichen Association soils. This reagent released considerably less Se for the Tarves Association soil (64 ng g^{-1}). The residual fraction was the largest for all 4 soils and varied between 364 and 443 ng g^{-1}. When the results were expressed as a percentage of the sum of the

individual fractions, over 77% of the Se was found to be unavailable for plant uptake (Figure 4.2.).

Table 4.9. Selenium concentration in soil fractions.

Sample	Selenium levels (ng g^{-1}) in Fraction				
	1 Anion exchange	2 Ligand-exchange	3 Acid-extractable	4 Oxidisable	5 Residual
Countesswells soil	9	23	80	138	440
Insch soil	10	21	63	162	443
Strichen soil	11	23	128	152	412
Tarves soil	9	15	99	64	364
Lsd (p = 0.05)	2.1	2.5	6.8	5.4	33.6

Lsd = Least significant difference for comparison between soils.

4.8.2. Sequential extraction of selenium in sewage sludge.

There was considerably more Se in each of the 5 fractions from the sewage sludge compared to the soils examined. This was particularly apparant in the oxidative extractable pool which was about 9 times greater than the average value for the soils.The low concentrations of soluble Se (42 ng g^{-1}) and ligand exchangeable Se (87 ng g^{-1}) indicate little Se is available for plant uptake (Table 4.10.). The Se contained in the unavailable Fractions 4 and 5 account for 90% of the Se of this sewage sludge (Figure 4.3.).

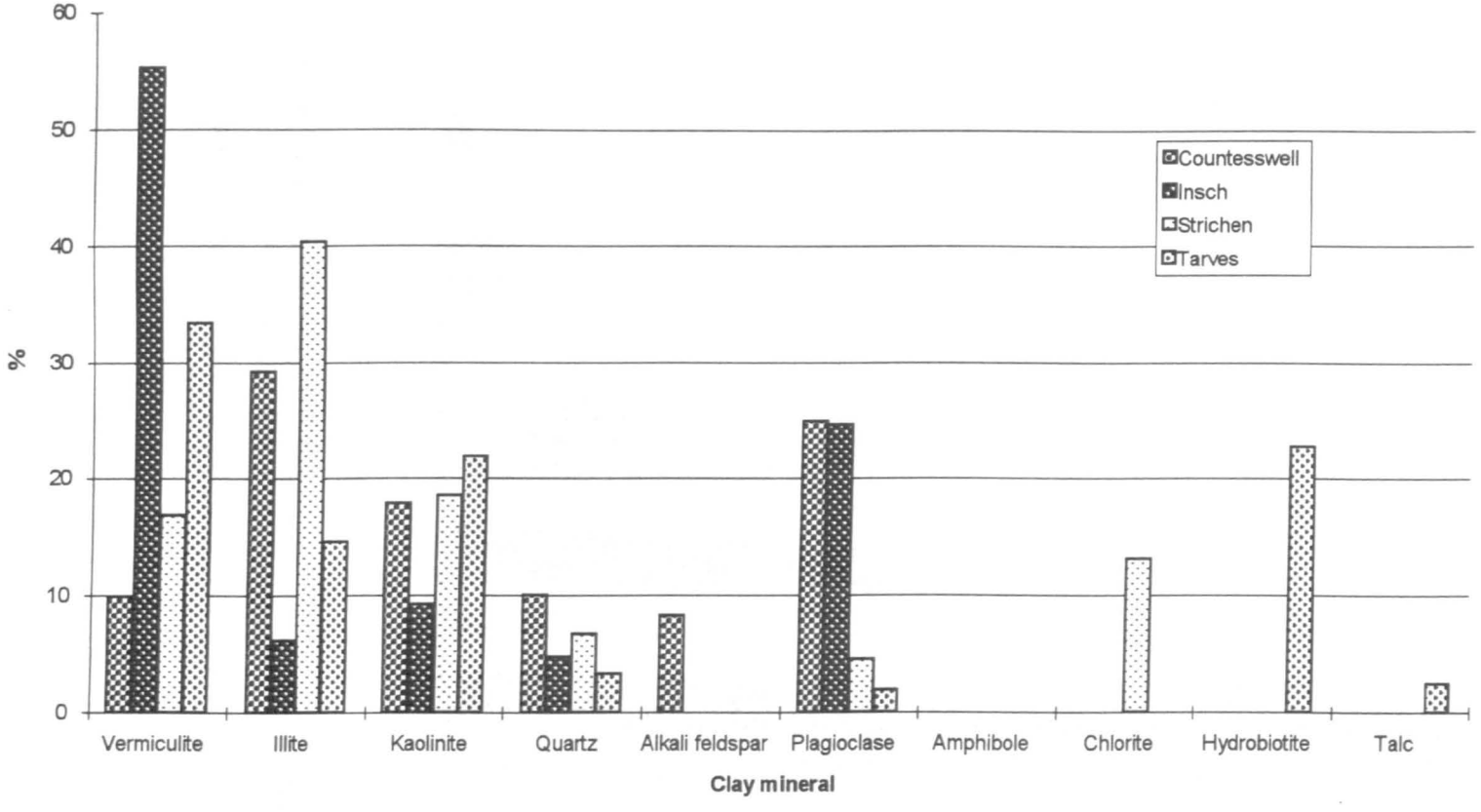
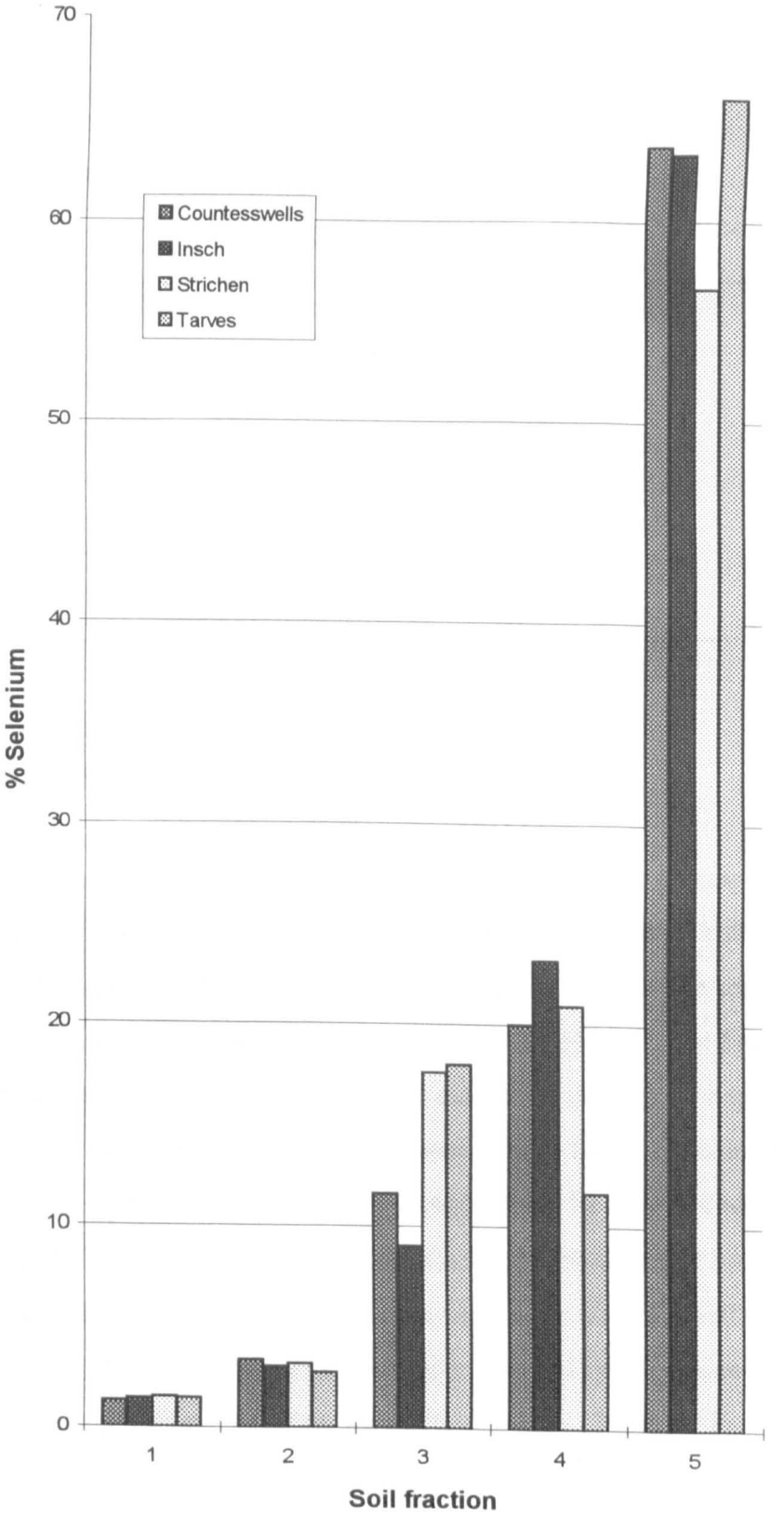


Figure 4.2. Predominant clay minerals in 4 N.E Scotland soils (<2 microns).



Fraction 1=soluble Se; Fraction 2=ligand exchange; Fraction 3=acid extractable; Fraction 4=oxidisable; Fraction 5=residual

Figure 4.3. Percentage of selenium in each soil fraction.

Table 4.10. Selenium in the London sewage sludge fractions.

Fraction	Se (ng g^{-1})	Se (% of total)
1-Soluble Se	42	1.2
2-Ligand exchangeable Se	87	2.5
3-Acid extractable Se	164	4.7
4-Oxidisable Se	1166	33.8
5-Residual Se	1988	57.7

4.8.3. Total selenium.

The Se total content of the soils were in the 0.5-0.8 μg g^{-1} range (Table 4.6). The highest value was for the Insch Association soil. Table 4.3 shows this was the least acidic soil and contained the most organic matter. The total Se in each soil and sludge and the total Se obtained from the sum of Se in Fractions 1 to 5 are shown in Table 4.11. The sequential extraction involved 5 steps and the recovery figures obtained showed good agreement with the total Se determined for each sample. The percentage recovery ranged from 92 to 108% with a mean recovery of 100%. The sewage sludge was used in this study had a Se concentration of 3.55 μg $^{-1}$g, which was much higher than the Se content of the soils. The sum of the Se in the 5 Fractions again showed good agreement with that obtained from a single digestion with 97% recovery (Table 4.11).

4.8.4. Reproducibility.

The data obtained for these Fractions shows good reproducibility in the overall totals (coefficient of variation =1-5%, Table 4.11). Reproducibility was least satisfactory within Fraction 1 (mean coefficient of variation = 11%), which

Table 4.11. Recovery of Se from soil and sewage sludge

Sample	ΣFraction 1-5 (ng g^{-1})	CV(%) n=3	Total Se (ng g^{-1})[a]	CV(%) n=3	Recovery(%) (ΣFraction(1-5)/Total)
Countesswells soil	690	3.0	637	4.8	108
Insch soil	699	4.3	761	1.6	92
Strichen soil	726	1.0	710	2.5	102
Tarves soil	550	4.1	546	4.5	101
Sewage sludge	3447	4.6	3550	1.8	97

[a]Total Se measured by single step digestion of whole soil with HNO_3 and HF.

n=Number of replicates.

Σ=Sum.

CV=Coefficient of variation.

was probably due to the small Se levels which were detected, but showed improvement as the Se concentration increased.

4.8.5.Clay mineralogy.

All the soils contained Se adsorbing minerals (Figure 4.4.). The clay fraction from the Insch and the Tarves soil Associations contained > 30% vermiculite. The clay fraction from the Strichen soil Association was dominated by illite. The clay fraction from Countesswells soil Association contained about equal amounts of vermiculite, illite and kaolinite.

4.9. Discussion.

4.9.1. Selenium concentrations in fractions 1-5 of soils and sewage sludge.

Aqueous potassium chloride extracts soluble Se which is readily available for plant uptake. Soluble Se was the smallest of the 5 fractions and accounted for less than 2% of the total Se of these soils (9-11 ng/g). Soluble Se in the sewage sludge accounted for 1.2% (42 ng/g). This indicates that little of the total Se is bioavailable for plants from the soils but the application of the sewage sludge to soil may provide some additional Se for plant uptake.

Aqueous potassium dihydrogen phosphate extracts the Se(IV) which is adsorbed on Fe and other mineral oxides and on clays (Balistrieri and Chao, 1989). Se(IV) is less mobile than Se(VI) but is available (to a lesser degree) for uptake by plants. The low Se concentration (15 ng/g) for the Tarves Association soil may be due to its higher clay content. The dominant clay was vermiculite which has a high adsorption capacity (Hamdy and Gissel-Nielsen, 1977). Vermiculite also predominates in Insch, but Insch contains less total clay. The

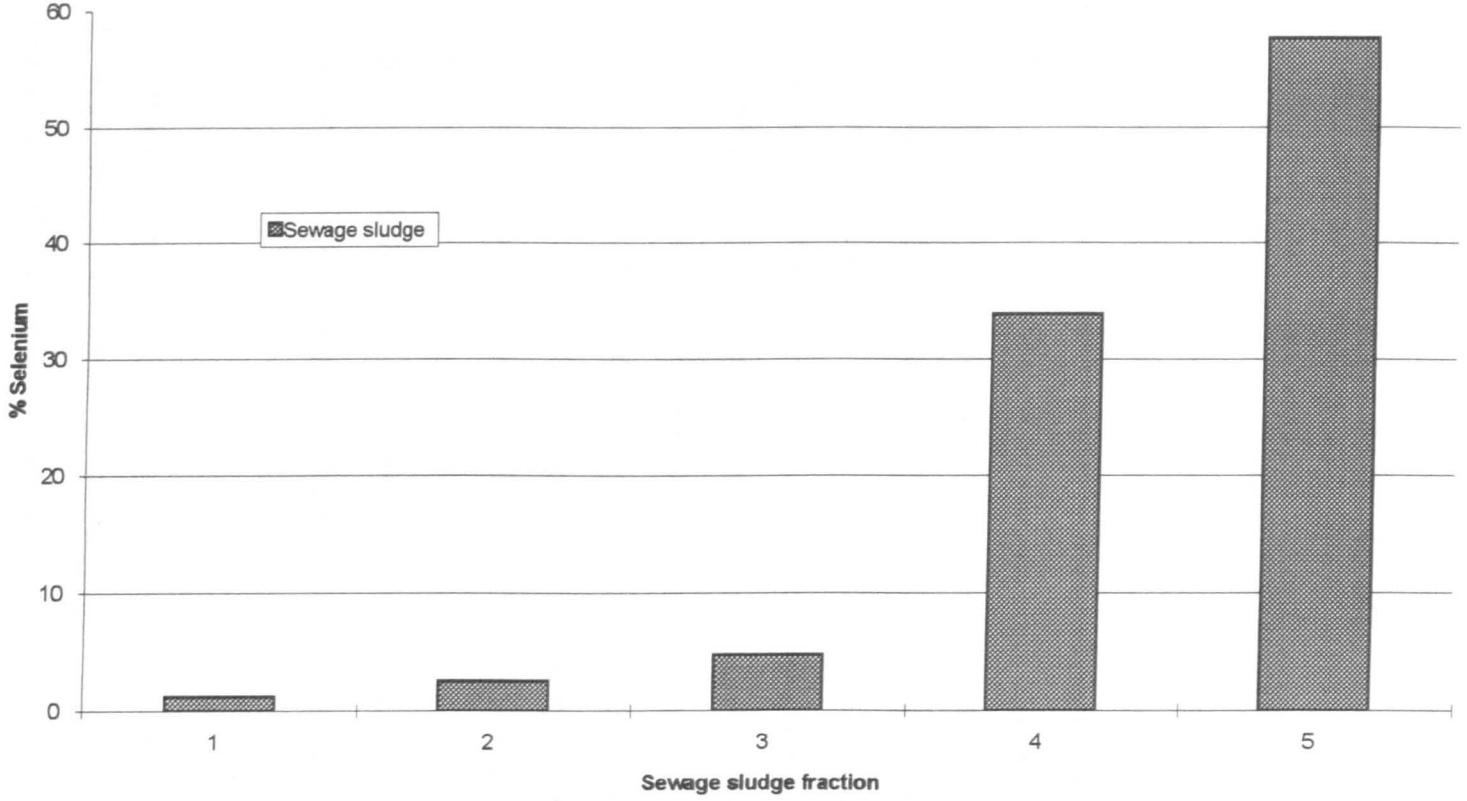


Figure 4.4. Percentage of selenium in each sewage sludge fraction.

dominant clay in the Countesswells and Strichen Asssociations was illite and was present in lesser quantities (Figure 4.2 and Tables 4.2 and 4.4). Selenium concentrations again indicate little Se is available for uptake and availability may be dependent upon soil pH changes and anion exchange reactions. The total of Se in Fraction 1 and Fraction 2 represents the available portion of the total Se pool. This may be determined by a single extraction with KH_2PO_4 to indicate the amount of the total Se which is available for uptake, saving time and reagents. The sewage sludge was found to contain 87 ng g^{-1} Se in Fraction 2.

