CHAPTER 1

Introduction

1. Chemical speciation

The last couple of decades of research have seen witness to an awareness change from that of believing that the biological response was proportional to the total amount of a trace element (Pleban et al., 1979) present to the recognition (i) that it is the actual chemical speciation of the metal complex which determines the bio-response and (ii) that overdose of essential trace element complexes can be detrimental to health.

For example the valence state of an element can profoundly affect its toxicity. Cr(III) is an essential element required for normal glucose metabolism but Cr(VI) is genotoxic and carcinogenic (Katz et al., 1994).

This growing realization has been accompanied by a move towards developing new analytical techniques and methods that are capable of monitoring chemical speciation at exceedingly low levels in complex matrix (May, 1995). For example, the ability to monitor trace metal complexes in blood plasma and serum involves selecting a desired species from many other species present in the same system (www.jate.u-szeged.hu/~spec98/abstr/williams.html).

The recognition that all chemistry, and especially that involved in the environment and in the human body, is governed by the laws of physical chemistry, and that such physical chemical constants can be used to extrapolate beyond the feasible levels of analysis leading to the development of an area of chemical speciation which is

closely-coupled with experimental data required for verification and validation of an analytical technique.

For the future, we must expect more legislation to involve details concerning chemical speciation as well as analytical data for the total amounts of a particular species present. It is also necessary to tackle the difficulties of convincing a non-chemically literate public of the subtleties of this subject and the benefits of the speciation chemistry, if correctly understood (www. jate. u-szeged. hu /~spec 98 / abstr / williams. html).

1.1 Heavy metal speciation

The earth's environment is a multi – dimensional area for a wide variety of living organisms and plants. The dynamics between all living things and their environment play a major role in their well being and survival. One of the most important aspects to consider is the role and interaction of trace elements and living matter. Trace elements play an important role in the functioning of life on our planet. Some elements can be highly toxic to a variety of life forms while others that are considered essential can become toxic at higher levels, different pathways and forms of exposure (Templeton et al., 2000).

Speciation is important but often employs complexed methods by which the effect of trace elements on the environment and all its living matter can be monitored (Whanger et al., 1994). Many of these effects depend strongly on the particular form in which the element is present in the system. For example as previously mentioned, Cr(VI) ions are far more toxic than Cr(III) (Katz et al., 1994) where as both methyl

mercury and inorganic mercury are toxic. They show different patterns of toxicity (Templeton et al., 2000).

Interactions of Metal-binding macromolecules

Most metals exist in biological fluids mainly in the form of macromolecules in equilibrium with a small, poorly defined, low molecular mass fraction. Most essential elements like Fe, Cu, Zn, and Mn are distributed among many dozens of enzymes where they provide structure, are redox active, or act as Lewis acids. By their nature, enzymes are generally present in catalytic amounts, and measurement of single enzymes is not usually part of biological speciation analysis. Rather, the macromolecular metal fraction is dominated by relatively few transport or storage proteins present in relatively greater amounts (Templeton, 1998).

Albumin is the protein present in blood plasma at the highest concentration, approximately 1mM. Therefore, for non-essential elements without specific binding sites on other proteins, non-specific binding to albumin may dominate speciation in the blood by mass action (Templeton, 1998; Wirth et al., 1985).

1.1.1 Speciation studies

The history of speciation, especially that of trace elements is not old. One of the main reasons for this was due to the inability of accurately quantifying trace elements. In fact the term 'trace' was only 1st used in the 19th century when a select number of element found in the human body could not be determined accurately with a high confidence level at low detection limits (Vanholder et al., 2002).

1.1.2 Techniques and methods for speciation analysis

Through the use of modern technology, development of new analytical procedures and optimised techniques, determination of metal speciation in many different types of sample matrices with a greater degree of accuracy is possible.

Speciation is difficult when it involves the accurate analysis of metal ions at trace level. It requires specific, versatile, accurate and sensitive enough methods. Furthermore most often the direct determination of metal species (specific oxidation, isotope or complex) is not possible due to the degree of complexity of the sample matrix or for low metal ion concentrations. The speciation would then require a two or multi step procedure whereby the species of interest are separated and or pre-concentrated prior to determination (Patriarca et al., 1998; Ndung'u, 1999). Speciation studies for future will serve an important framework in classifying and understanding the physiochemical properties of all substances especially those with a low $LD_{50.}$ A significant aspect of speciation is the role it plays in toxicology. It is known as fundamental rule of toxicology (Phipps, 1981; Duffs, 2002) that all substances, including carbon and all other elements and their derivatives, are toxic given in a high enough dose. The degree of toxicity of metals varies greatly from metal to metal and from organism to organism. Pure metals are rarely, if ever, very toxic (except as very fine powders, which may be harmful to the lungs from whatever substance they may originate). Toxicity, like essentiality, should be defined by reference to a dose–response curve for the species under consideration. This is another term that has been used loosely to refer to both the element and its compounds (Duffs, 2002).

1.2 Manganese

Found in a variety of foods, the trace element manganese is an essential element required for homeostatic and other important functions. Common food types that contain manganese include bread, nuts, cereals and green vegetables (such as peas and runner beans). It's also found in tea (Giles et al., 1983), probably the biggest dietary source (http://www. food. gov. uk/ healthiereating / vitaminsminerals / vitsminsaz / manganese /).

South Africa possesses approximately 82% of the world's manganese reserves in the form of manganese ores making it an important potential source of economic wealth (Viljoen et al., 1999). Manganese is currently used in a wide variety of applications, including: organomanganese compound as an anti-knock additive (Barceloux, 1999), manganese dioxide dry-cells batteries (Brent, 1956), steel, metal alloys (Venugopal et al., 1978), and fireworks. A high abundance of manganese ore in South Africa that is mined makes its potential for toxicity in miners and smelters important from an occupational health perspective. Myers et al., (2003) has shown the neurological effects of manganese on South African mineworkers. This implies that although only 14.9 % of the 82 % potential manganese ore reserves are being utilized, the exposure to manganese could gradually increase because of a greater demand due to the increased number of applications found for manganese in the future.

1.2.1 Biological importance of manganese

Manganese constitutes approximately 0.1 percent of the earth's crust and is therefore abundant. It is predominately found in the form of its oxides. Manganese is a transition metal, which occurs extensively in plants and animal tissues. It is however

found under normal conditions at trace levels in the human body. (www. healthandage. com / html / res / com / ConsSupplements / Manganesecs. html).

An average adult contains approximately a total of 10 – 20 mg of manganese found in tissue, fluids and bone (Underwood, 1977).

Manganese is one of many trace elements found in humans. Unfortunately its occurrence in the human body can lead to many adverse effects that include severe psychiatric and extrapyramidal motor dysfunction, which has in most cases been caused by oxidative damage (HaMai et al., 2004). It is also known that manganese is an essential nutrient for humans as its been found in a number of investigations using animals that deficiencies can result in abnormal brain functioning, indifferent skeletal and cartilage formation and impair reproductive and glucose tolerance (Keen et al., 1984).

1.2.2 Biological transport of manganese

As previously mentioned it has become more important for speciation studies to be carried out in order to investigate how different forms affect the toxicology. Once a metal species such as Mn(II) comes into contact with humans or animals various requirements are needed for it to affect that particular organism. Transport mechanisms of these ionic and molecular species across biological membranes have been researched vigorously and at present three major transport routes are known (Morrison et al., 1989). A select number of metal species can diffuse passively through aqueous cell membrane pores. The diffusion rate is dependant on the molecular size. This results in larger colloidal substances from being excluded whereas smaller free metal species diffuse at a faster rate. Membrane bound proteins

assist in the trans membrane movement of free metal ions. Lipid soluble species as passive diffusion transport enables for the rapid movement of metal ions across cell membranes. Lipid soluble complexes transport both the metal ion and its corresponding ligand into the cell, thus imposing an additional toxicity from the ligand, for example methyl mercury and other organometallics (Morrison et al., 1989).

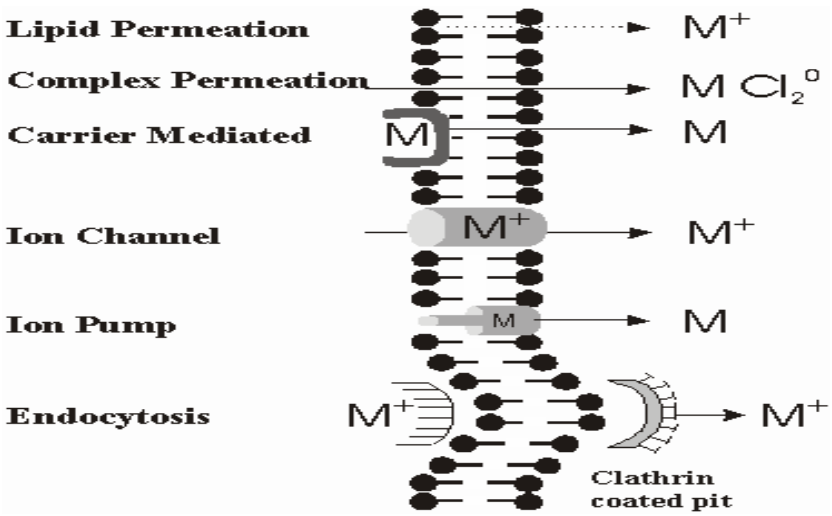

Figure 1 Mechanisms for the entry of bioavailable metal ions from the surrounding medium into the cells of bivalves (modified from Simkiss and Taylor, 1989). M, metal

i) Biological Role in Humans

Manganese plays an important role in the formation, and functioning of metalloproteins and mitochondrial enzymes that include: Superoxide dismutase (SOD) (McCord et al., 1969), Pyruvate Carboxylase (PC) (Venugopal et al., 1978) and the Glial specific enzyme, Glutamine Synetase (GS). Manganese also aids in the formation of connective tissue, fats and cholesterol, bones, blood-clotting factors, and

as mentioned above, proteins. Manganese Superoxide dismutase (MnSOD), is an anti oxidant that protects the body from toxic substances.

Just as the human body can be affected by an excess or deficiency in common life essential metals such as iron, zinc and copper, so to can manganese through excessive or insufficient consumption cause primary and secondary problems. The quantity of manganese present in the human body can also exaggerate or affect other illnesses. These include:

Diabetes. People who have diabetes often have significantly less manganese than healthy people. Manganese decreases blood sugar levels in some people who have diabetes.

Rheumatoid arthritis. One can have low levels of MnSOD when suffering from rheumatism. MnSOD helps protect the joints from damage during inflammation. A way to counter act this problem would be to administer manganese supplementation to increase MnSOD activity.

Epilepsy. An important study in the early 60's revealed that manganese deficiency is linked to and increase in susceptibility to seizures.

Schizophrenia. People who have schizophrenia may also respond well to manganese supplementation.

Osteoporosis. Bone loss occurs more rapidly after menopause and can lead to osteoporosis. Manganese increases bone density in postmenopausal women.

Other conditions. Manganese is also used to treat hardening of the arteries (atherosclerosis), osteoarthritis, tinnitus and hearing loss.

(www.healthandage.com/html/res/com/ConsSupplements/Manganese.html).

Manganese deficiency can lead to a wide range of conditions such as: growth retardation, skeletal (Raloff, 1986) and muscular abnormalities and abnormal

reproduction rates (Mervyn, 1985; Tolonen, 1990). On the other side of the scale, excessive intake of manganese can lead to brain abnormalities (A deficiency of the neurotransmitter dopamine, due to the manganese catalyzed oxidation of dopamine (Florence et al., 1987)) viewed on magnetic resonance imaging and in a few cases Parkinson's Disease-like symptoms (Donaldson et al., 1985; Leach, 1976; Witholt et al., 2000; Gwiazda et al., 2002). The average concentration of manganese commonly found in natural waters is in the range 0.1- 1.0mg l^{-1}. The current World Health Organization (WHO) guideline for manganese levels is 0.5mg l^{-1} for health and 0.1mg l^{-1} to avoid staining problems that manganese in the form of MnO_4^- can cause (Bowie et al., 1995). As previously mentioned, one of the most common forms of manganese toxicity related illnesses relates to the exposure of miners when mining manganese. The main reason for this is that miners are subjected to particulates containing high concentrations of manganese which has been linked to acute manganese intoxication, characterized by memory impairment, disorientation, acute anxiety and hallucinations (Donaldson et al., 1985; Leach, 1976). Manganese toxicity is a known problem in metal refining, manganese ore refinement and other industrial operations. Manganese concentrations in red blood cells are a greater deal higher than those found in blood serum. Concentrations of Mn in the red blood cells are higher than in plasma and have been used to assess status. For example, cows fed diets with 8mg l^{-1} Mn had 130ng of Mn/ml of whole blood, compared to 210ng of Mn/ml of whole blood in cows supplemented with 60mg l^{-1} Mn (Hidiroglou et al., 1978). This is a significant fact when testing individuals who have been subjected to long-term occupational exposure.

The primary site of manganese toxicity regardless of the route of exposure-by mouth, inhalation or injection by intravenous tube-in humans, monkey, rabbits and rats, is a

mass of nervous tissue buried deep within the cerebral hemispheres. This is the basal ganglia, part of the extra pyramidal system controlling bodily mechanics. The neuronal damage caused by the manganese tends to be more extensive in young, immature animals than in adults (http://www.westonaprice.org/soy/manganese.html; HaMai et al., 2004).

ii) Manganese binding biological ligands

Not much is known regarding the nature of manganese (Mn)-binding ligands in plasma and serum, and its transport mechanism across the blood-brain barrier (BBB). Mn has an affinity to bind to high molecular mass proteins, such as transmanganin, transferring (Tf), albumin and α_2-macroglobulin as well as to small proteins in the 10 000 to 20 000 Da molecular mass range. It is thought that this binding is dependant on the oxidation state of Mn (Hancock et al., 1973; Critchfield and Keen, 1992).

Systemic circulation: In the liver, a small essential fraction of the manganese adsorbed is oxidized by caeruloplasmin to the trivalent state and bound to transferrin; then, it remains in circulation and is distributed to other tissues.

Portal circulation: Manganese is mainly transported in the divalent state (Mn(II)). Mn(II) binds first to α_2-macroglobulin, where each molecule carries one atom of manganese. Albumin also plays an important role in the Mn(II) transport in the portal circulation (Hancock et al., 1973; Critchfield and Keen, 1992). Most of the portal Mn(II) is excreted in essentially one passage through the liver, where Mn is attached either to a storage protein, excreted in the bile or released back into plasma attached to

the same or different transport protein (Hancock et al., 1973; Critchfield and Keen, 1992).

Blood-Brain-Barrier-transferrin and transferrin receptors (TfR): Manganese transport across the Blood-Brain-Barrier (BBB) occurs both in the 2+ and 3+ oxidation states. The rate of transfer into the CNS is far slower than into other tissues (Aschner et al., 1999). Compared to Mn(II), Mn(III) has a slower elimination rate and therefore may have a greater tendency to accumulate in tissues.

The existence of a transferrin-independent transport system for Mn has been elucidated (Takeda et al., 1998; Takeda et al., 2000), however transferrin is shown to be required for the transport of manganese through the BBB (Suarez and Eriksson, 1993; Malecki et al., 1999). Manganese bound to transferrin is internalised into the cell through transferrin receptors expressed on the external surfaces of the cell (Suarez and Eriksson, 1993).

Incidents in two London hospitals alerted the medical community to the vulnerability of sick babies to manganese attack suffering liver disease. (http:// www. westonaprice. org/soy/manganese.html) The babies received nutrient solutions containing small amounts of manganese through intravenous tube feeding (http:// www.mercola. com/2001/jun/13/soy_formula.htm.)

Although the manganese concentration was no greater than that in soy formula, and considered safe by government standards, it caused brain damage after feeding periods lasting a few months to two years. Of 57 babies receiving “safe” amounts of manganese, two fell ill with movement disorders and six suffered damage to their

basal ganglia (www.healthandage. com / html / res / com / ConsSupplements / Manganese. html, www. westonaprice. org / soy / manganese. html).

1.3 Chemistry of manganese

When examining the chemistry of manganese and the differences each of the oxidation states exhibit, then the chemistry and in particular the speciation of this metal is complexed compared to other transition metals like Cr and Fe (Majors, 2004; Ottaway et al., 1986). From the Frost diagram in fig.2 below it is shown that the most common and most stable oxidation state of manganese is the divalent state (d^5) (Shriver et al., 1998).

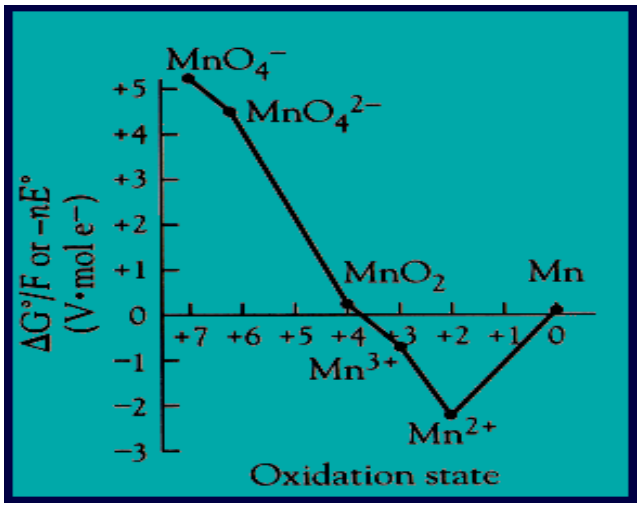

Figure 2 Frost diagram for manganese (Mackay et al., 1981)

Manganese metal is acquired from its oxides by reduction with aluminum. Typical ores of manganese are pyrolusite, MnO_2, and hausmannite, Mn_3O_4 (Liptrot, 1992). The main use of pure manganese is in ferromanganese of steels (Cotton et al., 1988). Manganese in its elemental form is present in more than 100 common salts and mineral complexes that are abundant in rocks, soils and on lake floors and oceans. The most common form this trace element is founds in is as manganese dioxide,

carbonate or silicate. Manganese in its +2 and +4 oxidation states is the most significant in aquatic systems (Canadian council of resource and environment ministers march, 1987).

Looking at an element that has similar chemistry features to manganese, iron falls into that category. The significant difference between the two metals is that manganese is harder and more brittle, but less refractory (mp 1247^{o}C). It is a fairly electropositive metal ion and dissolves with relative ease in dilute non-oxidizing acids. At room temperature it has a low reactivity towards non-metals, however at higher temperatures it reacts vigorously with a wide variety. One good example of this would be when Mn^{2+} is placed in $Cl_2(g)$ and burned; it reacts with the chlorine forming $MnCl_2$ (Cotton et al., 1988).

1.3.1 Manganese(II) complexes (d^5)

When comparing the equilibrium constant for complex formation in aqueous solution of Mn^{2+} with constants for Fe^{2+}- Cu^{2+} they are not high. The reason for this is due to Mn(II) being the largest of these ions and it has no ligand field stabilization energy in its complexes (except in the few of low spin). Manganese(II) complexes can have an extensive series of ligands, such as halide ligands ($MnCl^{+}$), (MnX_3^{-} octahedral) and MnX_4^{-} tetrahedral). Oxygen ligands for example, acetylactonate $[Mn(acac)_2]_3$. Sulphur ligands, for example, dithiocarbomate (18-D-I). Nitrogen ligands, for example, $[Mn(NH_3)]^{2+}$ and $[Mn(en)_3]^{2+}$. Phosphine ligands with general stoichiometery $MnX_2(PR_3)$ and finally, carbon ligands, which include alkoxides and dialkyamides.

1.3.2 Binary compounds

These compounds can include Manganese(II) Oxide, which is a gray-green to dark green powder obtained by roasting the carbonate in H_2 or N_2 or through the action of steam on $MnCl_2$ at 600^{o}C. The crystal structure is that of a rock salt type and the compound is insoluble in water. Another binary compound is Manganese(II) Hydroxide, that has an identical crystal structure to that of $Mg(OH)_2$. Other manganese binary compounds that exhibit a NaCl structure include, Manganese Sulphide, MnSe and MnTe (all strongly anti-ferromagnetic) (Cotton et al., 1988).

1.3.3 Divalent manganese and manganese (II) salts

The divalent oxidation state of Manganese is the most common and stable oxidation state in which manganese can occur. This is shown in figure 3 below (Kirk-Othmer, 1981) and previously in figure 2 (Frost diagram).

A wide variety of salts are formed by Mn(II) with all common anions, of which contain $[Mn(H_2O)_6^{2+}$ crystallize from water as hydrates. The sulphate, $MnSO_4$, made on fuming down H_2SO_4 solutions, is relatively stable and may be used for Mn analysis, provided no other cations giving non-volatile sulphates are present.

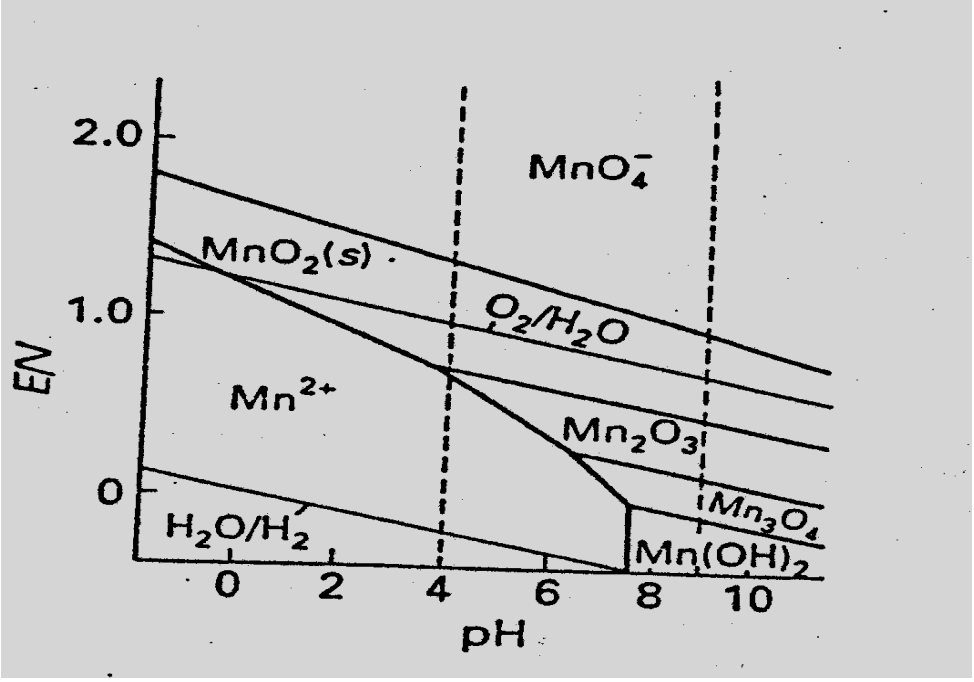

Figure 3 Pourbaix diagram for manganese species in diluted aqueous solutions (Kirk-Othmer, 1981)

1.4 Techniques and applications of biological speciation

Biological speciation studies are complexed. This is due to the complexity of biological matrix and sensitivity of the samples to various external changes such as temperature and pH. Various oxidation states of metals are dependent on pH. In order to carry out realistic speciation studies to determine biological pathways, bioaccumulation and toxicity of a particular metal species, the conditions at which the extractions and analysis are done at must mimic as close as possible to those of the real biological system in its natural state. A variety of different speciation methods exist (isotopic and oxidation state) depending on the nature of analyte species and sample matrix (Templeton et al., 2000).

Other analytical methods also used in speciation studies include: flow injection analysis in real samples by Diphenylcarbazide-Spectrophotometric determination

(Kargosha et al., 2003), Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP-AES) where manganese and copper were extracted by solid-phase extraction in milk with the intent of investigating the speciation of these metals as opposed to just determining their total concentrations as done in previous studies (Abollino et al., 1998; Lavi et al., 1990; Arruda et al., 1994; Favretto et al., 1994; Coni et al., 1990), and Resin titration followed by GF-AAS previously used in the speciation of Mn(II), Zn(II), Cu(II) and Al speciation in tea infusion (Alberti et al., 2003) also in fresh and sea water (Pesavento et al., 1998; Pesavento et al., 2000).

1.4.1 Extraction and sample preparation

There are many kinds of sample matrices (water, biological and geological). Many trace analytes in complex matrices often require extensive sample preparation to remove interferences and/or enrich the analytes many different preparation techniques exist (van de Merbel et al., 1993). Often samples cannot be run directly using an analytical determination technique but have to be extracted and/or preconcentrated. The speciation then becomes far more complexed due to time, cost, contamination and the reliability of the procedure. For this reason any sample preparation including extraction must be carefully selected and possibly optimized depending on the sample matrix. The major difficulty is when optimizing a method for biological samples. Due to biological samples having a complex matrix, optimal extraction conditions can differ from sample to sample.

i) Solvent extraction

Solvent extraction can be carried out by the transferal of a substance from any matrix to an appropriate liquid phase. Depending on the solubility of the solute analyte the

desired extractant solvent is chosen. In the case of the solute existing as an immiscible liquid were in the analyte is contained the separation is called a Liquid - Liquid extraction (LLE) (Rice et al., 1993). This method can preconcentrate more than one analyte simultaneously thus isolating unwanted elements (Morrison et al., 1957; Inczedy, 1995). Unfortunately there are a number of disadvantages using LLE as a batch separation method. These include (Sturgeon et al., 1980):

1. Time consuming.
2. Contamination through atmospheric exposure.
3. Large number of chemicals used often leads to high or/and random blanks.

ii) Dialysis techniques

Dialysis is a technique based on differences in mobility of ionic or molecular species in their liquid phase during their transport through a semi-permeable membrane into a secondary liquid phase. The second liquid phase does not have to necessarily be immiscible with the first liquid. Mass transfer then occurs between a donor and acceptor phase that are separated by a membrane, which selectively allows solutes to pass through by blocking the pathways of larger macromolecules or by differences in molecular diffusion. The concentration gradient set up in the transferable solute between the two phases is the responsible driving force for the mass transfer that occurs through the membrane (Ndung'u, 1999). This method of extraction has been successfully used in the determination of weakly acidic and basic pollutants in surface water. On-line electro dialysis was used as a sample pretreatment technique for the treatment of natural waters before column liquid chromatographic determination of polar pollutants (Groenewegen et al., 1994). Dialysis coupled with a suitable determination technique can also be used for online sample treatment and analysis of

both biomedical and environmental samples (Turnell et al., 1987; Aerts et al., 1988; Agasoster et al., 1990; van de Merbel et al., 1992; van der Merbel et al., 1994).

iii) Membrane extraction

There are currently many different types of membrane materials and designs used for extraction and sample preconcentration; however the most advantageous type of membrane is the liquid membrane (Ndung'u, 1999). There are many advantages associated with liquid membranes as compared to solid polymeric films. This is because liquid films offer much higher diffusivities and the liquid membranes can be made far thinner than the polymeric membranes. Furthermore, these liquid membranes can be custom made by the incorporation of an appropriate extractant in the membrane phase so that the separation properties of the membrane phase can be adjusted depending on the required application (Lindoy et al., 1989).

There are three types of liquid membranes all classified according to their configuration. These are: Bulk liquid membranes (BLM), surfactant or emulsion liquid membranes (ELM) and polymer-supported liquid membranes (SLM).

Bulk liquid membranes

These types of membrane are composed of a source and receiving phase, usually aqueous, separated by a bulk membrane phase normally organic held in a stirred system (Izaat et al., 1983). Unfortunately due to the thickness of these membranes it has been found that a low fluxation occurs through the membrane thus the use of this type of membrane is limited to liquid membrane mechanistic studies as opposed to other extraction applications (Dadfarnia et al., 1993; Zouhri et al., 1995).

Emulsion liquid membranes

An emulsion liquid membrane system exists in the form of a water-in-oil emulsion composed of the stripping solution with an extractant-containing organic solvent. These emulsions are then dispersed in a continuous aqueous solution (donor phase) from which the analytes are to be extracted. In order to maintain the emulsion phase wile separation occurs, surfactants are incorporated into the membrane phase. When this emulsion is dispersed through agitation in a continuous manner, a large number of small globules of emulsion are formed. The size of these globules can be altered by altering the surfactant concentration, emulsion viscosity, and type and degree of mixing. This enables for a larger number of emulsion globules to be produced thus increasing the overall surface area (Ding et al., 1991; Ndung'u, 1999).

This form of liquid membrane has been used in a number of different industrial and analytical applications in the recovery of metals such as from industrial waste (Izaat et al., 1987; Teresa et al., 1993) and even waste from water treatment processes (Raghuraman et al., 1994) and in preconcentration applications, the extraction of cobalt from simulated nuclear coolant water was achieved by Okamato et al., (1990) using a phosphonate extractant ELM (Okamoto et al., 1990). Strontium has been selectively extracted and enriched from industrial wastewater by Wang and Li (Wang et al., 1997).

There are unfortunately disadvantages in using this type of membrane. Difficulties in achieving stable emulsions, secondary emulsion formation and the need for multi parameter control. Furthermore long sample pretreatment is required prior to extraction (Ding et al., 1991).

Supported liquid membranes

Supported Liquid Membranes (SLM) consists of a water immiscible organic solvent trapped on a thin micro porous membrane and placed between two aqueous phases. These two aqueous phases are the donor and acceptor phases. The donor phase is the solution that contains the analyte of interest and to be extracted while the solution into which the analytes are captured is the acceptor phase. There are a number of different SLM configurations that exist with the most common being the planar and hollow fiber setups. SLM has been used in many different sample preconcentration and extraction applications. It has been used for the enrichment of carboxylic acids for samples with extremely low concentrations of the analyte (Shen et al., 1994), organic compounds in biological samples (Jonsson et al., 1994), determination of chlorinated phenols in natural water samples and extraction of aliphatic amines in human blood plasma (Knutsson et al., 1996; Lindegard et al., 1992) and for the extraction and preconcentration of Mn(II) from different sample matrix (Soko et al., 2003). Its application potential has also been demonstrated as a preconcentration technique for a number of other metal species such as Pb(II), Cu(II), Zn(II), and Cd(II) at trace levels (Keller et al., 2000).