Hydrochloric acid extracts Se from ferric, Mn and Al oxides but the amount of Se extracted varies and is thought to be incomplete (Chao and Sanzolone, 1989). Ferric oxides have been found adsorb more Se(IV) than the other mineral oxides and clays and therefore the level present plays a significant role in the Se levels found in this Fraction. The Insch Association soil was found to have the lowest Se concentration (63 ng g^{-1}) and has the highest concentration of oxalate extractable ferric oxides. The Countesswells Association soil had the next highest concentration of oxalate extractable ferric oxide and a Se level of 80 ng g^{-1}. Hydrochloric acid also hydrolyses organic matter and again the Insch and Countesswells Associations had the higher organic matter content (Tables 4.2 and 4.3). The other soils both had similar Se concentrations and similar oxalate extractable ferric oxide levels. This may indicate that not all the Se adsorbed onto these oxides has been released by the HCl. The sewage sludge was found to contain 164 ng g^{-1} Se. Se in this form is not readily available but can be released by microbial action, pH changes and reduction in redox potentials. The amounts present in the sewage sludge are double the amounts found in the soils, but are still relatively low and if released would be expected to provide little Se for plant uptake.

Potassium chlorate is a strong oxidising agent and when combined with 12 M HCl will release Se from sulphide minerals and humified organic matter. Se in this fraction is unavailable for uptake by plants. The amount available in the soils

varied between 11 and 23 % (Figure 4.3). The sewage sludge contained 34% Se in this form (Figure 4.4)

The mixture of HNO_3 and HF forms a strong oxidising agent which destroys silicate lattice in soils and sludges and releases Se in minerals which are contained in these lattices. This Se is highly unavailable. The soils and sludge all contained over half of their Se in this form.

Following the procedure of Chao and Sanzolone (1989), the soils and the sewage sludge were ground (<150 μm) prior to extraction. Grinding soils may expose fresh surfaces and change the relative contributions of each of the operationally defined fractions. Further studies are required to quantify the effects. The effect of autoclaving on the distribution of Se between the fractions in the sewage sludge is unknown.

4.9.2. Total selenium levels in soils and sewage sludges.

The Se content of the Scottish soils analysed were found to be in the 0.5-0.8 $\mu g^{-1}g$ range (Table 4.6.). The distribution of selenium in different soils is primarily a function of their parent material (Berrow and Ure, 1989). Although the values were similar, highest values were recorded for the Insch Association soil with parent material derived from gabbros and allied igneous rocks (Table 4.1). This soil had the highest loss on ignition and was the least acid possibly indicating an association with organic matter. Ure and Berrow have reported the mean value of 56 Scottish topsoils to be 0.69 with a range of 0.11-1.59 $\mu g\ g^{-1}$.

The sewage sludges contained significantly higher levels of Se than the soils (Table 4.6). The bioavailability of Se in these sludges is unknown and transfer to crop plants and ultimately the human food chain cannot be assessed without speciation studies.

Limited information is available on the Se content of sewage sludges, but there is evidence that the Se content of sewage sludges in the U.K. has decreased in the period between 1982/3 and 1990/1 from 5 to 1.6 $\mu g\ g^{-1}$ (ENDS, 1993).

The 4 soils used for sequential extraction of Se were selected for a pot experiment which involves the growth of a Se accumulator (the radish) on each of these soils which have been amended with different levels of the sewage sludge (Chapter 5). It was felt to be important to know the forms and availability of selenium in both the soils and sewage sludge being applied. This data at present indicates that little Se will be taken up by the plant from the sewage sludge amended soils.

4.10. Criticisms of sequential extraction procedures.

Criticisms of the use of sequential extractions as opposed to ion chromatography have been expressed Gruebel *et al* (1989). The authors expressed concern that although the Se released from ferric oxides (using H_2NOH-HCl as the extractant) was recovered, this was not the case for goethite and anatase minerals. The reason for this is the extractant was ineffective in releasing Se from these solid phases as after release, readsorption onto other minerals occurred. However, ferric oxides are known to adsorb more Se than any other mineral, perhaps this is not as critical as the authors suggest. The oxidation of complex organic matter was also examined. In this case the authors found that on release, the oxidation state of the Se changed [Se(IV) to Se(VI)]. This should not be problematic in the procedure used for our soils as all the Se is converted to Se(VI) prior to derivatisation. Diverse views are present for speciation methods in general and will perhaps not be resolved until a better understanding of speciation in soils is gained.

4.11. Conclusion.

The sequential extraction method has provided information on the amount of Se associated with different soil and sewage sludge fractions. The bioavailable fractions in both soils and sewage sludge represented < 5% of the total. Using the method of ID-MS, recovery of Se in the fractions ranged from 92-108% of the total determined Se by a single HF and HNO_3 extraction, providing further evidence of the reliability of the isotope dilution method. The determination of Se availability in sewage sludge amended soils has become important due to its increased application to agricultural land. The use of a single extraction with KH_2PO_4 quantitation by ID-MS can be applied to indicate the available Se pool. The 4M HCl extraction can indicate the potentially available Se. This may prove useful in aiding the safer application of sewage sludge to agricultural land.

Chapter 5. Study of selenium uptake by plants.

5.1. Introduction.

In order to assess the environmental impact of amending agricultural soil with sewage sludge containing Se, a series of pot experiments were carried out in which the availability of Se was determined using radish plants grown in amended soils. It is important to be able to assess whether soil has sufficient Se to provide dietary Se to livestock, or if there is too much present. The measurement of total Se in the soil alone does not provide a good measure of Se availability to plants and hence it has often been necessary to determine the actual Se content of the plants. The addition of Se to soils with Se deficient crops has previously been used to increase Se concentrations to overcome Se deficiency in livestock. Selenium accumulator plants may be used to reduce the amount of Se in Se toxic soils (Section 1.5.1).

The addition of Se to soil by sewage sludge application has received little attention. This may be because Se is not subject to the provisions of Directive 86/278/EEC which states maximum concentrations for potentially toxic metals in both the soils and sewage sludges (Section 1.6.2.). However, under the Code of Practice (Department of the Environment, 1989), it was suggested that the Se concentration in sewage sludge applied to agricultural land should not exceed 2 mg kg^{-1}.

Little is known about the plant availability of Se from sewage sludge. There has been some interest in measuring the amounts of Se taken up by plants grown in sewage sludge amended soil and relating this to solid speciation schemes, which classify total sewage sludge Se into readily available, potentially available or unavailable fractions to plants. The characterisation of Se in soil and sewage sludge (which reflects bioavailability of Se rather than total speciation)

was achieved and is discussed in Section 4. The same soils and sewage sludges were used in this plant uptake study. This Section examines firstly the uptake of Se by plants from soils to which Se was added in the form of sodium selenate or sodium selenite using both unlabelled Se and a radioactive isotope of Se (Section 5.4. and 5.7.) and secondly the uptake of Se by plants grown in sewage sludge amended soils (Section 5.8.).

5.2. Choice of plant species.

The radish (*Raphanus sativus* L) was used in this study for several reasons. Firstly, it is a Se accumulator which helps to overcome analytical difficulties with low Se soils. Secondly, the radish is quick growing and grows in acid soils of the kind used in this study. From the analytical point of view the radish is easy to analyse for Se because the bulb forms on the soil surface, soil contamination of this part is less likely than for other crop species.

5.3. Description of soils.

The 4 soils were from North East Scotland and met the rule based criteria for sewage sludge application (Towers, 1994). Their locations and descriptions are given in Table 5.1. Details of soil characteristics are given in Table 5.2 and the major ions present from acetic acid extractions are shown in Table 5.3. Analytical methodologies are discussed in Section 4.2. The soils were sampled during March 1994 and stored as described in Section 4.3.

5.4. Uptake of selenium by radish plants from soils treated with selenium.

This sections studies three experiments which looked at the uptake of Se by the radish plant. Firstly, a preliminary experiment where Se was added to the soil as sodium selenate, secondly where Se was added in the forms of sodium selenate and sodium selenite along with radioactive ^{75}Se and finally where Se was added in the form of sewage sludge.

5.4.1. Experiment 1. Uptake of selenium by radish plants from soils treated with sodium selenate.

The objective of this study was to determine the uptake of Se by radish plants from each of the 4 soils. This was a preliminary experiment to obtain data for the next larger experiments. The soils each received treatments of 0, 1, 2 and 3 $\mu g\ g^{-1}$ of Se as sodium selenate and each treatment was replicated 5 times.

Table 5.1. Location and description of soils.

Soil Association	Location (National Grid Reference)	Description[a]
Countesswells	NJ 850096	Freely drained humus-iron podzol
Insch	NJ 643312	Freely drained brown earth
Strichen	NO 671789	Freely drained humus-iron podzol
Tarves	NJ 855274	Freely drained brown earth

[a] Descriptions from Wilson *et al.* (1984).

Table 5.2. Soil properties.

Soil Association	pH (H_2O)	Loss on ignition (%)	Clay (%)	Oxalate extractable Fe ($g\ kg^{-1}$)
Countesswells	5.6	11.9	8.2	10.2
Insch	5.9	13.3	12.6	28.8
Strichen	5.2	8.5	12.2	9.6
Tarves	5.0	10.5	16.4	9.1

Table 5.3. Acetic acid extractable elements ($mg\ kg^{-1}$).

Soil Association	Ca	Na	K	Mg	P
Countesswells	2076	124	284	132	128
Insch	3208	168	212	104	60
Strichen	1172	148	92	112	88
Tarves	1556	172	208	108	80

5.4.2. Materials.

Sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3) were of analytical grade (Sigma Chemicals).

5.4.3. Pot experiments.

The soil which had been stored in plastic sacks for 8 weeks was seived (2 mm), homogenised, weighed (150 g) and placed in plastic pots (10 cm diameter) resting on saucers. To achieve soil Se concentrations of 1, 2 and 3 $\mu g\ g^{-1}$, 150, 300 and 450 μg of Se were required. A solution containing 9.5 g of Na_2SeO_4 in

500 cm^3 was prepared and diluted to give a Se concentration of 7.94 μg Se cm^{-3}. The amount added to the pots is shown in Table 5.4.

Table 5.4. Amount of selenium as Na_2SeO_4 added to pots containing 150 g soil.

Se concentration (μg g^{-1})	Volume Na_2SeO_4 added (cm^3)	Weight Se added (mg)
0	0	0
1	19	0.15
2	38	0.30
3	57	0.45

A nutrient solution containing N, P and K was prepared from NH_4NO_3 (6.8 g), KH_2PO_4 (5.0 g) and K_2HPO_4 (5.5 g) in 100 dm^3 of deionised water. This gave a N-P-K ratio of 1:2:2.

The selenate solution required per pot was mixed with 6 cm^3 of the nutrient solution and the volume made up to 75 cm^3 with water. The soil for each pot was emptied individually into a mixing bowl and the solution added. After thorough mixing with a spatula, the soil was returned to the pot (lined with Whatmans No 1 filter paper in the base). Since each soil was subjected to 4 levels of Se treatment (replication = 5), there was a total of 80 pots. A separate sample of each soil was weighed, dried at 30^0C and reweighed to obtain the gravimetric moisture content. This value was used to calculate the dry weight of soil taken (Table 5.5).

Table 5.5. Moisture content of soils after drying at 30^0C.

Soil	Moisture (%)	Dry weight of soil per pot (g)
Countesswells	5.5	142
Insch	6.2	141
Strichen	4.0	144
Tarves	5.4	142

5.4.4. Growth and harvesting of radish plants.

Three radish seeds were sown in each pot. The pots were laid out in a randomized block design generated using 'Genstat 5' (1990) as shown in Appendix 1. The pots were watered three times weekly (or as required) with 25 cm^3 of deionised water. After germination (about 2 weeks), the seeds were thinned to 1 per pot. Once a month 6 cm^3 of nutrient solution was added. Harvesting took place after 8 weeks. The soil was washed from the roots and each radish was placed in a preweighed resealable plastic bag. The radishes were freeze-dried (25^0C ambient) and their weights recorded.

5.4.5. Determination of selenium concentrations in whole radish plants.

Each whole radish was ground up using a coffee grinder and transferred to a Teflon bomb (Savillex, Minnesota, USA). After the addition of 100 mg of ^{76}Se isotope solution (concentration 18.507 $\mu g^{-1}g$), 4 cm^3 of HNO_3 (18.5 M) and 400 μl of H_2O_2 (30 volumes) were added and the samples left overnight to pre-digest. A microwave oven was used to complete digestion of the samples. The samples were heated on low power (420 W) for bursts of 0.5, 1, 2, 3 and 4 min. Between heating, the samples were cooled and vented. This digestion method is further

discussed in Chapter 3. After digestion, the Se in the samples was derivatised (Section 2.4) and the Se concentrations determined by ID-MS (Section 2.4).

5.4.6. Results.

Table 5.6. Selenium concentrations in radish plants grown on soil containing 3 μg Se g^{-1} added as selenate.

Soil Association and replicate number		Radish weight (g)	Se concentration in radish (μg/g)	Se uptake (μg)
Countesswells	1	0.710	0.64	0.451
	2	0.457	1.28	0.585
	3	0.487	1.10	0.536
	4	0.600	0.53	0.318
	5	0.447	0.98	0.438
Insch	1	0.558	1.30	0.725
	2	0.483	1.25	0.604
	3	0.817	1.02	0.833
	4	0.741	1.81	1.341
	5	0.435	1.20	0.522
Strichen	1	0.785	1.05	0.824
	2	0.514	1.19	0.617
	3	0.624	1.15	0.718
	4	0.555	0.91	0.505
	5	0.592	1.29	0.764
Tarves	1	0.526	1.15	0.605
	2	0.418	1.20	0.502
	3	0.537	1.47	0.789
	4	0.395	1.27	0.502
	5	1.049	0.83	0.871

The radishes grown in soils containing 0, 1 and 2 μg Se g^{-1} had Se concentrations which were too low to measure. The weights of the radishes were in general low, giving a small mass for analysis. The Se concentration of the radishes grown in soil containing 3 μg Se g^{-1} (Table 5.6) showed a large variation. The radishes which had accumulated the most Se (Table 5.7) were grown on the Insch Association soil (mean Se concentration 1.316 μg g^{-1}), those grown on the Countesswells Association soil showed the lowest Se concentrations (mean 0.906 μg Se g^{-1}). Radishes grown on soil of the Strichen and Tarves Associations had similar Se concentrations of 1.110 and 1.184 μg Se g^{-1} which were not significantly different ($p<0.05$).

Table 5.7. Mean Se concentration in whole radish plants grown in soils amended with 3 μg Se g^{-1} as selenate.

Soil	Mean Se conc. (μg g^{-1})	Mean Se uptake (μg)	C.V. (%)
Countesswells	0.91	0.466	34.7
Insch	1.32	0.805	14.9
Strichen	1.11	0.686	22.5
Tarves	1.18	0.654	19.6
Lsd (p=0.05)	0.132	0.053	

Lsd = least significant difference

5.4.7. Discussion.

Selenium was added to the soils in the form of selenate as it is known to be more available for plant uptake than selenite. The total Se concentration of each soil had been determined (Section 4.9.1.) and ranged from 0.5-0.8 μg g^{-1} with < 5% of the Se available for plant uptake. The Se concentrations in the radishes grown on soils amended with 0, 1 and 2 μg Se g^{-1} could not be measured. This was mainly the result of the low weights of the individual radishes. These low masses were thought to be due to sub-optimum addition of nutrients

such as phosphorus (Table 5.3). The soils had low to medium P status (MLURI Bulletin 1, 1983).

The radishes grown on soils amended with 3 μg Se g^{-1} showed Se mean concentrations (Table 5.7) ranging from 0.91 μg g^{-1} for the Countesswells Association soil to 1.32 μg g^{-1} for the Insch Association soil and these concentrations were significantly different ($p<0.05$). The Insch Association soil has the highest pH (5.9) of the four soils (Table 5.2). In acidic soils, Se is expected to be mainly in the form of selenite (Section 1.4 and Figure 1.1). Since Se was added in the form of selenate, it may be that the less acidic Insch soil was not sufficiently low pH to reduce the selenate form to selenite. Ferric oxides have a high Se(IV) adsorption capacity and Insch Association soil has high oxalate extractable iron levels (28.8 g kg^{-1}) compared to the other soils studied (9.0-10.2 g kg^{-1}) as shown on Table 5.2. The Insch Association soil also has the most organic matter (13.3%) which has the capacity to remove Se from soil solutions (Levesque, 1974; Bisbjerg and Gissel-Nielsen, 1969). Hence, it would be expected that more Se would be adsorbed onto organic matter and ferric oxides in the Insch Association soil than for the other soils.

There appears to be little reason why the radishes grown on Countesswells soil had the lowest Se levels The soil did not have the lowest total Se (Table 4.6) nor the least amount of available Se (Table 4.9). This soil had a pH of 5.6, and a low clay content (8.2%) with no dominant clay which strongly adsorbs Se. However, it did have the highest levels of extractable P (Table 5.3). Conflicting results on Se-PO_4^{3-} interaction have been found. Singh (1979) found that P additions reduced Se uptake and Levesque (1974) found the addition of P increased Se concentrations in some plants but not in others.

The other soils (Strichen and Tarves Associations) had similar pH values (5.0 and 5.2) and similar amounts of oxalate extractable Fe (9.6 and 9.1 g kg^{-1}). There was no significant difference in the Se concentrations in the radishes grown on these soils (1.11 and 1.18 μg g^{-1}, respectively) but the Se concentrations were

significantly different from the Se concentrations of the radishes grown on soils of the other Associations.

The average weights of the plants grown on the 4 different soils were not significantly different ($p<0.05$). Their overall growth was poor and there was a wide variation in the weights within each soil. The mean uptake of Se by the radish followed a similar trend to the Se concentration with those grown on the Insch Association showing the largest Se uptake, those grown on the Countesswells Association the lowest Se uptake and there was no significant difference between those grown on the Tarves and Strichen Association soils.

Previous Se uptake studies have used a variety of plants. Hurd-Karrer (1935) showed wheat grown on soil amended with 5 μg Se g^{-1} (as selenate) contained 1.19-1.24 μg Se g^{-1}. Banuelos and Meek (1990) found 5 different plant species contained Se concentrations in the range from 0.14-1.29 μg Se g^{-1} after growth in soil amended with 3.5 μg Se g^{-1} in the form of selenate. Banuelos *et al* (1993) found a Se concentration of 2 μg g^{-1} in wild mustard (a *Brassica* species) grown in soil containing 1.17 μg Se g^{-1} as selenate. In a field study Gupta *et al* (1993) determined Se concentrations in barley grown in the field where the soil was amended with 10, 20 and 40 g Se per hectare (4, 8 and 16 mg Se kg^{-1}). When the Se was in the form of selenate, the concentrations of Se in the barley were 0.2, 0.4 and 0.9 μg g^{-1}, respectively. The addition of Se as selenite was found to have little effect on the Se concentration in the barley. The Se concentrations of 42, 57 and 76 μg g^{-1} were in the same range as the control (33 μg g^{-1}). These results were obtained by pooling 8 plants (to increase the total mass of plant material) to determine the Se concentrations. The studies discussed in this section show a range of Se concentrations in the plant species which are around 1 μg Se g^{-1}.

5.4.8. Conclusion.

Radish plants grown on the soils which received no added N-P-K fertilizer were small (Table 5.6). The Se concentrations in plants grown in soils amended with 0, 1 and 2 μg Se g^{-1} was insufficient for detection using ID-MS.

The results obtained for the radish plants grown in soils amended with 3 μg Se g^{-1} showed that the plants which contained the highest Se concentrations were those grown on the Insch soil. This leads to the propositions that soil pH or soil phosphate levels or a combination of these may be the controlling factor when Se is in the form of selenate.

The plant feeding regime needs to be reviewed for future experiments with healthier plants giving a larger mass for analysis. The decision was also made to measure the Se levels by growing more than 1 plant per pot and pooling these together if necessary to obtain measurable Se amounts.

5.5. Preparation of soils and solutions for study of selenium uptake by radish using radioactive ^{75}Se.

5.5.1. Objectives.

The objective of this study was to measure the uptake of Se by the radish from the four soils using ^{75}Se as a radiotracer. The use of radioisotopes overcomes sensitivity problems experienced by ID-MS methods and a greater number of samples can be processed. Selenium was to be added to the soils in two forms; sodium selenate or sodium selenite. The treatments to be applied were to give Se concentrations of 1 and 2 μg g^{-1} at two different pH levels. Radiotracers only measure the uptake from the added Se and hence all the results

obtained in this experiment refer only to net uptake of added Se rather than natural Se.

5.5.2. Adjustment of soil pH by the addition of lime.

The effect of liming on the uptake of Se was investigated. The recommended pH for the growth of *Cruciferae* such as the radish and the turnip is pH 6.2 (The Scottish Agricultural Colleges, 1985). As a rule of thumb to raise the pH of 1 hectare of soil (approximately 2,500 tonnes) by 0.1 pH units, 8 tonnes of lime are required. Hence, to raise the pH of 1 kg of soil by 0.1 pH units, 320 mg of lime were required. The amount of lime (as ground limestone) added to each soil is shown in Table 5.8. The soils (seived (2 mm) and homogenised) and lime were mixed thoroughly and stored in polythene bags for 3 weeks prior to planting to allow reaction to occur.

Table 5.8. Amount of ground limestone added to each soil to raise the pH to 6.2.

Soil Association	pH (H_2O)	Soil weight (kg)	Weight lime added (g)
Countesswells	5.6	16.5	31.6
Insch	5.9	21.0	19.3
Strichen	5.2	20.0	64.0
Tarves	5.0	20.0	76.7

5.5.3. Preparation of sodium selenate and sodium selenite solutions.

(1) Sodium selenate: 1.8956 g of Na_2SeO_4 was dissolved in 100 cm^3 of deionised water. A tenfold dilution of this solution gave a solution with a Se concentration of 0.079 mg cm^{-3}.

(2) Sodium selenite: 1.6972 g of Na_2SeO_3 was dissolved in 100 cm^3 of deionised water. A tenfold dilution of this solution gave a solution with a Se concentration of 0.079 mg cm^{-3}.

5.5.4. Preparation of $Na_2{}^{75}SeO_3$ and conversion to $Na_2{}^{75}SeO_4$.

Radiolabelled ^{75}Se was obtained from Amersham Life Science (Little Charlton, England) as an aqueous solution of sodium selenite with an activity of 84 Mbq cm^{-3}. This is a gamma emitter with a half life of 120 days. The solution contained a small amount of carrier (115 μg Se cm^{-3}).

(1) Selenite: The ^{75}Se was supplied as aqueous sodium selenite. An aliquot (0.2 cm^3) was diluted to 10 cm^3 with deionised water.

(2) Selenate: The conversion of sodium selenite (0.2 cm^3) to sodium selenate was achieved by heating the solution with 2 cm^3 HNO_3 (18.5 M) at 95^{0}C for 30 min (Chao and Sanzolone, 1989). This solution was also diluted to 10 cm^3 with deionised water.

5.5.5. Preparation of $Na_2{}^{75}SeO_4$ and $Na_2{}^{75}SeO_3$ solutions.

(1) $Na_2{}^{75}SeO_4$ stock solutions: 3 cm^3 of the radioactive $Na_2{}^{75}SeO_4$ solution was made up to 250 cm^3 using 0.079 mg cm^{-3} sodium selenate solution. A further 3 cm^3 of the radioactive sodium ^{75}selenate solution was made up to 500 cm^3 with 0.079 mg cm^{-3} sodium selenate solution.

(2) $Na_2{}^{75}SeO_3$ stock solutions: Two 3 cm^3 samples of the radioactive sodium ^{75}selenite solution were made up to 250 and 500 cm^3 using the sodium selenite solution as described for $Na_2{}^{75}SeO_4$.

A further set of solutions were prepared by diluting 3 cm^3 of the $Na_2{}^{75}SeO_4$ radioactive solution (from 10 cm^3 solutions prepared in Section 5.5.5) to 250 cm^3 with deionised water. These was used in a preliminary experiment to study the transfer of the radioisotope from soil to plant. All the solutions were stored in plastic bottles.

5.5.6. Selenium additions to soil.

The soil Se concentrations required for the plant uptake study were 1 and 2 μg/g. The initial weight of soil in each pot was 400 g, but this was corrected for moisture content (Table 5.9).

Table 5.9. Weight of soil used for ^{75}Se uptake study.

Soil	Initial soil weight (g)	Moisture content (%)	Dry soil weight (g)	Se concentration in soil after addition of selenate and selenite solution (μg g^{-1})	
Countesswells	400	5.5	378	1.0	2.1
Insch	400	6.3	375	1.0	2.1
Strichen	400	5.9	376	1.0	2.1
Tarves	400	6.5	374	1.0	2.1

Hence the additions of Se were approximately 0.4 and 0.8 mg. The amounts of Se added to each pot are as follows:-

Se as $Na_2{}^{75}SeO_4$

1 μg g^{-1} Se addition: obtained from 250 cm^3 $Na_2{}^{75}SeO_4$ solution.

Se content = 78.5 μg cm^{-3}

Addition to pot = 5 cm^3

Total Se added = 0.393 mg

2 μg g^{-1} Se addition: obtained from 500 cm^3 $Na_2{}^{75}SeO_4$ solution.

Se content = 78.5 μg cm^{-3}

Addition to pot = 10 cm^3

Total Se added = 0.785 mg

Se as $Na_2{}^{75}SeO_3$

No addition of Se to soil for preliminary experiment to study transfer percentage from soil to plant.

Se content = 0.03 μg cm^{-3}

Addition to pot = 5 cm^3

Total Se added = 0.15 μg

1 μg g^{-1} Se addition: obtained from 250 cm^3 $Na_2{}^{75}SeO_3$ solution.

Se content = 78.1 μg cm^{-3}

Addition to pot = 5 cm^3

Total Se added = 0.390 mg

2 μg g^{-1} Se addition: obtained from 500 cm^3 $Na_2{}^{75}SeO_3$ solution.

Se content = 78.1 μg cm^{-3}

Addition to pot = 10 cm^3

Total Se added = 0.781 mg

5.5.7. Radioactivity of $Na_2{}^{75}SeO_4$ and $Na_2{}^{75}SeO_3$ stock solutions.

$Na_2{}^{75}SeO_4$:

The original volume of $Na_2{}^{75}SeO_4$ was 0.2 cm^3. This was made up to 10 cm^3 to give a solution containing 16.8 MBq radioactivity. After dilution, the amount of radioactivity added to each pot was 0.1008 MBq.

$Na_2{}^{75}SeO_3$:

The original volume of $Na_2{}^{75}SeO_3$ was 0.2 cm^3. This was made up to 10 cm^3 to give a solution containing 16.8 MBq radioactivity. After dilution, the amount of radioactivity added to each pot was 0.1008 MBq.

5.5.8. Conversion of radioactivity from MBq to disintegrations per minute (DPM).

Radioactivity added to each pot was 0.1008 MBq or 6,038,400 DPM. The radioactivity was calculated as DPM to relate to the analytical instrumentation output which is measured as CPM.

5.5.9. Preliminary ^{75}Se uptake study, optimum harvesting time, the effect of freeze-drying and soil homogenity tests.

5.5.9.1. Transfer percentage of solutions containing ^{75}Se.

For this study, 5 pots for each soil were lined with filter paper and placed in saucers. Each of the soils (400 g) were mixed with 5 cm^3 of the $Na_2{}^{75}SeO_3$ solution (to give soil Se concentration of 0 $\mu g\ g^{-1}$ from Se added) and placed in the pots. The soils were watered (100 cm^3) and fed with 'Phostrogen' (10 g/10 L; N-P-K ratio 1:2:1.5). Two radish plants per pot were planted and these were thinned to 1 per pot after germination. The radish plants were harvested after 7 weeks. This was achieved by pulling the radish from the soil, washing any remaining soil from the radish, removing the roots, and placing plant parts into a preweighed vial. The radioactivity of each sample was measured using an auto-gamma counter (Section 5.6).

5.5.9.2. Uptake studies to calculate optimum harvesting time, the effect of freeze drying and homogenity of selenium in soil.

In the preliminary ^{75}Se uptake study, 4 pots were lined with filter paper and placed in saucers. Only the Insch soil was used (400 g) and this was mixed with 10 cm^3 of $Na_2{}^{75}SeO_3$ stock solution and placed in the pots. The radishes were planted (as described in Section 5.5.9.1) and harvested after 5, 6, 7 and 8 weeks. The radioactivity of the 'bulb' and leaves were measured in an auto-gamma counter (Section 5.6). The samples were freeze-dried and the radioactivity remeasured. Subsamples of soil from one pot were also taken and their radioactivity measured.

5.5.10. Results and discussion of preliminary selenium uptake experiments.

The transfer percentages (Table 5.10) range from 0.6-3.4%. The radishes grown on the Tarves Association soil showed the highest transfer, followed by the Countesswells Association soil. There was no significant difference for those radishes grown on the Tarves or Strichen Association soils.

Table 5.10. Percentage transfer of ^{75}Se to the radish plant.

Soil Association	Transfer (%)[a] ^{75}Se as selenite
Countesswells	2.2
Insch	1.1
Strichen	1.5
Tarves	3.4
Lsd (p = 0.05)	0.288

[a]Based on total radioactivity of 6038400 DPM added.

Table 5.11. Amount of radioactivity (^{75}Se) in the radish from preliminary study.

Week of sampling	Activity in radish (CPM)		Corrected activity* (DPM)	
	Bulb	Leaves	Bulb	Leaves
5	42074	302941	55517	399733
6	81959	302005	112608	414940
7	110187	672997	157639	962820
8	84902	671704	128687	1018113

*Corrected for background, efficiency and time.

The results obtained (Table 5.11) for optimum harvesting time showed that the level of radioactivity increased for weeks 5 to 7 but the Se concentration in the bulb fell in week 8. This was a preliminary experiment and there was no replication. Hence these results were indicative only. The level of activity was found to be greater in the leaves than the bulb. The activity corrected to zero time was obtained from the equation :

$$N_0 = N\exp(t/t_{1/2}.\ln 2)$$

where N_0=corrected activity, N=measured activity, t=time after initial radioactive decay date, $t_{1/2}$=half life of radioisotope. Two of the radish plants measured for radioactivity were freeze-dried and their radioactivity re-measured (Table 5.12). The results were similar showing that any ^{75}Se lost during freeze drying (from volatile organoselenium compounds) was negligible(+/- 5%). The percentage of ^{75}Se taken up by the radish was calculated (Table 5.13). Hence ^{75}Se had been taken up by the plant whether it had been supplied with carrier (0.079 mg cm^{-3} sodium selenite solution) or without carrier as an aqueous solution. Finally, 5 subsamples of soil (5 g each randomly taken from a pot which had been emptied onto a clean surface) were counted (Table 5.14) and found to vary between

22377 and 32143 counts per minute (CPM) showing the distribution of ^{75}Se was not homogeneous.

Table 5.12. Effect of freeze-drying on radioactivity in radish.

Radish part	Corrected activity before freeze drying (CPM)	Corrected activity after freeze drying (DPM)	Change on drying (%)
Bulb (plant 1)	55517	53454	-4.0
(plant 2)	37101	36075	-2.8
Leaves (plant 1)	399733	405099	+1.3
(plant 2)	329190	332135	+1.0

Table 5.13. Percentage uptake of ^{75}Se by the radish (Activity added to soil = 6038400 CPM).

Radish section	Week	Activity in radish (DPM)	^{75}Se uptake (%)
Bulb	5	55517	0.92
	6	112608	1.86
	7	157639	2.61
	8	128687	2.13
Leaves	5	399733	6.62
	6	414940	6.87
	7	962820	15.94
	8	1018113	16.86

5.14. ^{75}Se activity in 5 subsamples of soil (5 g) from 1 pot.

Subsample	Radioactivity (CPM) in 5 g
1	22377
2	32143
3	28577
4	29070
5	22649

5.5.11. Conclusions.

The results of the preliminary experiment led to the following conclusions:

(1) Adding about 0.1 MBq of ^{75}Se to each pot was sufficient to allow radioactive counting of the radish sample in a short time.

(2) The addition of isotope solution to the soil for each pot should be made up to 100 cm^3 with deionised water prior to mixing with the soil. This should lead to a more homogeneous distribution of ^{75}Se in the soil.

(3) Harvesting of the radish should be performed after 7 weeks of growth.

(4) Freeze-drying does not result in a loss of radioactive ^{75}Se confirming no loss of organoselenium compounds.