SLM techniques are still relatively new in comparison to other sample preparation techniques and their analytical applications are not as wide. They provide an advantage of performing LLE in a flow system, are easily automated and are highly adaptable to field sampling. The main advantage is because of the reduced number of reagents employed, the selectivity of the extraction can be easily adapted by using custom reagents (Lindoy et al., 1989).

1.5 Sampling and sample storage

Sampling is the process of selecting a representative bulk sample from the total quantity (Harris, 1998). The use of new methods of sample preparation, extraction and the advent of modern technology, makes it possible to measure a broad range of trace elements at concentrations as low as parts per trillion. Unfortunately being able to have such high detection levels comes at a price as it means sampling and sample storage has to be carried out ensuring both uncontamination and representation of all samples (Horowitz, 1997).

This means that a large percentage of analytical errors originate from sampling. These include: contamination, losses, and physical or chemical perturbation (Riley et al., 1975).

1.5.1 Sampling of biological fluids

A wide variety of safety precautions need to be implemented during biological fluid sample collection. The main reason for this is due to contamination and losses of the trace element to be analysed. Contaminants themselves can affect results by changing the amount of bound analyte to a specific fraction. Other factors such as individual variations, occupational exposure and physiological state of the subject may also affect the chemical speciation and concentration of a specific trace element found in the body (Ndung'u Sweden, 1999).

Often the time of sampling plays an important role in the representation and specificity of the sample. This particularly applies in the case of biological sampling for toxicological purposes as many toxins including metals have specific bioaccumulation regions and varying detection periods before excretion or any other dilution effects. A good example of sampling selectivity would be the sampling of

urine. As described by (Das et al., 1996) it is common practice for urine samples to be taken over a 24-hour time frame. It has been suggested that the reason for this could be due to most of urines typical constituents showing diurnal variation and because the volume of liquids passing through the body varies according to drinking patterns there follows a variable peak excretion of a specific toxic species.

Biological sample storage, depending on the species to be tested for is also of great significance to the overall analysis. Most samples are best collected and stored in polyethylene or polypropylene air tight containers and kept refrigerated at a temperature range of $2^{o}C$ to $-20^{o}C$. Certain processes such as lyophilisation and centrifuging can be used for preserving certain biological fluids for long periods (Gardiner, 1993).

1.6 Experimental design and chemometric data analysis

Chemometrics and data processing are increasingly being used in research throughout the world and more specifically in analytical chemistry for data analysis and experimental design, univariate signal/data processing, multivariate calibration and pattern recognition (Brereton, 2003). For example the two-dimensional and orthogonal information can be compared by using evolving factor analysis after the unreduced data has been treated with an algorithm for the elimination of technique specific variations (http: // www. hicstatistics. Org / 2003StatsProceedings / Kurt%20Varmuza. pdf).

International trends in chemometrics involve diverse research projects in chemical measurements, software and hardware for various applications. Signal processing has been improved for analytical measurements with respect to chemical or physical information involving time series analysis, digital filtering, smoothing, deconvolution,

background correction and image analysis. Structure/activity relations are of high interest, especially in the areas of relating the chemical structure to the biological activity (www.analytisk.kemi.uu.se/AktuellForskning.htm).

CHAPTER 2

Analytical techniques used in the study

2. Accuracy in trace analysis

Throughout the advancement of science and technology there has become an increased emphasis placed on the importance towards the consideration and detection of extremely small quantities of elements in chemical, physical and biological systems. This has lead to far greater demands and expectations regarding more reliable and sophisticated analytical infrastructure and analysis. The analytical criteria imposed by the minute quantities and furthermore, the complexed systems involved have lead to the development of instrumentation and analytical methodology so specialized that the need for a distinct field of analytical chemistry is required, known today as trace analysis.

The term trace can have a number of connotations depending on its origins. So it has become clear that in order to avoid ambiguity, it is herein defined as a constituent making up only a small fraction of the sample to be considered. Depending on the trace or micro constituent being considered, measurements are more than often made at the parts per million (mg l^{-1}) level. However with an increasing demand for even a higher level of elemental detection analysis has ventured into the parts per billion (μg l^{-1}) region. This is the ultra trace detection limit. It is important to note that the detection limits defined above depend on the nature of the sample to be analyzed, the analytical technique employed and the analyst. One discerning feature of trace analysis that separates it from the other macro analysis is defined in the final step of a trace analysis in the final step of trace analysis and may be far more micro than

ordinary out of date micro analysis, involving microgram and nanogram quantities of material (Morrison 1965, http: www.archeotrace.co.uk/services/trace.html).There are also many problems, which arise when considering the procedure of trace analysis. In summary they are:

1) The direct determination of a trace species in a relatively large sample with regards to the sample size.
2) Preconcentration of a micro constituent into a small, relatively matrix free sample to improve detectability and/or eradicate matrix interference.
3) Using probe techniques to study small-localized concentrations of impurities in a larger sample.
4) The determination of major and minor species in a minute initial sample.

Prior to performing a trace analysis, another important, yet often overlooked factor, is the question of which trace technique is to be implemented for a specific analysis. The main criteria used in the selection of a suitable trace analytical technique are:

Sensitivity

Accuracy and precision

Selectivity

Although trace analysis plays an important role in many disciplines of quantitative analysis, it's through speciation that makes this form of analysis such a powerful tool. Speciation is used to determine the different physiochemical forms of a particular element that make up its total concentration (www.uilondon.org/ures.htm).

There are a number of techniques that have been developed for analytical speciation using trace analysis. An analytical determination method that involves voltammetry, a complexed electro analytical application is widely used and has a broad range of

applications, depending on the analysis required. Many forms of voltammetry exist and depending on the detection limit required and sample composition; all play an important role in the electro analytical trace analysis in speciation chemistry (Skoog et al., 1992).

2.1 Sampling and sample storage of biological fluids

2.1.1 Milk

The chemistry of milk

Milk is an excellent food for the very young, but humans have also adapted to using milk, especially cow's milk, as a food source for persons of all ages. Many specialized milk products like cheese, yogurt, butter, and ice cream are staples of our diet. Milk is probably the most nutritionally complete food that can be found in nature. This property is important, since it is the only food young mammals consume in the nutritionally significant weeks following birth. Whole milk contains vitamins (principally thiamine, riboflavin, pantothenic acid, and vitamins A, D, and K), minerals (calcium, potassium, sodium, phosphorus, and trace metals), proteins (which include all the essential amino acids), carbohydrates (chiefly lactose), and lipids (fats) (Pavia et al., 2000).

Fats: Whole milk is a type of oil-water emulsion; containing approximately 4% fat dispersed as very small (5-10 microns in diameter) globules. These globules are so small that a drop of milk contains about a million of them. Because the fat in milk is so finely dispersed, it is digested more easily than fat from any other source. The fat

emulsion is stabilized to some extent by complex phospholipids and proteins that are adsorbed on the surfaces of the globules (Pavia et al., 2000).

Proteins: Albumins are globular proteins that are soluble in water and in dilute salt solutions. They are, however, denatured and coagulated by heat or under acid conditions. Lactalbumins are the second most abundant protein types in milk. Once the caseins have been removed, and the solution has been made acidic, the lactalbumins can be isolated by heating the mixture to precipitate them. A typical albumin protein has a molecular weight of approximately 41,000. A third type of protein in milk is the lactoglobulins. These are present in smaller amounts than the albumins and generally denature and precipitate under the same conditions as the albumins. Lactoglobulins posses the immunological properties of milk. They protect the young mammal until its own immune systems have developed (Pavia et al., 2000).

Carbohydrates: When the fats and the proteins have been removed from milk, the carbohydrates remain, as they are soluble in aqueous solution. Lactose is the main carbohydrate found in milk (Pavia et al., 2000).

2.1.2 Blood serum

Blood is obtained from sites, the most likely being an individual person's arteries, veins (region inside the elbow) or capillaries. Whole blood is centrifuged to convert it into blood serum. Before the sample is collected, the skin is cleaned using alcohol and a tourniquet is placed a few centimetres above the sampling site to prevent the blood from flowing to the heart thus causing the veins to become more visible (Tietz, 1987).

Serum, originating from plasma in which blood cells are made into a suspension and transported through the body with clotting factors removed, contains 60 to 80mg of

protein per ml. Serum also contains various small molecules including salts, lipids, amino acids, and sugars (Burtis et al., 2001). Albumin, immunoglobulins, transferrin, haptoglobin, and lipoproteins are the major protein constituents of serum (Burtis et al., 2001; Turner et al., 1970). In addition to these major constituents, serum also contains many other proteins that are synthesized and secreted, shed, or lost from cells and tissues throughout the body (Schrader et al., 2001; Kennedy, 2001). Approximately 10,000 proteins may commonly occur in serum, most of which would be present at very low relative abundances (Wrotnowski, 1998).

2.1.3 Sample preparation

The difficulties caused by sample matrix in trace analysis are often overcome by separation or isolation of trace elements from the matrix prior to determination (Morrison, 1965). Different types and forms of extraction techniques have been employed for many years. Depending on the complexity of the sample matrix, each technique is used accordingly.

2.2 Supported liquid membrane (SLM) extraction

2.2.1 SLM fundamentals and mechanisms

Supported liquid membrane technology is an extremely promising alternative to existing sample pre-treatment methods for metal ions due to its selective enrichment capabilities of such ions from different complex matrices (Danesi, 1988). The technique is based on a porous PTFE (poly-tetrafluoroethylene) membrane, impregnated with a water immiscible organic solvent (i.e. DEHPA). The hydrophobic liquid membrane forms a barrier between the sample and the analytical instrumentation. The extraction can be controlled by the pH of the two phases, the

donor and the acceptor. Under correct pH conditions, acid or basic analytes can be uncharged and extractable from the donor solution and charged and non-extractable in the acceptor solution. The uncharged analyte species diffuse across the membrane and are then back-extracted into the acceptor; where the pH is such that the analytes are charged and as a result captured in the acceptor phase. For a flow system where the sample passes the donor channel with the acceptor kept stagnant, enrichment of the analyte in the acceptor is possible. The use of SLM technology for analytical pre-concentration in order to improve the detection limit of metal ions is limited in the types of species (Papantoni et al., 1995). The diffusion transport of metal species across a membrane containing a hydrophobic liquid in its pores is achieved by forming uncharged metal complexes either by adding complexing reagents to the donor solution containing the analyte or to the membrane liquid, where the added reagent acts as a carrier (Malcus et al., 1996).

Extraction mechanism and fundamentals

In the SLM extraction technique an ionic species to be extracted such as the metal ion (Mn^{2+}), is transported across the PTFE membrane using an extractant liquid that is loaded and held inside the membrane pores. This extractant is capable of forming an uncharged ion association complex with the metal ion present in the donor solution. DEHPA is one type of extractant already used for the extraction of trace metals in different matrix (Komasawa et al., 1983). After transportation of the neutral complex through the membrane, the metal ion is released at the membrane-acceptor interface as a result of protonation of the extracting agent. A schematic representation adapted from Ndung'u, 1999 of this mechanism is shown in figure 4 below.

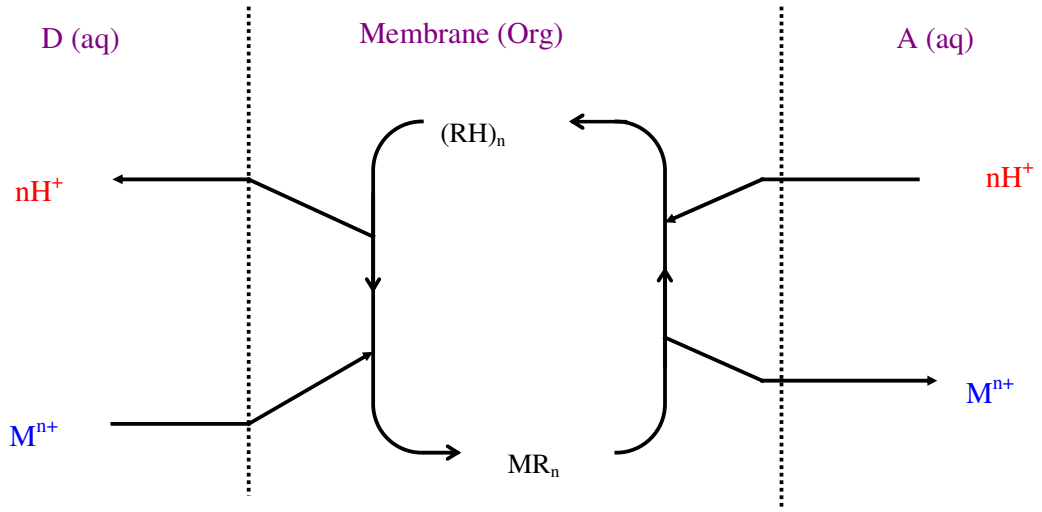

Figure 4 Mechanism of SLM extraction scheme of the metal ion transport across the membrane, where M^{n+} and RH_n represent metal species and acidic extractant, respectively. MR_n is metal-extractant complex. nH^+ is hydrogen ions.(Djane et al., 1997).

One of the major difficulties for the use of SLM technologies on an industrial scale is the relatively short lifetime the membrane has due to the loss of the organic solvent from the substrates. As a result there has been much emphasis focused on better and new ways of improving the lifespan of these membranes (Kemperman et al., 1996).

2.2.2 Extraction efficiency and enrichment factor

Extraction efficiency refers to the recovery process and is generally defined as the fraction of analyte that is transported from the donor phase through the membrane and thus located in the acceptor phase:

$$E = C_A V_A / C_i V_i \qquad \text{Eq 1}$$

Where C_i and C_A are the concentrations of the analyte input to the system during the extraction time period and collected in the acceptor, respectively. The extraction efficiency is a function of a number of thermodynamic and kinetic parameters including the donor flow rate, and the partition coefficient of the analyte.

There are two distinguishable situations namely membrane controlled extraction and donor controlled extraction. The former is related to the rate limiting step of the diffusion of analyte through the membrane while the later occurs when the partition coefficient of the analytes K, is larger than about 10, while the mass transfer is mainly membrane controlled when K<1.

Enrichment factor indicates the extent to which the extraction process increases the analyte concentration of the original sample:

$$E_e = C_A / C_D \qquad \text{Eq 2}$$

C_A and C_D represent the concentrations of the analyte in the acceptor and donor phases, respectively. E and E_e are related by the following expression:

$$E_e = E_{VI} / V_A \qquad \text{Eq 3}$$

Where V_I and V_A are the volumes of the sample (donor phase) input to the system and the acceptor phase collected, respectively.

2.3 Size exclusion chromatography

Size-Exclusion Chromatography (SEC), also called gel-permeation chromatography (GPC), uses porous particles to separate molecules of different sizes. It is generally used to separate biological molecules and to determine molecular weights and molecular weight distributions of polymers. Molecules that are smaller than the pore size can enter the particles and therefore have a longer path and longer transit time than larger molecules that cannot enter the particles. All molecules larger than the pore size are unretained and elute together. Molecules that can enter the pores will have an average residence time in the particles that depends on the molecules size and shape. Different molecules therefore have different total transit times through the column (Hunt, 1979; Yau et al., 1979). The main reason for the use of this technique will be to separate different biological fractions and then determine the concentration of a particular metal species in each fraction. This will give vital information with regards to the transport and bioavailability of each individual species (www.elchem.kaist.ac.kr/vt/chem-ed/sep/Ic/size-exc.htm).

Shown below in figure 5, is the schematic representation of a typical size-exclusion chromatography column used.

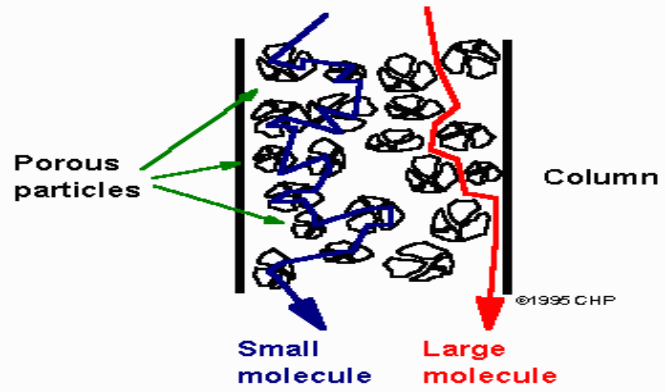

Figure 5 Schematic representation of a size exclusion column (www . elchem. kaist. ac. kr)

2.4 Electrochemical techniques

2.4.1 Voltammetry

Potentiometry, conductometry and electro analysis have been frequently applied to a number of different electrochemical research fields. Polarography, is a branch of voltammetry, where a relationship between potential and current is characterized through the use of a dropping mercury electrode (working electrode) (Meites, 1967) The current is usually proportional to the concentration of the analyte being determined. Voltammetry is one of a wide variety of electrochemical methods used and refers to an analytical technique in which a relationship between voltage and current is observed during an electrochemical process (Crow, 1969). Voltammetry is thus a broad name describing all electrochemical methods used in the analysis of solutions containing reducible or oxidizable species (www.chem.ch.huji.ac.il). In voltammetry, the dropping mercury electrode can also be used as the working electrode due to the drop continually generating a fresh surface for each growth stage that results in reproducible current-potential data (Skoog et al., 1992; Harris, 1998; Delahay, 1954). Voltammetry is one of the most sensitive analytical techniques available which have a unique capability to distinguish oxidation states that can affect a substance's toxicology (Willard et al., 1988).

There are different types of polarographic modes or techniques used in speciation and electro analytical chemistry depending on the relationship between the pulse output and signal used. The most discerning difference that separates them is the type of current recorded and voltage ramp employed. The main are:

Direct current polarography

Sampled current polarography

Normal pulse polarography

Differential pulse polarography

It should be noted that the last three techniques are as a result of improvements towards polarographic detection on sensitivity (Skoog et al., 1996; Bauer et al., 1978; Heyrovsky et al., 1968; Harris, 1999; Harris et al., 1974).

Direct current employs a linear voltage ramp. The voltage is scanned with the ramp from positive to negative at a constant rate. The current is measured continuously resulting in an oscillating wave. The oscillations in the wave are as a result of the mercury drop growing and then falling. Only a small current flows at the most positive potentials. The area of the curve preceding the residual current is defined as the polarographic step or wave where the current increases steeply. The difference between the residual current and the peak plateau gives rise to the limiting current. This is proportional to the concentration of a polarographically active species in solution (Bauer et al., 1978; Harris et al., 1974).

Sampled current, the working electrode suspends one mercury drop of constant size, per second and then takes a measurement on the drop. For this form of polarography a staircase voltage profile is produced. There is a voltage step up to a more negative value than the previous voltage for each new drop. The current is recorded for example, 17 milli- seconds (sampling time) before the drop is dislodged. One of the most significant advantages of this method is that the signal to noise ratio is increased (Harris, 1999).

Normal current polarography has a much lower detection limit of 10-6 mol dm-3 compared to the other direct and sampled current methods. Voltage pulses are utilized and the current at the end of each pulse is measured and also corresponds to the end of

each drop life. At the start of each drop the potential is below that required for reduction of analyte (species). Once the analyte is reduced there is a high faradic current because the concentration of the reduced species at the surface of the drop is far higher than was the case for sampled current polarography. At the end of each pulse the current is measured so as to allow for the charging current to decrease substantially. After each drop falls the potential is stepped back to its initial value and the process starts again (Harris, 1999).

In **Differential pulse** polarography the superimposition of small pulses on a linear voltage ramp takes place. Increasing the pulse height or modulation amplitude increases the signal height, however decreases resolution of neighboring peaks. Current is measured once before the pulse and then for the final 17 milliseconds of the pulse. The resulting polarogram is a subtraction of the initial current at the beginning of the pulse application from the current value taken at the end of each voltage step plotted against the potential measured prior to the voltage pulse. A differential pulse polarogram is approximately the derivative of a direct current polarogram.

2.4.2 Voltammetric instrumentation

i) Cell

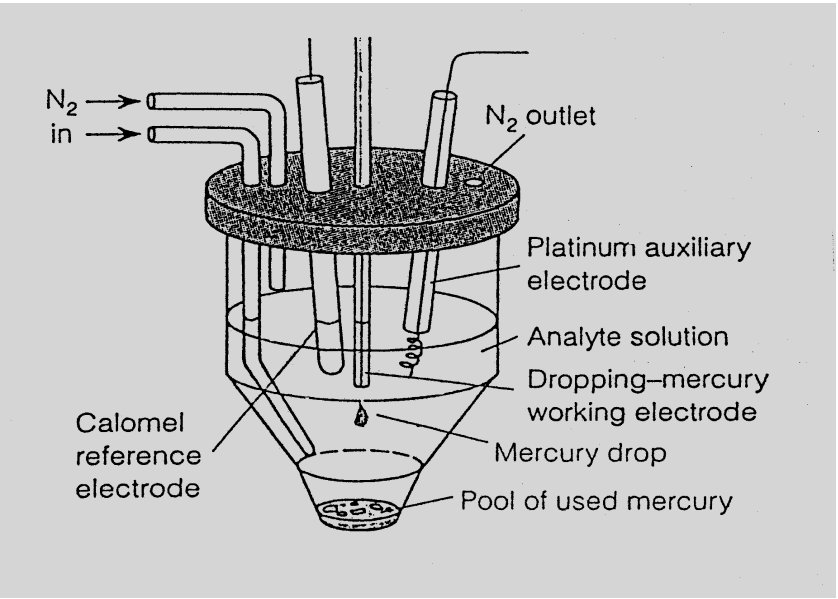

Figure 6 Typical electrochemical cell (Harris, 2003)

ii) Working electrodes

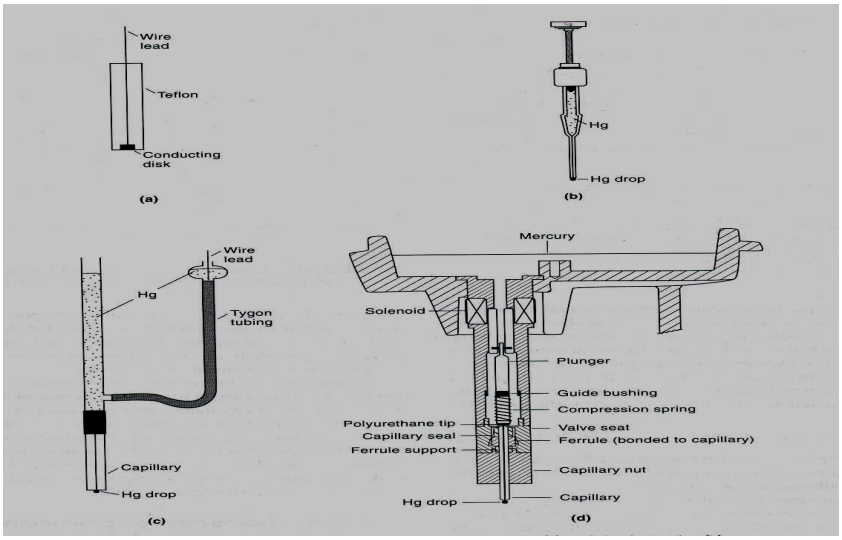

Figure 7 Working electrodes. Common types of microelectrodes: (a) disk electrode; (b) hanging mercury drop electrode; (c) dropping mercury electrode; (d) static mercury dropping electrode (Skoog et al., 1996).

Static mercury drop electrode

These forms of electrodes use a hanging mercury drop were the mercury drop is delivered through a capillary (from a plunger). The entire experiment is performed on a single mercury drop held on the capillary tip. After each new experiment the drop is dislodged and replaced for the next experiment. A static electrode area results in elimination of the charging current caused by a changing surface area (dropping mercury electrode). This enhances sensitivity (Willard et al., 1988).

2.4.3 Stripping techniques

Stripping Voltammetry consists of a number of electrochemical techniques that are used routinely in various analytical applications. The main reason for this can be attributed to their increased sensitivity compared with classical polarographic methods. There are many different types of electrochemical stripping analytical techniques, consisting predominantly of anodic stripping voltammetry (ASV), adsorptive stripping voltammetry (AdSV) and cathodic stripping voltammetry (CSV) (Saterlay, 1999). These techniques consist of two main steps. An initial preconcentration step, the analyte of interest is accumulated from solution on to a suitable working electrode substrate. According to the nature of the analyte, this can be accomplished in two ways:

1) Electrochemical deposition
2) Physical adsorption

The potential of the working electrode is then adjusted in such a way as to release the analyte as an ion from the electrode. Faradic current resulting from this adjustment is a direct quantification of the amount of analyte present in the sample (Saterlay, 1999). In general, stripping analysis involves analyte from a dilute solution being

concentrated into a single mercury drop by electro reduction. The electro active species is then stripped from the electrode by reversal of the scanned voltage sweep. The result is a more positive potential developing, oxidizing the species back into solution. The current recorded during this oxidation process is related to the amount of analyte initially deposited. This is the most sensitive voltammetric technique developed, as the analyte is concentrated from a dilute solution. Furthermore the longer the period of concentration, the more sensitive the analysis (Harris, 1998).

i) Cathodic Stripping Voltammetry

Analyte is concentrated into a single mercury drop by oxidation at a fixed voltage for a fixed time. The potential is then made more negative, and current is measured as analyte is spontaneously reduced (Harris, 1999). CSV is not as widely used as ASV. It can also be performed without the use of mercury, which is an advantage as no interferences occur from dissolved oxygen (Olga et al 2003). A variety of different solid electrodes have been used in the trace analysis of manganese (Labuda et al., 1989; Jin et al., 2000; Vos et al., 1986)

ii) Anodic Stripping Voltammetry

Analyte is concentrated into a single mercury drop by reduction at a fixed voltage for a fixed time. The potential is then made more positive, and current is measured as analyte is spontaneously oxidized (Harris, 1999). ASV has been the most commonly used form of electro-chemical stripping analysis for the determination of various trace metals (van den Berg, 1991; Brainina et al., 2000; Olga et al., 2003). Mn(II) has also been determined using this technique (O'Halloran, 1982; Ghoneim et al., 2000;

Locatelli et al., 2001). There are however three main problems for manganese determination with ASV:

a) Manganese has a relatively low solubility in mercury

b) The Mn(II) deposition potential by reduction is close to the reduction potential for the hydrogen ion (-1.7V vs. SCE).

c) The formation of intermetallic compounds at the mercury electrode (Brett et al., 1989; Jin et al., 2000).

iii) Adsorptive Stripping Voltammetry (Cathodic Stripping Voltammetry)

Cathodic adsorptive stripping methods are quite similar to cathodic stripping, except for the fact that in CAS, a hanging mercury drop is employed and deposition of the analyte then occurs by physical adsorption on the surface rather than by electrolytic deposition. The physical adsorption (preconcerntration step), involves a complexing ligand, which forms a stable complex with the metal species of interest and is accumulated by adsorption on the working electrode surface. The stirring rate becomes a more important factor in AdSV (good stirring results in rapid adsorption). CSV is effectively the electrochemical inverse of ASV, where the analyte of interest is accumulated as an oxidized species (rather than reduced as in anodic stripping voltammetry) on the electrode at a positive potential, and then quantified during the stripping step as the electrode potential is swept cathodically. In summary, the working electrode behaves as an anode during deposition and as a cathode during stripping (Skoog et al., 1992; Saterlay et al., 1999; Paneli et al., 1993).

The mechanism of adsorption adopted in the cathodic stripping voltammetry as described by Wang, (1982). Three main variations to the mechanism exist and are summarized below:

1) The metal-ligand reaction forming the complex in solution.

$$M^{n+} + nL = ML_n^{n+}$$

The complex that is formed is then adsorbed onto the electrode surface.

$$ML_n^{n+}\ (soln) = ML_n^{n+}\ (ads)$$

2) The adsorbed ligand is produced first, and then the complexation reaction occurs.

$$NL\ (soln) = nL(ads)$$

$$M^{n+} + nL\ (ads) = ML_n^{n+}\ (ads)$$

3) The metal cation does not form a surface active complex with the ligand, instead, a reduced or oxidized electrochemical product of the metal cation forms a complex.

$$M^{n+} \pm me = M^{n\pm m}$$

$$M^{n\pm m} + (n\pm m)\ L = M^{n\pm m}L_{(n\pm m)}$$

$$M^{n\pm m}\ L_{(n\pm m)}(soln) = M^{n\pm m}L_{(n\pm m)}(ads)$$

4) A process containing mechanisms 2 and 3 have been obtained from Neiman et al (1989).

$$NL(soln) = nL(ads)$$

$$M^{n+} \pm me = M^{n\pm m}\ (soln)$$

$$M^{n\pm m} + (n\pm m)\ L = M^{n\pm m}L_{(n\pm m)}(ads)$$

The stripping procedure occurs via three mechanisms described by van den Berg (1991) as the potential is scanned towards more negative values.

a) Reduction of the elemental species in the adsorbed complex.

$$ML_n^{+n}\ (ads) = M^{+n-1} + nL = ML^{+n-1}$$

b) Reduction of the ligand in the adsorbed complex.

$$ML_n^{+n}\ (ads) = M^{+n} + nL$$

c) Catalytic production of hydrogen

$$2H^{+}+2e^{-}=H_2$$

2.5 Spectroscopic analysis

2.5.1 Inductively Coupled Plasma - Optical Emission Spectroscopy

Compared to ordinary flames in spectroscopic methods, inductively coupled plasma attains much higher temperatures. In addition most of the new and advanced analytical techniques are also able to eliminate many of the common interferences caused by the sample or flame itself. ICP is no exception as its high temperature and stability and chemical inert argon environment eliminate much of the interferences encountered with ordinary combustion flames (Harris, 1999).