5.6. Optimisation of gamma counter parameters.

A Canberra Packard autogamma counting system (Cobra Series, Model 5003) was used. This is equipped with a thallium activated sodium iodide crystal detector (3 inches).

5.6.1. Parameters set for Cobra II autogamma counter.

A counting window for ^{75}Se in the range 80-467 KeV was used. Counting was continued until the error (% Sigma) was < 1 % up to a maximum of 20 min counting time (Haygarth *et al*, 1995). Prior to each use, the response of the photomultiplier was calibrated using a ^{137}ceasium reference. The radioactivity of a blank solution was also determined for each run. The vials used (5 cm^3) were obtained from Canberra Packard.

5.6.2. Calculation of efficiency of autogamma counter.

Two samples (0.1 cm^3) from each of the $Na_2{}^{75}SeO_4$ and $Na_2{}^{75}SeO_3$ solutions were made up to 1 cm^3. The activity of each of the stock isotope solutions was 0.1008 MBq /5 cm^3. Hence, the activity of these solutions was 5.45 x 10^{-2} mCi cm^{-3} (120990 DPM cm^{-3}). This level of radioactivity was equivalent to 2% of the amount of radioactivity which was added to each radish pot. The count time was 2 min (which was found to be sufficient for the error to be < 1 % sigma) and the elevation postion set at 1. This was repeated for elevator positions 2, 3 and 4. The results (Table 5.15) show that maximum efficiency occurred at elevator position 2. The mean count obtained (to calculate efficiencies) for ^{75}Se as sodium selenate was 103025 CPM and for ^{75}Se as sodium selenite was 90706 CPM. Hence the efficiencies for the ^{75}Se and ^{75}Se solutions were 85% and 75% respectively, leading to the proposition that some of the radioactivity from the ^{75}selenite solution had been lost by surface absorption during preparation of solutions.

5.6.3. Investigation of elevator positions for volumes of 1, 2, 3, 4 and 5 cm^3.

The radioactivity of the ^{75}Se solutions was measured at elevator positions 1-4. The solutions were made up to 2 cm^3 with deionised water and measured at each elevator position. This was repeated for volumes of 3, 4 and 5 cm^3. The results (Table 5.16) show maximum efficiency was obtained at elevator position 2 for volumes of 1, 2 and 3 cm^3 and elevation position 3 gave maximum efficiency for 4 and 5 cm^3.

5.6.4. Use of ^{75}Se calibration standards.

Solutions were prepared from each $Na_2{}^{75}SeO_4$ and $Na_2{}^{75}SeO_3$ stock solutions (Section 5.5.6). An aliquot of 0.1 cm^3 was diluted to 1 cm^3 for each of the 4 stock solutions. Table 5.17 shows the radioactivity levels, the values obtained both originally and after a 6 week period, and the values after correction for background, efficiency and time. After a 6 week interval, there was negligible changes in the radiation levels detected. These calibration standards were used to check that the instrument was performing satisfactorily prior to analysis of the radish samples.

5.15. Radioactivity recorded for 1 cm^3 ^{75}Se solutions at elevation positions 1-4.

Sample	Radioactivity (CPM) for elevator positions*			
	1	2	3	4
$Na_2{}^{75}SeO_4$	99606	101966	96926	93143
$Na_2{}^{75}SeO_4$	99366	104083	97586	92602
$Na_2{}^{75}SeO_3$	92123	90903	88806	86039
$Na_2{}^{75}SeO_3$	87523	90509	88890	85218

*% sigma <1%.

5.16. Radioactivity for 1-5 cm^3 ^{75}Se solutions which are at elevation positions 1-5.

Sample volume (cm^3)	Radioactivity (CPM) for elevator positions*			
	1	2	3	4
1	94402	95971	93990	90594
2	94170	95649	94851	92330
3	92967	95173	94603	92491
4	90645	94337	94968	92699
5	85621	92658	93937	93738

*% sigma <1%.

5.17. Radioactivity recorded for calibration standards.

Calibration standard from selenate and selenite solutions (Section 5.5.6)	Initial radioactivity* (CPM)	Radioactivity* after 6 weeks (CPM)
$Na_2{}^{75}SeO_4$ (500 cm^3)	115195	114172
$Na_2{}^{75}SeO_4$ (250 cm^3)	57832	57408
$Na_2{}^{75}SeO_3$ (500 cm^3)	122066	122672
$Na_2{}^{75}SeO_3$ (500 cm^3)	61420	61400

*Corrected for background, efficiency and time.

5.7. Experiment 2. Study of selenium uptake by the radish using ^{75}Se.

5.7.1. Planting procedure used for selenium uptake study using ^{75}Se.

The initial weight of soil taken for each pot was 400 g. The air dry weights are given in Table 5.8. The soil was placed in a mixing basin. The required volume of the stock isotope solutions (activity = 0.1008 MBq) was made up to 100 cm^3 and added to the soil. After thorough mixing, the soil was returned to its pot (with

filter paper in base). Each soil was treated with two Se levels at two pH values and replicated 5 times. This resulted in 80 pots for each of the sodium selenate and sodium selenite treatment.

The radish seeds were planted (2 per pot) and were watered three times a week. The radishes received a nutrient solution of 'Phostrogen' (10 g/10 L) weekly (50 cm^3). Randomisation of the layout of the pots was achieved using 'Genstat 5' (1990) as shown in Appendix 2.

5.7.2. Harvesting and radioactive counting of radish plants.

Harvesting took place after 7 weeks of growth. The plants were extracted from the soil and the roots removed. The radish 'bulbs' were washed and placed in resealable polythene bags. The leaves were placed in pre-weighed plastic vials. Samples of the roots for each treatment were washed and placed into the pre-weighed vials. The samples were freeze dried and the radish bulbs transferred to the pre-weighed vials. The samples were placed in a Cobra II autogamma counter (Canberra Packard, Conneticut, USA) and the radioactivity measured. The concentrations of Se in the leaves, bulb and radish were obtained by correcting the radioactivity reading for background, counting efficiency and radioactive decay. The recorded plant weights allowed DPM mg^{-1} to be calculated as follows :

DPM in plant / DPM added to pot x weight of added Se / dry weight of plant

5.7.3. Results.

The concentration of Se in the plants grown on soils containing 1 mg Se kg^{-1} was obtained by using the relationship that 395 μg Se as sodium selenite or selenate equated to 6,038,400 CPM and hence 15287 CPM equated to 1 μg of

Se. Similarly, for plants grown on soil containing 2 mg Se kg^{-1}, 7643 CPM equated to 1 μg of Se. The results were divided into selenate and selenite groups, followed by the concentrations of Se found in the leaves, bulb and the whole radish less the roots (Tables 5.18-5.23). Roots samples from radishes grown in each soil were randomly selected, the soil washed off and the radioactivity of the roots counted. This was time consuming and it was difficult to totally remove the soil stuck to the roots. The results ranged between 600 and 800 CPM and it was decided that this was neglible (< 0.5 %) compared to the CPM obtained for bulb and leaves. It can be seen that the concentration of Se in the form of selenate is 2 to 3 times higher than for Se as selenite. It was also observed that the leaves contained a higher concentration of Se than the bulb. An increase in the amount of sodium selenate added to the soil led to an increase in the Se concentration in the radish. This trend was not observed when sodium selenite was added to the soil. The adjustment of soil pH to pH 6.2 gave mixed results, with both increases and decreases in Se uptake being observed. In order to assess the effect of soil type, pH and level of Se added on Se uptake, a 3 way analysis of variance (ANOVA) was performed using 'Genstat 5' (1990) as shown in Appendix 3.

5.7.3.1. Effect of soil type on plant uptake of selenium as sodium selenite.

The radishes grown on the Tarves Association soil showed the largest Se concentrations (Table 5.24) when soil pH and the amount of Se added to the soil are considered as a whole. The radish plants grown on the Insch Association soil had the lowest Se concentration. The Se concentration in radishes grown on the Strichen Association soil was not significantly different ($p<0.05$) from the Tarves Association.

Table 5.18. Concentration of selenium (from added selenium) in radish leaves after treatment of soil with sodium selenate.

Soil	pH	Se added (mg kg^{-1})	Se conc. (mg kg^{-1})	Coefficient of variation (%) n=5
Countesswells	5.6	1	49.8	3.8
		2	57.9	3.5
	6.2	1	22.2	9.4
		2	46.4	4.2
Insch	5.9	1	33.9	5.4
		2	127.6	2.7
	6.2	1	43.7	4.2
		2	115.7	3.0
Strichen	5.2	1	54.9	2.7
		2	87.5	2.9
	6.2	1	65.1	2.1
		2	75.3	4.8
Tarves	5.0	1	27.5	10.1
		2	121.8	3.1
	6.2	1	25.1	12.9
		2	47.1	6.7

Table 5.19 Concentration of selenium (from added selenium) in radish bulb after treatment of soil with sodium selenate.

Soil	pH	Se added (mg kg^{-1})	Se conc. (mg kg^{-1})	Coefficient of variation (%) n=5
Countesswells	5.6	1	15.5	6.0
		2	27.4	10.1
	6.2	1	14.1	6.2
		2	20.9	2.8
Insch	5.9	1	19.0	7.2
		2	35.6	3.4
	6.2	1	22.1	3.3
		2	37.8	2.9
Strichen	5.2	1	13.6	3.0
		2	35.3	3.2
	6.2	1	17.5	4.8
		2	36.5	2.7
Tarves	5.0	1	10.0	13.1
		2	34.9	3.6
	6.2	1	8.5	9.3
		2	22.9	5.4

Table 5.20. Concentration of selenium (from added selenium) in radish (bulb and leaves) after treatment of soil with sodium selenate.

Soil	pH	Se added (mg kg^{-1})	Se conc. (mg kg^{-1})	Mean Se uptake (μg)	Coefficient of variation (%) n=5
Countesswells	5.6	1	26.5	60.8	25.8
		2	37.8	77.0	10.0
	6.2	1	16.1	31.5	7.1
		2	28.9	48.8	7.3
Insch	5.9	1	25.9	35.6	9.0
		2	70.4	103.8	9.2
	6.2	1	32.1	33.4	6.2
		2	71.9	95.6	9.6
Strichen	5.2	1	25.9	45.2	10.1
		2	56.9	106.2	8.9
	6.2	1	35.2	57.8	8.5
		2	53.1	82.6	11.6
Tarves	5.0	1	17.0	30.6	18.9
		2	81.0	130.8	9.1
	6.2	1	14.0	25.8	9.0
		2	34.4	46.9	10.7

Table 5.21. Concentration of selenium (from added selenium) in radish leaves after treatment of soil with sodium selenite.

Soil	pH	Se added (mg kg^{-1})	Se conc. (mg kg^{-1})	Coefficient of variation (%) n=5
Countesswells	5.6	1	11.3	3.0
		2	40.8	2.2
	6.2	1	8.6	11.3
		2	8.6	10.5
Insch	5.9	1	10.7	15.6
		2	7.9	13.6
	6.2	1	5.7	13.6
		2	4.3	11.8
Strichen	5.2	1	23.3	7.6
		2	28.5	8.3
	6.2	1	15.3	2.6
		2	15.8	35.4
Tarves	5.0	1	11.3	8.2
		2	13.6	7.9
	6.2	1	14.6	4.1
		2	28.7	3.6

Table 5.22. Concentration of selenium (from added selenium) in radish bulb after treatment of soil with sodium selenite.

Soil	pH	Se added (mg kg^{-1})	Se conc. (mg kg^{-1})	Coefficient of variation (%) n=5
Countesswells	5.6	1	4.0	3.0
		2	10.8	5.9
	6.2	1	4.6	7.0
		2	9.1	17.3
Insch	5.9	1	4.9	8.3
		2	3.8	8.5
	6.2	1	4.3	3.5
		2	2.3	7.7
Strichen	5.2	1	6.1	10.6
		2	11.6	2.5
	6.2	1	5.5	49.3
		2	6.4	11.1
Tarves	5.0	1	5.9	8.1
		2	7.8	16.7
	6.2	1	7.3	8.6
		2	18.0	6.7

Table 5.23. Concentration of selenium (from added selenium) in radish (bulb and leaves) after treatment of soil with sodium selenite.

Soil	pH	Se added (mg kg^{-1})	Se conc. (mg kg^{-1})	Mean Se uptake (μg)	Coefficient of variation (%) n=5
Countesswells	5.6	1	5.8	13.6	23.8
		2	18.1	39.1	7.1
	6.2	1	5.8	11.6	10.9
		2	9.1	18.4	16.8
Insch	5.9	1	6.7	8.2	10.8
		2	5.1	6.6	9.1
	6.2	1	4.9	7.4	6.0
		2	2.9	3.4	7.4
Strichen	5.2	1	10.9	18.4	10.1
		2	17.3	22.8	12.1
	6.2	1	7.6	10.4	31.2
		2	10.8	15.2	11.4
Tarves	5.0	1	7.8	15.2	3.7
		2	9.7	15.3	7.5
	6.2	1	9.2	13.6	3.5
		2	21.4	39.6	4.0

Table 5.24. Mean concentrations of Se in radishes (roots + bulb) grown on soils at 2 pH values in soils containing additions of 1 and 2 mg Se kg^{-1}as selenite.

Soil Association	Se concentration in radish (mg kg^{-1})
Countesswells	9.63
Insch	4.88
Strichen	11.68
Tarves	12.03
Lsd (p=0.05)	0.740

Lsd = Least significant difference for comparison between soils

5.7.3.2. Effect of liming and amount of selenium added (as sodium selenite).

The addition of lime to the soils produced variable results for the concentration of Se by the radish (Table 5.25). The Se concentration in the radish decreased for each soil Association apart from Tarves which showed a large increase.

Increasing the soil Se level from 1 mg kg^{-1} to 2 mg kg^{-1} increased the Se concentrations in the radish in all cases apart from Insch (Table 5.25). There was no significant differences ($p<0.05$) in the concentration of Se between plants grown on Countesswells and Insch Association soils or Strichen and Tarves Association soils containing 1 mg Se kg^{-1}. When the soils contained 2 mg Se kg^{-1}, again no significant differences were found between Strichen and Tarves soils.

The decrease in the Se levels on plants grown on the Insch soil as the Se supplied increased from 1 to 2 mg Se kg^{-1} was also found when both normal pH and pH 6.2 were considered (Table 5.26). At 1 mg Se kg^{-1}, no significant differences were observed between Countesswells and Insch Associations, but this

was not repeated at 2 mg Se kg^{-1} as the Se levels in those plants grown in Insch soil decreased.

Table 5.25. Concentration of selenium (from added selenium) in radish (mg kg^{-1}) dry weight in limed and unlimed soils.

Soil Association	unlimed	limed	Se added to soil	
	Se mg kg^{-1}		1 mg kg^{-1}	2 mg kg^{-1}
Countesswells	11.95	7.31	5.68	13.58
Insch	5.89	3.87	5.77	3.99
Strichen	14.13	9.23	9.32	14.05
Tarves	8.75	15.31	8.5	15.55
Lsd (p=0.05)		1.046		

Lsd = Least significant difference for comparison between soils and treatments

Table 5.26. Concentration of selenium (from added selenium) in radish (mg kg^{-1}) dry weight in limed and unlimed soils.

Soil Association	umlimed		limed	
	1 mg Se kg^{-1}	2 mg Se kg^{-1}	1 mg Se kg^{-1}	2 mg Se kg^{-1}
Countesswells	5.80	18.10	5.56	9.06
Insch	6.74	5.04	4.81	2.93
Strichen	10.99	17.28	7.64	10.82
Tarves	7.80	9.69	9.20	21.42
Lsd (p=0.05)		1.578		

Lsd = Least significant difference for comparison between soils.

5.7.3.3. Effect of soil type on plant uptake of selenium as sodium selenate.

The radishes which had the highest concentration of Se (51.3 μg g^{-1}) were those grown on the Insch Association soil. The least Se (22.5 μg g^{-1}) was found in the radishes grown on the Countesswells Association soil which was not

significantly different ($p<0.05$) from the Tarves Association soil (Table 5.27). This was consistent with the results found in Section 5.4.6 which studied the uptake of Se (non radiolabelled) supplied as selenate.

Table 5.27. Mean concentrations of selenium (from added selenium) in radishes grown on soils at two pH values in soils containing 1 and 2 mg Se kg^{-1} as selenate.

Soil Association	Se concentration in radish (mg kg^{-1})
Countesswells	22.47
Insch	51.34
Strichen	44.12
Tarves	24.18
Lsd (p=0.05)	2.624

Lsd = Least significant difference for comparison between soils

5.7.3.4. Effect of liming and amount of selenium added (as sodium selenate).

An increase in soil pH produced insignificant changes ($p < 0.05$) in plant Se concentration for plants grown on the Insch and Strichen Association soils, and a decrease in Se concentrations for those grown on Countesswells and Tarves soil.

Increasing the level of Se added from 1 to 2 mg kg^{-1} produced large increases in the Se concentrations in the plant. The effect of doubling the Se added led to more than twice the uptake apart from those grown on the Countesswells soil (Table 5.28). This was also found to be the case when the effect on doubling the Se added to the soil whilst keeping the pH constant was examined. At normal pH, the effect of doubling the Se added was particularly pronounced for the plants grown on the Tarves soil which produced increases of over 400% (Table 5.29).

Table 5.28 Concentration of selenium (from added selenium) in radish (mg kg^{-1}) dry weight in limed and unlimed soil.

Soil Association	unlimed	limed	Se conc. in soil	
			1 mg kg^{-1}	2 mg kg^{-1}
Countesswells	32.18	22.47	21.31	33.34
Insch	48.16	51.34	28.33	71.17
Strichen	41.43	44.12	30.57	54.98
Tarves	49.03	24.18	15.48	57.73
Lsd (p=0.05)			3.712	

Lsd = Least significant difference for comparison between soils

Table 5.29 Concentration of selenium (from added selenium) in radish (mg kg^{-1}) dry weight in limed and unlimed soils.

Soil Association	unlimed		limed	
	1 mg Se kg^{-1}	2 mg Se kg^{-1}	1 mg Se kg^{-1}	2 mg Se kg^{-1}
Countesswells	26.56	37.80	16.06	28.88
Insch	25.92	70.40	30.74	71.94
Strichen	25.96	56.90	35.18	53.06
Tarves	16.98	81.08	13.98	34.38
Lsd (p=0.05)			5.250	

Lsd = Least significant difference for comparison between soils and treatments

5.7.4. Discussion.

All plants can accumulate Se when grown in soil containing moderate levels of water soluble Se (Arvy, 1992). The uptake of Se by plants is greater if the Se present is in the form of selenate as opposed to selenite. This is because Se(IV) is adsorbed with much greater affinity than Se(VI) by ferric oxides, organic matter and clays, and is less soluble than Se(VI) in soil fractions (Figure 1.4, Hamdy and Gissel-Nielsen, 1977).

The weights of plants obtained showed variation within each soil but overall the average plant weights between soils was not significantly different ($p<0.05$). Disregarding the effects of liming or level of Se added to the soil, the results obtained for Se added as selenite (Table 5.24) showed radish plants grown on the Insch soil contained the lowest concentrations of Se (4.88 $\mu g\ g^{-1}$). The Insch Association (pH 5.9) has the highest oxalate extractable Fe (28.8 mg kg^{-1}) and the highest organic matter content (loss on ignition = 13.3%) (Table 5.2). The Se concentration of radish plants grown on the Strichen (pH 5.2) and Tarves (pH 5.0) soils were the greatest (11.68 and 12.03 $\mu g\ g^{-1}$ respectively) and not significantly different ($p<0.05$). These soils have similar ferric oxide levels (9.6 and 9.1 mg kg^{-1}) and similar organic matter levels (LOI = 8.5 and 10.5%). Radishes grown on Countesswells soil (pH 5.6) contained 9.63 μg Se g^{-1}. This soil has slightly higher organic matter levels (LOI = 11.9%) and oxalate extractable Fe (10.2 mg kg^{-1}) than Strichen and Tarves soil (Table 5.2).

The plants with the lowest Se concentrations were grown on the soil with the highest pH and those with the greatest Se concentrations on the soils with the lowest pH. The high levels of oxalate extractable Fe present in the Insch soil may be the major factor as the adsorbent of Se(IV) and this is in agreement with Hamdy and Gissel-Nielsen (1977). Organic matter appears to play a greater role than clay in these soils for Se(IV) adsorption.

The addition of lime to raise the soil pH to 6.2 reduced the Se uptake by all the plants except for those grown on the Tarves soil. The reason for this is unclear. Neal *et al.*, 1987 found the addition of lime was likely to reduce the adsorption capacity of clays since as soil pH increases, ^{-}OH replace Se(IV) ions on adsorption sites, releasing Se(IV) into solution and available for plant uptake. This does not however explain why the other three Associations showed a decrease in plant available Se. Adding lime can alter soil pH which may give a long term response of oxidising Se(IV) to Se(VI) but this does not appear to have

happened over this time period (Geering *et al.*, 1968) as there was no large increase in Se concentration found in the radish plants.

The increase in the amounts of Se added to the soils from 1 to 2 mg Se kg^{-1} resulted in increased uptake of Se by plants grown on all the soils except the Insch Association. The Insch soil has much higher amounts of oxalate extractable Fe and this may have contributed by simply adsorbing more of the selenite applied. The effect of increasing the amount of Se added to limed and unlimed soil showed the same trend with only those plants grown on the Insch soil showing a decrease in Se concentration.

The application of Se as selenate rather than selenite produced higher Se concentrations in the radish plants. An overview of the results show that the plants grown on the Tarves and Countesswells soils contained the lowest Se concentrations (24.18 and 22.47 $\mu g\ g^{-1}$ respectively) and these were not significantly different. Plants grown on the Insch soil contained the highest Se concentrations. This soil had the highest pH (5.9). The Tarves Association had the lowest pH (5.0) and the highest clay levels (16.4%) which may have adsorbed some of the Se. The high extractable P (80 mg kg^{-1}) levels of the Countesswells Association soil may also have a negative correlation on Se uptake and although there is still debate on the effects of P in the soil, it should be noted that the Insch soil has the lowest P levels (1.54 mg kg^{-1}).

Liming did not significantly change Se plant uptake (for Strichen and Insch soils) or resulted in a decrease in the Se plant uptake. This was unexpected and requires further investigation.

Increasing the amount of Se added to the soil from 1 to 2 mg kg^{-1} produced large increases in Se plant concentration, particulary for those grown to the Tarves soil (15.4 to 57.7 $\mu g\ g^{-1}$). The reasons for this are unclear but the increase was more marked in plants grown in unlimed soils than limed soils. For

all the soils, an increase in pH was not observed to be effective in increasing plant Se uptake when Se added to the soil was increased from 1 to 2 $\mu g\ g^{-1}$.

Initial studies of Se adsorption isotherms for these soil Associations were carried out by Heiko Krehl, an MSc student (Krehl, 1996). The addition of Se as selenite to the soil, showed that the Insch soil adsorbed the most Se ($k_D = 34.8$). Countesswells and Strichen soils showed the least Se adsorption ($k_D = 19.3$ and 22.3 respectively). The results obtained when Se was added as selenate were similar with the Insch soil showing the greatest adsorption and Countesswells soil the least. These results do not correlate with the experimental results obtained from the radish plants.

5.7.5. Conclusion.

The uptake of Se supplied as selenite to these acidic soils produced concentrations in the radish which were dependent on the oxalate extractable Fe contents, organic matter and clay content of the soils.

Increasing the soil pH by liming increased Se concentration in plants grown on Tarves soil only and indeed decreased Se uptake by plants grown on the other soils. Although the soils were limed 3 weeks prior to planting, this perhaps was not long enough for the limestone to react with the soil before the radish plants were planted.

Doubling the amount of Se added to the soils as selenate led to a doubling of the concentration of Se apart from those plants grown on Insch soil where Se concentration decreased.

When Se was supplied as selenate, it is uncertain what the controlling factors are and a complex picture involving pH, P status and clay content all appear to play a role in determining Se concentration in the plants.

Plants grown on the Insch Association soil contained the highest Se concentrations if Se was supplied as selenate, and contained the lowest concentrations when Se was supplied as selenite.

Se adsorption studies were expected to help explain some of these studies but the results obtained did not correlate with the Se concentrations in the radishes and further work is needed in this area.

5.8. Experiment 3. Study of selenium uptake by the radish grown in sewage sludge amended soils.

In this pot experiment, the sewage sludge was added to each of the 4 soils to give 0, 10 and 20% (w/w) of sewage sludge amended soil.

5.8.1. Treatment of sewage sludge.

The sewage sludge (from storage near London) was autoclaved three times (120^0C, 3 h) to eliminate pathogens. It was freeze-dried and sieved (2 mm). The total Se content was 3.55 $\mu g\ g^{-1}$ (Section 4.6).

5.8.2. Addition of sewage sludge to the soil.

The soils were seived (2 mm) and homogenised. The dry weight for each soil and the amount of sewage sludge added are shown in Table 5.30. For each

pot the soil was mixed thoroughly with the sewage sludge. The soils were left for 1 week before planting radish seeds.

Table 5.30. Amount of sewage sludge added to each soil (g).

Soil	Dry weight	Addition level		
	(g)	0%	10%	20%
Countesswells	566	0	56.6	113.2
Insch	564	0	56.4	112.8
Strichen	565	0	56.5	113
Tarves	562	0	56.2	112.4

5.8.3. Planting of radish seeds.

The pots were laid out according to a randomised design generated using 'Genstat 5' (1990) as shown in Appendix 4 . Six radish seeds were sown in each pot. Deionised water (150 cm^3) was added. The pots were watered three times a week and a nutrient solution of 'Phostrogen' applied weekly (10 g/10 L, 50 cm^3). After 2 weeks, the radishes were thinned to 4 plants per pot. Each treatment was replicated 5 times.

5.8.4. Harvesting of radish plants.

Harvesting took place after 8 weeks. The soil was washed from the roots and the 4 radish plants from each pot were placed in a pre-weighed resealable plastic bag. The radish plants were freeze-dried and their weights recorded.

5.8.5. Determination of selenium concentrations in the radish plants.

The set of 4 radish plants from each pot were ground using a coffee grinder and placed in a Teflon bomb. Thereafter, 100 µl of ^{76}Se isotope solution, 5 cm^3 HNO_3 and 500 µl of H_2O_2 were added. After predigestion overnight, the samples were digested in a microwave oven on low power (420 W) for bursts of 2, 3, 4 and 5 min until a clear digest was obtained. In between heating, the samples were cooled and vented. The samples were derivatised and the Se concentrations determined by ID-MS as described in Section 2.4.

5.8.6. Results.

Although 4 radishes were used for each analysis, no measurable Se was detected. The detection limit for this method was 10 ng, hence less than 10 ng of Se was in the 4 radishes (approx 6 g). This was the case for all treatments of 0, 10 and 20% sewage sludge amended soils.

5.8.7. Discussion.

The low amounts of available Se in the sewage sludge found by sequential extraction (Chapter 4) are reflected in the radish plants. The sequential extraction procedure showed that the soluble Se(VI) extracted (using KCl) was 42 ng g^{-1}. This is the Se that is readily available for plant uptake. The extractant KH_2PO_4 indicated that the level of exchangeable Se(IV) present was 87 ng g^{-1}. The availability of this Se is dependent on soil pH and anion exchange reactions as this Se is adsorbed on ferric oxides and clays. The pH of the soils indicated that Se as selenite would not be readily available.

Plant uptake studies indicated a maximum Se transfer of 3.4% of added Se which equates to 1.4 ng g^{-1} for Se as selenite, and the indigenous soil Se levels (9-11 ng g^{-1}) are very low. Hence the concentrations of Se in the radishes were expected to be below the detection limits.

5.8.9. Conclusions.

Although the total amount of Se in the sewage sludge was 3.55 mg kg^{-1}, the amount of Se in the radish plants was not able to be measured as it was below the detection limit of 10 ng. Thus the importance of determining the forms of Se present is important in uptake studies. The results indicate that the sewage sludge could be applied to agricultural land with little increase in plant Se uptake. However it would be of no use to alleviate Se deficiency in soils.

5.9. Overall conclusions on the study of selenium uptake by plants.

The Se concentrations in the radish plants gave an indication of the important factors to be considered when studying plant Se uptake. These include amounts of oxalate extractable Fe, organic content and clay content. The use of radioactive tracers allowed a more rapid analysis of a large number of plants. As expected more Se was taken up when Se was added as selenate rather than selenite. Doubling the amount of Se added to the soil increased the plant uptake by up to 4 times when added as selenate, but twice or less as selenite. Increasing soil pH was not generally effective in increasing Se uptake by the plants. The Se concentrations in plants grown after the application of sewage sludge to the soils could not be measured (hence was less than 10 ng detection limit) although the sludge applied had a Se concentration of 3.55 mg kg^{-1}. The use of pot experiments confirmed the results obtained for the speciation of Se in soil and sewage sludge samples.

Chapter 6. Discussion.

6.1. General discussion.

The increased application of sewage sludge to agricultural land from 1998 has raised worries on the effects of metal contaminants entering and accumulating in plants and animals and hence into man. Although much work has been carried out on the impact of heavy metals in the environment, Se has received little attention and its measurement in sludges and soils is not compulsory from EC Directive 86/278/EEC.

The disposal of sewage sludge containing high levels of Se onto agricultural land provides the possibilities of alleviating Se deficiency in soil or creating Se toxicity problems. The determination of total Se in the sludge does not enable the assessment of the levels of Se which is available for plant uptake. Selenium exists in 4 oxidation states, namely selenide, elemental Se, selenite and selenate. The form of the Se in the soil or sewage sludge is dependent primarily on the pH and redox potential of the medium. Hence it would be useful if the Se forms present could be identified prior to sludge application to land. Selenium in selenate form is readily available for plant uptake and one method of measuring the available Se is the use of plants which can accumulate Se. The radish plant used is a member of the *Cruciferae* species which has been found to accumulate Se, is easy to cultivate and grows to a size suitable for experimental analysis.

The measurement of Se has previously been problematic. The volatile nature of Se and the presence of potentially interfering ions in the sample matrix has resulted in difficulties in achieving accurate results with good precision. The use of stable and radioisotopes overcomes many of these problems.

The determination of Se by ID-MS has been developed for biological samples. The methodology involves sample digestion followed by derivatisation to

the piazselenol Se-NPD. The adaptation of the method for the measurement of Se concentrations in plants soils and sewage sludges has been discussed in Chapter 2. The analytical instrumentation used was a standard benchtop GC-MS. The stable isotope solution (^{76}Se) was added to the sample prior to digestion following the principle that any Se volatile losses from the samples should be reflected in the spike and thus the overall sample Se:spike Se ratio would be unchanged. The chemical and isotopic purity of the spike solution was determined by reverse ID-MS. The derivatisation of the volatile Se-NPD species dissolved in $CHCl_3$ allowed direct injection into the GC-MS. Validation of the methodology was achieved by the use of plant and soil CRMs. The Se concentration in the CRMs were calculated from the Pickup and McPherson equation and lay within the confidence levels given. A detection limit of 10 ng per 2 μl injection was found. However, since the procedure preconcentrates the sample, the 10 ng limit may come from a large sample size. Overall the method was found to give accurate results with good reproducibility.

The use of inorganic ^{76}Se as the spike solution led to a worry that the method would only work providing the organically bound Se was also freed during sample digestion (as discussed in Chapter 3). Selenomethionine and selenocysteine are the prevalent organic Se compounds and hence it was decided to investigate various digestion methods which would liberate Se from these compounds and which would also not be time consuming. There was little variety for the digestion of soil and sewage sludge samples as the use of HF was necessary to break down the silicon lattice network, and the use of a closed digestion system reduced the dangers associated with using HF. Four digestion methods were studied and the amount of ^{76}Se added calculated to give m/z 229/225 ratio approaching unity for maximum accuracy. The use of H_3PO_4-HNO_3-H_2O_2 in a closed microwave digestion system was found to give recoveries of around 100% for selenomethionine and selenocysteine respectively. The use of microwave digestion was also able to decrease the time necessary for complete digestion and hence increase sample throughput. It had been reported that other

scientists working on Se determinations in biological samples (Ducros, personal communication) had observed column deactivation after as few as 10 to 20 samples. This did not seem to occur with the digestion methods employed for the samples in this project, with over 200 samples being injected before column degradation was observed.

The experimental work carried out in Chapters 2 and 3 resulted in a high level of confidence that the methodology and use of ID-MS could produce accurate results in the determination of Se concentration in plants, soils and sewage sludges.

One of the aims of this project was to develop a method which would indicate the levels of Se which were readily available for uptake from both soils and sewage sludges. The results from this would be compared to the results from a pot experiment where radishes were grown in soils amended with a Se rich sewage sludge. The method employed used successively stronger oxidising agents to extract the Se associated with different soil and sewage sludge fractions. After extraction, the Se in each fraction was derivatised to Se-NPD and analysed by ID-MS as before. The results identify how much Se is likely to be readily available, potentially available or unavailable rather than quantifying the chemical species present.