The inductively coupled plasma (argon) torch is a unique type of plasma that obtains its sustaining power by induction from a high frequency magnetic field. Initially the argon gas passes through a 25-mm quartz tube and is then enclosed by an induction coil once it arrives at the tip of the quartz tube. An alternating current flows through this coil at a frequency of approximately 30MHz and at power levels of 2kW.

The argon stream is initially seeded once it has entered the coil. This is done with free electrons from a Tesla discharge coil. These electrons interact with the magnetic field set up by the coil and gain sufficient energy to ionize argon atoms by collision

excitation. The cations and electrons generated by the initial Tesla spark are accelerated by the magnetic field in a circular flow pattern perpendicular to the stream that flows through the torch tip. The high temperature generated by the plasma also requires a second stream of gas (argon) to provide a vortex flow to cool the inner quartz walls of the torch. The excitation and emission zones of ICP are well defined and spatially separated mainly due to no electrode contact existing in the source. This results in a high signal to noise ratio of the analyte emission that in turn gives low detection limits.

When it comes to the introduction of the sample (liquid) into the plasma source, there are three main practical considerations:

1) The maximum drop size for introducing the sample into the atomizer.

2) The rate of solvent flow must fall within a specific range of values.

3) The analytical precision of an ICP system is strongly dependent on careful control of these parameters over a long period of time (Willard et al., 1988).

Sensitivity using inductively coupled plasma is enhanced by an order of magnitude through the observation of the emission along the length of the plasma as apposed to across the diameter of the plasma (Alavosus et al., 1995).

2.6 Microscopic methods

2.6.1 Scanning electron microscopy

In Scanning Electron Microscopy (SEM), Backscattered Electron (BSE) imaging is used to generate an image of the distribution of relatively high atomic number material located on or within a sample. When a primary beam electron interacts with one or more atomic nuclei which reverses its direction of travel with low energy loss (elastic scattering) and causes it to escape from the surface of the specimen it is referred to as a backscattered electron. BSE are emitted from the specimen with energies ranging from 50eV up to the energy of the interacting primary beam (Everhart et al., 1959; Richards et al., 1999). Secondary emitted electrons (SE) have less than 50eV of energy, with the majority having only around 10eV. BSE travel in straight lines at high velocities. Therefore, the majority of these electrons are not attracted to the positive charge on the conventional Everhart Thornley SE detector. However, some do travel in that direction and are detected, providing a BSE derived signal within a 'SE' image (Everhart et al., 1959). The amount of BSE produced during the inter-action of the primary beam with the sample increases proportionally to the average atomic number (Z) of the specimen (Palluel, 1947).

CHAPTER 3

3.1 Aims and objectives for the study

For many years minerals have formed the backbone of the South African economy. In particular, South Africa is the world's leading producer of precious metals (platinum-group metals and gold) and important steel producing metals such as vanadium and chromium, and is ranked as one of the top producers of aluminium, iron ore, manganese, titanium and zirconium (Viljoen et al., 1999). This is shown in table 1 below:

Table 1 South Africa's ranking in the world as a supplier of some major mineral commodities (Viljoen et al., 1999).

	Reserves		**Production**	
	%	**Rank**	**%**	**Rank**
Gold	39.1	1	19.9	1
PGMs	55.7	1	47.1	1
Chromite	68.3	1	45.6	1
Manganese ore	80.0	1	14.9	3
Titanium	30.5	1	24.1	1
Vanadium	44.8	1	56.9	1

In light of the above statistics, the mining and minerals industries contribute significantly to employment in South Africa, with almost a third of the population depending on mining directly or indirectly (Viljoen et al., 1999). The concern for the health of workers in this sector is justified.

The aim of this work was to develop new and reliable techniques simple to implement and use for the enrichment and extraction of the trace metal, manganese (Mn(II)) in blood serum.

A Supported Liquid Membrane technique was used and optimized for the extraction of Mn(II) from blood serum. An Adsorptive Stripping Voltammetric method for the determination of Mn(II) was used and also optimized.

Aims:

- Optimize the SLM (flat spiral disk) extraction method in order to extract and preconcentrate Mn(II) from blood serum.
- Compare the effect of different sample matrices (water, milk and blood serum) have on free Mn(II).
- Develop and optimize a SLM probe micro extraction method.
- Optimize an Adsorptive Stripping Voltammetric method (Wang et al., 1993) for the trace determination of Mn(II) in blood serum.
- Apply the optimized methods of extraction and determination to investigate the binding and biological uptake of Mn(II) in blood serum (aged blood serum and blood serum fractions).

Experimental

CHAPTER 4

Extraction methods

4.1 Nature of samples

All samples, except the unfractionated blood serum, were obtained and prepared individually. The blood serum (approximately 40ml in total) was obtained from the National Center of Occupational Health, while all long life fat free clover cow milk was purchased from a local supermarket. All samples analyzed were prepared and stored according to established analytical protocols.

4.2 Supported Liquid Membrane extraction (flat spiral disk)

4.2.1 Instrumentation

A membrane unit which was designed and obtained from the Department of Analytical Chemistry, Lund, Sweden consisting of two circular polytetrafluoroethylene (PTFE) blocks (diameter: 120mm, thickness: 15mm), with machined grooves in the shape of an Archimedes spiral (depth: 0.25mm, width: 1.5mm, length: 250cm and total volume of approximately 0.95mL). A porous PTFE membrane (containing 115μm polyethylene backing) having pore size of 0.2μm and porosity of 0.70 obtained from Millipore (Millipore, Bedford, MA, USA) was used as the membrane support for the organic phase (extractant). The Cole-Parmer Instrument Company manufactured the peristaltic pump and associated acid-resistant tubing used. pH measurements were recorded using a Beckman 50 pH meter with a Metrohm glass electrode.

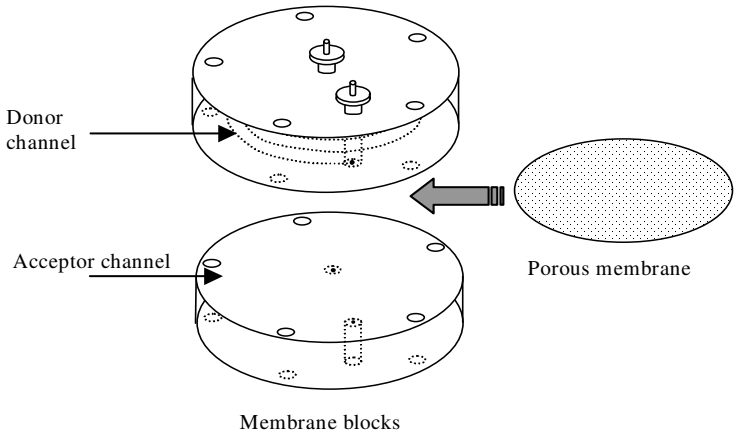

Figure 8 Schematic representation of flat spiral SLM

4.2.2 Reagents

All apparatus were cleaned extensively and throughout the procedure that follows, analytical grade quality reagents and Millipore water were used. The general extraction procedure required the following: 0.2mol dm^{-3} HNO_3 obtained from analytical grade 55% Nitric acid from Saarchem Pty Ltd. (Johannesburg, South Africa) and 0.1mol dm^{-3} NaOH solutions for all pH adjustments. Membrane liquid consisting of 15% di-2-ethylhexyl phosphoric acid (DEHPA) (v/v) and 10% Tri-n-octylphosphine oxide (TOPO)(w/v) dissolved in kerosene from Sigma-Aldrich (Steinheim, Germany).

A 1mol dm^{-3} HNO_3 solution for washing membrane unit and a 1000mg l^{-1} Mn(II) stock solution was prepared using the required weight of $Mn(NO_3)_2 \cdot 6H_2O$ every 2-3 weeks.

4.2.3 SLM extraction procedure

The PTFE membrane pores were saturated with organic phase by replacing the membrane for approximately 15minutes in a beaker containing 15% DEHPA (v/v) in a kerosene solution. This was repeated for both sides of the membrane. The beaker was then covered with a watch glass to prevent evaporation and any organic phase from escaping or being contaminated. The membrane was thereafter placed between the spiral circular PTFE blocks, and the complete membrane unit subsequently secured. From the manufactures instructions and outlines obtained in the literature, the pumping system was connected (Djane, 1997; Djane et al., 1997b; Papantoni et al., 1995). A 1mol dm^{-3} HNO_3 solution was pumped for 15 minutes at a flow rate of 1mL/minute washing out any excess of the organic solution from the two channels of the membrane unit. There after, the system was used to extract manganese from known diluted water, milk and unfractionated blood serum samples (spiked with Mn (II)) and also none spiked samples in the case of milk. The acceptor phase employed in all cases was a 0.2mol dm^{-3} HNO_3 aqueous solution while the diluted sample constituted the donor phase on each separate occasion. The donor phase pH was adjusted to 3.0, thus setting up a proton gradient. The specific parameters such as volume of sample extracted, extraction time, flow rate, Mn concentration spiked, etc are mentioned in the results and discussion section. The non-spiked or blank samples were run at an early stage of the sample extraction to prevent any residual contamination from the membrane unit and supporting system.

4.3 Supported Liquid Membrane probe (SLM probe)

4.3.1 Instrumentation and construction

Instrumentation used for the membrane probe was different to the flat spiral disk SLM method initially used. The probe was made from a polypropylene tube with dimensions; 13mm i.d, 16mm o.d and 92mm length. One end of this tube was closed with a thin porous PTFE membrane filter and sealed with PTFE tape (As shown in figure 9). A porous PTFE membrane with 115um polyethylene backing, pore size of 0.2µm, total thickness of 175µm and porosity of 0.70 was obtained from Millipore FG, Bedford, MA, USA. The electrochemical equipment used to analyze the extracts obtained was the same as that used for the conventional SLM extraction method.

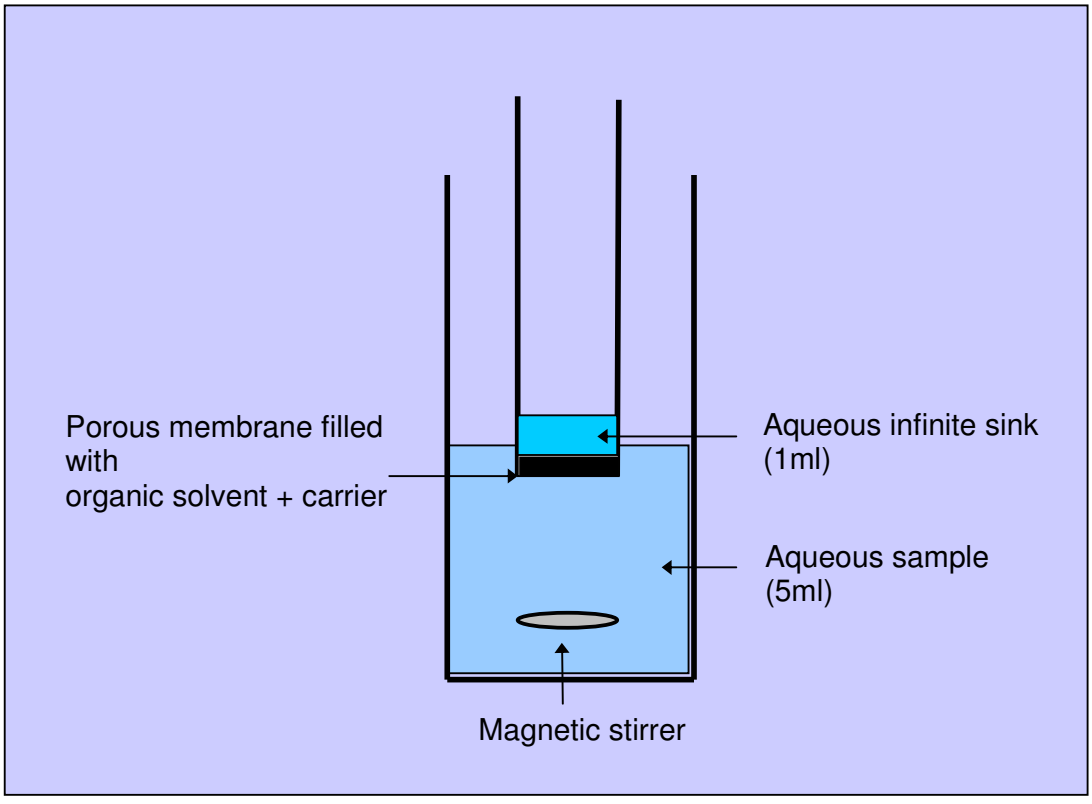

Figure 9 SLM probe

Membrane probe and micro- extraction

The principle of the membrane probe is based on that of the supported liquid membrane technique employing a stagnant acceptor phase. Unlike the previous SLM configuration used (as shown in figure 8), extraction is carried out using significantly smaller volumes of both the acceptor and donor phases. The principle mechanism of extraction is identical to the mechanism used in the flat spiral disk SLM extraction method described above.

The major factors that influence the extraction efficiency is the phase composition of the two aqueous phases. These factors include: Composition of the organic solvent impregnated into the membrane and the donor flow rate. The donor solution is stirred in the membrane probe technique; hence the significance of the stirring rate plays a major role as a transport parameter. This is in contrast to the donor flow rate of the conventional SLM set up transport mechanism to the membrane surface. An additional factor is probe depth in the donor solution. This plays a significant role as it affects the mass transfer from the bulk donor phase to the membrane surface.

In flat spiral disk SLM, longer extraction times and high sample flow rates obtain high enrichment factors. The disadvantage to this approach is that it demands large sample volumes with dilutions often being required in order to achieve these larger volumes. This can pose as a significant problem when the analyte of interest is found in extremely low concentrations in the samples. This in turn adds additional requirements to the extraction method such as the need for even higher extraction efficiencies in order to compensate for the dilution. In the membrane probe extraction method, stirring rate and time are the main parameters controlling high enrichment

factors. Another advantage of the membrane probe is that only small samples are required therefore making miniaturization of a method using this configuration easier.

4.3.2 Reagents

All apparatus were cleaned extensively and throughout the procedure that follows, analytical grade quality reagents and Millipore water were used. The general extraction procedure required the following: 0.2mol dm^{-3} HNO_3 obtained from analytical grade 55% Nitric acid from Saarchem Pty Ltd. (Johannesburg, South Africa) and 0.1mol dm^{-3} NaOH solutions for all pH adjustments.

Membrane liquid consisting of 15% di-2-ethylhexyl phosphoric acid (DEHPA)(v/v) and 10% Tri-n-octylphosphine oxide (TOPO)(w/v) dissolved in kerosene from Sigma-Aldrich (Steinheim, Germany).

1mol dm^{-3} HNO_3 solution for washing membrane unit.

1000mgl^{-1} Mn(II) stock solution was prepared using the required weight of $Mn(NO_3)_2 \cdot 6H_2O$ every 2-3 weeks.

4.4 Optimization of SLM (flat spiral disk) extraction of Mn(II)

There are a number of SLM experimental set-ups currently adopted, however the membrane set-up used in this research consisted of a porous polytetrafluoroethylene (PTFE) membrane, separating two aqueous solutions. The membrane was impregnated with an organic solvent and mounted between two flat blocks made of PTFE. In these blocks, corresponding spiral grooves are machined forming a flow channel on each side of the membrane.

4.4.1 Optimization in Water

Deionized water was spiked with 40µg l^{-1} Mn(II) (donor solution). An average extraction efficiency of 68% was achieved for the deionised water as shown in table 2 below.

Table 2 Average extraction efficiencies for three different samples types

Sample	**Average extraction efficiency/%**	**Mn^{2+} Spiked concentration / µg l^{-1}**
Water	68.0	40
Milk	5.05	40
Blood serum	20.5	40

The results in table 2 above show how different sample matrix affects the extraction efficiency. It can therefore be seen that blood serum has a far less complexed matrix when compared to milk.

4.5 Optimization of SLM extraction conditions for different samples matrices

4.5.1 Donor volume

The donor volume and its effect on extraction efficiency was investigated using fresh fat free milk with only minor changes being done to the experimental conditions such as increasing the amount of donor solution collected. This was performed on the fresh milk sample spiked to contain 10µg l^{-1}.

Table 3 Relationship between extraction efficiency and extracted donor volume

Milk extract # (spiked with 10µg l^{-1} Mn(II))	Average extraction efficiency (%)	Extracted donor volume /ml
1	5.9	150
2	3.3	150
3	8.5	200

The data given above also shows that the extraction efficiency was independent of the donor volume extracted. Slight variations were observed in the results as shown by the standard deviation of 4.531.

4.5.2 Carrier % (v/v)

In most extraction techniques, carrier (mostly organic) molecules play a vital role in the transport of the desired metal ion across the membrane surface. This experiment was no different. However, having identified the importance of a carrier, optimization of the % carrier becomes important.

This was carried out simply by holding all other parameters constant that influence extraction efficiency and change the % carrier concentration.

Table 4 Average extraction efficiency variations with % carrier concentration (extraction of fresh milk spiked with 100µg l^{-1})

% Carrier (DEHPA: Kerosene v/v)	**Average extraction efficiency (%)**	**RSD(%)**
5	2.45	5.88
15	3.60	1.94
40	2.84	6.22

Conditions (flat spiral SLM):

Dilution factor = 30

Flow rate = 1ml.min^{-1}

Extraction time = 30min

There was an increase in the extraction efficiency with an increase in the carrier (extractant) concentration from 5 to 15%, after which there is a drop at an extractant concentration of 40%. The decrease could be as a result of the formation of higher order ion association complexes that according to Djane et al., (1997b) slow down the diffusive mass transfer process. Another reason could be the increased viscosity of the

liquid inside the membrane leading to lower diffusion coefficients. From the above extractant concentrations tested, it was decided that an extractant of 15% (v/v) would be used for further extractions in order to achieve optimal results (Djane et al., 1997b)

4.5.3 Sample dilution

By diluting a sample prior to extraction, the matrix effect is reduced. This can have many implications both advantageously and disadvantageously. Dilution can cause not only a decrease in the matrix of a particular sample, but can also decrease the detection limit at which the method can operate thereby reducing the reproducibility of the analysis.

4.6 Samples extracted

4.6.1 Optimization in milk

16.00ml milk was diluted with deionised water to make up a 500ml solution (1:30 dilution). Extraction was repeated three times for each spiked solution prepared. Each solution was spiked to contain 10, 40 and 100µg l^{-1} respectively. The pH was adjusted to 3.00 as described in chapter three. Each of the above solutions then constituted the donor phase. The SLM extraction was carried out for 100 minutes (sample flow rate: 1.0 ml/min), thereby giving the volume of 100ml for the donor phase extracted. The acceptor phase was as described in chapter three. 2ml of the extract was collected in the acceptor phase.

Varying the spiked concentration in the diluted sample before extraction had a minimal influence on the extraction efficiency of the SLM technique. This can be

observed in the data pertaining to the extraction of Mn(II) in fat free fresh milk given below.

Table 5 SLM (flat spiral disk) extraction of different spiked Mn(II) in fresh fat free milk

Spiked Mn(II) concentration /µg l^{-1}	**Average extraction efficiency /%**	**RSD%**
10	5.26	18.0
40	5.05	29.4
100	3.65	1.94

The trend followed above indicates that at lower spiked concentrations, better extraction efficiencies are obtained. However the total spiked concentration for any given sample is expected to yield the same results in terms of the extraction efficiency. Although the above extraction efficiencies also vary (For higher concentrations extracted, linearity is lost), the RSD values show relatively good precision. The extraction efficiencies obtained show relatively good stability in a wide range of concentrations (10-100µg l^{-1}). This shows that the SLM technique is reproducible for the extraction of Mn(II) in milk.

4.6.2 Optimization in aged milk (48 hours)

16 ml of fat free milk solution was prepared using the same method as the first sample of fresh fat free milk. The two sample solutions were spiked with 40µg l^{-1} and 100µg l^{-1} concentrations of manganese(II) respectively and their pH adjusted to 3.00.

Results obtained reflect a similar trend to that of the fresh milk. For a Mn(II) spiked concentration of 40µg l^{-1} an extraction efficiency of 6.02 % was obtained were as for the 100µg l^{-1} the extraction efficiency was much lower at 1.68 %.

The average extraction efficiencies obtained show a similar trend to that of the fresh milk extraction efficiency were for higher concentrations extracted, linearity is lost.

Aging conditions for milk

During sample preparation and extraction the temperature of the surrounding was 25^{o}C and kept constant. However it was observed that during the sample dilution and pH adjustment stage for the aged 48-hour and fresh fat free milk samples, the addition of the 1mol dm^{-3} HNO_3 that is used to achieve a pH of 3, catalyzed the precipitation of albumin (water soluble protein) and other proteins present in milk (Pavia et al., 2000)**.** It is not known whether this precipitation has any effect on the spiked concentration of Mn(II) in the sample. However it suspected that during precipitation, a fraction of the labile complexed Mn(II) co precipitates, resulting in decreased concentrations of labile Mn(II). When the 48-hour aged milk sample was stored in a refrigerator at 4^{o}C, it was possible that the co precipitated Mn(II) cations dissolved back into solution due to equilibrium being established. For the fresh milk sample that was spiked and not stored in the refrigerator prior to extraction, lower extraction efficiency resulted. This could be a possible explanation as to why the 48-hour aged milk achieved higher extraction efficiency than its counterpart fresh milk.

Table 6 Average extraction efficiencies of fresh and 48 hour aged milk

Spiked Mn(II) concentration/μg l^{-1}	**48 hour aged milk average extraction efficiency %**	**Fresh milk average extraction efficiency %**
40	1.7	5.1
100	3.6	6.0

The aged milk has much lower average extraction efficiencies for both spiked Mn(II) concentrations compared to fresh milk samples. This decrease in extraction efficiency with time could be due to the quenching of free Mn(II) in the sample by the fat proteins that have more time to bind to the Mn(II).

4.6.3 Blood serum

8ml whole blood serum was diluted with milli pore deionised water to make up a 240ml solution (1:30 dilution). The pH of the solution was adjusted to 3.00 and the solution was spiked with 40μg l^{-1} Mn(II). This constituted the donor phase. SLM extraction was then performed for 30 minutes (sample flow rate: 1.0ml / min); hence the volume of the donor phase extracted was 30ml. As before 2ml was then collected forming the acceptor phase. A total of three extractions were performed for statistical validation.

Table 7 Average extraction efficiencies for whole blood serum diluted 30x

Blood serum extract (spiked with 40µg l^{-1} Mn(II))	**Average extraction efficiency (%)**
1	10.4
2	18.3
3	8.30

The unfractionated whole blood serum results shown in table 7 tend to show some differences. In any reproducible SLM extraction process performed, similar extraction efficiencies should be achieved relative to each extract collected providing certain important parameters such as pH of the donor and acceptor phases, donor flow rate and mass transfer kinetics in the donor solution is kept constant (Norberg, 2000). It has also been noted from previous research that often, the optimum extraction efficiency is achieved from the second extract collected, thereafter it decreases using the same membrane. This is due to the extractant or carrier (DEHPA) achieving optimal functionality on the 2nd extraction. There after the membrane can either becomes damaged or the pores could block and loose their selective capability for effective separation. This lose in efficiency through membrane alterations is commonly observed in the extraction of biological samples that have complexed matrix components such as fatty acids and convoluted protein molecules. These complex matrices interfere and bind to the membrane pores. An average extraction efficiency range of 8.3 – 18.3 was obtained.

4.6.4 Extraction efficiency of Mn(II) at different concentrations spiked in blood serum

Table 8 Extraction of Mn(II) from blood serum at different spiked concentrations using a SLM flat spiral unit (Soko et al., 2003)

Sample condition	Concentration of Mn(II) spiked (µg l^{-1})	Organic carrier composition	Concentration of Mn(II) extracted (µg l^{-1})	Average extraction efficiency (%)	RSD (%)	Total concentration of Mn (ICP-AES) (µg l^{-1})
Fresh	5	A	ND	-	-	5.0
Fresh	10	A	31.9	35.4	14.8	10.0
Fresh	25	A	88.0	39.1	17.3	25.0
Fresh	40	A	147.6	41.0	14.8	39.8
Fresh	5	B	14.0	25.5	17.4	4.9
Aged	10	B	36.5	40.6	19.6	9.9
Aged	30	B	129.9	48.1	11.2	30.1
Aged	50	B	216.9	45.7	12.3	49.9

Conditions: flow rate = 0.3ml min^{-1}, extraction time = 60min, dilution factor = 20, A = 15% DEHPA in kerosene, B = 10% TOPO in 15% DEHPA/kerosene.

The table above shows blood samples which were diluted 20 times and spiked with different Mn(II) concentrations and categorized according to samples extracted immediately and samples left for ageing for 24 hours (refrigerated at 4^{o}C). The extraction efficiencies achieved were much higher than for milk**.** A possible reason

for this could be attributed to the fact that milk contains a greater concentration of fat thus quenching free Mn(II) from solution.

Using two different organic carrier % compositions different extraction efficiencies were obtained. For the lowest concentration of Mn(II) spiked, 5μg l^{-1} was not detected after using 15% (v/v) DEHPA in kerosene (type A). However the addition of 10% TOPO (type B) increases the membranes polarity and improved the extraction efficiency. Thus the aged blood serum samples were extracted using membrane solution type B. This resulted in slightly higher extraction efficiencies with improved reproducibility.

4.6.5 Extraction and comparison of different sample matrices (milk vs. blood)

A mean extraction efficiency (for both samples spiked to contain 40μg l^{-1}) of 9.4 % (RSD% = 16.5) was obtained for the blood serum samples while the milk achieved 5.05 % (RSD% = 29.4).

The extraction efficiency for blood serum is larger than that for the fresh milk. There are numerous reasons for this difference in extraction efficiencies of which the most important being the effect of the sample matrix. The matrix of blood serum is less complexed and contains fewer components such as fatty acids and other biological lipids that contribute to the overall matrix effect. This results in far higher extraction efficiencies being achieved mainly due to the membrane pores remaining intact (also evident from micrographs in Figure 10a and b). The membrane pores are however blocked by the matrix of milk (Figure 10b) thus accounting for it's low extraction efficiency.

4.7. Scanning Electron Microscopy

i) Instrumentation

A scanning electron microscope (JOEL, JSM-840, Tokyo) was used for the study of matrix effects by analyzing membrane filters exposed to water, milk and blood serum.

ii) Procedure

The main aim of using a scanning electron microscope was to qualitatively investigate the effect different types of matrix (water, milk and blood serum) have on the membrane pores and structure. After each different extraction the membrane was carefully removed from it's mounting and dried at room temperature. The center region of the membrane filters which were the most heavily exposed to the sample were then analyzed with a JSM-840 scanning electronic microscope instrument manufactured by JOEL (Tokyo, Japan).

4.7.1 Matrix effect in SLM analysis

As observed for the fresh milk and blood serum extraction efficiencies, matrix plays an important role in membrane performance. In order to support the above experimental evidence a closer look was taken into the membrane structure preceding the extraction of Mn (II) in water, milk and blood serum.