For the soils examined, the concentrations of soluble, readily available Se (extracted by KCl) found were between 9 and 11 ng g^{-1}. Plant uptake of selenate was found to be a maximum of 3.4%, hence very low levels of Se would be readily available from these soils. Available Se (extracted by KH_2PO_4) was the Se (selenite) adsorbed on clays and mineral oxides. The levels found were low (15 to 23 ng g^{-1}). The acidic nature of these soils was unlikely to free the adsorbed selenite into the soluble and readily available selenate. However, for soils in general, the calculation of available Se from the Se pool covers the Se extracted by these chemicals. This fraction of the Se pool may be determined by a single

KH_2PO_4 extraction. The Se which is strongly bonded to the ferric oxides was labelled as potentially available. The extractant used was HCl and the Se associated with this fraction is thought only to become available following microbial action, pH changes or reduction in redox potential. The concentrations of Se in this fraction ranged from 63 to 128 ng g^{-1}. The Se associated with humidified organic matter was extracted by $KClO_3$ and HCl and the Se contained in the silicon lattice network extracted with HF and HNO_3. This Se is unavailable to plants. The Se in these residual fractions accounted for over 77% of the Se sum for the total of the individual fractions. Hence from these results, the uptake of Se from these soils would be expected to be low.

The sewage sludge examined had a total Se concentration of 3.55 μg g^{-1}. This concentration could result in high Se uptake if it were in a form available for plants. The results of sequential extraction showed only 42 ng g^{-1} as highly available for plant uptake, with 87 ng g^{-1} adsorbed on clays and ferric oxides. The soils amended with this sewage sludge were acidic and it is unlikely that Se from this fraction would be available for plant uptake. Potentially available Se from the sludge accounted for 164 ng g^{-1}. The Se contained in fractions 4 and 5 accounted for over 90% of the total sum of Se from the individual fractions. Hence for this particular sludge low Se uptake by plants would be expected.

The recovery of Se in both the soils and sewage sludge was in the 92-108% range, showing little Se losses during sample preparation. Reproducibility for the overall totals showed C.Vs from 1 to 5 %.

To confirm the propositions from the sequential extraction procedure, a pot experiment was set up with the 4 soils amended with 0, 10 and 20% sewage sludge. A total of 4 radishes were grown per pot and these were ground together to give a mass of around 6 g per pot. After digestion and derivatisation to Se-NPD, the levels of Se in all these plants lay below the detection limit of 10 ng,

showing the Se concentration to be less than 1.7 ng g^{-1}. Hence the amounts of Se available were low, confirming that little Se was available for plant uptake.

Initial pot experiments in the first year were set up to examine the uptake of Se (as selenate) by radish plants. Unfortunately the growth of the plants was poor and results were only obtained from those grown in soil containing 3 mg Se g^{-1}. However it was established that Se would be taken up and enabled a better growth regime to be implemented for the next pot experiments. The results obtained were able to be related to some of the soil characteristic properties.

Radioactive tracers were used for Se determinations in the final pot experiments. The use of ^{75}Se allowed for a rapid sample analysis for an increased number of radish plants. The transfer factors for selenate and selenite were determined and gave maximum transmissions of 2.5 and 3.4% respectively. The uptake of Se as selenate was 2 to 3 times higher than Se as selenite. In both cases, the 'bulb' of the radish was found to have lower Se concentrations than the leaves (in most cases the leaves contained at least twice as much Se). Increasing the level of Se added to the soil from 1 to 2 mg Se g^{-1} increased the Se in the radish if added in the form of selenate but this did not always happen if added as selenite. The addition of lime to increase soil pH was expected to produce higher plant Se concentrations. This was not observed. Se added as selenate produced on significant difference in uptake for plants grown on 2 of the soils and a decrease for plants grown on the other 2. Se added as selenite produced a decrease in Se plant uptake for all but one of the soils. Initial adsorption studies produced results showing little difference in adsorption coefficients between soils treated with selenate and selenite and those with pH 6.2 compared with normal pH. Further investigations are needed in this area.

The uptake of Se by plants grown in soils treated with selenite could be related to the soil characteristics, with ferric oxide levels, clay levels and organic matter content being the important factors. The uptake of Se by plants in the soils

treated with selenate were less obviously related to soil properties but pH and P levels both appear to be important factors. Good reproducibility was obtained in all cases.

The different amount of Se taken up by plants depending upon the type of soil used, the pH and the soil properties may be able to assess the form and level of Se which should be added to allievate Se deficient soil. The ability to assess the form of Se present in sewage sludges and soils should indicate possible benefits or problems which may arise on its application to agricultural land.

The impact of potential pollutants in the environment is increasingly become a public concern. There is already widespread awareness of the effect of the addition of chemicals to agricultural practices. The new EC legislation for the disposal of sewage sludges onto farmland must be accompanied by information on the consequential outcomes of this practices. Reassurances on the effects of the organic and inorganic constituents may be called for. The growing research agenda in pollution monitoring and bioremediation in EEC countries needs to use standardised methodology. The use of stable and radioisotopes are particularly suitable for Se determinations. The use of stable isotopes in particular have been shown to provide a simple, relatively inexpensive and accurate method for determination of Se.

6.2. Suggestions for future work.

1. The uptake by plants of Se added as selenite has been well documented and can be related to soil properties. The uptake of Se as selenate appears more complex. The use of a wider variety of soils at different pH levels and an analysis of further ions present in the may be necessary for better understanding of the results.

2. Further work is needed in the use of adsorption isotherms to determine the levels of Se available in soil solution compared to those adsorbed by soil particles. Comparisons between soils for both selenate and selenite application, along with the effect of adding lime to increase soil pH should provide further information for uptake studies.

3. In this study only 1 sewage sludge was examined in detail. The examination of other sludges containing higher levels of bioavailable Se would be of interest. The impact of a sewage sludge with high levels of Se in a form readily available for plant uptake needs to be investigated.

4. The intrinsic radioactive labelling of Se in sewage sludge would perhaps yield the most useful information on the impact of Se on the environment.

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Appendix 1

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Genstat 5  Release 3.1  (Sun/Unix)                    Wed Oct  9 14:10:42 1996
Copyright 1994, Lawes Agricultural Trust (Rothamsted Experimental Station)

  1  JOB 'Randomization radish pot experiment, July 94'
  2  units [nvalues=80]
  3  variate [values=1...80] pot_ref; dec=0
  4  factor [levels=5] block
  5  factor [levels=4; labels=!t(a,b,c,d)] soil
  6  factor [levels=!(0,1,2,3)] se; dec=0
  7  generate block,soil,se
  8  factor [levels=16]plot
  9  generate plot
 10  randomize [blockstructure=block/plot; seed=318611] soil,se
 11  for aa=1
 12  page
 13  print 'randomization of soil selenium trts to pots : july 94'
 14  print pot_ref,block,soil,se
 15  endfor
```

randomization of soil selenium trts to pots : july 94

pot_ref	block	soil	se
1	1	c	1
2	1	d	3
3	1	b	1
4	1	a	1
5	1	d	0
6	1	d	2
7	1	a	2
8	1	b	0
9	1	a	3
10	1	c	3
11	1	d	1
12	1	a	0
13	1	b	3
14	1	c	2
15	1	c	0
16	1	b	2
17	2	b	1
18	2	a	2
19	2	d	1
20	2	a	3
21	2	d	0
22	2	c	2
23	2	c	1
24	2	a	1
25	2	b	0
26	2	d	2
27	2	b	3
28	2	a	0
29	2	c	0
30	2	c	3
31	2	d	3
32	2	b	2
33	3	d	2
34	3	a	2
35	3	d	3
36	3	c	0
37	3	c	1
38	3	d	0
39	3	b	3
40	3	c	2
41	3	a	1
42	3	b	2
43	3	c	3
44	3	b	1
45	3	d	1
46	3	b	0
47	3	a	0
48	3	a	3
49	4	d	2
50	4	b	1
51	4	a	0
52	4	b	2
53	4	a	1
54	4	c	2
55	4	c	1
56	4	a	3
57	4	b	3
58	4	c	3

59	4	d	3
60	4	d	0
61	4	c	0
62	4	b	0
63	4	a	2
64	4	d	1
65	5	d	1
66	5	b	3
67	5	a	2
68	5	c	3
69	5	d	3
70	5	c	2
71	5	a	0
72	5	b	2
73	5	d	0
74	5	a	1
75	5	b	0
76	5	c	1
77	5	b	1
78	5	c	0
79	5	a	3
80	5	d	2

Appendix 2

randomization of soil selenium trts to pots : july 96

pot_ref	block	soil	se	ph
1	1	c	2	0
2	1	a	0	0
3	1	c	1	0
4	1	c	0	0
5	1	b	1	0
6	1	b	2	1
7	1	a	0	1
8	1	c	1	1
9	1	d	1	0
10	1	b	2	0
11	1	a	2	0
12	1	d	0	0
13	1	d	2	1
14	1	c	0	1
15	1	a	1	1
16	1	b	0	0
17	1	a	2	1
18	1	b	0	1
19	1	d	0	1
20	1	a	1	0
21	1	d	2	0
22	1	d	1	1
23	1	b	1	1
24	1	c	2	1
25	2	a	0	1
26	2	d	2	1
27	2	b	1	0
28	2	a	0	0
29	2	c	2	0
30	2	b	0	0
31	2	a	2	0
32	2	c	1	1
33	2	a	2	1
34	2	b	2	1
35	2	c	0	1
36	2	b	2	0
37	2	d	1	1
38	2	b	1	1
39	2	c	0	0
40	2	d	1	0
41	2	d	0	1
42	2	c	2	1
43	2	d	2	0
44	2	a	1	0
45	2	c	1	0
46	2	a	1	1
47	2	b	0	1
48	2	d	0	0
49	3	c	1	0
50	3	c	2	1
51	3	c	1	1
52	3	b	1	0
53	3	b	1	1
54	3	c	0	0
55	3	d	2	1
56	3	b	2	0
57	3	d	2	0
58	3	b	0	0

59	3	b	2	1
60	3	d	0	1
61	3	c	0	1
62	3	a	2	0
63	3	a	0	0
64	3	a	1	1
65	3	b	0	1
66	3	a	1	0
67	3	d	1	0
68	3	a	2	1
69	3	d	1	1
70	3	c	2	0
71	3	a	0	1
72	3	d	0	0
73	4	d	0	1
74	4	a	1	0
75	4	d	2	1
76	4	a	1	1
77	4	c	2	0
78	4	b	2	0
79	4	d	2	0
80	4	a	0	1
81	4	a	2	0
82	4	c	1	0
83	4	b	0	1
84	4	a	0	0
85	4	d	1	0
86	4	b	2	1
87	4	c	1	1
88	4	b	0	0
89	4	c	2	1
90	4	d	1	1
91	4	b	1	0
92	4	a	2	1
93	4	d	0	0
94	4	c	0	0
95	4	b	1	1
96	4	c	0	1
97	5	d	2	0
98	5	b	2	0
99	5	c	2	0
100	5	a	2	1
101	5	c	0	0
102	5	a	1	1
103	5	d	0	0
104	5	d	0	1
105	5	a	0	1
106	5	c	2	1
107	5	a	0	0
108	5	b	2	1
109	5	a	1	0
110	5	b	1	1
111	5	d	1	1
112	5	b	0	0
113	5	c	0	1
114	5	c	1	1
115	5	b	1	0
116	5	d	2	1
117	5	c	1	0
118	5	b	0	1
119	5	a	2	0
120	5	d	1	0

Appendix 3

```
Genstat 5  Release 3.1  (Sun/Unix)                    Thu Nov 21 11:30:07 1996
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  1
  2
  3
  4  UNITS [NVALUES=80]
  5  FACTOR [LEVELS=4; LABELS=!T(Count, Insch, Strichen, Tarves)] Soil
  6  FACTOR [LEVELS=!(5, 6)] Ph
  7  FACTOR [LEVELS=!( 1, 2)] Se
  8  FACTOR [LEVELS=5;] Rep
  9  OPEN 'sefract2.dat'; CHANNEL=2
 10  READ [CHANNEL=2] Soil, Ph, Se, Rep, Conc

 Identifier    Minimum       Mean    Maximum     Values    Missing
       Conc      2.630      9.556     22.750         80          0

 Identifier     Values    Missing     Levels
       Soil         80          0          4
         Ph         80          0          2
         Se         80          0          2
        Rep         80          0          5

 11  Print Soil, Ph, Se, Rep, Conc

      Soil          Ph          Se         Rep        Conc
     Count       5.000       1.000           1       5.910
     Count       5.000       1.000           2       3.430
     Count       5.000       1.000           3       6.230
     Count       5.000       1.000           4       6.430
     Count       5.000       1.000           5       6.980
     Count       5.000       2.000           1      16.230
     Count       5.000       2.000           2      17.770
     Count       5.000       2.000           3      19.550
     Count       5.000       2.000           4      17.920
     Count       5.000       2.000           5      19.050
     Count       6.000       1.000           1       5.740
     Count       6.000       1.000           2       4.810
     Count       6.000       1.000           3       6.130
     Count       6.000       1.000           4       5.680
     Count       6.000       1.000           5       5.440
     Count       6.000       2.000           1       7.190
     Count       6.000       2.000           2       8.190
     Count       6.000       2.000           3      11.150
     Count       6.000       2.000           4       9.010
     Count       6.000       2.000           5       9.780
     Insch       5.000       1.000           1       6.540
     Insch       5.000       1.000           2       6.140
     Insch       5.000       1.000           3       7.970
     Insch       5.000       1.000           4       6.350
     Insch       5.000       1.000           5       6.680
     Insch       5.000       2.000           1       4.880
     Insch       5.000       2.000           2       4.700
     Insch       5.000       2.000           3       5.470
     Insch       5.000       2.000           4       5.580
     Insch       5.000       2.000           5       4.590
     Insch       6.000       1.000           1       4.820
     Insch       6.000       1.000           2       4.470
     Insch       6.000       1.000           3       5.390
     Insch       6.000       1.000           4       4.700
     Insch       6.000       1.000           5       4.650
     Insch       6.000       2.000           1       2.630
     Insch       6.000       2.000           2       2.930
     Insch       6.000       2.000           3       3.010
```

Insch	6.000	2.000	4	2.910
Insch	6.000	2.000	5	3.180
Strichen	5.000	1.000	1	10.050
Strichen	5.000	1.000	2	9.640
Strichen	5.000	1.000	3	12.170
Strichen	5.000	1.000	4	11.440
Strichen	5.000	1.000	5	11.650
Strichen	5.000	2.000	1	19.110
Strichen	5.000	2.000	2	19.660
Strichen	5.000	2.000	3	15.770
Strichen	5.000	2.000	4	17.120
Strichen	5.000	2.000	5	14.730
Strichen	6.000	1.000	1	8.210
Strichen	6.000	1.000	2	8.990
Strichen	6.000	1.000	3	7.960
Strichen	6.000	1.000	4	3.550
Strichen	6.000	1.000	5	9.510
Strichen	6.000	2.000	1	10.580
Strichen	6.000	2.000	2	10.870
Strichen	6.000	2.000	3	8.870
Strichen	6.000	2.000	4	11.950
Strichen	6.000	2.000	5	11.850
Tarves	5.000	1.000	1	7.700
Tarves	5.000	1.000	2	7.420
Tarves	5.000	1.000	3	7.790
Tarves	5.000	1.000	4	8.210
Tarves	5.000	1.000	5	7.890
Tarves	5.000	2.000	1	9.730
Tarves	5.000	2.000	2	10.230
Tarves	5.000	2.000	3	8.400
Tarves	5.000	2.000	4	9.970
Tarves	5.000	2.000	5	10.110
Tarves	6.000	1.000	1	8.930
Tarves	6.000	1.000	2	9.150
Tarves	6.000	1.000	3	8.670
Tarves	6.000	1.000	4	9.880
Tarves	6.000	1.000	5	9.370
Tarves	6.000	2.000	1	20.840
Tarves	6.000	2.000	2	21.340
Tarves	6.000	2.000	3	20.480
Tarves	6.000	2.000	4	21.700
Tarves	6.000	2.000	5	22.750

```
12  BLOCK Rep
13  TREAT Soil * Ph * Se
14  ANOVA [FPROB=Y] Conc
```

***** Analysis of variance *****

Variate: Conc

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	4	3.587	0.897	0.66	
Rep.*Units* stratum					
Soil	3	650.289	216.763	158.64	<.001
Ph	1	31.163	31.163	22.81	<.001
Se	1	401.005	401.005	293.47	<.001
Soil.Ph	3	432.427	144.142	105.49	<.001
Soil.Se	3	288.263	96.088	70.32	<.001
Ph.Se	1	0.966	0.966	0.71	0.404
Soil.Ph.Se	3	241.579	80.526	58.93	<.001
Residual	60	81.985	1.366		
Total	79	2131.264			

* MESSAGE: the following units have large residuals.

Rep 2	*units* 10	2.58	s.e. 1.01
Rep 4	*units* 11	-4.06	s.e. 1.01
Rep 5	*units* 10	-2.88	s.e. 1.01

***** Tables of means *****

Variate: Conc

Grand mean 9.56

Soil	Count	Insch	Strichen	Tarves
	9.63	4.88	11.68	12.03
Ph	5.00	6.00		
	10.18	8.93		
Se	1.00	2.00		
	7.32	11.79		

Soil	Ph	5.00	6.00
Count		11.95	7.31
Insch		5.89	3.87
Strichen		14.13	9.23
Tarves		8.75	15.31

Soil	Se	1.00	2.00
Count		5.68	13.58
Insch		5.77	3.99
Strichen		9.32	14.05
Tarves		8.50	15.55

Ph	Se	1.00	2.00
5.00		7.83	12.53
6.00		6.80	11.06

	Ph	5.00		6.00	
Soil	Se	1.00	2.00	1.00	2.00

Count	5.80	18.10	5.56	9.06
Insch	6.74	5.04	4.81	2.93
Strichen	10.99	17.28	7.64	10.82
Tarves	7.80	9.69	9.20	21.42

*** Standard errors of differences of means ***

Table	Soil	Ph	Se	Soil Ph
rep.	20	40	40	10
s.e.d.	0.370	0.261	0.261	0.523

Table	Soil Se	Ph Se	Soil Ph Se
rep.	10	20	5
s.e.d.	0.523	0.370	0.739

```
  15  aplot f,n
```

```
Genstat 5  Release 3.1  (Sun/Unix)                    Wed Nov 27 09:25:08 1996
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  1
  2
  3
  4  UNITS [NVALUES=80]
  5  FACTOR [LEVELS=4; LABELS=!T(Count, Insch, Strichen, Tarves)] Soil
  6  FACTOR [LEVELS=!(5, 6)] Ph
  7  FACTOR [LEVELS=!( 1, 2)] Se
  8  FACTOR [LEVELS=5;] Rep
  9  OPEN 'sefract.dat'; CHANNEL=2
 10  READ [CHANNEL=2] Soil, Ph, Se, Rep, Conc

 Identifier    Minimum       Mean    Maximum     Values    Missing
       Conc      11.80      39.11      90.20         80          0

 Identifier     Values    Missing     Levels
       Soil         80          0          4
         Ph         80          0          2
         Se         80          0          2
        Rep         80          0          5

 11  Print Soil, Ph, Se, Rep, Conc

      Soil          Ph          Se         Rep        Conc
     Count       5.000       1.000           1       19.50
     Count       5.000       1.000           2       29.40
     Count       5.000       1.000           3       37.10
     Count       5.000       1.000           4       23.70
     Count       5.000       1.000           5       23.10
     Count       5.000       2.000           1       35.10
     Count       5.000       2.000           2       32.90
     Count       5.000       2.000           3       40.20
     Count       5.000       2.000           4       38.60
     Count       5.000       2.000           5       42.20
     Count       6.000       1.000           1       14.50
     Count       6.000       1.000           2       15.60
     Count       6.000       1.000           3       16.00
     Count       6.000       1.000           4       17.50
     Count       6.000       1.000           5       16.70
     Count       6.000       2.000           1       27.40
     Count       6.000       2.000           2       29.60
     Count       6.000       2.000           3       30.50
     Count       6.000       2.000           4       26.00
     Count       6.000       2.000           5       30.90
     Insch       5.000       1.000           1       21.90
     Insch       5.000       1.000           2       26.10
     Insch       5.000       1.000           3       27.20
     Insch       5.000       1.000           4       26.80
     Insch       5.000       1.000           5       27.60
     Insch       5.000       2.000           1       74.70
     Insch       5.000       2.000           2       62.90
     Insch       5.000       2.000           3       77.00
     Insch       5.000       2.000           4       73.50
     Insch       5.000       2.000           5       63.90
     Insch       6.000       1.000           1       27.90
     Insch       6.000       1.000           2       30.40
     Insch       6.000       1.000           3       32.90
     Insch       6.000       1.000           4       30.70
     Insch       6.000       1.000           5       31.80
     Insch       6.000       2.000           1       75.10
     Insch       6.000       2.000           2       67.80
     Insch       6.000       2.000           3       68.50
```

Insch	6.000	2.000	4	82.60
Insch	6.000	2.000	5	65.70
Strichen	5.000	1.000	1	22.90
Strichen	5.000	1.000	2	25.50
Strichen	5.000	1.000	3	29.90
Strichen	5.000	1.000	4	26.70
Strichen	5.000	1.000	5	24.80
Strichen	5.000	2.000	1	53.00
Strichen	5.000	2.000	2	52.70
Strichen	5.000	2.000	3	54.80
Strichen	5.000	2.000	4	64.10
Strichen	5.000	2.000	5	59.90
Strichen	6.000	1.000	1	35.40
Strichen	6.000	1.000	2	30.80
Strichen	6.000	1.000	3	34.30
Strichen	6.000	1.000	4	36.50
Strichen	6.000	1.000	5	38.90
Strichen	6.000	2.000	1	43.80
Strichen	6.000	2.000	2	49.80
Strichen	6.000	2.000	3	57.10
Strichen	6.000	2.000	4	58.90
Strichen	6.000	2.000	5	55.70
Tarves	5.000	1.000	1	14.20
Tarves	5.000	1.000	2	16.50
Tarves	5.000	1.000	3	22.50
Tarves	5.000	1.000	4	15.90
Tarves	5.000	1.000	5	15.80
Tarves	5.000	2.000	1	70.00
Tarves	5.000	2.000	2	84.20
Tarves	5.000	2.000	3	80.20
Tarves	5.000	2.000	4	90.20
Tarves	5.000	2.000	5	80.80
Tarves	6.000	1.000	1	14.00
Tarves	6.000	1.000	2	14.80
Tarves	6.000	1.000	3	11.80
Tarves	6.000	1.000	4	14.70
Tarves	6.000	1.000	5	14.60
Tarves	6.000	2.000	1	31.30
Tarves	6.000	2.000	2	40.70
Tarves	6.000	2.000	3	33.00
Tarves	6.000	2.000	4	34.30
Tarves	6.000	2.000	5	32.60