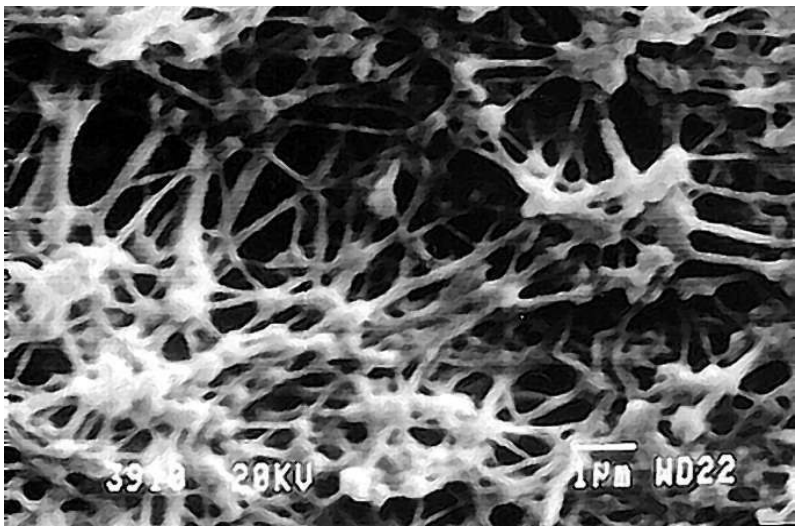

Figure 10a Micrograph of a SLM (flat spiral disk) used in the extraction of Mn(II) in water taken using a Joel Jsm-840 Scanning electron microscope. Magnification: x 6500, Peak Current: 6E-10 mA, Accelerator Voltage: 20 kV

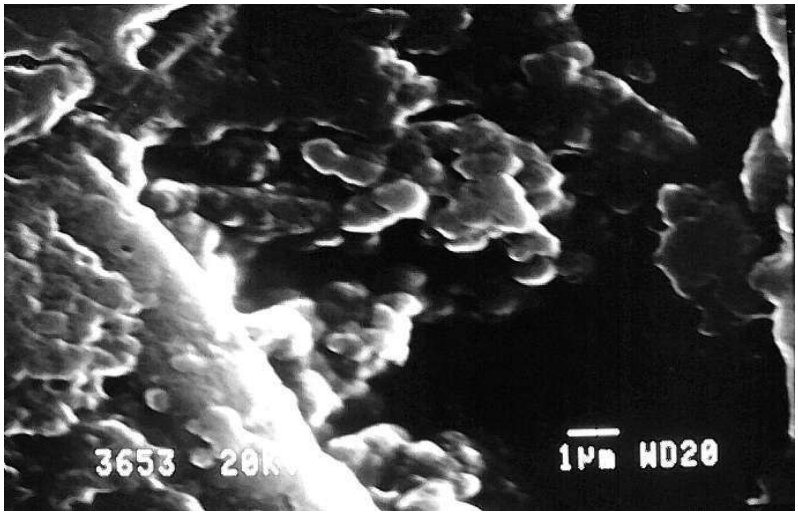

Figure 10b Micrograph of a SLM (flat spiral disk) used in the extraction of Mn(II) in milk taken using a Joel Jsm-840 Scanning electron microscope. Magnification: x 7000, Peak Current: 3E-10 mA, Accelerator Voltage: 20 kV

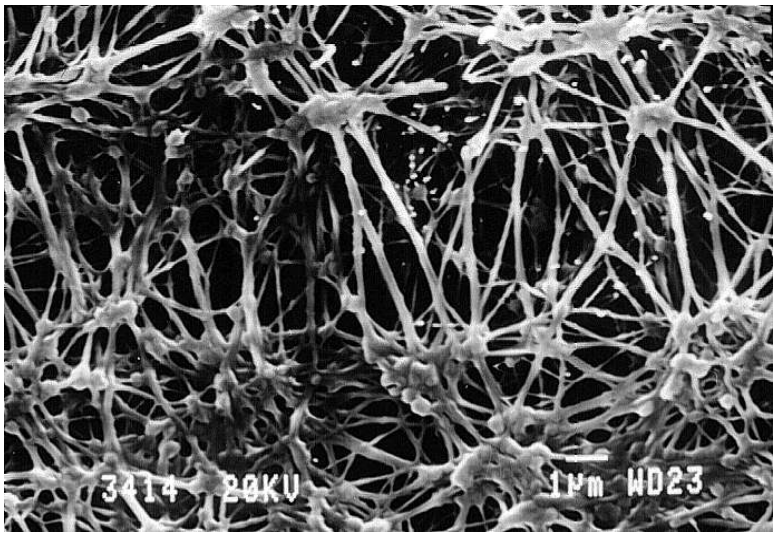

Figure 10c Micrograph of a SLM (flat spiral disk) used in the extraction of Mn(II) in whole blood serum taken using a Joel Jsm-840 Scanning electron microscope. Magnification: x 7000, Peak Current: 3E-10 mA, Accelerator Voltage: 20 kV.

After the extraction of water, milk and blood serum samples membrane filters were examined using a scanning electron microscope. From the images it is clear that the sample matrix that was deposited the most was that of milk. The blocking of the membrane pores resulted in a dramatic drop of extraction efficiencies compared with water and blood serum samples. However it is visible from the scanning electron microscope images that blood serum did deposit some organic matter (not to the extent of milk).

4.8 SLM probe

4.8.1 Development and construction

The principle of the membrane probe is based on that of the supported liquid membrane technique employing a stagnant acceptor phase. Unlike the SLM (flat

spiral disk) configuration previously used (as shown in figure 8), extraction is carried out using significantly smaller volumes One of the major difficulties for the use of SLM technologies on an industrial scale is the relatively short lifetime the membrane has due to the loss of the organic solvent from the substrates. As a result there has been much emphasis focused on better and new ways of improving the lifespan of these membranes (Kemperman et al., 1996) for both the acceptor and donor phases. The principle mechanism of extraction is identical to the mechanism used in the flat spiral SLM extraction method described above.

The major factors that influence the extraction efficiency is the phase composition of the two aqueous phases. These factors include: Composition of the organic solvent impregnated into the membrane and the donor flow rate. The donor solution is stirred in the membrane probe technique; hence the significance of the stirring rate plays a major role as a transport parameter. This is in contrast to the donor flow rate of the conventional flat spiral SLM set up transport mechanism to the membrane surface. An additional factor is probe depth in the donor solution. This plays a significant role as it affects the mass transfer from the bulk donor phase to the membrane surface.

There are a number of SLM set-ups currently adopted, however the membrane set-up used in this research consists of a porous polytetrafluoroethylene (PTFE) membrane, separating two aqueous solutions. The membrane is impregnated with an organic solvent and mounted between two flat blocks made of PTFE. In these blocks, corresponding spiral grooves are machined forming a flow channel on each side of the membrane.

Optimisation of Probe depth

All conditions and parameters for the following can be found in previous chapters except for the probe depth that was varied accordingly in order to determine its optimum depth. A polypropylene tube fixed to a thin porous PTFE membrane (soaked in 15% (w/v) DEHPA) was attached, and positioned at different levels inside the donor solution. This was done in order to try and determine an optimum probe depth in the sample.

Table 9 Average extraction efficiency vs. membrane probe depth

Average extraction efficiency %	Probe depth / mm
50	Less than 1
96	2

The average extraction efficiency improved greatly as the membrane probe was positioned deeper into the donor solution. This could perhaps be justified by the fact that as the probe gets closer to the stirrer and the center of the solution; the membrane is kept in contact with the analyte for a longer time thus minimizing transport as a limiting factor. The stirring produces a constant flow rate of fresh unextracted solution thus allowing more analyte to be extracted.

4.9 SLM spiral disk vs. SLM probe

The conditions used for the membrane probe, were used as previously described where the sample was spiked to contain 40µg l^{-1} Mn(II) (donor phase) 5ml of which was placed into a glass vial and stirred continuously during extraction (shown in figure 9). The acceptor solution consisted of 0.2mol dm^{-3} HNO_3. An extraction time

of 60 minutes was employed. For the flat spiral SLM procedure a flow rate of 1.00mL min^{-1} was used.

Table 10 Comparison of two different SLM extraction methods

	SLM (flat spiral disk method) Average extraction efficiency %	**SLM (membrane probe) Average extraction efficiency %**
Water	68.0	92.0
Whole blood serum	9.4	43.0

In table 10 above it can be observed that the average extraction efficiencies for water and whole blood serum are higher for the SLM membrane probe method as compared to the flat spiral disk SLM method. There is also a noticeable decrease of the extraction efficiencies in each sample type for both methods. This difference is due to the different complexities of each sample's matrix with whole blood serum having the most complex matrix (Vrouwe et al., 2004; Magner, 1998; Clark et al., 1962) while water's is almost negligible.

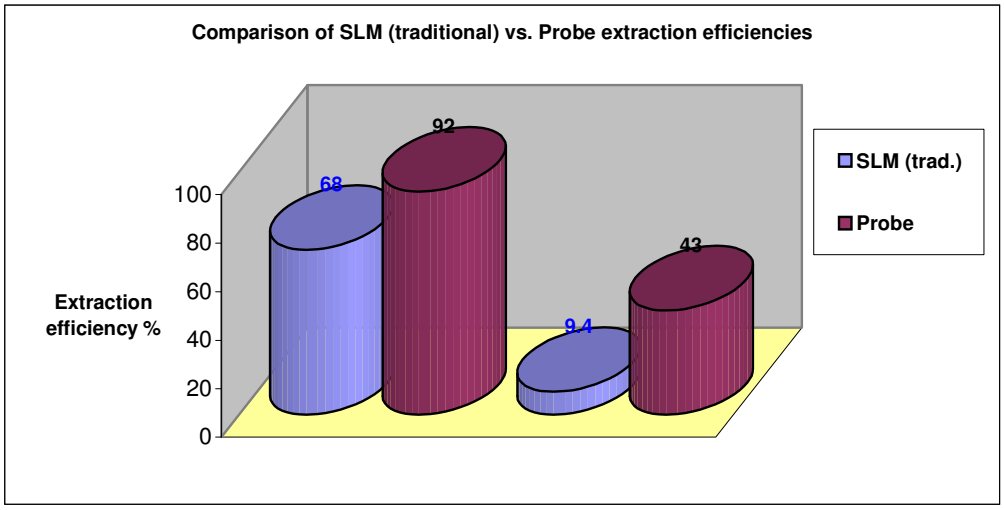

Figure 11 Membrane probe vs. flat spiral disk SLM average extraction efficiencies for whole blood, serum and water comparison

It was discovered when examining the finer details of the SLM results and investigating new improved methods to optimize the extraction efficiency of Mn(II). One way of achieving this was found by decreasing the sample (donor) size through modifications such as those apparent in the membrane probe set-up and procedure. This improved the extraction efficiencies of Mn(II) in water and blood serum two-fold. Compared to the traditional SLM configurations, this has many advantages. It is the simplest configuration to be reported thus far, inexpensive since no pumps are required, possible to perform many probe extractions simultaneously, an important aspect in routine analysis and drug screening processes. Since the sample is stirred, it allows for the same sample to be extracted many times thus giving higher extraction efficiencies. This means that only small sample volumes are required as extraction occurs from the bottom of the probe (shown in figure 9). Depending on the sample matrix, the SLM probe can be used repeatedly. Furthermore regeneration of the probe itself is easy and more cost effective.

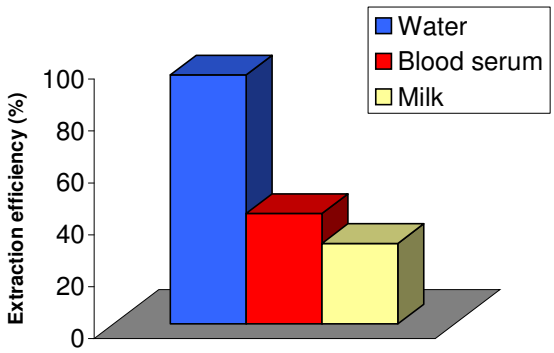

Figure 12 Influence of sample matrix on the average extraction efficiency (%) for SLM probe. Conditions: Extraction time = 60 minutes.

CHAPTER 5

Determination techniques for Mn(II) in different matrices

5.1 Initial manganese determination method by AdSV

Analysis and interpretation of results

The technique of standard addition has been utilized as well as data correlation and comparative analysis of the output data obtained. This form of data analysis was then used to compare and evaluate certain voltammetric and extraction parameters. There were two main areas of emphasis; the first on the optimization of extraction parameters using SLM and membrane probe extraction methods and the second was the further optimization of the electro analytical technique (Adsorptive Stripping Voltammetry) for the trace and ultra trace analysis of Mn(II) in biological fluids especially blood serum. These optimized parameters would then later be used in the analysis of aged spiked blood serum and finally fractionated blood serum. Both the voltammetric analysis and sample preparation via preconcerntration using SLM were collaborately used in the determination of labile complexed manganese(II) present in each sample analysed. The optimization of the extraction technique was primarily determined by a graphical technique that allowed for the concentration of the species being analysed to be quantified. This enabled for the extraction efficiency to be calculated as shown in chapter two.

For each sample, data points plotted were obtained from voltammetric analysis using a standard addition method. These were fitted linearly and extrapolated to the X-axis (which satisfied the condition y = 0). This value yielded the concentration of Mn(II) in the cell. Depending on the reversibility characteristics of the voltammograms

obtained either the peak area or peak height was plotted as a function of concentration in the voltammetric cell. Initially for the first part of the project (Water, milk and blood serum) the peak area was used, however once the electro analytical technique was further optimized, peak height was then used for improved accuracy. The product of the concentration value as determined by extrapolation to the X-axis and the dilution factor of the sample was calculated, giving the concentration of Mn(II) in the sample. A considerable amount of care was taken in this part of the voltammetric analysis as any analysis carried out beyond the region of linearity, fits a second order polynomial. This occurs because at higher manganese(II) concentrations (also for other metals like lead and copper) the electrode surface becomes saturated and is covered by a secondary layer of adsorbed Mn(II) complex causing a gradual decline in the increase of the peak area and peak height (Fischer et al., 2001; Li et al., 2004). Due to reproducibility and the ability to compensate for fairly complex matrix effects, the standard addition technique can be regarded as one of the best methods for sample analysis (Harris et al., 1999).

5.1.1 Instrumentation

A Metrohm 663 VA stand, connected to an Autolab potentiostat/galvanostat (PGSTAT 12) integrator through an Eco Chemie IME 663 interface, was used in performing all adsorptive stripping voltammetry measurements. The signal was amplified using a PGSTAT 12 differential electrometer amplifier. The pH was measured using a Beckman 50 pH meter while the cell was kept at 25^{o}C from a Labcon low temperature water bath by circulating water around the cell. The reference electrode was a silver/silver chloride (Metrohm) electrode and the auxiliary electrode was a platinum (Metrohm) electrode. The above electrodes and pH electrode

was placed in a glass encased jacketed layered vessel and all subsequent measurements were performed within. All dissolved oxygen was removed from solution using high purity nitrogen gas.

5.1.2 Reagents

All chemicals used were of analytical grade. All water used was of ultra pure quality and obtained from a milli-Q (Millipore, Bedford, Ma, USA) water purification system. Glassware and relevant apparatus was extensively cleaned by washing with a common brand name detergent and then soaking in 2mol dm^{-3} nitric acid for at least a 12-hour period. There after Millipore water was used in rinsing just prior to analysis.

1000mg l^{-1} Mn (II) stock solution was prepared using the required weight of $Mn(NO_3)_2 \cdot 6H_2O$ (Every 2-3 weeks).

0.1mol dm^{-3} borax stock solution was prepared daily

5% (w/v) ascorbic acid stock solution was prepared daily

0.2% (w/v) gelatin stock solution prepared daily.

40% NaOH (w/v) solution for pH adjustment.

All samples where necessary, were stored temporarily in a refrigerator at a temperature of 3-4^{o}C.

Supporting electrolyte

Initially all analysis performed using AdSV was carried out using a three component supporting electrolyte, comprising of gelatin, borax and ascorbic acid present in solution. Borax acts as a buffer maintaining the optimal pH conditions required. The gelatin and ascorbic acid forms a ternary complex of Mn / ascorbic acid / gelatin as reported by Wang et al (1993). This ternary complex serves as a reinforcement

mechanism not fully understood at this stage, for the adsorption process. It was later determined through different optimization and correlation experiments that the gelatin can interact with the blood serum matrix. Gelatinases found in blood serum have an extra domain that contributes to their high affinity for gelatin. The domain is located next to the catalytic domain, and it is a 58-amino acid sequence that is repeated three times (Overall, 1991). This sequence is identical to the extra cellular matrix (ECM) fibronectin type II motif, which gives the gelatinases their high affinity for binding to and degrading gelatin-denatured collagen a chains (Overall, 1991). It was also shown by Basset et al., (1997) and Murphy et al (1997) that certain matrix metalloproteinases (MMP) present in blood serum namely MMP-9 (gelatinase-B) and MMP-2 (gelatinase-A) can degradate gelatin (Basset et al., 1997; Murphy et al., 1997) .

It is thought this interaction could perhaps prevent optimal adsorption of the metal ligand complex. It was thus only determined advantageous in leaving the gelatin out of the analysis solution. For this reason gelatin was only used in all initial experiments for SLM optimization investigations in different sample matrix (water, milk and some pilot whole blood serum samples). As reported by Wang et al., (1993), 0.03% (w/v) ascorbic acid is employed as the optimum ligand concentration.

5.1.3 Procedure

Prior to commencement of all measurements, and between solutions, 1mol dm^{-3} HNO_3 was used to rinse the cell (glass) and electrodes followed by rinsing with Millipore deionised water to prevent residual contamination. The pH meter was standardized using pH = 4.0±0.02 (25^oC) and a pH = 7.0±0.05 (25^oC) phosphate buffer solutions.

The Luggin capillary of the reference electrode was filled with 0.5mol dm^{-3} $NaNO_3$ solution. A total volume of 10ml was analyzed for each sample measured that was made up of the sample extract itself and the relevant amount of supporting electrolytes used. Before the sample was run, a precautionary blank run was carried out which contained only the supporting electrolyte made up to the required 10ml cell volume (or 5ml cell volume for membrane probe extracts). The extract composition of the 10ml and 5ml (Volume of supporting electrolyte: Volume of sample) was not altered drastically except in some milk and blood serum samples where the volume of ascorbic acid was tripled to that given below. Typically the final concentration in the 5ml and 10ml cell would be: 0.005% borax, 0.03% ascorbic acid and 0.001% gelatin (all w/v), as reported by Wang et al., (1993). Wherever the composition of each solution analyzed was altered, mention is made in the results and discussion section.

When required, the pH of the mixture in the cell was adjusted to fall within the optimised range of 7.2-7.6. This adjustment was achieved using 40% (w/v) NaOH.

Voltammetric determination of the solution under investigation was then carried out using the optimised parameters already determined by Wang et al., (1993) for the determination of Mn(II) in water. These same parameters were used in the determination of Mn(II) in long life fat free milk and unfractionated blood serum samples.

5.2 Spectroscopic determination of total manganese (ICP-OES)

5.2.1 Instrumentation

An Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES Ciros) with charged-coupled detection device obtained from Spectro Analytical Instruments

Inc. (Kleve, Germany). The system was controlled with Smart Analyzer Windows software. Purging of the system was carried out using pure Argon gas.

Optimized instrumental parameters:

Plasma power: 1200W

Coolant gas flow rate: 12.01min^{-1}

Nebulizer flow rate: 1.0ml min^{-1} at wavelength of 257.610nm.

Auxiliary flow rate 1.01min^{-1}

5.2.2 Reagents

Multi-element solutions standards (Spectro-chemically pure) were used to make the calibration standard concentration range used.

5.2.3 Procedure

Standards were made using ultra pure milli pore water. Standards made: 0.05mg l^{-1}, 0.1mg l^{-1}, 0.2mg l^{-1}, 0.5mg l^{-1} and 1.0mg l^{-1}. Samples analyzed were diluted 10, 25 and 50 times depending on the concentration initially determined in the first scan.

5.3 Initial parameters used in SLM optimisation

As reported by Wang et al (1993), a pH range of 8-10 showed extremely high stability with respect to the peak current and due to the initial study not involving optimization of the analysis technique (this range was applied). It has also been determined in previous studies at a fixed deposition potential of –1.3V the optimum range under the same local conditions used in this study was found to be pH = 8-9. These results show that the pH is a reproducible parameter, thus was not initially optimized and used in the determination of Mn(II) in samples after SLM optimization.

5.3.1 Sample matrix effects

The ability to compensate for complex matrix effects illustrates that the standard addition procedure is an acceptable and reliable method for the analysis of biological samples, which often have relatively complex matrix consisting of different proteins and fatty acids. It was found that the complexity of this matrix differs greatly depending on the type and nature of biological sample analyzed. Although Mn(II) was initially determined in aqueous samples, this was an initial optimization. The aim was to develop and optimize an extraction and determination method for the trace analysis of Mn(II) in biological samples with more complexed matrices (Initially fat free clover milk and then whole unfractionated blood serum). Once the SLM and AdSV procedures were optimized further, Mn(II) was then extracted and determined in aged blood serum and fractionated blood serum. As seen in table 11 below, the blood serum showed higher extraction efficiency compared to the milk sample. The implications of this have a direct effect on the analysis of these samples based on the fact that AdSV measures the manganese(II) concentration in the form of free Mn(II) and labile complexes. From the fact that Mn(II) binds to many types of different proteins that constitute a large percentage of a biological samples' matrix, shows that the sample containing a more complex matrix can yield a lower percentage of labile Mn(II). The Mn(II) is known to form a labile complex with ascorbic acid (Berkessek et al., 1999). This complex adsorbs onto the surface of the mercury drop.

Table 11 Blood serum and milk spiked with 40µg l^{-1} Mn(II)

Sample type	**Whole blood serum**	**Fresh milk**
Average extraction efficiency / %	20.5	5.05
Peak position / mV	-1.481	-1.486

5.4 Application of AdSV in sample analysis

The methodology pertaining to the analysis and determination of the concentration of Mn(II) in the water, milk, aged (48-hour) milk and blood serum has been described below as well as all parameters used for the determination of Mn(II) in water and milk. Although these parameters have not yet been completely optimised for blood serum, due to the similarities in composition of matrix and overall structure, these parameters were varied slightly to enhance detection and peak measurements.

5.4.1 Determination of Mn(II) in water (Std. Addition)

Conditions

Total volume 10ml that consisted of 2ml sample and 8ml supporting electrolyte (constituent concentrations as in procedure in chapter three adjusted for total cell volume). The parameters used were identical to those described previously. Approximately 4µl 40 % NaOH for pH adjustment (negligible). pH = 8.45-8.82.

Results

Table 12 Determination of Mn(II) in water

Volume. 10mg l^{-1} Mn(II) added / µl	**Total Volume / ml**	**Mn(II) concentration of std addition (In cell) / µg l^{-1}**	**Peak area (V/A)**	**Peak position (E/V)**
0.00	10.00	0.00	2.06E-10	-1.512
40.00	10.04	40.00	2.24E-10	-1.512
80.00	10.08	80.00	2.85E-10	-1.512
120.00	10.12	120.00	3.03E-10	-1.512
160.00	10.16	160.00	3.66E-10	-1.512
200.00	10.20	200.00	3.84E-10	-1.512

The data above was used to plot the following graph (standard addition method) allowing for the concentration of Mn(II) to be determined in a water sample spiked with 40µg l^{-1}.

Determination of Mn (II) in water using Std. addition. method

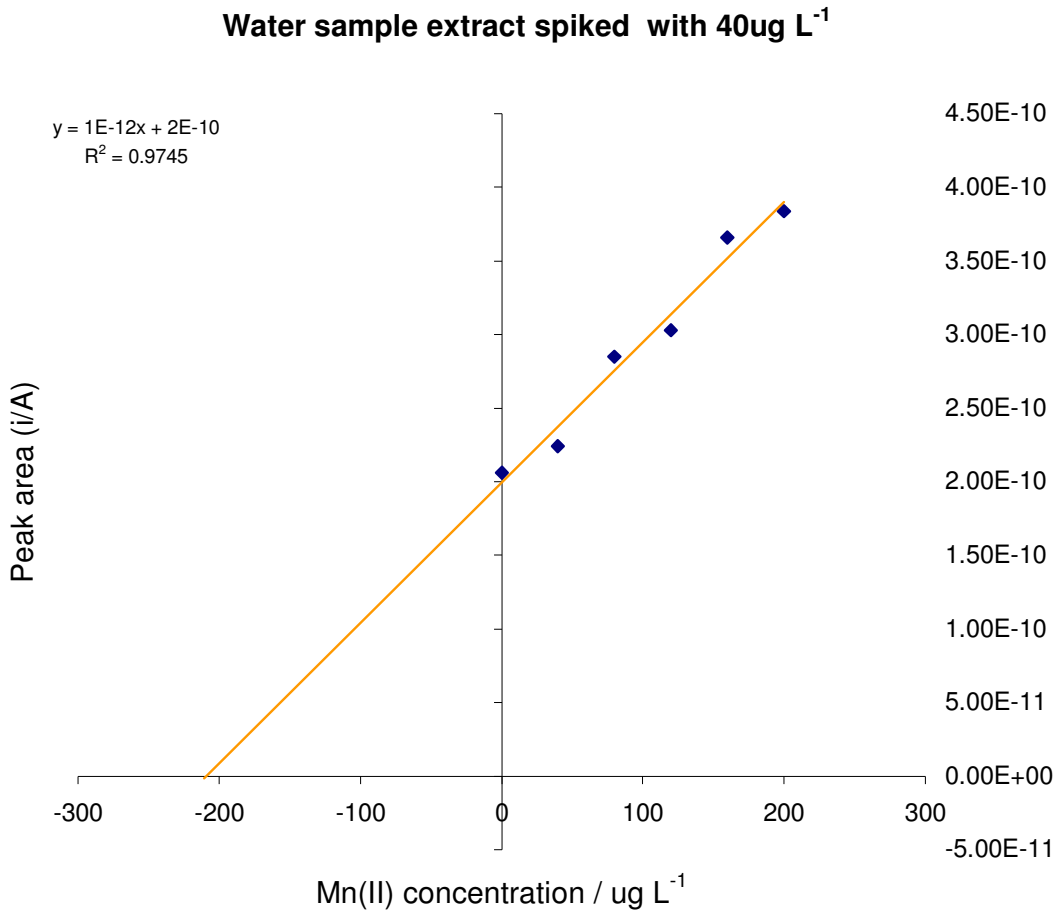

Figure 13 Determination of Mn(II) concentration in water

The Mn(II) concentration in the water sample was found to be 1.05mg l^{-1}. The R^2-value of 0.975 indicates a good linear fit. The uncertainty associated with the determination was therefore 2.4%. This analysis was initially optimised in water. It showed that measurements obtained were reproducible and reliable allowing for the comparison of data later on. It also served as a natural control for comparing concentrations of Mn(II) in biological samples such as milk and blood serum. This analysis also provided confirmation as to the applicability of the experimental parameters used.

5.4.2 Determination of Mn(II) in milk

From previous studies long life fat free cow milk was analysed. As mentioned in the methodology, long life fat free milk was used. The reason for using fat free milk was to p use a sample with a similar matrix complexity to blood serum. The fat in the milk would also block the 0.22µm membrane pores during extraction. Other components that could play a role in the extraction process also had to be considered such as protein and carbohydrate concentrations which both play a role in matrix effects. It is for this reason that a description of the product contents of the Clover long life fat free milk is given in table 13 below.

Table 13 Milk composition (according to label)

Mean value	Per 100ml
Carbohydrate (lactose)	5.1g
Fat	0.5g
Protein	3.5g
Energy	160kJ

The brand of milk was not chosen based on its fat content or product label due to the fact that the matrix effect was of particular interest to this project and in order to optimise the analysis of other biological samples such as blood serum often with far less complexed matrix, it was first optimised for the more complexed sample matrix.

Conditions

A total volume that constituted the electrochemical cell consisting of 8ml of supporting electrolyte and 2ml of sample (note: concentration of the ascorbic acid was increased three-fold). The pH was adjusted using 40% NaOH. pH = 8.55-8.60.

Results

Table 14a Analysis of Mn(II) in milk (flat spiral disc SLM) extract 1 and 2 spiked with 40µg l^{-1} Mn(II) (*Extract 1)*

Vol. of 10mg l^{-1} Mn(II) added /µl	**Total volume /ml**	**Mn(II) concentration of std addition (In cell) /µg l^{-1}**	**Peak area (V/A)**	**Peak position (E/V)**
0.00	10.00	0.00	7.95E-12	-1.481
20.00	10.02	20.00	2.47E-11	-1.486
40.00	10.04	40.00	3.93E-11	-1.481
60.00	10.06	60.00	5.16E-11	-1.486
80.00	10.08	80.00	6.82E-11	-1.481

Table 14b Extract 2 (fresh milk)

Vol. of 10mg l^{-1} Mn(II) added /µl	**Total volume /ml**	**Mn(II) concentration of std. addition (In cell) /µg l^{-1}**	**Peak area (V/A)**	**Peak position (E/V)**
0.00	10.00	0.00	1.96E-11	-1.476
60.00	10.06	60.00	7.81E-11	-1.486
80.00	10.08	80.00	8.81E-11	-1.486

The corresponding graphs for both the above milk extract is plotted below using the same procedure previously used.

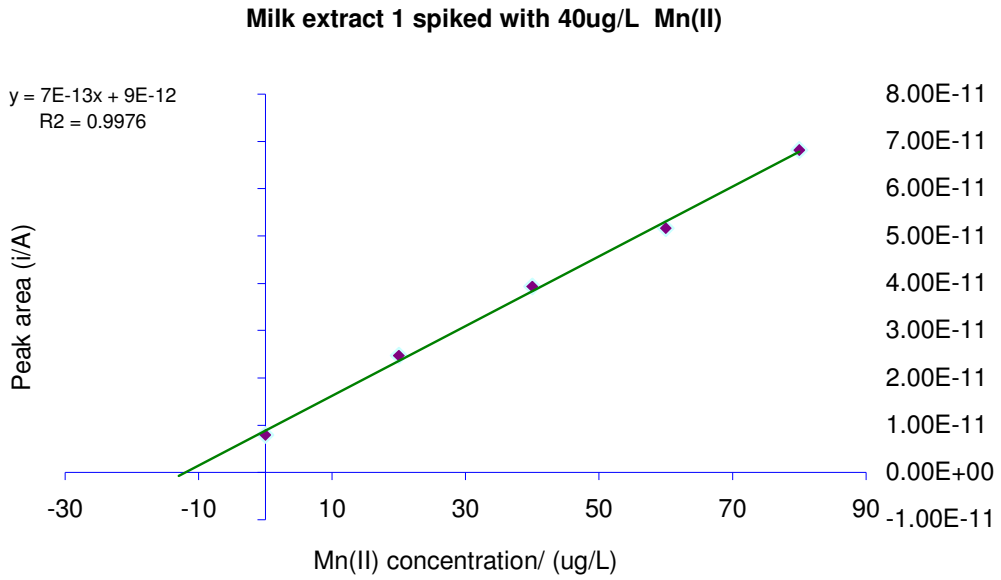

Figure 14a Determination of Mn(II) concentration in milk SLM (flat spiral disk) extract1

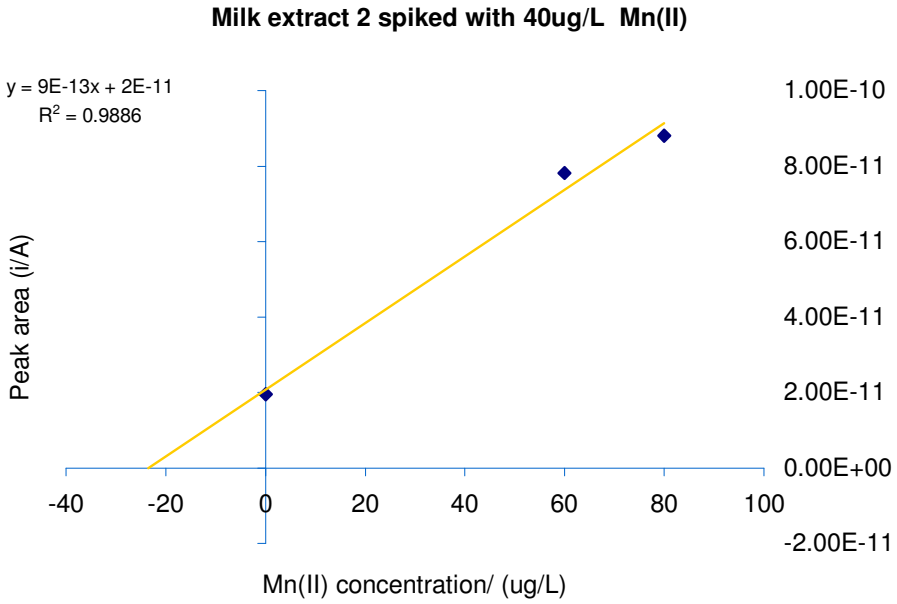

Figure 14b Determination of Mn(II) concentration] in milk SLM (flat spiral disk) extract 2

From the graphs, the concentrations of Mn(II) in each extract were calculated as previously described taking into consideration the dilution factor of the electrochemical cell.

Extract 1: 80.00µg l^{-1} (0.080mg l^{-1}) 16 x 5 (dilution factor)

Extract 2: 122.5µg l^{-1} (0.1225mg l^{-1}) 24.5 x 5 (dilution factor)

The R^2- values for both extracts are indicative of good linear fits with the uncertainty in the determination of 0.24% and 1.1% respectively. The overlaid voltammograms showing the standard additions for extract 1in the above analysis and that the results are reproducible, are included in appendix 3.

The above experimental procedure was performed on milk that was spiked with 10 and 100µg l^{-1} Mn(II) in order to show that the method is reproducible for milk at different spiked concentrations.

Milk extract spiked with 10µg l^{-1} Mn(II) = 89µg l^{-1}, 44µg l^{-1}, 25µg l^{-1} for each respective extract (diluted five times). 100µg l^{-1} Mn(II) = 35µg l^{-1} , 36µg l^{-1} for extracts 1 and 2 respectively.

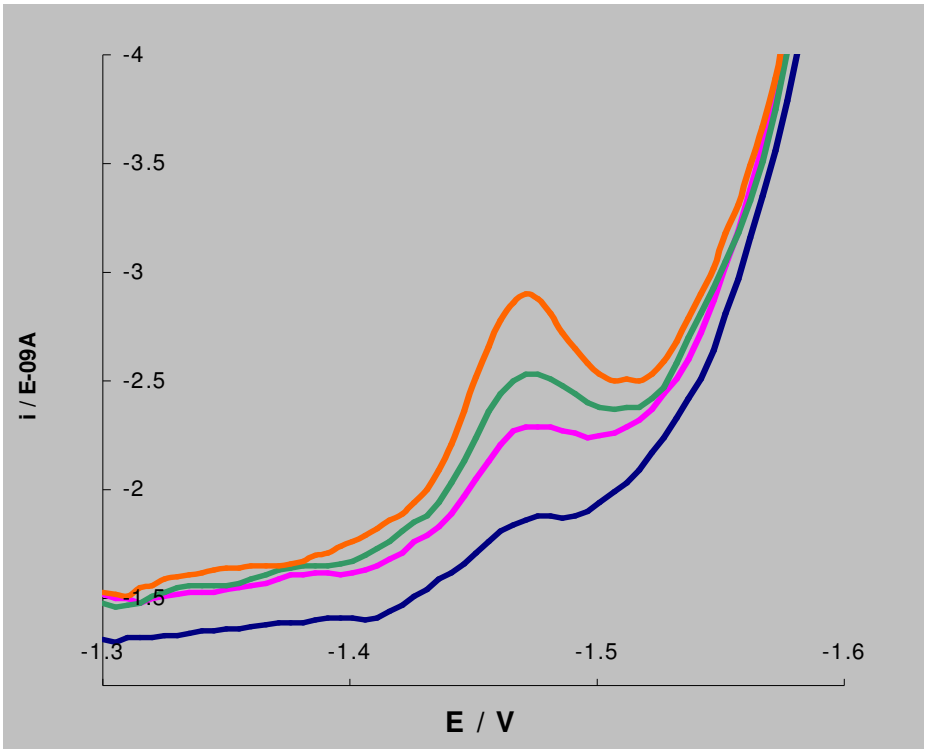

Figure 15 Typical voltammograms of milk

Conditions: Extraction of 40µg l^{-1}Mn(II) in milk

a = extract; **b** = extract +20µg l^{-1} Mn(II); **c** = b+20µg l^{-1} Mn(II); **d** = c +20µg l^{-1} Mn(II)

5.4.3 Determination of Mn(II) in aged milk (48-hour)

Conditions

As previously carried out, 8ml of supporting electrolyte and 2ml sample extract (constituent concentrations as described in the procedure in chapter 5). Again the ascorbic acid concentration was tripled. AdSV parameters can be viewed in appendix 2 and in chapter 5 pH was adjusted using 40 % NaOH, pH = 8.23-8.38.

Results

Mn(II) concentration = 285µg l^{-1}, 317µg l^{-1} for extract 1 and 2 respectively calculated using the method used previously of standard addition. (Dilution factor 5)

5.4.4 Determination of Mn(II) in whole blood serum

Conditions

10ml total was placed in the electrochemical cell of which 8ml constituted the supporting electrolyte and 2ml the extracted sample spiked with 40µg l^{-1} Mn(II). Refer to chapter three for further details. pH was adjusted using 40% NaOH and kept in range 8.69-8.72.

Table 15 Determination of Mn(II) in blood serum after extract

Vol. of 10mg l^{-1} Mn(II) added /ul	**Total volume /ml**	**Mn(II) concentration of std. addition (In cell) /µg l^{-1}**	**Peak area (V/A)**	**Peak position (E/V)**
0.00	10.00	0.00	1.86E-11	-1.476
10.00	10.01	10.00	2.62E-11	-1.476
30.00	10.03	30.00	4.04E-11	-1.481
40.00	10.04	40.00	4.26E-11	-1.481

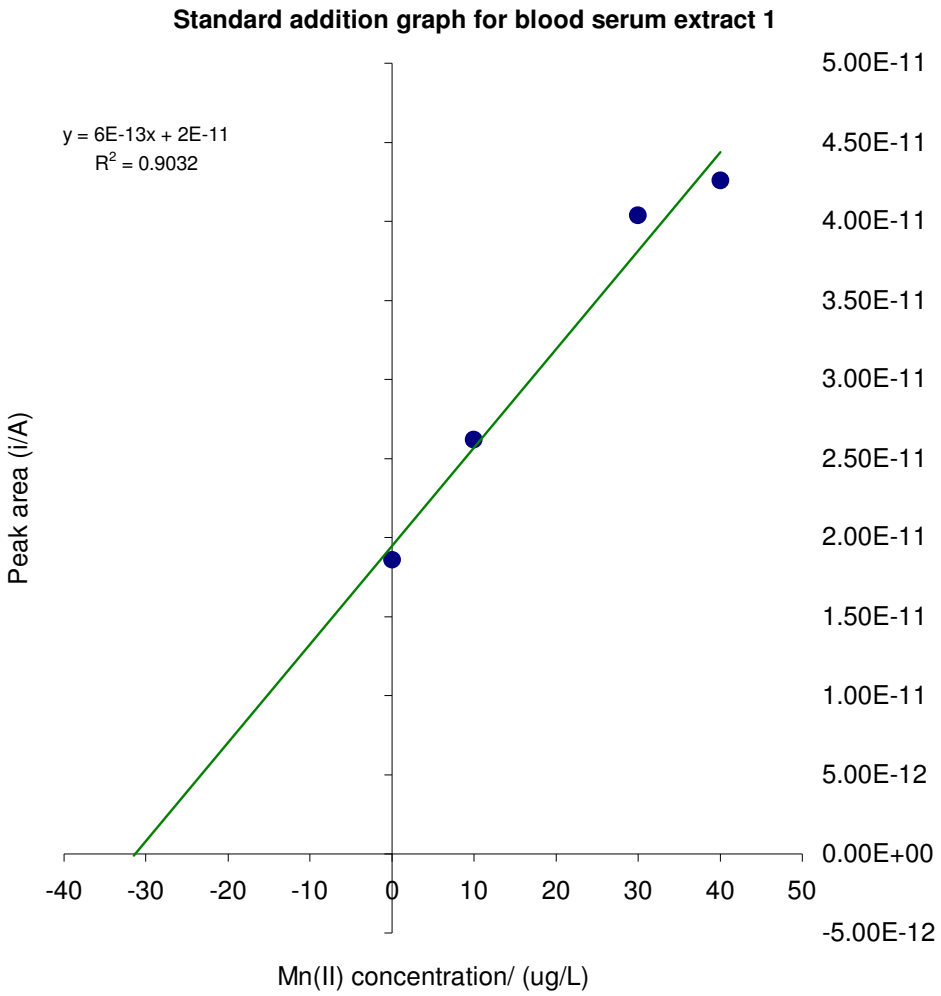

Figure 16 Determination of Mn(II) in SLM (flat spiral disk) blood serum extract 1

The concentration of Mn(II) determined in the blood serum:

These results tend to show high concentrations of Mn(II). The R^2- value for extract 1 is indicative of a fairly good linear fit with the uncertainty in the determination of 8.3%. Figure 17 below shows typical blood serum voltammograms overlaid, showing the standard addition method.

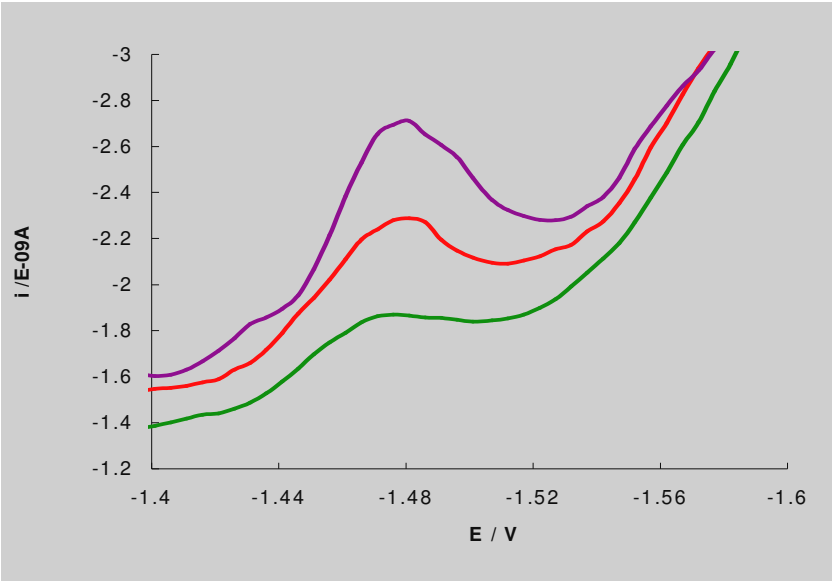

Figure 17 Typical voltammograms for Mn(II) extracted from the blood serum

Conditions: Extraction of 40µg l^{-1}Mn(II) in blood serum **a** = extract; **b** = extract + 10µg l^{-1}Mn(II) and **c** = b + 10µg l^{-1}Mn(II).

5.5 Determination of labile complex Mn(II) in blank milk extractions

In order to determine the total labile complexed amount of Mn(II) in any sample, it is imperative that a blank extraction be performed. This was carried out for a fresh milk sample. The extraction efficiency used in the calculation was the average taken of all the 10µg l^{-1} spiked Mn(II) sample extracts.

Extraction Conditions

16ml of fresh milk was diluted accordingly (1:30) to make up a 500ml solution. As previously described pH was adjusted to 3. 200ml of donor solution was extracted and 2ml collected (acceptor solution).

Table 16 Blank extracts 1 and 2-voltammetric analysis respectively.

Vol. of 10mg l^{-1} Mn(II) added /µl	**Total volume/ml**	**Mn(II) concentration of std addition (In cell) /µg l^{-1}**	**Peak area (V/A)**	**Peak position (E/V)**
0.00	10.00	0.00	7.58E-12	-1.461
10.00	10.01	10.00	8.43E-12	-1.466
20.00	10.02	20.00	1.41E-11	-1.471
30.00	10.03	30.00	1.53E-11	-1.466
50.00	10.05	50.00	2.09E-11	-1.466
0.00	10.00	0.00	7.22E-13	-1.456
10.00	10.01	10.00	9.87E-13	-1.471
20.00	10.02	20.00	1.89E-12	-1.476

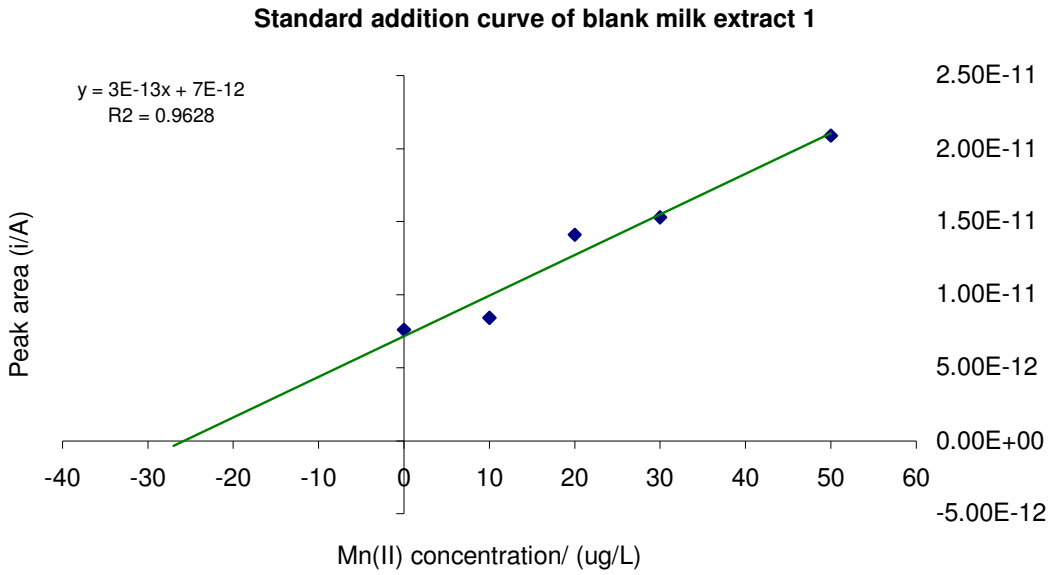

Figure 18a Determination of total labile complexed Mn(II) using Standard addition method.

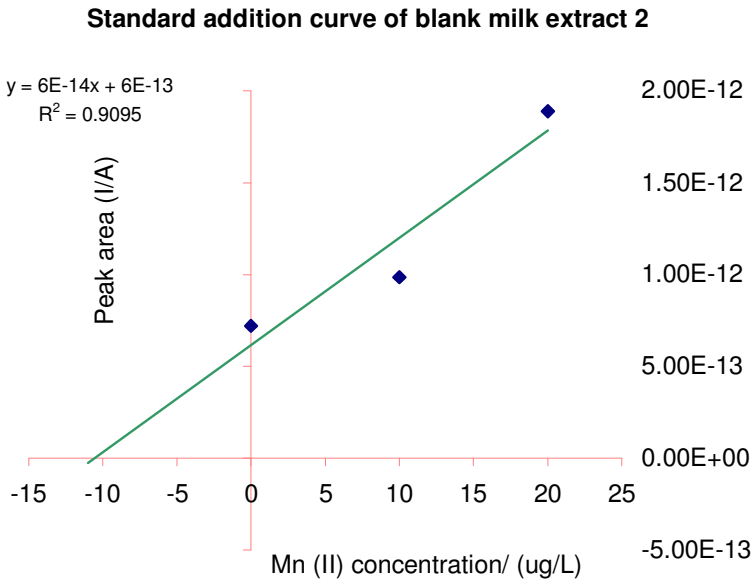

Figure 18b Determination of total labile complexed Mn(II) using Standard addition method

From figure 18a and figure 18b, the extrapolated value was determined. After taking the dilution factor (x5) of each sample into account, the following extrapolated value

(C_A = the concentration of the acceptor solution) was calculated: Using the mean extraction efficiency of the fresh milk spiked with 10µg l^{-1} Mn(II) and the required formula in chapter two and considering the final dilution factor (x30); the concentration of labile complexed Mn(II) was determined for each blank milk extract. The acceptor solution of extract one was found to contain 130µg l^{-1} and extract two 54.5µg l^{-1}. The mean final concentration value was then calculated and reported below.

Final labile complexed Mn(II) concentration in milk

The final concentrations determined for blank extract one and two were 8.39µg l^{-1} and 3.51µg l^{-1} respectively. The average final concentration of the above blank extracts was therefore calculated with a, standard deviation of 5.91.

Optimization of AdSV analysis for the determination of Mn(II) in aqueous and biological fluids

5.6 Initial optimisation performed

Conditions

All conditions used can be found in chapter three. All optimization was initially done for deionised water and then used in further optimization for biological matrix.

5.6.1 pH optimisation

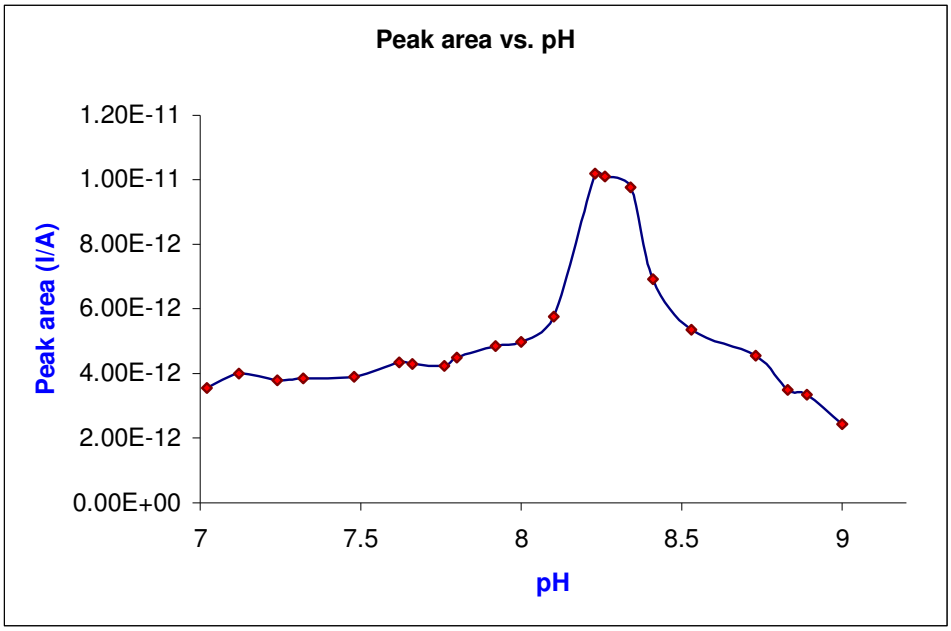

Figure 19 Peak area vs. pH

Table 17 pH optimization

pH	Peak position E/V	Peak area /A	RSD%
7.02	-1.481	3.56E-12	1.26
7.12	-1.476	4.00E-12	10.96
7.24	-1.476	3.79E-12	20.14
7.32	-1.476	3.85E-12	5.46
7.48	-1.476	3.91E-12	1.43
7.62	-1.471	4.35E-12	18.8
7.66	-1.466	4.30E-12	7.43
7.76	-1.466	4.25E-12	3.26
7.80	-1.466	4.50E-12	2.49
7.92	-1.466	4.85E-12	2.92
8.00	-1.471	4.98E-12	2.27
8.10	-1.471	5.76E-12	1.47
8.23	-1.486	1.02E-11	1.54
8.26	-1.486	1.01E-11	1.54
8.34	-1.486	9.76E-12	0.435
8.41	-1.486	6.93E-12	2.24
8.53	-1.486	5.36E-12	1.58
8.73	-1.481	4.55E-12	1.55
8.83	-1.486	3.50E-12	3.72
8.89	-1.481	3.34E-12	5.93
9.00	-1.481	2.43E-12	17.46

AdSV Conditions:

Deposition potential: -1.4V

Purge time: 100s

Equilibrium time: 10s

Deposition time: 25s

Spiked concentration: 40µg l^{-1}

No stirring during deposition time period

At pH 8.35 a maximum peak current for deionised water spiked to contain 40µg l^{-1} is attained. This is however a 2d (one factor) optimization experiment so any contributions or correlation between pH and deposition potential are not considered. This was therefore only performed to get a rough idea of which pH range needed to be focused on when designing the two factor (systematic) response matrix experiments.

5.6.2 Deposition potential

A 2d or single factor response optimization experiment was run to establish an optimum deposition potential for a fixed pH and Mn(II) concentration of 24µg l^{-1} with all the other voltammetric parameters as described in chapter three. One of the main problems encountered when optimizing electro analytical parameters was that each parameter couldn't be independently optimized as many of these parameters are correlated and thus require correlation optimization methods. This 2d deposition potential optimization was thus done to get an idea of the region in which the true optimized value could be located. From this rough data correlation matrix experiments could then be performed.

Table 18 Deposition potential and peak height

Deposition potential /V	**Peak position E/V**	**Peak height /A**	**RSD (%)**
-1.1	-1.476	3.73E-12	3.79
-1.15	-1.471	3.36E-12	2.53
-1.2	-1.476	4.52E-12	40.67
-1.25	-1.471	3.08E-12	3.67
-1.3	-1.471	3.24E-12	10.05
-1.35	-1.471	2.59E-12	4.91
-1.4	-1.471	2.32E-12	18.29
-1.5	-1.471	4.69E-12	2.11
-1.6	-1.471	5.80E-12	4.75
-1.7	-1.471	7.05E-12	1.00
-1.8	-1.471	6.97E-12	1.42
-1.9	-1.471	1.53E-11	2.77
-2.0	-1.476	6.03E-11	0.469
-2.1	-1.471	5.56E-11	7.34
-2.2	-1.471	4.18E-11	6.10

AdSV Conditions:

pH: 8.17

Purge time: 50s

Equilibration time: 5s

Deposition time: 25s

Mn(II) concentration: 40μg l^{-1},with no stirring during the deposition time period.

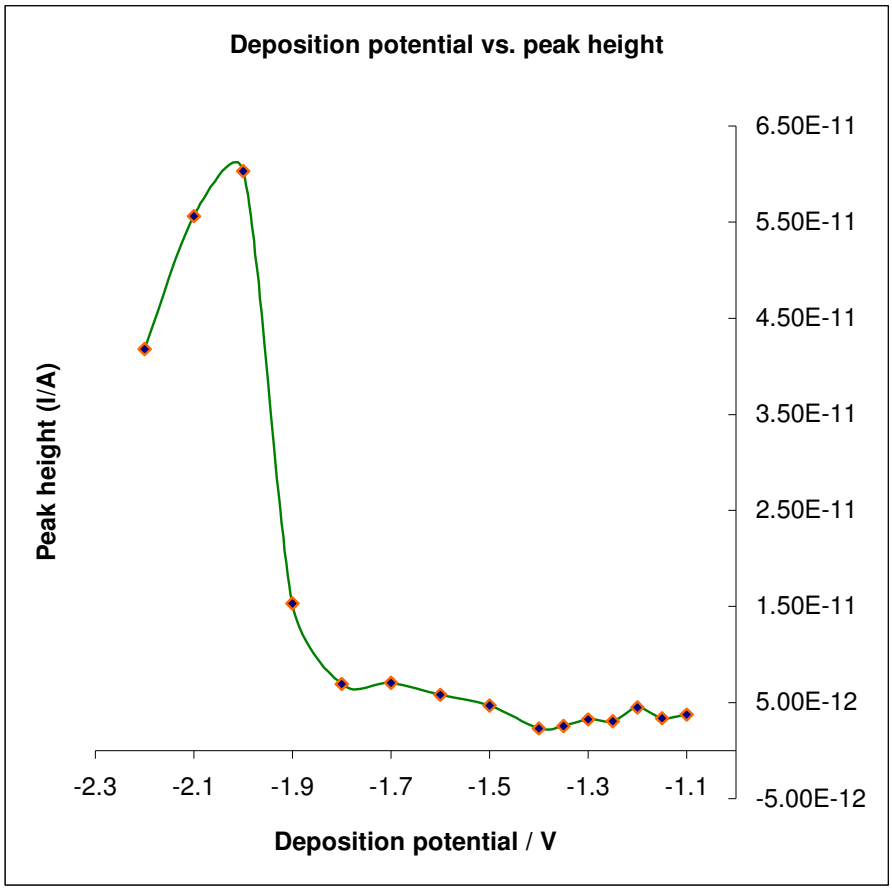

Figure 20 Deposition potential and peak current relationship

From the plot above, it can be observed that the optimum deposition potential was attained at –2.0V. This value is quite significant as all deposition potentials optimized in previous research falls in a range of –1.3V to –1.6V (Wang et al., 1993), which is below –2.0V. One problem that can occur at mercury electrodes is the formation of a calomel film on the surface (Vydra et al., 1976). The calomel film greatly lowers the potential for hydrogen evolution. This can cause serious interference during stripping of very negatively deposited metals (Florence, 1972) Furthermore another significant factor is that above a deposition potential of approximately –1.7V hydrogen gas evolution occurs. Although AdSV addresses such problems (encountered in Anodic Stripping Voltammetry (ASV)), at very negative deposition potentials hydrogen

evolution can still present difficulties (Wang et al., 1994). The potential at which hydrogen evolution occurs is highly dependent on the type of electrode material (working electrode) and metal being adsorbed onto the working electrode (Pletcher, 1991). At –1.9V hydrogen evolution was visible to the naked eye.

Hydrogen evolution at a level that does not cause interference of the signal could be advantageous. This could be as a result of surface or surface energy modifications (adsorption sites) by the hydrogen atoms (Pletcher, 1991; Lipkowski, 1998) enhancing the adsorption of the manganese-ascorbic acid complex. Several new phenomena can occur if hydrogen molecules or atoms accumulate on certain material (mercury drop). In a multiparticle interaction, neighboring H species interact with each other (mainly repulsive mutual interactions dominate), causing a decrease in adsorption energy with increasing hydrogen surface concentration (Lipkowski, 1998).

5.6.3 Deposition time

In order to determine the optimal deposition time, the deposition time was changed while keeping all other parameters constant and the resulting peak current was measured as shown in table 20.

Table 19 Deposition time and peak current

Deposition time /s	**Peak position E/V**	**Peak area /A**	**RSD %**
10	-1.486	3.75E-12	3.77
20	-1.486	5.50E-12	2.55
25	-1.486	7.27E-12	1.36
30	-1.486	6.11E-12	1.62
35	-1.476	4.50E-12	6.22
40	-1.481	4.03E-12	1.42
45	-1.481	3.04E-12	6.50
50	-1.476	3.10E-12	7.21
60	-1.481	2.16E-12	10.48
100	-1.481	6.32E-13	2.45

AdSV Conditions

pH: 8.11

Purge time: 100s

Equilibrium time: 10s

Deposition potential: -1.4V

Mn(II) concentration: 40μg l^{-1} with no stirring during the deposition time period

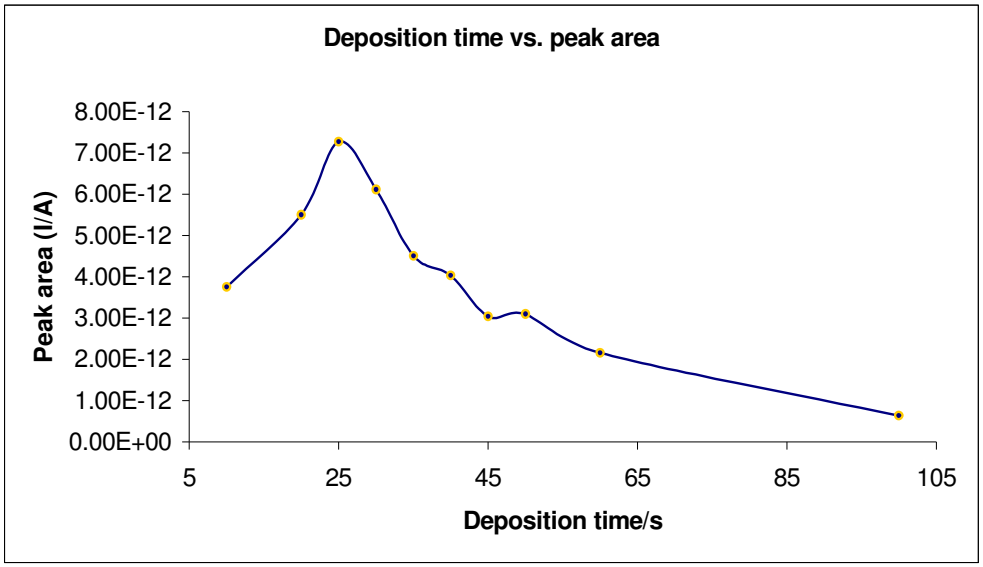

Figure 21 Deposition time vs. peak area

A maximum peak area was obtained at a deposition time of approximately 25s. As the process of deposition was carried out without stirring this low deposition time maxima is expected due to the limited transport (diffusion) of the metal ions to the surface of the working electrode.