```
12  BLOCK Rep
13  TREAT Soil * Ph * Se
14  ANOVA [FPROB=Y] Conc
```

***** Analysis of variance *****

Variate: Conc

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	4	265.73	66.43	3.86	
Rep.*Units* stratum					
Soil	3	5436.06	1812.02	105.23	<.001
Ph	1	1028.90	1028.90	59.75	<.001
Se	1	18461.93	18461.93	1072.10	<.001
Soil.Ph	3	2616.88	872.29	50.66	<.001
Soil.Se	3	3342.56	1114.19	64.70	<.001
Ph.Se	1	1067.99	1067.99	62.02	<.001
Soil.Ph.Se	3	1548.89	516.30	29.98	<.001
Residual	60	1033.22	17.22		
Total	79	34802.15			

* MESSAGE: the following units have large residuals.

Rep 3 *units* 1 8.84 s.e. 3.59

***** Tables of means *****

Variate: Conc

Grand mean 39.11

Soil	Count	Insch	Strichen	Tarves
	27.33	49.75	42.78	36.60

Ph	5.00	6.00
	42.70	35.53

Se	1.00	2.00
	23.92	54.31

Soil	Ph	5.00	6.00
Count		32.18	22.47
Insch		48.16	51.34
Strichen		41.43	44.12
Tarves		49.03	24.18

Soil	Se	1.00	2.00
Count		21.31	33.34
Insch		28.33	71.17
Strichen		30.57	54.98
Tarves		15.48	57.73

Ph	Se	1.00	2.00
5.00		23.86	61.55
6.00		23.99	47.06

	Ph	5.00		6.00	
Soil	Se	1.00	2.00	1.00	2.00
Count		26.56	37.80	16.06	28.88
Insch		25.92	70.40	30.74	71.94

Strichen	25.96	56.90	35.18	53.06
Tarves	16.98	81.08	13.98	34.38

*** Standard errors of differences of means ***

Table	Soil	Ph	Se	Soil Ph
rep.	20	40	40	10
s.e.d.	1.312	0.928	0.928	1.856

Table	Soil Se	Ph Se	Soil Ph Se
rep.	10	20	5
s.e.d.	1.856	1.312	2.625

15 aplot f,n

Appendix 4

randomization of soil selenium trts to pots : july 95

pot_ref	block	soil	se
1	1	a	0
2	1	b	0
3	1	d	2
4	1	c	1
5	1	b	1
6	1	b	2
7	1	d	0
8	1	a	2
9	1	c	0
10	1	c	2
11	1	d	1
12	1	a	1
13	2	c	0
14	2	a	0
15	2	d	2
16	2	c	1
17	2	b	2
18	2	d	0
19	2	a	1
20	2	d	1
21	2	c	2
22	2	a	2
23	2	b	1
24	2	b	0
25	3	b	1
26	3	a	1
27	3	d	2
28	3	d	0
29	3	c	0
30	3	d	1
31	3	c	1
32	3	c	2
33	3	b	0
34	3	a	0
35	3	a	2
36	3	b	2
37	4	c	1
38	4	d	1
39	4	a	2
40	4	d	2
41	4	b	0
42	4	a	0
43	4	c	2
44	4	a	1
45	4	c	0
46	4	b	2
47	4	b	1
48	4	d	0
49	5	a	1
50	5	b	1
51	5	c	2
52	5	c	0
53	5	b	2
54	5	a	2
55	5	d	2
56	5	b	0
57	5	d	1
58	5	d	0

59	5	a	0
60	5	c	1
61	6	a	2
62	6	d	1
63	6	c	0
64	6	c	1
65	6	b	0
66	6	a	0
67	6	d	2
68	6	d	0
69	6	a	1
70	6	b	1
71	6	c	2
72	6	b	2
73	7	c	0
74	7	d	1
75	7	c	2
76	7	c	1
77	7	a	0
78	7	a	1
79	7	b	2
80	7	a	2
81	7	d	2
82	7	b	1
83	7	b	0
84	7	d	0
85	8	d	2
86	8	a	1
87	8	c	0
88	8	a	2
89	8	b	0
90	8	d	0
91	8	a	0
92	8	b	2
93	8	c	1
94	8	d	1
95	8	b	1
96	8	c	2
97	9	b	1
98	9	c	2
99	9	b	0
100	9	c	1
101	9	c	0
102	9	a	1
103	9	b	2
104	9	d	2
105	9	a	0
106	9	a	2
107	9	d	0
108	9	d	1
109	10	c	1
110	10	d	0
111	10	d	1
112	10	a	1
113	10	b	1
114	10	a	2
115	10	c	2
116	10	b	0
117	10	b	2
118	10	d	2
119	10	a	0
120	10	c	0

Appendix 5

Publications resulting from this thesis.

MacLeod, F., McGaw, B.A. and Shand, C. A. (1997) Use of a benchtop GC-MS for determination of selenium in environmental samples by stable isotope dilution-mass spectrometry. *PEAK* .1:1-4.

MacLeod, F., McGaw, B. A. and Shand, C. A. (1996) Stable isotope dilution-mass spectrometry for determining total selenium levels in plants, soil and sewage sludge. *Talanta*.43:1091-1098.

MacLeod, F., McGaw, B.A. and Shand, C. A. (1996) Quantitation of selenium in plants and soils by isotope dilution GC-MS. Proceedings of the 44th Annual Conference on Mass Spectrometry and Allied Topics, Portland, Oregon, USA, May 12-16, 1996. (p20).

MacLeod, F., McGaw, B. A. and Shand, C. A. (1996) Sequential extraction of selenium from four Scottish soils and a sewage sludge. *Communications in Plant and Soil Science.*(submitted).

Additional publication.

MacLeod, F. and McGaw, B. A. (1996) Iodine bioavailability. In: Bioavailability of minerals and trace elements (Fairweather-Tait, S. and Hurrell, R. F. eds). *Nutrition Research Reviews*. 9:295-324.

ELSEVIER

Talanta 43 (1996) 1091–1098

Talanta

Stable isotope dilution—mass spectrometry for determining total selenium levels in plants, soils and sewage sludges

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Abstract

Quantitation of selenium in plants, soils and sludges was achieved by isotope dilution-mass spectrometry using a benchtop instrument. Samples for analysis were spiked with ^{76}Se isotope solution. Plant material was digested on a heating block at 150°C using a mixture of nitric acid and hydrogen peroxide. Selenium in soils and sludges was released by treatment with nitric acid followed by digestion with nitric and hydrofluoric acids. Selenium in the digests was reduced to Se(IV) with hydrochloric acid and derivatised with nitro-1, 2-phenylenediamine to 5'-nitropiazselenol. Analysis by gas chromatography mass spectrometry using selected ion monitoring was validated using certified reference materials (CRMs) and gave results within the certified range with a low standard deviation. The CRMs plant (Chinese cabbage leaves) and soil (Chinese soil) were found to contain ($\pm$95% confidence limits) 0.091($\pm$0.007) $\mu g\ g^{-1}$ and 1.67($\pm$0.04) $\mu g\ g^{-1}$ Se respectively. The certified values were 0.083($\pm$0.008) $\mu g\ g^{-1}$ and 1.56($\pm$0.12) $\mu g\ g^{-1}$ respectively. The selenium content of four different freely drained acid Scottish soils under grasslands was in the range 0.5–0.8 $\mu g\ g^{-1}$ air-dried soil. Sewage sludges were found to contain measurably more selenium than the soils, and samples of three sludges taken from sites in the UK contained between 1.1 and 3.5 $\mu g\ g^{-1}$ dry matter.

Keywords: Selenium; Soil; Sewage sludge; Isotope dilution-mass spectrometry

1. Introduction

Selenium is an element which has aroused much interest in the last decade. It is an essential trace metalloid for animals [1]. The role of selenium in the body is as a co-factor in two enzymes. The first, glutathione peroxidase, acts as a antioxidant by destroying peroxides which attack cellular membranes. The second, iodothyronine 5'-deiodinase, converts thyroxine (T_4) to triiodothyronine (T_3) with the release of iodine [2]. Toxic amounts of selenium can result in symptoms ranging from loss of hair and nails to fibriosis of the kidney and myocardial congestion [1]. In China, diets deficient in selenium have resulted in "Keshan disease" [2].

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Selenium in the diet is obtained from plants and animal products and the level in these is dependent largely on the selenium content and the chemical species present in local soils [2]. Surface waters seldom contain enough selenium to provide a significant amount for the diet [3]. The concentration of selenium is low (0.3–0.5 mg kg^{-1}) [3] in many soils of the world, including parts of Scotland [4]. The addition of selenium fertilisers, selenium supplementation of feeds and direct injection of selenium into livestock have been used to overcome deficiency problems. New EC legislation, reviewed by Matthews [5], coming into forced in 1998 banning the discharge of sewage sludge into the sea will result in its increased application to agricultural land. These sludges could also be exploited as a source of selenium for addition to deficient soils. In other situations, addition of sludge could conceivably lead to selenium toxicity problems. Although there is no statutory requirement [6] to have analytical data for selenium in soils and sludges before disposal, analysis is specified in the code of practice [7].

The analysis of selenium is notoriously difficult since it is potentially volatile and present in very low levels in most soils (<2 μg g^{-1}) and plant (<100 ng g^{-1}) [2]. Current methods include fluorimetry, neutron activation analysis (NAA) and atomic absorption spectrometry (AAS). Fluorimetry is an established low-cost method but involves a digestion step and care has to be taken to avoid interference [8,9]. Solid samples can be analysed by NAA, and although accurate it is not widely available, involves a long irradiation period (100 h) and a post-irradiation period of 60 days [10]. Various AAS methods have been employed. Flame AAS has high detection limits and lacks precision [11]. Hydride generation AAS suffers from copper and arsenic interferences and also requires careful sample preparation [12]. Graphite furnace AAS (GFAAS) has good sensitivity, but suffers from interferences which need to be overcome by the use of matrix modifiers and background correction [13]. Gas chromatography of volatile derivatives with electron capture detection has given accurate results for selenium levels in plant and animal tissues but involves laborious sample preparation [14]. Isotope dilution-mass spectrometry (ID-MS) by gas chromatography–mass spectrometry (GC–MS) as an alternative method has the advantage of not requiring quantitative recovery and giving accurate and precise selenium measurements. The method was first employed by Reamer and Veillon [15] for biological samples and has since been extended by Ducros and Favier [16] to human body fluids. Sample preparation is relatively straightforward but care should be taken to ensure that complete mixing of isotope spike and sample occurs and that the levels of spike added are in the same range as endogenous levels. This paper describes the first application of this method for the quantitation of selenium in plants, soils and sewage sludges and evaluates the analytical performance of a standard benchtop GC–MS instrument. An alternative ID-MS method using thermal ionisation mass spectrometry (TIMS) has been employed by Tanzer and Heumann [17] to measure selenium in environmental water samples. This technique offers high precision and accuracy but is not generally available to analytical laboratories.

2. Experimental

2.1. Instrumentation

A 5890 gas chromatograph coupled to a 5791A quadrupole mass spectrometer (Hewlett-Packard, Chesire, UK) was used. A capillary DB-1 GC column (Jones Chromatography, Mid Glamorgan, UK) of 25 m length, 0.2 mm i.d. and 0.33 μm film thickness was connected to the ion source. The carrier was helium at a flow rate of 5 cm^3 min^{-1}. The injections were in splitless mode and the initial temperature was 120°C and was set to rise to 200°C at a rate of 5°C min^{-1}. The injector and transfer line temperatures were 250 and 280°C respectively, with a purge gas flow rate of 20 cm^3 min^{-1}. Electron impact ionisation was used (electron energy = 70 eV).

The mass spectrometer was programmed using MS Chemstation 1989/90 software (Hewlett-Packard) in selected ion monitoring (SIM) mode to

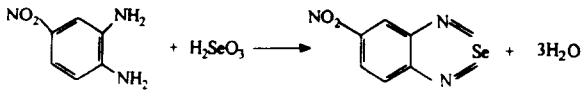

Fig. 1. Formation of 5-nitropiazselenol (Se-NPD).

monitor the peaks at m/z 225 and 229 which corresponded to the 4-nitro-1,2-phenylenediamine (NPD) derivatives of ^{76}Se and ^{80}Se respectively (Fig 1). The scanning time was 4.1 cycles s^{-1}. The autointegrate command was used and the integration time for each selected peak was 420 ms.

2.2. Materials and reagents

Glassware and plasticware were cleaned by soaking for 16 h in reagent grade 4 M HNO_3, and then for 24 h in deionised water and finally by drying at 50°C. Reagents were of AristaR grade: HNO_3 (18.5 M), HCl (12 M), HF (28.9 M) (Merck, UK), ammonium hydroxide (14.8 M) and H_2O_2 (30 volume) (Fisons, UK). Standards were prepared from 1000 mg dm^{-3} selenium, as Se(IV), atomic absorption standard solution (Fisons, UK).

Elemental ^{76}Se (19.8 mg, certified isotope enrichment = 98.5% ± 0.2%) in elemental form (Europa Scientific, Cheshire, UK) was dissolved in 4.5 cm^3 concentrated HNO_3 and diluted with 99.5 cm^3 deionised water to give a "spike" solution with a concentration of approximately 186.5 $\mu g\ g^{-1}$.

The derivatising agent, 4-nitro-1,2-phenylenediamine (Merck, Germany) (100 mg), was dissolved in 25 cm^3 of 0.1 M HCl and shaken for 0.5 h. It was purified by washing with cyclohexane and kept at 4°C in darkness for up to 2 weeks.

The indicator solution [16] consisted of a solution containing 20 mM EDTA, 10 mg l^{-1} bromocresol purple and 7 M ammonium hydroxide.

2.3. Soils

Freely drained soils of the Countesswells, Insch, Strichen and Tarves Associations [18] from the North East of Scotland (Table 1) were chosen for

Table 1
Parent rocks and properties of four soils from the North East of Scotland

Soil Association	Parent material	National Grid Reference	pH (in water)	Loss on ignition (%)
Countesswells	Drifts derived from granite and granitic rocks	NJ 850096	5.2	11.7
Insch	Drifts derived from gabbros and igneous rocks	NJ 643312	5.0	15.4
Strichen	Drifts derived from arenaceous schists and strongly weathered argillaceous schists of the Dalradian Series	NO 671789	5.6	8.05
Tarves	Drifts derived from intermediate rocks or mixed acid and basic rocks, both metamorphic and igneous	NJ 855274	5.8	11.4

Table 2
Isotopic compositions of natural and ^{76}Se as Se–NPD

Selenium isotope	Se–NPD ion (m/z)	Measured % abundance Se–NPD (natural Se) $n = 10$[a]	RSD (%)	Theoretical % abundance (natural Se)[b]	Measured % abundance Se–NPD (^{76}Se soln.) $n = 10$[a]
74	223	0.77	7.79	0.80	1.07
76	225	8.45	0.24	8.29	91.35
77	226	7.54	0.26	7.61	6.84
78	227	22.47	0.4	22.22	0.63
80	229	46.56	0.19	45.93	0.11
82	231	8.35	0.6	8.85	0.0

[a] n = number of replicates.
[b] Calculated on the basis of the known isotopic composition of C, H, N, O and Se.

this study. These soils were selected because such soil types meet the suitability criterion for sewage sludge application [19]. Soil was taken to a depth of 25 cm, seived in a 6 mm seive to remove stones and visible roots and air-dried at 30°C. Each soil was homogenised and subsampled by coning and quartering. The soil CRM was Chinese soil (C74-05, Laboratory of the Government Chemist, Middlesex, UK).

Soil pH was determined in water using 1:2 soil-to-water ratio with a glass electrode. Loss on ignition was determined by weighing before and after heating at 450°C for 16 h.

2.4. Sewage sludges

Sludge samples were taken from three locations in the UK, namely London, Birmingham and the Scottish Border region. The first two samples were anaerobically digested. Prior to analysis these sludges were autoclaved three times (120°C), freeze dried, seived (2 mm) and homogenised by coning and quartering.

2.5. Plant material

This work forms part of a project investigating the uptake of selenium from sewage sludge amended soils by agricultural plants. The CRM used was cabbage leaves (C85-04, Laboratory of the Government Chemist) and was selected because this is one of the few plant CRMs with certified selenium levels. Cabbage is also a selenium accumulator plant.

2.6. Digestion of soils and sludges

The method used for digestion of soils and sludges is a modification of that used by Nham and Brodie [20]. Soil or sludge (0.5 g) was placed in a 50 cm^3 Teflon bomb (Savillex, MN) and spiked with 0.1 g ^{76}Se solution (concn. = 18.51 $\mu g\ g^{-1}$). Nitric acid (18.5 M, 2 cm^3) was added and the mixture placed in an 800 W microwave oven. The sample was heated on low power for bursts of 0.5, 1, 1.5, 2 and 3 min. In between times the samples were cooled and vented. This eliminated organic material from the sample. Hydrofluoric acid (28.9 M, 10 cm^3) was added and the lid replaced and hand tightened. The sample was heated on a hotplate at 180°C for 6 h and cooled for 16 h. The top was then removed and the HF evaporated at 120°C for approximately 6 h. After cooling, 10 cm^3 of 18.5 M HNO_3 was added and evaporated at 120°C for $\approx$6 h. The residue was redissolved in 4 cm^3 of 3 M HNO_3.

2.7. Plant digestion

The CRM cabbage leaves were digested using a heating block at 150°C. The CRM (5.0 g) was

Table 3
A comparison of measured and theoretical isotope ratios across a range of mole ratios of unlabelled to labelled Se

Mixture	Mole ratio unlabelled to labelled Se	Measured mean m/z 229/225 ratio	RSD (%)	Theoretical m/z 229/225 ratio[a]
1	0.53	0.25	0.33	0.26
2	0.70	0.33	0.15	0.34
3	1.06	0.48	0.45	0.49
4	2.63	1.07	0.30	1.08
5	4.16	1.55	0.43	1.53

[a] Based on chemical and isotopic purity of the ^{76}Se spike and AAS standard.

spiked with 0.1000 g of the isotope solution and the sample digested with 30 cm^3 18.5 M HNO_3 and 3 cm^3 H_2O_2. This was heated for 10 h or until a clear digest was obtained. This method was adapted from that of Dong et al. [21].

2.8. Derivatisation

The digest was transferred to a Pyrex tube (50 cm^3) and 200 μl of 6 M HCl was added. The tube was heated for 5 min at 80°C in a water bath. This step was repeated until 1 cm^3 of HCl has been added which ensured that Se(VI) was converted to Se(IV). The tube was cooled and 2 cm^3 of the indicator solution was added. This ensured that the solution was of the correct pH prior to derivatisation. Heating was resumed at 80°C for 1.5 h. After cooling, 5 cm^3 of 0.1 M HCl was added and the tubes left for 16 h in darkness at room temperature [15,16]. The following day 0.5 cm^3 of the derivatising agent was added. The tube was heated (40°C) in a water bath (30 min) and, after cooling, 5 cm^3 of chloroform was added and the tube shaken for 5 min to extract the piazselenol complex (Se–NPD) (Fig. 1). The organic phase was transferred to a round-bottom flask and the $CHCl_3$ evaporated using a rotary film evaporator. The residue was transferred to Eppendorf tubes (1 cm^3) and redissolved in 25 μl of $CHCl_3$ prior to anlaysis. The selenium standard and ^{76}Se spike solutions were derivatised as above and the Se–NPD dissolved in 200 μl of the $CHCl_3$. In all cases a 2 μl sample injection ensured that a minimum of 10 ng Se was loaded onto the column.

2.9. Isotope analysis

Selenium AAS solution [0.2 cm^3 (200 μg Se)] and ^{76}Se spike solution [0.5 cm^3 (92.3 μg Se)] were derivatised to their NPD derivatives and their molecular ion (M^+) regions analysed in the SIM mode by GC–MS. The concentration of the ^{76}Se spike solution was determined accurately by reverse ID-MS using the Se AAS solution as the internal standard [16]. For this work, the m/z 229/225 ratio was approximately one in order to maximise analytical precision. From these data it was possible to calculate the isotopic and chemical purity of the selenium spike solution.

3. Results and discussion

3.1. Isotope composition of natural and enriched selenium

The isotope compostions of the M^+ region of both natural abundance selenium (AAS solution) and isotopic ^{76}Se spike solution are shown in Table 2. These data show good agreement between the isotopic composition of the natural abundance Se solution and the theoretical values calculated on the basis of Se, C, H, N and O isotope distributions. The measured m/z 229/225 ratio for the natural abundance Se–NPD was 5.5116 ± 0.0212 ($n = 10$) (Table 2), which is close to the theoretical value of 5.5365. The M^+ region of the spike solution is also useful because it permits the calculation of the isotopic purity of the Se spike (certified isotope enrichment =

Table 4
Practical detection limits for quantitation of selenium

Sample size (ng) Se loaded onto column[a]	Mean m/z 229/225 ratio ($n = 4$)[b]	RSD (%)
100	5.59	2.3
50	5.59	2.3
20	5.53	2.1
10	5.61	2.2
5	4.32	5.2
1	3.59	6.2

[a] These weights refer to Se and not Se–NPD. The concentration of the stock Se–NPD solution was confirmed by GFAAS.
[b] n = number of replicates.

98.5 ± 0.2%). From the data in Table 2 it can be calculated that the Se in the spike is >99% ^{76}Se.

The chemical purity of the spike is also an important criterion for ID-MS; the presence of impurities would lead to errors in determining the concentration of the spike solution if this was done by gravimetric methods alone. To confirm the chemical purity, reverse isotope dilution of the spike solution was done using the natural abundance AAS solution and the concentraiton of the spike solution was found to be 184.7 ± 7 μg g^{-1} (± SD, $n = 5$). This was further diluted to 18.5 μg g^{-1} for all ID-MS quantitation experiments described in this paper. The validation of this figure was tested with a range of mole ratios of ^{76}Se spike (unlabelled) to natural abundance Se (labelled) (AAS solution). These data are presented in Table 3 and show excellent agreement between

Table 5
Selenium levels in CRM cabbage leaves (C85-04) and CRM soil (C74-05) (certified selenium levels in cabbage leaves = 0.083 (± 0.008) μg g^{-1}; certified selenium levels in Chinese soil = 1.56 (± 0.12) μg g^{-1})

CRM	Mean Se concentration (μg g^{-1}) ($n = 3$)[a]	RSD (%)
Cabbage leaves a	0.087	5.5
Cabbage leaves b	0.094	2.2
Cabbage leaves c	0.092	<0.05
Soil a	1.67	1.5
Soil b	1.65	2.4
Soil c	1.68	0.3

[a] n = number of replicates.

Table 6
Selenium levels in selected soils from North East Scotland

Soil association	Mean Se concn. (μg g^{-1}) ($n = 3$)[a]	RSD (%)
Countesswells	0.637	4.8
Insch	0.761	1.6
Strichen	0.710	2.5
Tarves	0.546	4.5

[a] n = number of replicates.

theoretical and measured m/z 229/225 ratios across an acceptable working range of ratios (i.e. close to unity where maximum precision is achieved) [22].

For quantitation of Se by ID-MS the parameters used in the Pickup and McPherson equation [16] were taken from the results presented in Table 2. The equation can be written as $x = y(RQ_l - Q_k)/(P_k - RP_l)$ where x = amount of selenium in the sample in moles, y = amount of selenium in the spike added in moles, R = observed m/z 229/225 ratio in the mole mixture and P_k, P_l, Q_k and Q_l are the relative abundance of the isotopes of natural selenium and the spike solution at m/z 229 and 225 respectively. From the values in Table 2 this formula can be rewritten as

$$x = y(91.35R - 0.11)/(46.56 - 8.45R)$$

3.2. Detection limits

Table 4 shows that with injection sizes of >10 ng natural abundance selenium, it is possible to obtain m/z 229/225 values that are not significantly different ($p < 0.1$) from theoretical values. With injection sizes of <10 ng Se the ratio be-

Table 7
Selenium levels in sewage sludges

Sludge	Mean concentration (μg g^{-1}) ($n = 3$)[a]	RSD (%)
London	3.55	1.8
Scottish Borders	1.15	1.8
Birmingham	3.13	2.9

[a] n = number of replicates.

came increasingly inaccurate and imprecise. In all subsequent experiments attempts were made to ensure that >10 ng selenium was available for each SIM analysis. As the m/z 229 225 ratio approaches unity in ID-MS experiments it may be possible to obtain good data with lower sample sizes

3.3. Selenium quantitation in CRMs and samples

The selenium content of the cabbage leaves was determined from three replicate samples analysed three times each to give a mean of means value (±95% confidence interval) of 0.091 ± 0.007 $\mu g\ g^{-1}$ (certified value = 0.083 ± 0.008 $\mu g\ g^{-1}$) (Table 5). The soil CRM was analysed in the same manner to give a mean of means value of 1.67 ± 0.04 $\mu g\ g^{-1}$ (certified value = 1.56 ± 0.12 $\mu g\ g^{-1}$) (Table 5). For both CRMs the random errors for our measurements are lower than the uncertainty values given for the certified materials and the mean selenium concentrations lie within their 95% confidence intervals. In both cases the mean values are <10% higher than the certified concentrations.

The selenium contents of the Scottish soils analysed were found to be in 0.5–1 $\mu g\ g^{-1}$ range (Table 6). The distribution of selenium in different soils is primarily a function of their parent material [3]. Although the values were similar, highest values were recorded for the Insch Association soil with parent material derived from gabbros and allied igneous rocks. This soil had the highest loss on ignition and was the least acid (Table 1) possibly indicating an association with organic matter. Ure and Berrow [23] have reported the mean selenium content of 56 Scottish topsoils to be 0.69 $\mu g\ g^{-1}$ with a range of 0.11–1.59 $\mu g\ g^{-1}$.

The sewage sludges contained significantly higher levels of selenium than the soils (Table 7) and would increase the selenium content of the soils if applied. The bioavailability of selenium in these sludges is unknown and transfer to crop plants and ultimately the human food chain cannot be assessed. Limited information is available on the selenium content of sewage sludges, but there is evidence that the Se content of sewage sludges in the UK has decreases in the period between 1982/1983 and 1990/1991 from 5 to 1.6 $\mu g\ g^{-1}$ [24].

4. Conclusions

This study has shown that ID-MS using a benchtop instrument can be used to determine selenium in plants, soils and sludges with good precision and accuracy and at a relatively low cost. Four soils of different parent material in NE Scotland were found to contain between 0.5 and 0.8 $\mu g\ g^{-1}$ selenium. Three sewage sludges were found to contain between 1.0 and 4.0 $\mu g\ g^{-1}$ selenium. If the application of sewage sludge to agricultural land leads to toxic levels of selenium entering the food chain then it may be necessary to include the analysis for selenium as a statutory requirement.

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