5.7 Optimization of interrelated parameters

A major draw back in using the one factor at a time approach is that it is unable to reveal any information about interactions between factors. Interactions are interdependencies among the factors, present when the effect of the factor is dependent on the level of one or more of the other factors. In the case of the systematic approach, one of the factors is varied in a series of experiments while all of the other factors are kept constant. This is what is referred to as the 'one-factor-at-a-time' approach (Barker, 1985).

5.7.1 Experimental design

These experiments were all carried out using 2-factor response systematic approach. A three or four square matrix plot was used to determine the experimental points to be used in each run. Once a surface or contour plot was generated, the maximum was determined. The area around the immediate region surrounding the maxima was then concentrated on and in this way sequential optimization using matrix experiments was performed for the new region selected. Response values can be related to factor levels by a mathematical function (model). Such a model can be graphically represented by a "response surface." An example of a response surface can be seen in figure 22 were a response factor is a function of two other factors. Response surfaces can exhibit a wide variety of shapes and are not always symmetrical (Hutzinger, 1995)

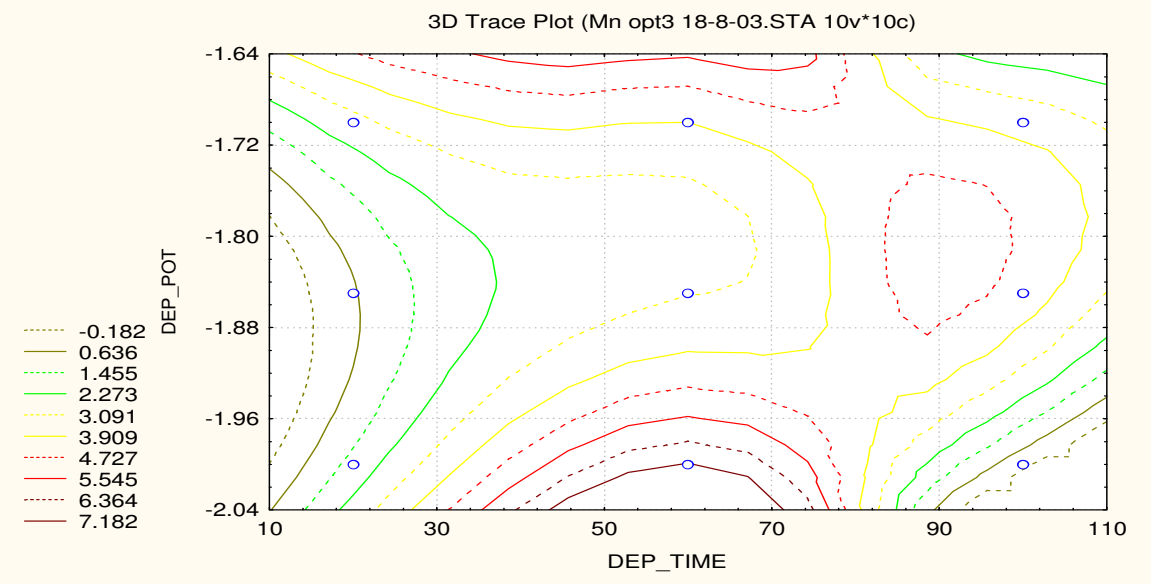

Figure 22a Two-factor contour plot of deposition time vs. deposition potential surface response

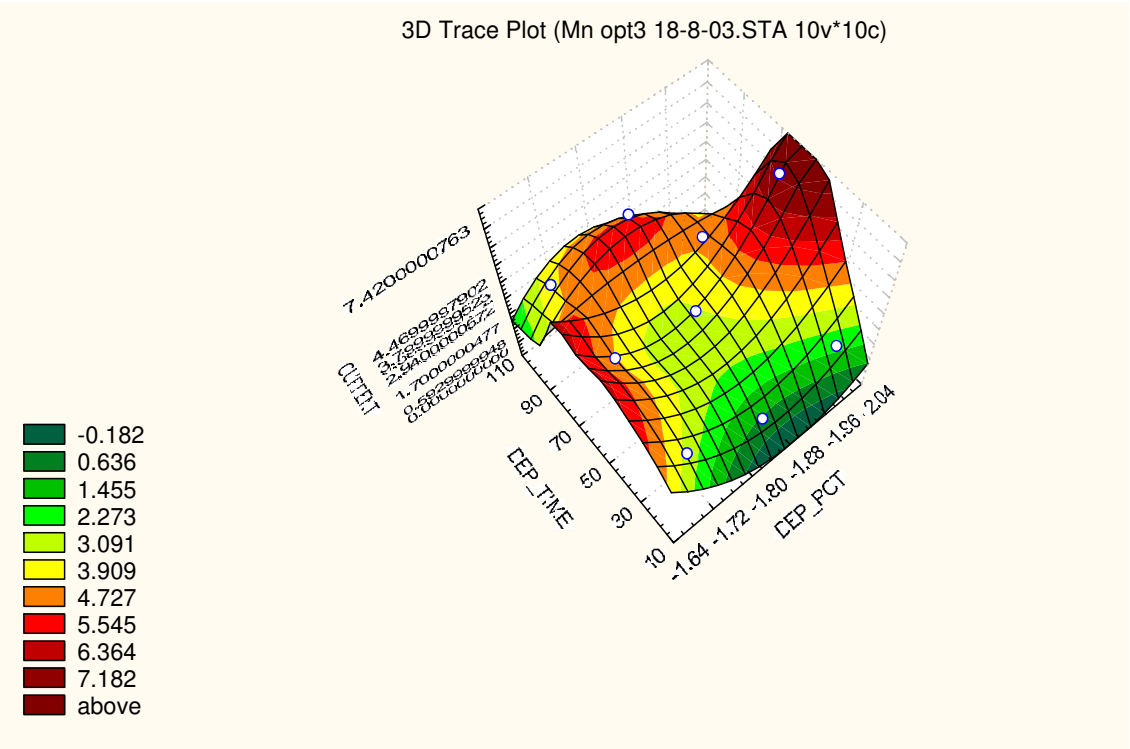

Figure 22b Two-factor response surface for deposition time vs. deposition potential

5.7.2 Deposition time vs. deposition potential

Once each of the above parameters was optimized individually, it was decided to use a systematic correlation plot / experiment to determine the relationship each parameter has with respect to other parameters and then determine the optimum peak current.

Table 20 Deposition potential and deposition time relationship

Deposition potential /V	**Peak current /A**	**Deposition time /s**	**RSD%**
-1.7	2.85E-10	20	5.66
-1.7	3.80E-10	60	6.23
-1.7	3.59E-10	100	1.45
-1.85	5.93E-9	20	10.32
-1.85	2.94E-10	60	3.45
-1.85	4.47E-10	100	4.52
-2.00	1.70E-10	20	7.56
-2.00	7.42E-10	60	3.56
-2.00	0.000	100	2.11

From the above two-factor response plots it was observed that no distinct maxima exist for the deposition potential with regards to deposition time. However there is an incomplete maximum that occurs between 45 – 70s in a deposition potential range of negative 1.9-2.04V. Peak noise and poor reproducibility was observed in other systematic response experiments incorporating longer deposition times with more negative deposition potentials. A possible reason for this is thought to be hydrogen

evolution, which was observed to dominate at deposition times greater than 70s for reduction potentials greater than –2.0V. Although only one experimental point occurs at the surface maximum, it was reproducible in other experiments. Perhaps as a result of the improved efficiency of the reduction step, double layer formation of the analyte on the mercury drop occurs at a shorter deposition time. The advantage of short deposition times is shorter sample determination times.

5.7.3 pH vs. deposition potential

After 2d experiments were performed to determine the rough ranges in which the maxima for each variable relative to peak current occurs, it was decided to combined certain parameter and perform systematic correlation matrix experiments following a grid symmetry approach. After each matrix was designed using the systematic approach, the local maxima were focused on using more points and smaller correlation ranges. It should be noted that in a number of correlation matrices the maximum peak current was difficult to attain due to the effect of hydrogen evolution on peak stability and catalytic current.

A two factor 4x4 matrix response experiment was performed in order to determine the optimum pH and deposition potential without allowing the evolution of hydrogen gas to interfere with the stability and reproducibility of the signal. Relativity good peaks were achieved and with good reproducibility. There was no visible evidence of hydrogen evolution although it is generated at these potentials (Pletcher, 1991). When the hydrogen became visible and small bubbles started to appear on the surface of the working electrode, the hanging mercury drop dislodged before the deposition time had lapsed. The data below shows how a typical matrix experiment is designed and

plotted to yield different types of representations for graphical analysis of the optimum parameters used.

4x4 matrix table

This optimization covered a two-factor range (deposition potential and pH) just before peak distortion and visible noise was generated from hydrogen gas evolution

Table 21 Two-factor response surface analysis for pH and deposition potential

(8.73, -1.9) **1.15E-11 (8.99)**	**(8.73, -2.0)** **4.03E-11 (6.82)**	**(8.73, -2.1)** **1.22E-11 (14.67)**	**(8.73, -2.2)** **2.26E-11 (6.71)**
(8.24, -1.9) **1.26E-11 (6.62)**	**(8.24, -2.0)** **4.36E-11 (6.33)**	**(8.24, -2.1)** **1.33E-11 (2.66)**	**(8.24, -2.2)** **2.11E-11 (10.54)**
(7.74. –1.9) **1.27E-11 (4.82)**	**(7.74, -2.0)** **5.17E-11 (28.30)**	**(7.74, -2.1)** **1.62E-11 (8.92)**	**(7.74, -2.2)** **2.38E-11 (12.21)**
(7.14, -1.9) **9.07E-12 (14.94)**	**(7.14, -2.0)** **1.55E-11 (19.29)**	**(7.14, -2.1)** **1.76E-11 (8.19)**	**(7.74, -2.2)** **2.24E-11 (11.02)**

Legend:

Red = Peak current

Blue = RSD %

Black = pH, deposition potential experimental point

3D Scatterplot of pH vs deposition potential related to peak current (4v*16c)

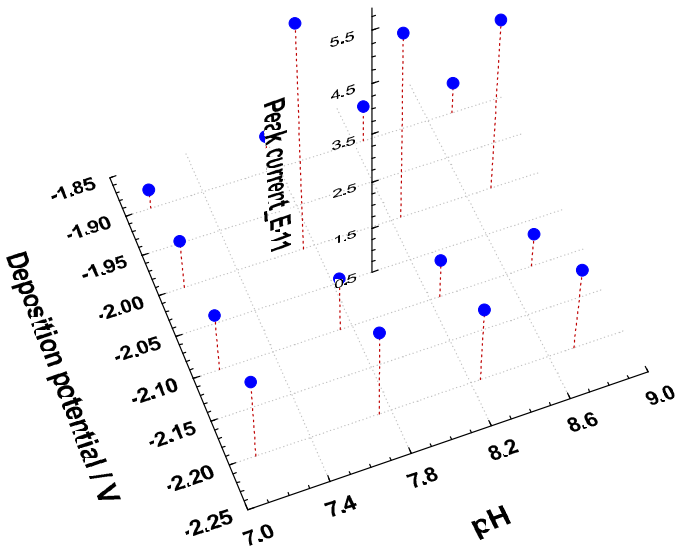

Figure 23a Two factor scatterplot for pH vs. deposition potential

The 3-d scatter plot above (Figure 23a) shows the relationship between pH and deposition potential for 24ug l^{-1} Mn(II) in the cell. As a typical 3-d scatter plot gives the relationship between three variables it can be used to find the optimum of all these variables correlated. In the above scatter plot a maxima for the peak current was found at a pH value of approximately 7.8 and deposition potential of –2.0V.

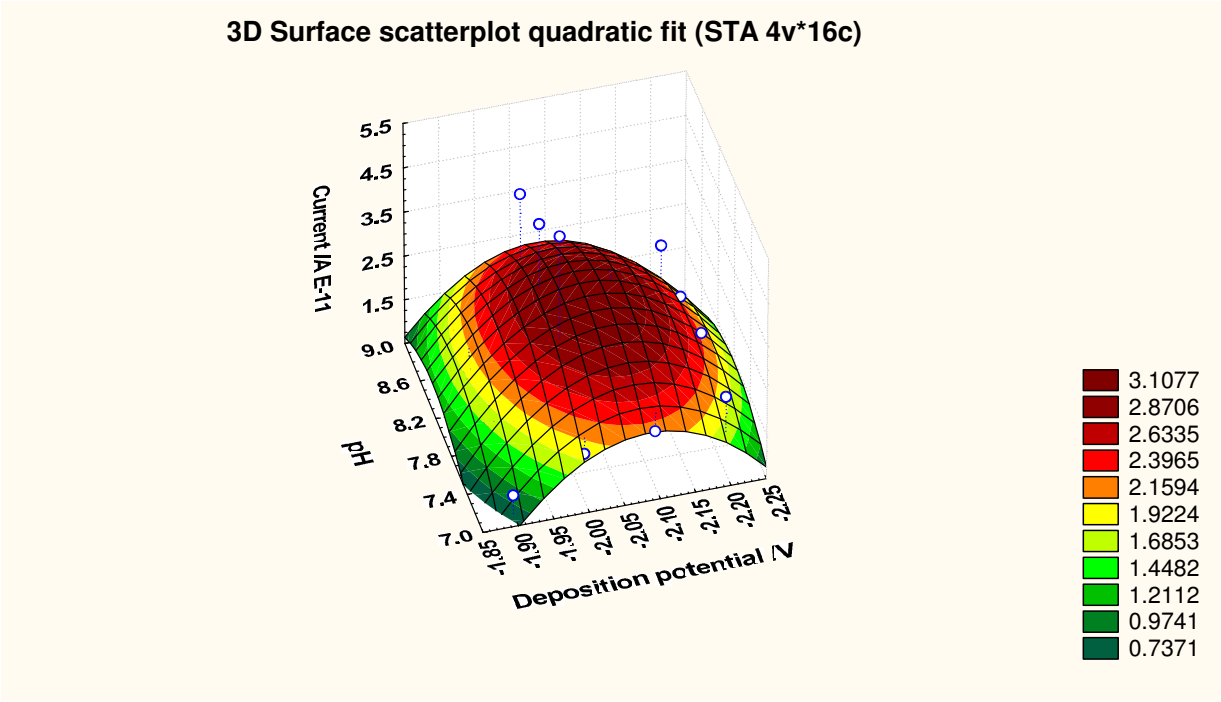

Figure 23b Two-factor response surface scatterplot of pH vs. deposition potential

From the above 3-d quadratic surface scatter plot a clear maxima was located where both the optimum pH and deposition potential can be found that yield the maximum peak height (current). It is interesting to note that the optimum dark red region for pH is far broader than the optimum deposition potential range. This therefore shows that the deposition potential is a more important parameter to consider when optimisation of the analysis of Mn(II) is carried out. It does however show that pH affects the adsorptive current to deposition potential.

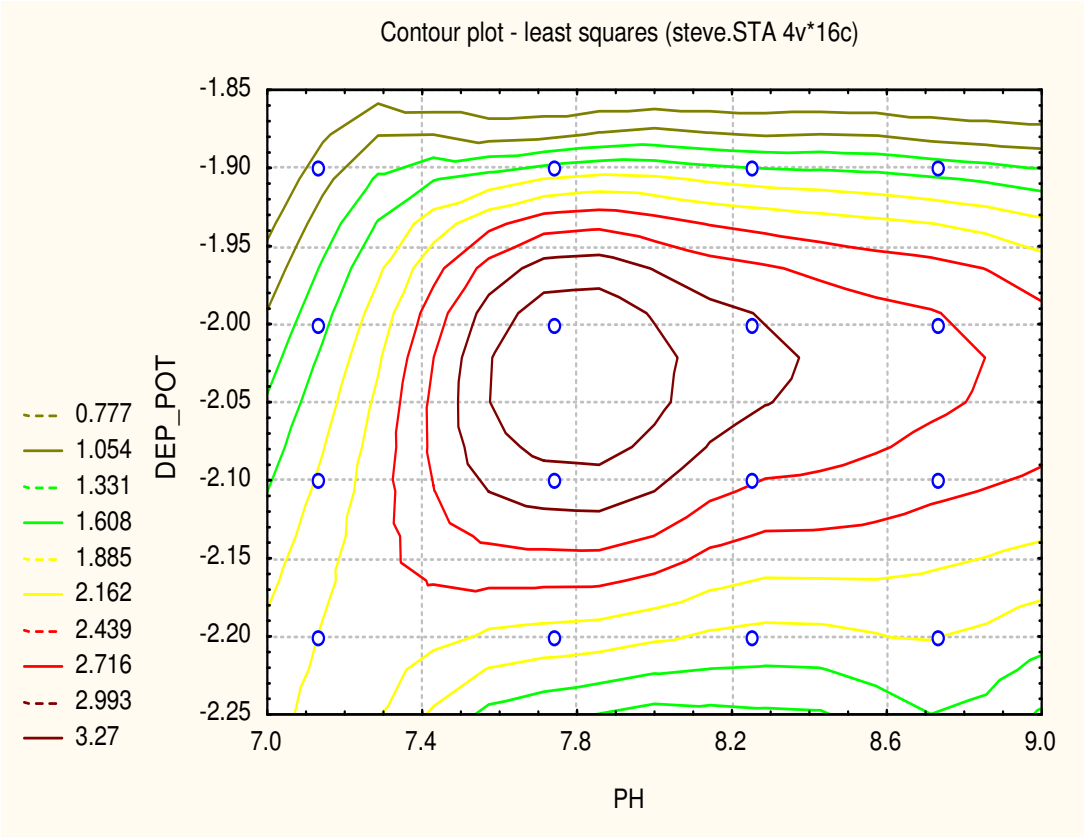

Figure 23c Two-factor contour plot of a 4x4 correlation matrix of pH and deposition potential related to peak current

5.7.4 Deposition time relationship

Using the parameters listed below Table 22 a 2d experimental optimisation was performed to determine the optimal deposition time.

Table 22 2-d Deposition time vs. peak area relationship

Deposition time/s	Peak position E/V	Peak area /A	RSD%
10	-1.486	2.22E-11	7.01
15	-1.481	3.00E-11	2.83
25	-1.486	4.14E-11	5.81
45	-1.486	7.58E-11	7.46
60	-1.481	9.36E-11	9.22
* 80	-1.481	1.44E-10	13.31
* 90	-1.476	1.45E-10	11.70
* 100	-1.476	1.54E-10	0.452
* 120	-1.481	1.60E-10	<50

Visible hydrogen evolution in electrochemical cell

AdSV Conditions

pH: 7.75

Deposition potential: -2.0V

Purge time: 50s

Spiked sample concentration: 24µg l^{-1}

Equilibrium time: 10s

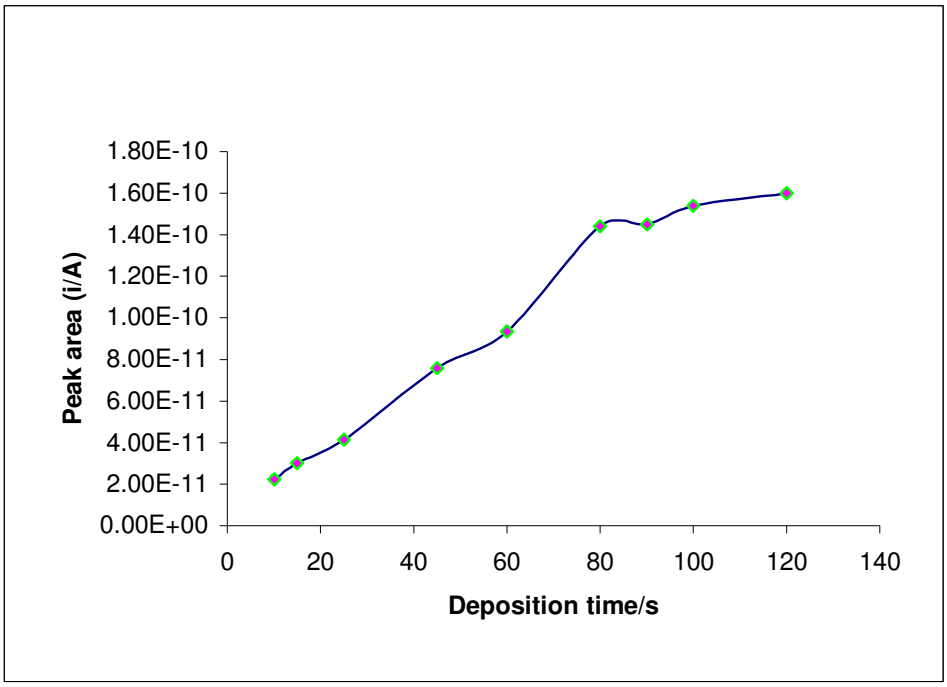

Figure 24 2-d Single factor deposition time vs. peak current response

The optimal deposition time for AdSV analysis is always dependant on the sample type, e.g. matrix involved and concentration of analyte expected. An optimization experiment for the deposition time was however carried out to try and establish the a rough time and the effect deposition time has on the reproducibility of the analysis using the new optimized pH, stirring and deposition potential. It was established that as the deposition time is increased for a sample (deionised water) spiked to contain 24µg l^{-1} Mn(II), there is no maxima for the time range selected. Hydrogen evolution was clearly visible after 60 seconds of deposition and this although peak interference was not visible, a reduction does of the increase in peak current did occur. This is perhaps as a result of analyte double layer formation on the mercury electrode causing a current linearity loss. From figure 24 it can be seen that a transition or intermediate maxima occurred at approximately 80 seconds thus giving further evidence hydrogen evolution is mainly dependant on the deposition potential, deposition time also plays a role in determining the effect hydrogen has on the surface of the mercury electrode

(As discussed in 5.7.3). As time is increased, more hydrogen is evolved at the working electrode. At the initial inception of hydrogen the catalytic properties favour the enhancement of the signal for Mn(II) detection due to possible surface modifications on the working electrode (Pletcher, 1991; Lipkowski, 1998).

5.7.5 Equilibration time

The equilibration time was changed as shown below and the peak area was then measured while all other parameters such as deposition potential and pH were kept constant.

Table 23 Equilibration time optimization

Equilibration time/s	Peak position E/V	Peak area /A	RSD%
2	-1.476	8.50E-12	1.33
3	-1.476	9.66E-12	7.12
4	-1.476	1.10E-11	10.40
5	-1.481	1.83E-11	0.760
7	-1.491	1.65E-11	6.62
9	-1.486	1.45E-11	2.93
13	-1.486	9.12E-12	1.86
18	-1.486	7.63E-12	1.67
23	-1.481	5.56E-12	5.09
30	-1.491	4.34E-12	1.63
40	-1.486	3.57E-12	11.10
60	-1.486	1.15E-12	14.76

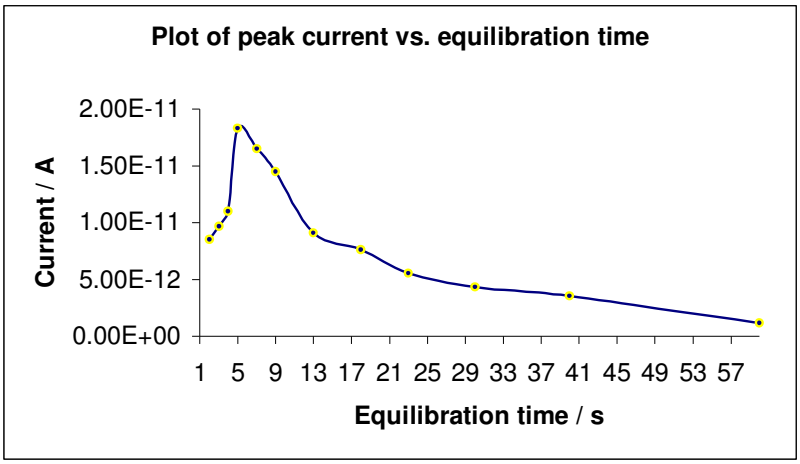

Figure 25 Equilibration time optimization

AdSV Conditions

pH: 7.75

Deposition potential: -2.0V

Purge time: 50s

Spiked sample concentration: 24µg l^{-1}

A maximum peak current was achieved for an equilibration time of 5 seconds. Thereafter as the equilibrium time was increased, the peak current decreased. The equilibrium time optimization was carried out using previously optimized parameter prior to them being optimized here again. A deposition potential of –1.4V was used for this optimization to avoid interferences from hydrogen evolution initially. Once the other parameters (deposition time, pH and deposition potential) were optimized the optimized equilibration time was checked and found to be unchanged to the above optimization.

It is also believed that the optimum equilibration time is shorter than that used previously (Wang et al., 1993) due to it limiting the amount of matrix interferences from the sample. In the case of deionised water, prior to the removal of gelatin as one

of the background electrolytes it is thought that the shorter equilibrium time minimized the interference contribution from the gelatin. As the equilibration time was increased, the amount of inferences that could affect the signal increases which can decrease the peak current response.

AdSV Conditions

pH: 8.17

Deposition time: 25s

Deposition potential: -1.4V

Purge time: 50s

Spiked sample concentration: 40µg l^{-1}

5.8 Transport parameters

For most analytes the adsorption at the surface of the electrode is fast and the overall rate of accumulation is governed by mass transport. As in anodic stripping voltammetry various forms of forced convection (stirring, rotation, ultrasound and flow) are used during the preconcentration step (Wang, 1982). In the AdSV method used (Wang et al., 1993) for the determination of Mn(II) in chapter five no stirring was performed during deposition. When stirring was performed during deposition, the peak current increased almost two fold as compared to no stirring. Reproducibility of the peak current was also good. When analysing samples for ultra trace amounts of Mn(II), stirring was used during deposition and the optimised stirring speed determined. In the plot below four different stirring speeds were selected while all the other voltammetric parameters were kept constant. An optimum stirring rate during deposition was found to be 2000rpm as shown in figure 26 below.

5.8.1 Stirring (reproducibility and optimization)

It was found that stirring during deposition improved increased the peak current obtained for different samples as stirring increases the amount of analyte at the surface of the working electrode (Pletcher, 1991). The stirring speed was then optimised by taking a number of measurements for the same concentration of Mn(II) in a deionised water sample at different stirring speeds as shown in table 24 below.

AdSV Conditions: Purge time: 50s, pH: 7.80, deposition time: 25s, deposition potential: -2.0V, equilibrium time: 5s

Table 24 Stirring optimisation

Stirring speed /rpm	Peak position E/V	Peak Area /A	RSD%
0	-1.481	2.88E-11	2.95
1000	-1.476	3.31E-11	5.13
2000	-1.476	3.91E-11	3.62
3000	-1.476	2.89E-11	0.489
4000	-1.471	3.03E-11	1.40

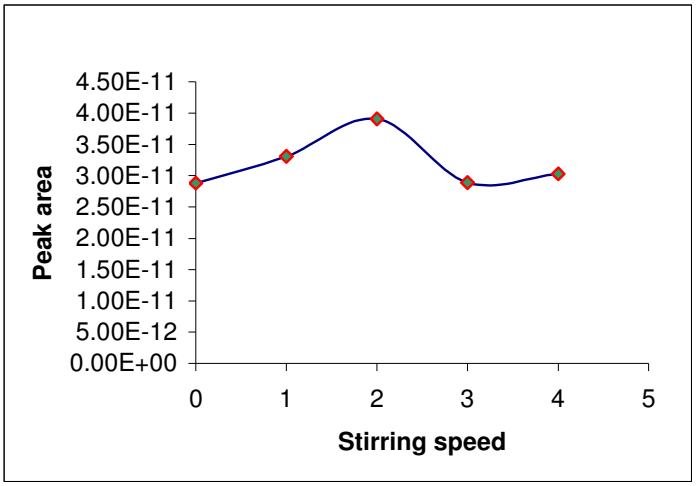

Figure 26 Stirring optimization

5.9 Supporting electrolyte

5.9.1 Gelatin

All analysis done for the optimization of the SLM (flat spiral disk) and SLM probe extraction techniques was carried out as described as previously described using various background components. One in particular is gelatin, which is used as a maxima peak suppressor and also as a peak enhancer. It works by forming a ternary complex with the Mn(II)–L (ascorbic acid) in solution and then adsorbed onto the hanging mercury drop on the working electrode more efficiently.

Gelatin is one of the most common suppressors used. A concentration of 0.01% has been reported as adequate for the complete elimination of maximum (Fried, 1973; Wang, 1993).

When optimizing the concentration of gelatin used in the cell, it was found that when gelatin was excluded from the method using the new optimized parameters (deposition potential of –2.0V, equilibration time, pH) the largest peak current was produced and with no visible maxima present. High concentrations of gelatin are undesirable since they may suppress the whole peak, distort its shape and even shift the reduction potential of a metal ion in the presence of a ligand (competitive complexation by the ligand and gelatin) (Fried, 1973; Crow, 1969). However it is unclear as to the exact reason why the use of gelatin is unfavourable under these conditions. It is possible that under the newly optimized parameters the gelatin behaves differently or decomposes in solution.

Furthermore it was also noticed that the addition of gelatin (even small concentrations) affected the stability of the drop itself by causing it to detach from the capillary, acting as a typical surfactant (Crow, 1969; Fried, 1973; Fisher, 1996).

Table 25 Gelatin optimisation

Gelatin % concentration (w/v) in cell	Peak position E/V	Peak height /A	RSD%
0	-1.461	2.13E-9	15.27
0.0006	-1.471	1.34E-9	23.22
0.001	-1.476	4.91E-10	17.86
0.002	-1.466	2.54E-10	11.14

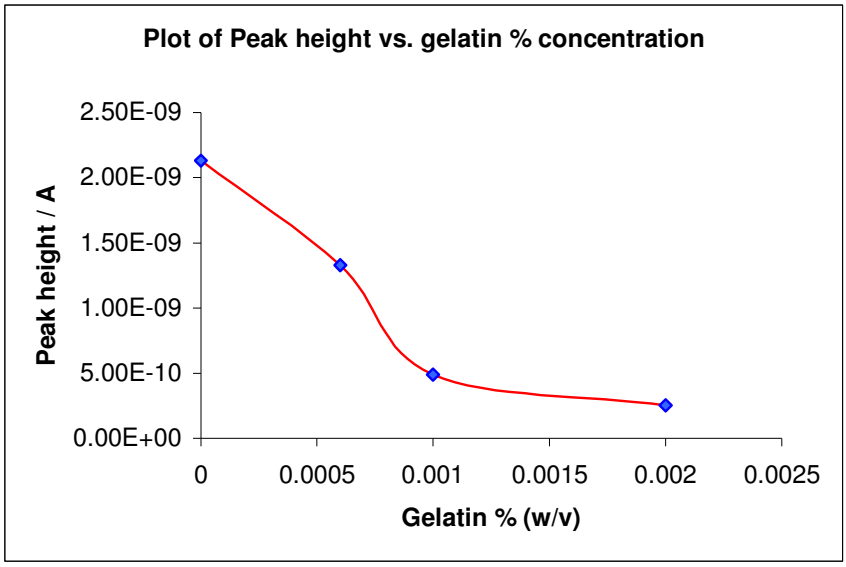

Figure 27 Gelatin optimization

AdSV Conditions

Purge time: 50s, pH: 7.75, deposition time: 45s, deposition potential: -2.0V, stirring speed: 2rpm, equilibration time: 5s, Mn(II) concentration: 24µg l^{-1}

Deposition potential

It was decided to determine the relationship between deposition potential and the presence of gelatin as one of the background electrolyte components. This was done by measuring the peak current generated at different deposition potentials for a water sample containing the previous concentration of gelatin used (Wang et al., 1993; Soko et al., 2002) and for a water sample with no gelatin added.

Deposition potential for gelatin vs. no gelatin (no stirring for both)

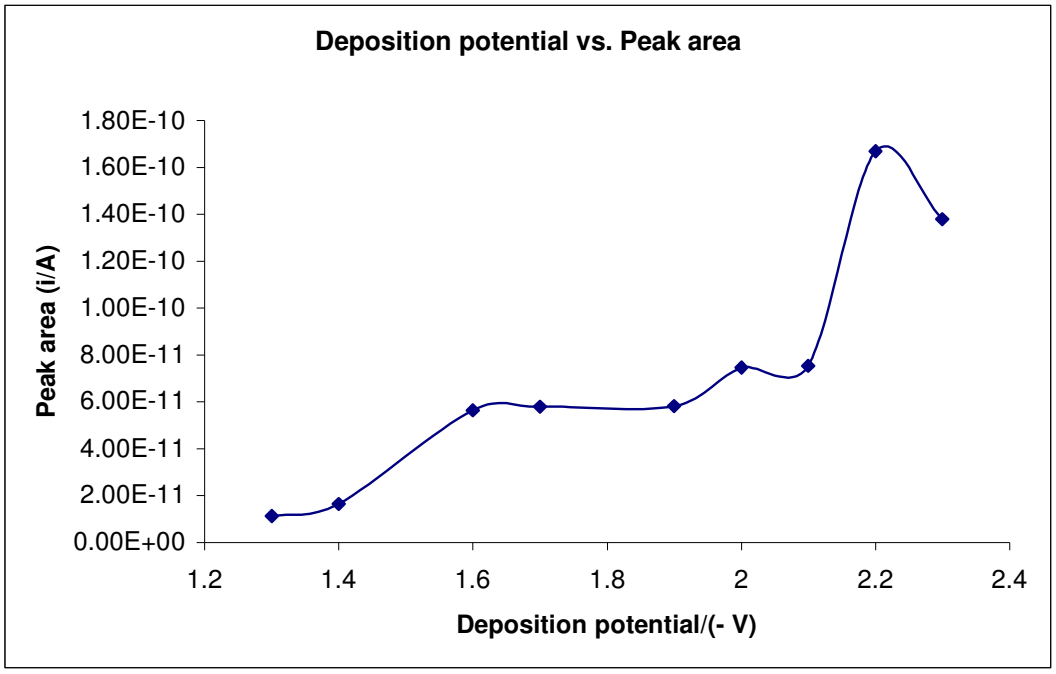

Figure 28a Deposition potential for solution with no gelatin

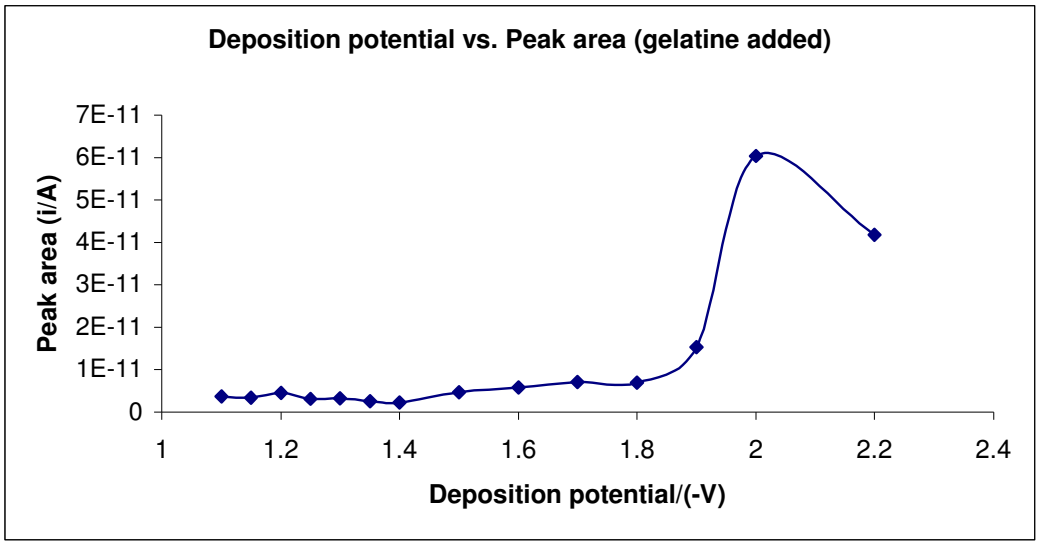

Figure 28b Deposition potential for solution with gelatin present

The two figures above (Figure 28a and b) show a difference in response of the peak area related to deposition potential when comparing gelatin vs. no gelatin in the solution. For solution containing gelatin (0.01% w/v) it is observed that the peak currents obtained applying different deposition potentials were considerably lower than for the non-gelatin solution. For the gelatin solution a maximum peak current occurred at –2.0V whereas for no gelatin the optimum deposition potential was –2.2V. This shows that although the gelatin was previously believed to enhance peak current, it also has a shifting effect on the optimum deposition potential (Crow, 1969).

The non-gelatin plot has more local maxima in the same region (deposition potential –1.3 – 2.0V) were the gelatin plot was almost flat. This demonstrates the use of gelatin in suppressing maxima and lowering the current-noise level. The improvement in peak current for the non-gelatin solution can however be seen and is one order of magnitude larger. This is significant for trace analysis of biological samples with complex matrixes.

AdSV Conditions

Stirring: 2000rpm

Equilibration time: 5s

Deposition time: 50s

pH: 7.70

Purge: 50s

Mn(II) concentration: 24µg l^{-1}

When comparing the two plots for gelatin vs. no gelatin added, it is clear there are both similarities and differences.

5.9.2 Ascorbic acid

It was decided to also check to see whether the concentration of ascorbic acid used was the optimal one prior to the optimization already performed.

AdSV Conditions

Purge time: 50s

Deposition time: 60s

Deposition potential: -2.0V

Stirring: 2000rpm

Gelatin: Non added

Equilibrium time: 5s

Spiked concentration of Mn (II): 24µg l^{-1}

Table 26 Ascorbic acid optimization

Ascorbic acid % (w/v)	Peak position E/V	Peak Area /A	RSD%
0.005	-1.466	7.64E-11	69.63
0.015	-1.466	8.81E-11	14.45
0.035	-1.466	1.15E-10	2.46
0.065	-1.461	1.14E-10	13.65
0.115	-1.461	1.11E-10	2.56

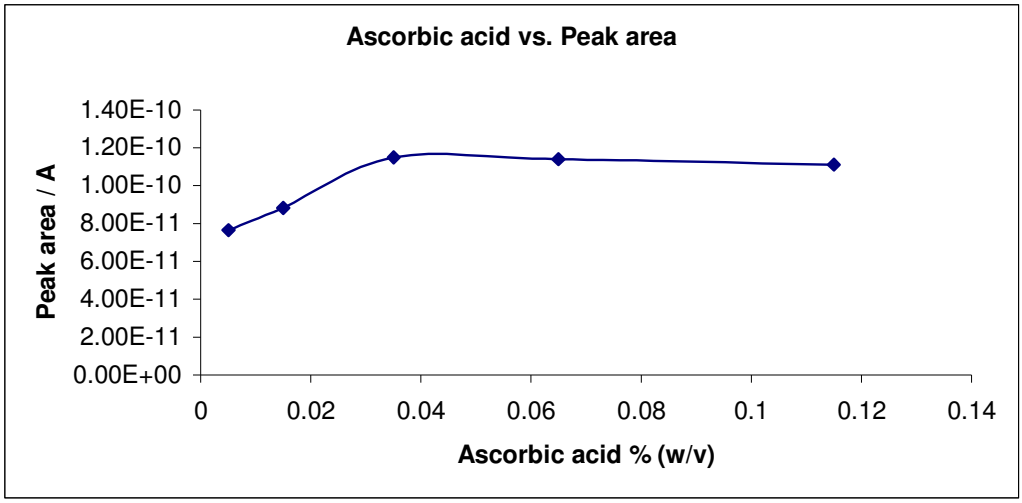

Figure 29 Ascorbic acid concentrations vs. peak current

A deionised water sample was run without the addition of any ascorbic acid as the blank to see whether a peak is generated. As predicted, there was a peak however its position was not the same as for the expected Mn(II) reduction potential. Once an initial concentration (%w/v) of ascorbic acid was added the peak shifted to the expected reduction potential. After the initial addition of 0.005% w/v, four subsequent additions were made each time measuring the peak current response. As the

concentration of ascorbic acid was increased, the peak area showed a direct proportionality with a maximum occurring at 0.035%. The slope thereafter does not change considerably thus it has been attained that for trace analysis of Mn(II) the minimum volume of ascorbic acid to be added to the solution is 0.035%. This also confirms that the amount of ascorbic acid previously used by Wang et al., (1993) is also suitable for the AdSV optimised determination method for Mn(II).

5.10 Summary of final instrumental parameters used

Summary of instrumental and analytical parameters used in the optimized AdSV procedure for the determination of Mn(II)

Table 27 Optimized analytical parameters used

Parameter	Value
Pretreatment	
N_2 (g) purge time(s)	50
Conditioning potential(V)	0
Duration(s)	0
Deposition Potential	-2.0
Duration(s)	45 /100
Equilibrium time(s)	5
Measurement	
Cell off after measurement	Yes
Modulation time	0.05
Interval time	0.25
Potentials	
Initial Potential(V)	-1.3
End Potential(V)	-1.6
Step Potential(V)	0.0051
Modulation amplitude(V)	0.02505
Standby potential(V)	0

CHAPTER 6

Size Exclusion Chromatography

6.1 Size Exclusion Chromatography

6.1.1 Instrumentation

A High Performance Liquid Chromatography (HPLC) System which consists of a Spectra System SCM 1000 liquid chromatograph, equipped with an AS3000 Auto sampler, UV6000 detector with ChromQuest software (all Premier Technologies /SMM instruments) and a 2112 Radirac fraction collector were used. Size exclusion chromatography was performed using a BIOSEP-SEC-S2000 column of 300x7.8mm (from Phenomenex).

6.1.2 Reagents

Preparation of Buffer (HPLC mobile phase) was carried out as follows: The mobile phase was prepared by weighing out 31.202g of NaH_2PO_4, 23.275g NaCl and 1.000g NaN_3 into a 2000ml volumetric flask. 1000ml of Millipore water was added and the solutes were dissolved by continuous stirring using a magnetic stirrer. The pH of the solution was adjusted to 7.00 using a saturated sodium hydroxide solution. Millipore water was then added up to the 2000ml mark on the volumetric flask and thoroughly mixed to yield a uniform solution. The solution was filtered through a 0.22μm filter paper using a pressure pump to remove any small solid particles and suspensions. This resulting phosphate buffer of pH 7.00 was then stored at 3-4^{o}C.

Protein standards also had to be prepared. Five different protein standards were prepared as follows each in a 1.5ml tube in a phosphate buffer solution as described above.

0.100g of Albumin (Human), alpha$_2$-macroglobulin, transferring, alpha & beta globulin and gamma globulin were each dissolved in a 1.0ml buffer solution.

Table 28 Purity of reagents used for SEC.

Materials Used	Purity	Supplier
Albumin (Human)	99%	Sigma
Ethanolamide	98%	Sigma
Alpha & Beta Globulin	99%	Sigma
Gamma Globulin	99%	Sigma
Manganese Chloride	>95%	BDH
Alpha$_2$-Macroglobulin	98%	Sigma
Sodium azide (NaN_3)	99.9%	BDH
Sodium chloride	99.9%	BDH
Sodium-Orthophosphate-Dihydrogen	99.9%	BDH
Transferrin	98%	Sigma
Deionised water	Resistivity 18.2MΩ.cm, 0.22µm filtered.	

6.1.3 Procedure

Blood was centrifuged at 4^{o}C at 2000rpm for 10 minutes. This step was carried out to isolate the serum from the whole blood. The upper yellowish layer is the easily identifiable serum. This serum is then aspirated from the whole blood into a sterile clean vial and sealed until further use.

Sampling techniques

Whole blood is centrifuged at 4°C for 10 minutes at 2000rpm. This step isolates serum from the whole blood. Serum is then aspirated from the whole blood into a clean vial.

Sample Preparation

1. 2ml of serum sample was diluted with 2ml of phosphate buffer in a clean vial.
2. 1ml of the above mixture was then spiked with 1ml of 4mg l^{-1} $MnCl_2$.

Once the serum was separated from the whole blood, 2ml of this serum sample and 2ml of the phosphate buffer were pipetted into a clean vial additively making up a dilution of 1:1 of the two liquids. 1ml of the above mixture was then spiked with $MnCl_2$ to give a concentration of 2500µg l^{-1} in a 1.5ml sterile clean vial. The mixture was then filtered using a 1.5ml disposable plastic syringe and a 0.22µm filter into a 1.5ml auto-sampler vial, which was then run through the size exclusion column in a High performance liquid chromatography instrument for analysis and separation of the desired fractions. A 30µl sample was injection into the column, and this was followed by elution of the column with phosphate buffer at a flow rate of 0.25ml/min to enable efficient separation and collection of pure fractions. Elution was performed at room temperature and proteins were detected by monitoring column flow at 280nm. Six 1ml fractions were collected and stored in sealed plastic vials and stored at 3-4^{o}C.

<u>The following protein concentrations were prepared in a phosphate buffer solution:</u>

- 0.01g/ml albumin (human)
- 0.01g/ml γ-globulin
- 0.01g/ml transferin

- 0.01g/ml trypsin inhibitor
- 0.005g/ml α_2-macroglobulin
- 0.025g/ml globulins (predominantly α & β)
- 0.002g/ml dextran
- 0.004g/ml β- amylase
- 0.01g /ml bovine serum albumin
- 0.003g/ml carbonic anhydrase
- 0.002g/ml cytochrome c
- 0.005g/ml alcohol dehydrogenase

Preparation of Test (serum) specimens:

(1) Serum samples were diluted 1:1 with phosphate buffer.

(2) Spiked with different concentrations of Manganese (Mn).

(3) Filtered through a 0.22µm filter

(4) A volume of 30µl was loaded into the column to obtain protein profiles.

(5) The total run time was 70min.

(6) 1ml fractions were collected.

Instrumental Parameters (Size Exclusion)

Column: size exclusion

Flow rate: 0.25min/ml

Run time: 70minutes

Wavelength: 280nm

Mobile phase: 100% Phosphate buffer

Injection volume: 30µl or 60µl

Fraction volume: 1ml

Only 6 fractions contain protein fractions of interest = 6ml

Serum samples

Un - Serum = Unspiked serum

Un – Serum 1 : Serum diluted 1:1 with phosphate buffer.

Un – Serum 2 : One of the serum fractions from the HPLC after injection of 60µl of Un – Serum 1.

Sp – Serum = Serum spiked with $MnCl_2$

Sp - Serum 1 : 1ml of serum spiked with 1ml of 4000µg l^{-1} $MnCl_2$

Sp - Serum 2 : One of the serum fractions from the HPLC after injection of 60µl of Sp – Serum 1.

Sp – Serum 3 : One of the serum fractions from the HPLC after injection of 30µl of Sp - Serum 1.

CHAPTER 7

Applications of extraction and determination using optimised parameters

7.1 Extraction of aged blood serum

In order to investigate the effect time of Mn(II) in the sample(donor phase) has on extraction efficiency due to the possible binding of free Mn(II) to proteins within the blood, the blood serum was spiked to contain 50μg l^{-1} adjusted to pH of 3 and left to age for: 0, 24, 48 and 72 hours in a refrigerator at 3-4^{o}C. The results below in table 29 show the effect of aging on the extraction efficiency of Mn(II) from whole blood serum.

Table 29 Average extraction efficiency and serum aging time

Time /hours	Extract #	Average extraction efficiency /%	RSD /%
0	1	8.20	17.39
	2	10.5	
24	1	2.82	12.55
	2	2.36	
48	1	1.40	34.39
	2	2.30	
72	1	0.93	16.16
	2	1.17	

From table 29 above it is observed that the longer whole blood serum is aged (refrigerated), the lower the extraction efficiency becomes. As time progresses it is also apparent that the difference in extraction efficiencies decreases. As the time of the spiked serum sample increases, the amount of free unbound Mn(II) decreases as it has more time to interact with the complex protein matrix of the serum and consequently becomes bound to these proteins. As time progresses the supported liquid membrane extraction of the unbound or free Mn(II) thus decreases. With time (e.g. 48-72 hours) this decrease of Mn(II) becomes less apparent due to the maximum available sites for Mn(II) already being occupied (linearity is reached).

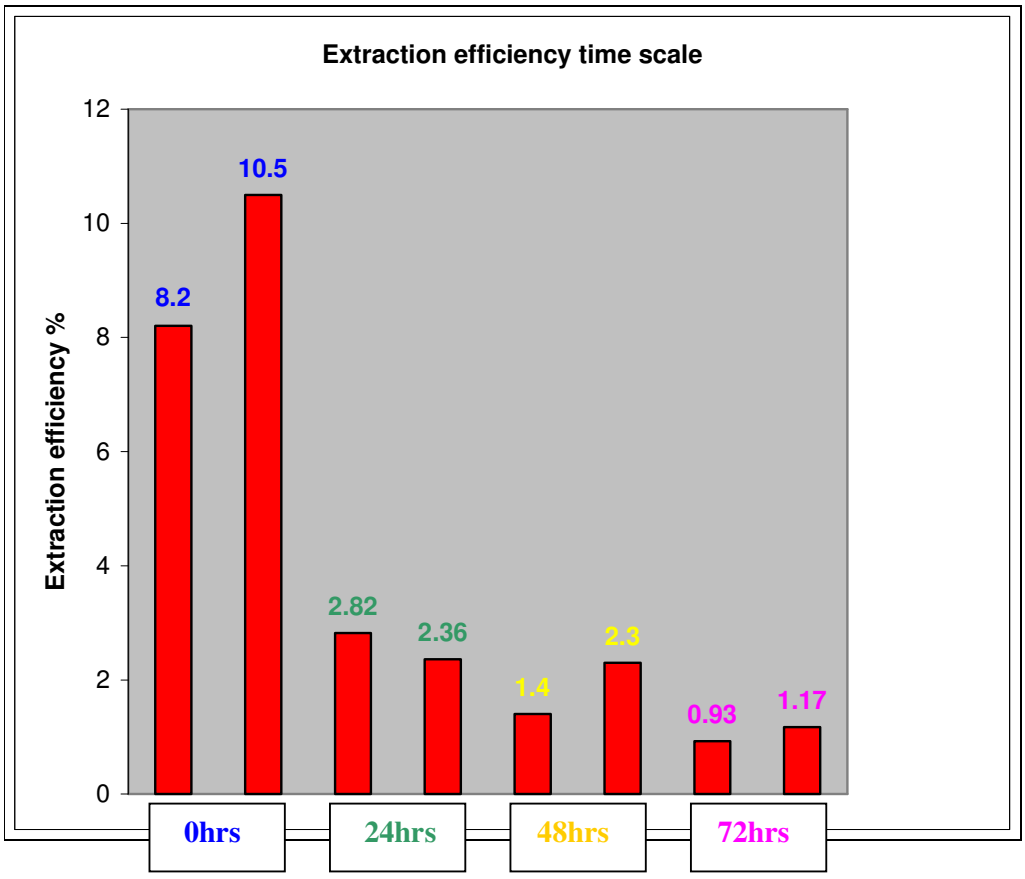

Figure 30 Average extraction efficiency and time relationship for Mn(II) in blood serum

7.1.1 Extraction conditions

Conditions

For all aged serum extracts the same experimental procedure was followed so as to minimize variation to obtain consistent results for all aged extracts. The required amount of whole unfractionated blood serum was diluted 35x and spiked to contain 50µg l^{-1} and pH adjusted to 3.00 according to experimental procedure in chapter three. Sequential extraction was performed on all aged samples in replicates of three in 30minute intervals. During the aging process, the spiked whole blood serum was stored in a sealed beaker in a refrigerator at 3-4^{o}C. All voltammetric parameters varied from sample to sample were specified in each case.

7.1.2 Determination of Mn(II) in aged serum

Determination of Mn(II) in 0-hours aged serum

Extract 1

AdSV conditions

Purge time: 50s (5s for between each standard addition)

Deposition Potential: -2.0V

Deposition time: 60s

Equilibrium time: 5s

Adjusted pH: 7.93

Cell volumetric composition:

Sample: 1.0ml

Deionised H_2O: 3.70ml

L-Ascorbic acid: 0.075ml

Borax: 0.250ml

NaOH: 0.035ml

Total cell volume: 5.06ml

Extract 1

Table 30a 0-hours aged serum

Mn(II) concentration added /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	3.31E-9	-1.456	2.56
10	5.32E-9	-1.456	0.266
20	6.60E-9	-1.451	5.57
30	9.18E-9	-1.451	1.39

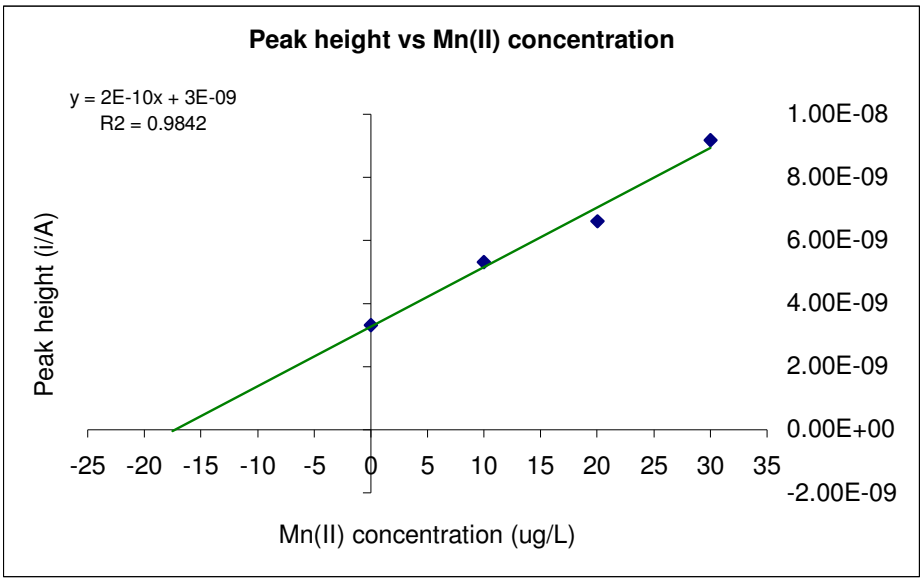

Figure 31a 0-hours aged serum.

From the intercept of 17.5 a Mn(II) concentration of 6.13mg l^{-1} was obtained.

Extract 2

Table 30b 0-hour aged serum

Mn(II) concentration added /µg l^{-1}	Peak height /A	Peak position E/V	RSD%
0	4.37E-9	-1.451	6.24
10	6.32E-9	-1.456	1.79
20	8.11E-9	-1.451	1.05
40	1.21E-8	-1.451	1.17

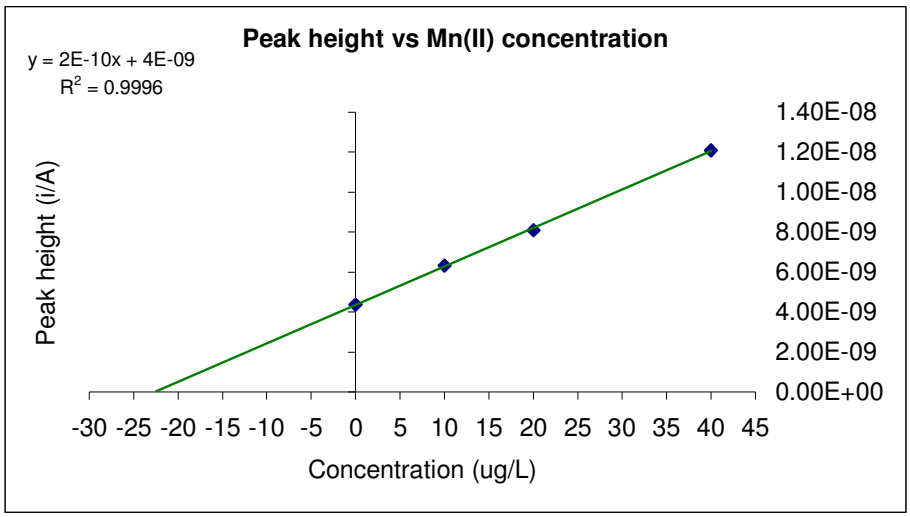

Figure 31b 0-hours aged serum

The concentration of Mn(II) in the above extract was thus determined to be 7.88mg l^{-1}

Determination of Mn(II) in 24-hour aged blood serum

Extract 1

Table 31a 24-hour aged serum

Mn(II) concentration added/µg l^{-1}	Peak height /A	Peak position E/V	RSD%
0	3.42E-9	-1.456	1.65
10	6.74E-9	-1.456	2.10
20	1.07E-8	-1.456	5.29
30	1.35E-8	-1.456	2.10

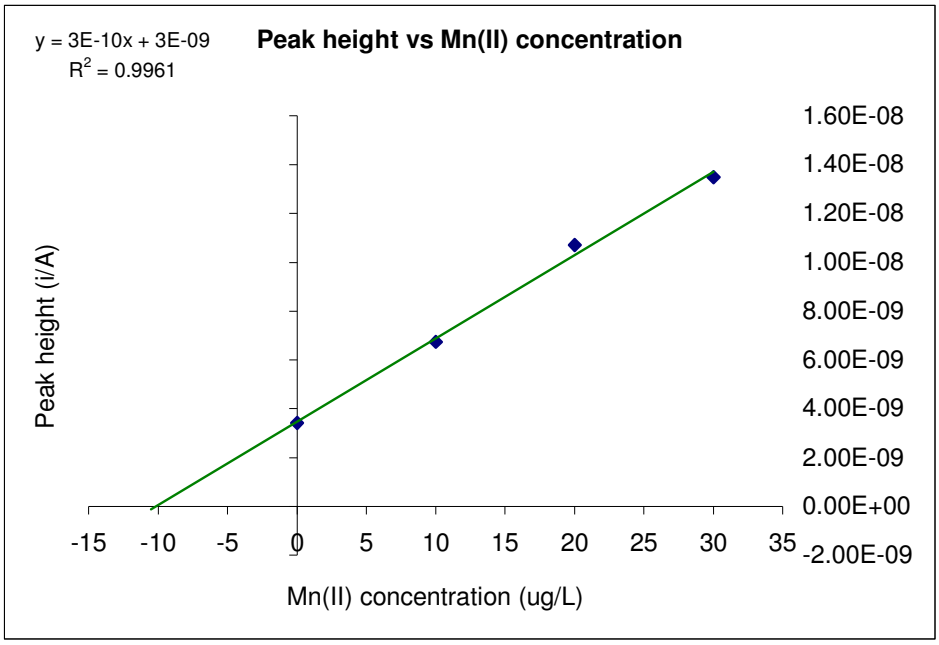

Figure 32a 24-hour aged serum

A Mn(II) concentration of 1.771mg l^{-1} was found which, is lower than the previous O-hours aged serum and is thus consistent with what is expected as the age of the sample increases.

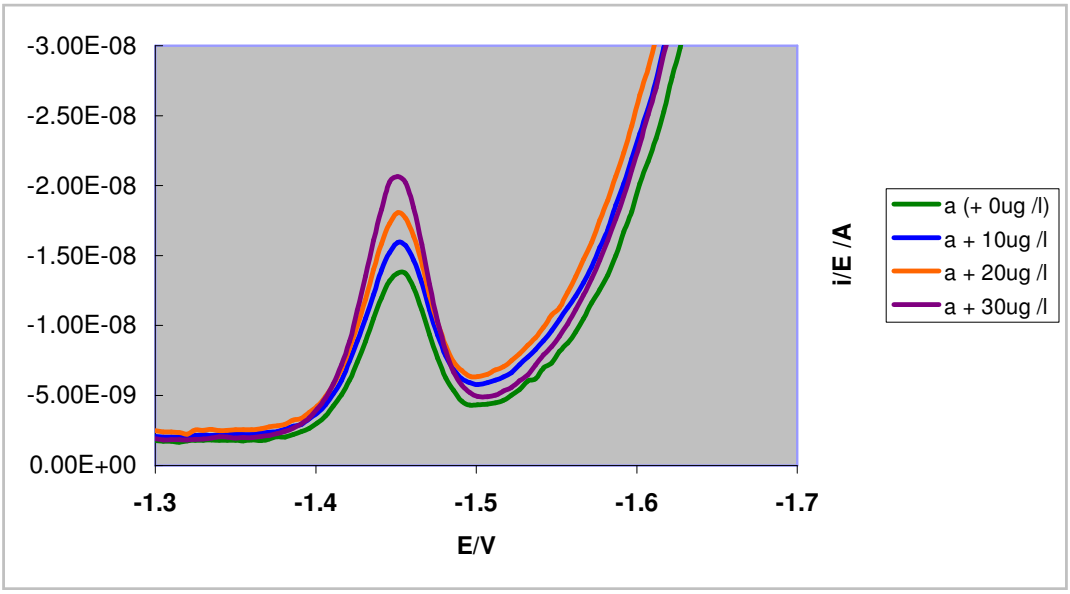

Figure 32b Typical overlaid voltammograms showing good peak (kinetic) reversibility from the determination of Mn(II) in a 24hour aged blood serum extract.

Extract 2

Table 31b 24-hours aged serum

Mn(II) concentration added /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD %**
0	1.11E-9	-1.446	5.76
10	3.05E-9	-1.446	13.45
20	5.76E-9	-1.451	7.86
30	7.28E-9	-1.451	0.389

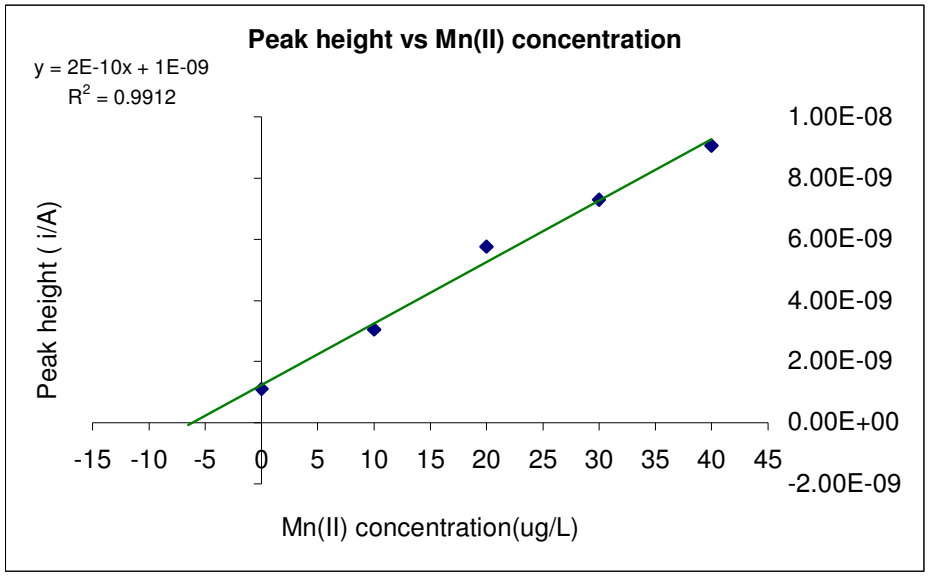

Figure 32c 24-hours aged serum

A Mn(II) concentration of 2.121mg l^{-1} was determined.

Determination of Mn(II) in 48-hour Aged blood serum

Extract 1

Table 32a 48-hour aged serum

Mn(II) concentration added /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	2.59E-9	-1.456	7.64
10	7.88E-9	-1.451	13.81
30	1.73E-8	-1.451	2.87

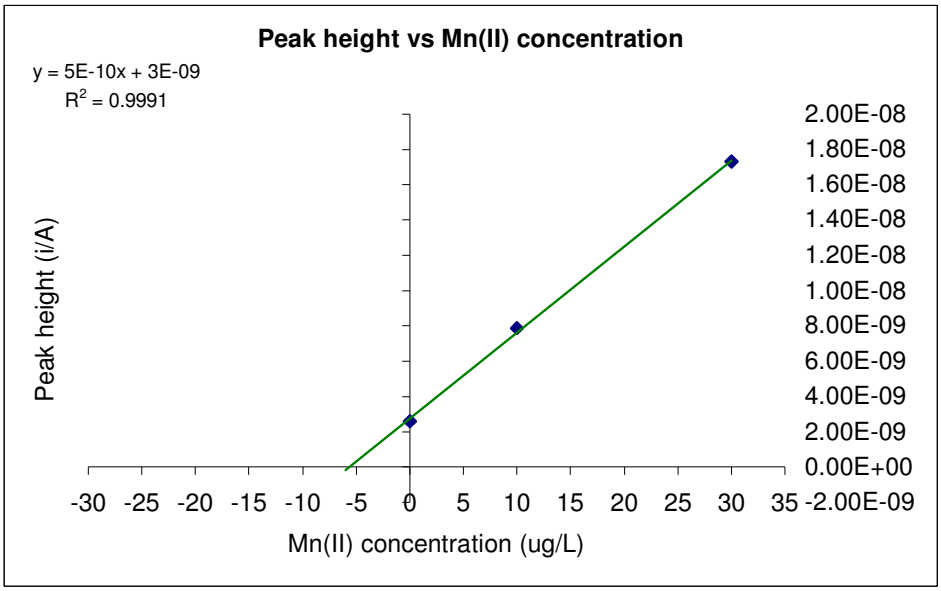

Figure 33a 48-hour aged serum

Concentration of Mn(II) in extract 1 is 1.050mg l^{-1} .

Extract 2

Table 32b 48-hour aged serum

Mn(II) concentration added /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	3.97E-9	-1.451	23.9
10	8.06E-9	-1.451	5.61
30	1.47E-8	-1.451	9.62
40	1.88E-8	-1.451	3.76

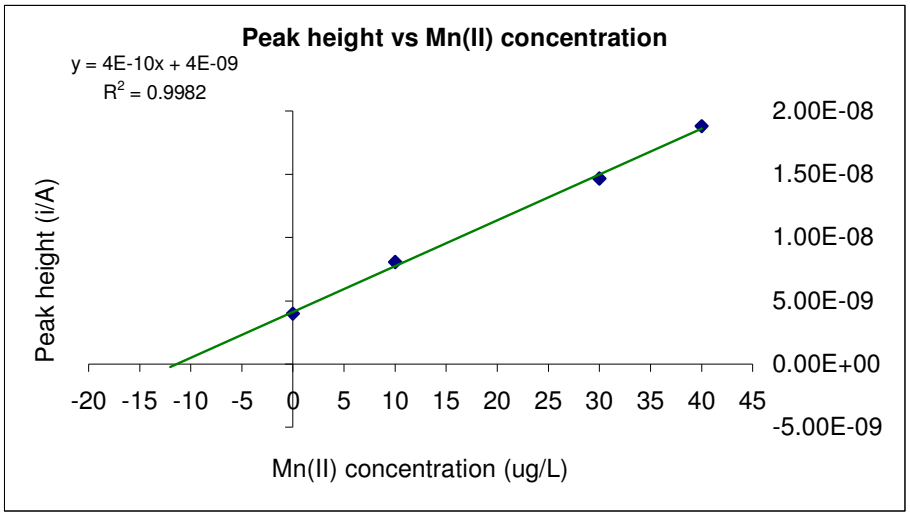

Figure 33b 48-hour aged serum

A Mn(II) concentration of 1.750mg l^{-1} was determined in the above extract.

Determination of Mn(II) in 72-hour Aged blood serum

Extract 1

Table 33a 72-hour aged serum

Mn(II) concentration added /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	1.15E-9	-1.476	8.03
10	6.34E-9	-1.471	5.35
20	1.05E-8	-1.471	2.02
30	1.42E-8	-1.471	31.87

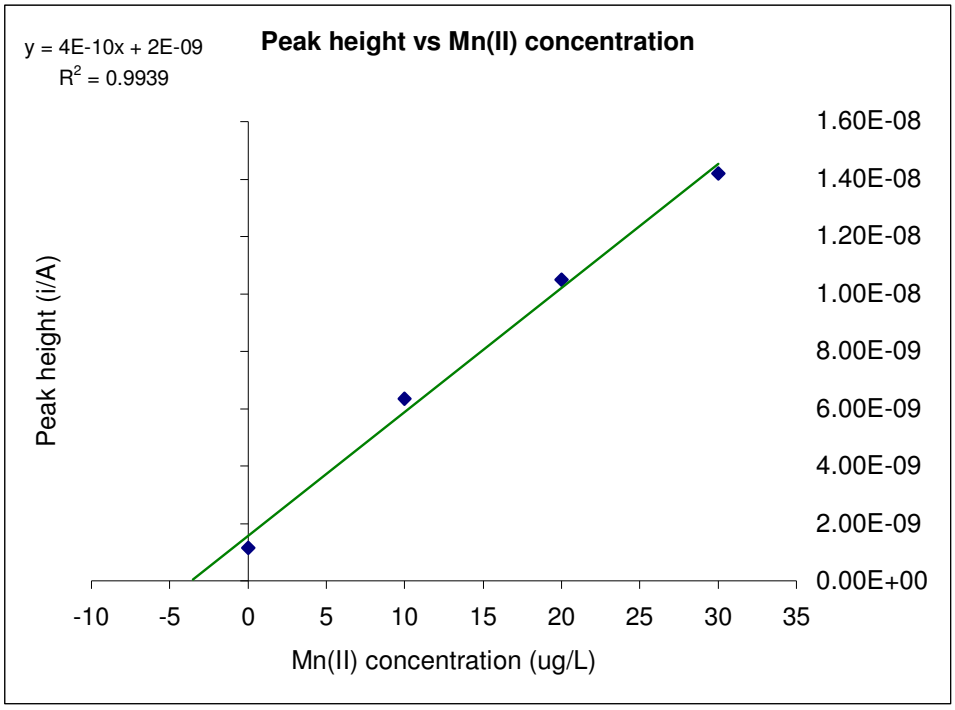

Figure 34a 72-hour aged serum

A Mn(II) concentration of 0.700mg l^{-1} was thus determined for extract 1 above.

Extract 2

Table 33b 72-hour aged serum

Mn(II) concentration added /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	3.78E-9	-1.451	4.49
10	9.42E-9	-1.451	6.01
30	2.42E-8	-1.451	3.06
40	3.01E-8	-1.451	33.4

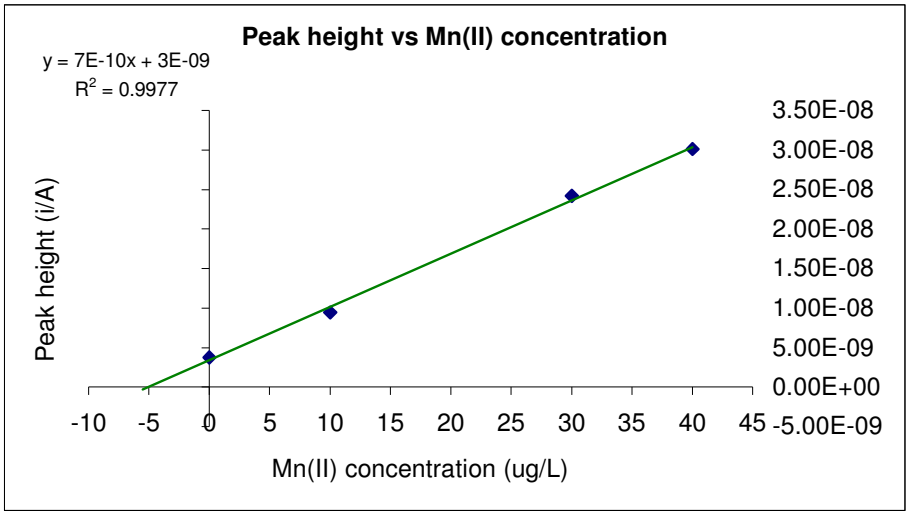

Figure 34b 72-hour aged serum

A Mn(II) concentration of 0.875mg l^{-1} was determined.

7.1.3 Blank recovery

This was performed using deionised water and the background electrolytes only before and after each day's analysis in order to quantify any residual Mn(II) contamination. The standard addition curve plotted below is obtained from the average current values from each respective standard addition. The residual contamination of Mn(II) was found to be almost negligible.

Table 34 Blank recovery for aged serum Mn(II) determination

Mn(II) concentration added /µg l^{-1}	Peak height /A	Peak position E/V	RSD%
0	7.42E-11	-1.451	9.24
10	2.60E-9	-1.451	1.09
20	5.47E-9	-1.451	4.14
30	7.88E-9	-1.451	0.359

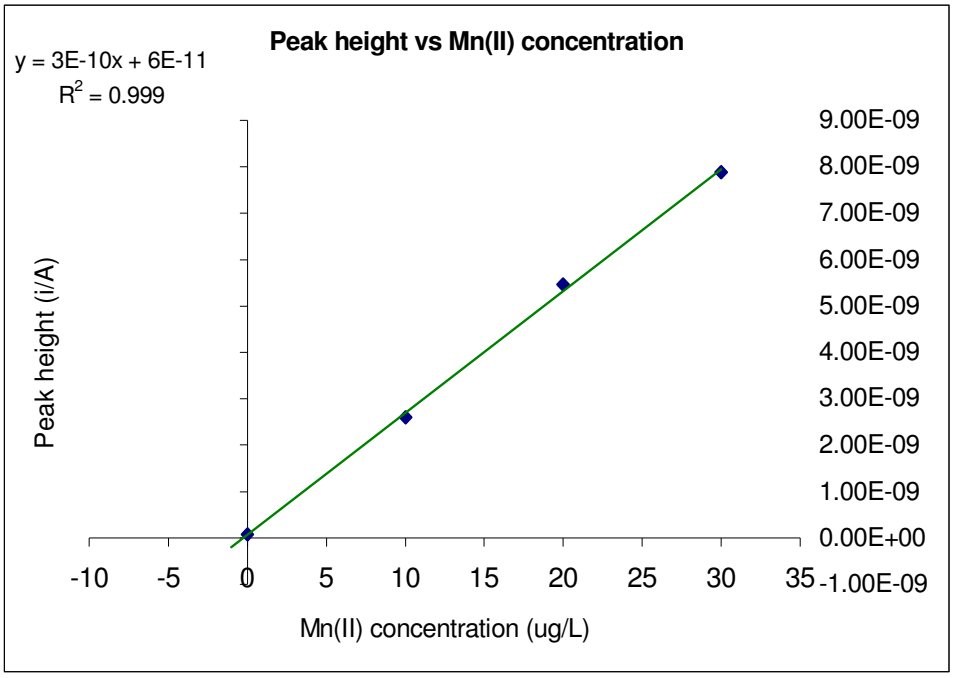

Figure 35 Blank recovery standard addition plot

A concentration of 0.2µg l^{-1} of Mn(II) shows that the contribution of Mn(II) to each extract from residual contamination was almost negligible. This accounts for between 2-21% of the total Mn(II) with respect to each extract.

7.2 Blood serum fractions (extraction and sample preparation)

Blood serum fractions of human blood serum were collected using an HPLC-size exclusion column. To investigate the feasibility of the study using fractions, it first was determined whether the concentrations the fractions were spiked with before being run through the column were sufficiently high after dilution through the column and collection, to be detected once the samples have been extracted using SLM (probe) and then using AdSV for the Mn(II) determination method. So this is just an initial study to extract a combination of 6 fractions combined and spiked with 2000µg l^{-1} and 800µg l^{-1} respectively prior to being run through the column. 2ml of the combined fraction was diluted 25x in order to eliminate as much matrix as possible and increase the volume so as to have a sufficient amount for a 2x sequential extraction. A donor volume of 4ml was used and acceptor volume was 1ml as used before. The donor volume was decreased from 5ml to 4ml to prevent over dilution of the fractionated serum. Extraction was done twice sequentially (only replacing the donor solution each time) in 30minute sessions. Three membrane probes were used for each extraction.

7.2.1 Extraction of 800µg l^{-1} Mn(II) blood serum fraction

Table 35 Summary of 800µg l^{-1} Mn(II) spiked fraction extraction and determination.

Extract #	Average extraction efficiency %	Mn(II) concentration /mg l^{-1}
1	23.4	1.500

2	11.7	0.750
3	19.6	1.253

An average Standard deviation of 0.382 was found for the three Mn(II) concentrations determined in table 35 above. A RSD of 32.7 % was achieved.

7.2.2 Extraction of 2000µg l^{-1} Mn(II) blood serum fraction

Table 36 Summary of 2000µg l^{-1} Mn(II) spiked fraction extraction and determination

Extract #	Average extraction efficiency %	Mn(II) concentration /mg l^{-1}
1	6.9	1.100
2	8.8	1.400
3	9.1	1.450

An average Standard deviation of 0.189 was found for the four Mn(II) concentrations determined in table 36 above. A RSD of 14.4 % was achieved.

Extraction efficiency

The extraction efficiencies in table 35 are higher than those in table 36, however the extraction efficiencies for the 2000µg l^{-1} Mn(II) blood serum fraction show good reproducibility (precision). The 800µg l^{-1} Mn(II) spiked fraction produced far lower extraction efficiencies, however these showed poor reproducibility as seen by the RSD and average standard deviation values for each respective spiked blood serum fraction.

7.3 AdSV determination for blood fractions

7.3.1 Determination of 800µg l^{-1} Mn(II) blood serum fraction

The method used for the 2000µg l^{-1} fractions above was repeated for the 800µg l^{-1} fraction so as to keep all parameters constant.

Extract 1

Table 37a 800µg l^{-1} Mn(II) blood serum fraction

Mn(II) concentration /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	3.13E-8	-1.451	2.26
30	3.67E-8	-1.451	1.54
60	3.92E-8	-1.451	1.80
100	4.51E-8	-1.451	3.14
140	5.77E-8	-1.451	2.45
180	6.59E-8	-1.451	2.58

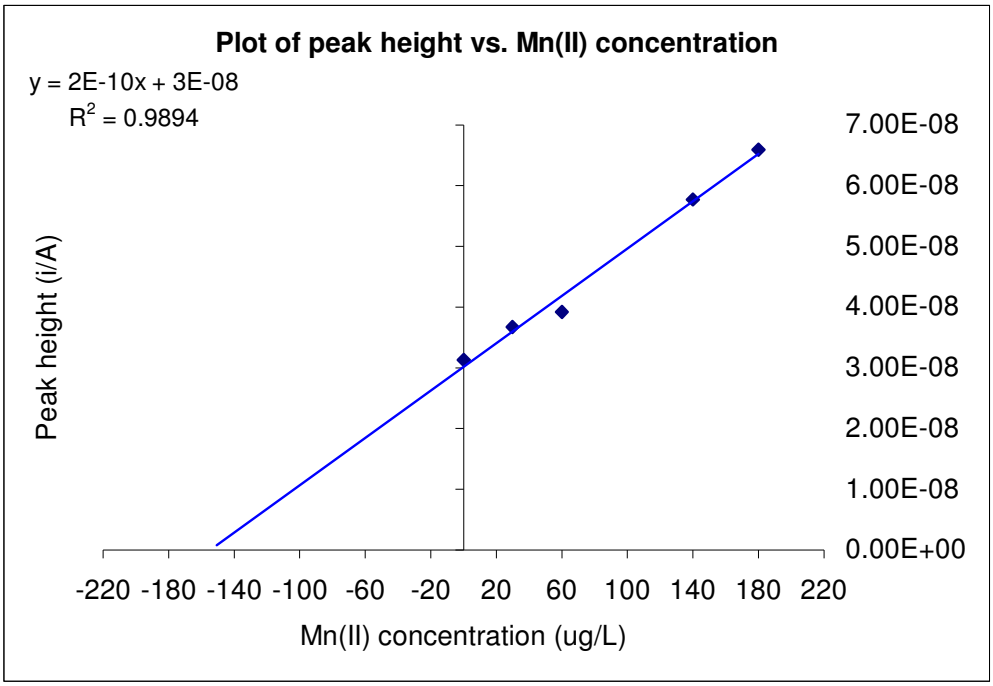

Figure 36a 800µg l^{-1} Mn(II) blood serum fraction

The concentration of Mn(II) in extract 1 after dilution was 1.500mg l^{-1}.

Extract 2

Table 37b 800µg l^{-1} Mn(II) blood serum fraction.

Mn(II) concentration /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	1.50E-8	-1.446	6.60
20	2.88E-8	-1.446	0.982
40	3.88E-8	-1.446	2.94
60	4.69E-8	-1.446	3.02

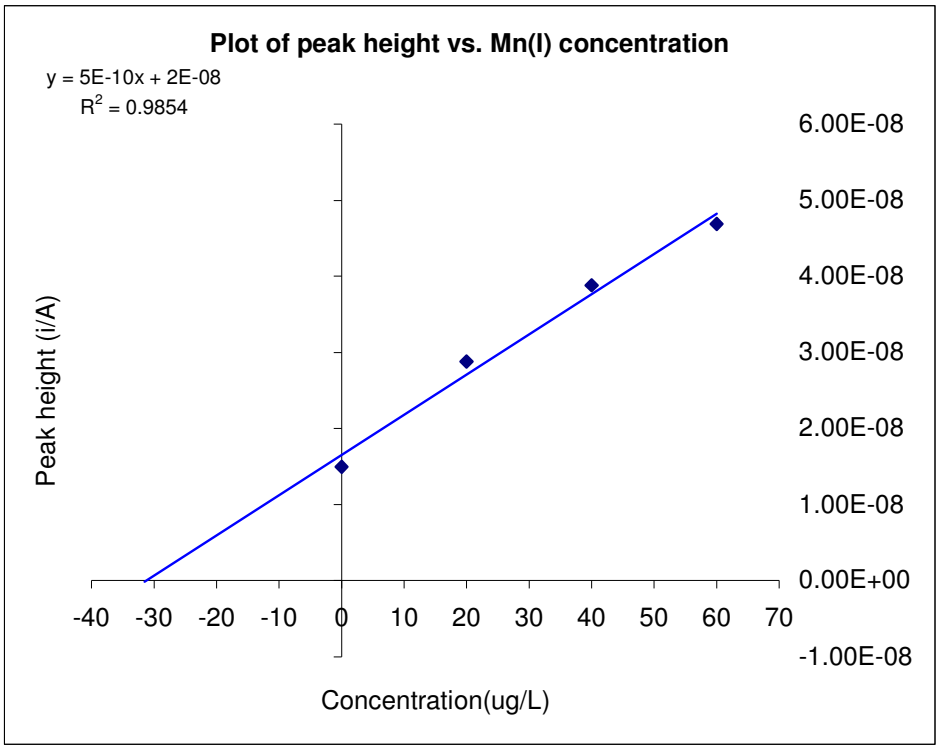

Figure 36b 800µg l^{-1} Mn(II) blood serum fraction.

The concentration of Mn(II) was determined to be 0.750mg l^{-1}

Extract 3

Table 37c 800µg l^{-1} Mn(II) blood serum fraction.

Mn(II) concentration /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	2.20E-8	-1.441	3.52
10	2.36E-8	-1.446	0.599
15	3.35E-8	-1.446	24.25
30	3.47E-8	-1.446	0.001
60	4.27E-8	-1.446	1.99
90	5.00E-8	-1.446	1.31

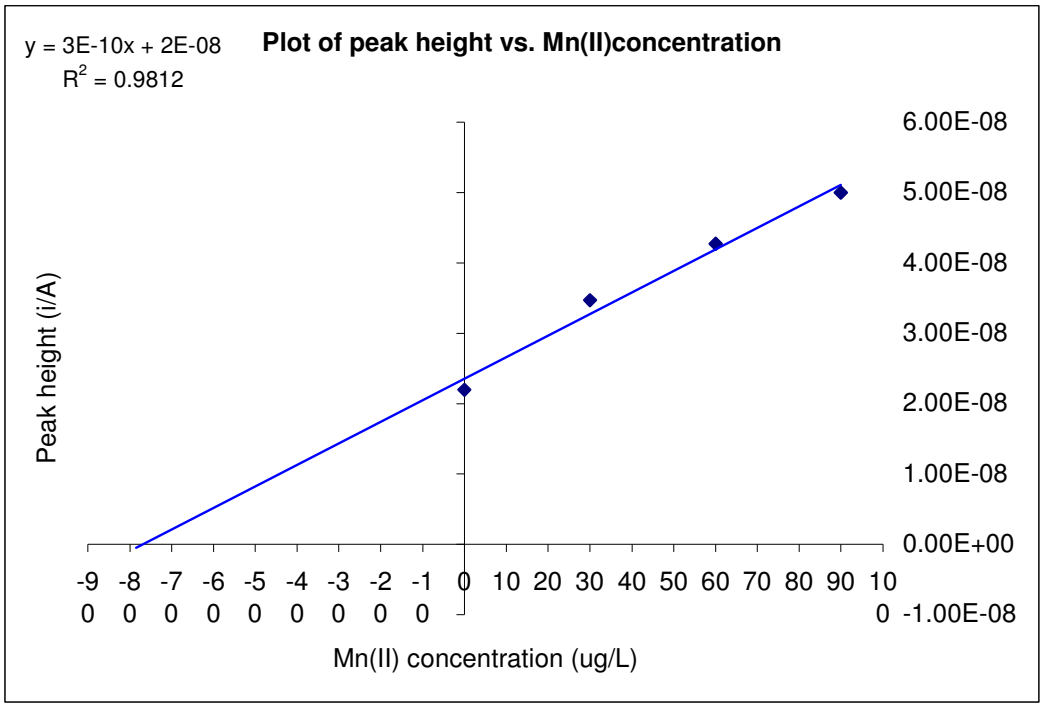

Figure 36c 800µg l^{-1} Mn(II) blood serum fraction.

From an intercept of 75 the concentration of Mn(II) was 1.253mg l^{-1}.

Blank recovery

Table 38 Blank recovery for 800µg l^{-1} Mn(II) blood serum fractions

Mn(II) concentration /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	2.47E-10	-1.491	4.01
10	5.04E-9	-1.486	4.21
20	9.56E-9	-1.486	0.444
30	1.43E-8	-1.486	3.96

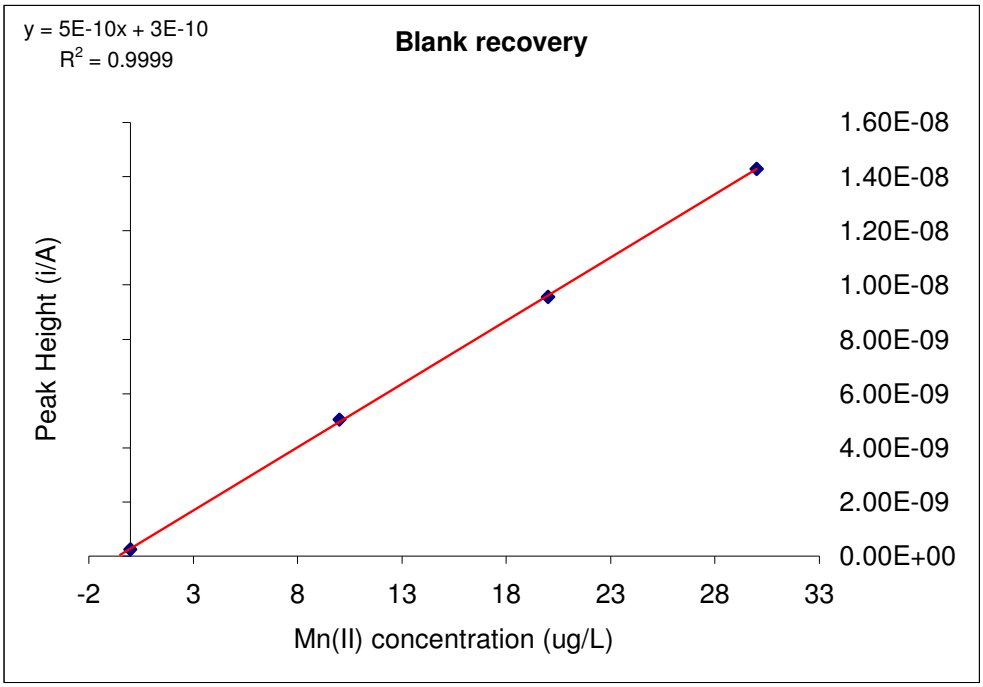

Figure 37 Blank recovery for 800µg l^{-1} Mn(II) blood serum fraction

Mn(II) concentration of 0.6µg l^{-1} was determined for the blank recovery.

7.3.2 Determination of 2000µg l^{-1} Mn(II) blood serum fraction

Extract 1

Table 39a Extract 1 of 2000µg l^{-1} Mn(II) blood serum fraction

Mn(II) concentration /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	6.29E-8	-1.446	2.01
50	7.42E-8	-1.446	5.39
90	8.43E-8	-1.446	0.839
130	9.89E-8	-1.446	0.143
170	1.06E-7	-1.446	1.33

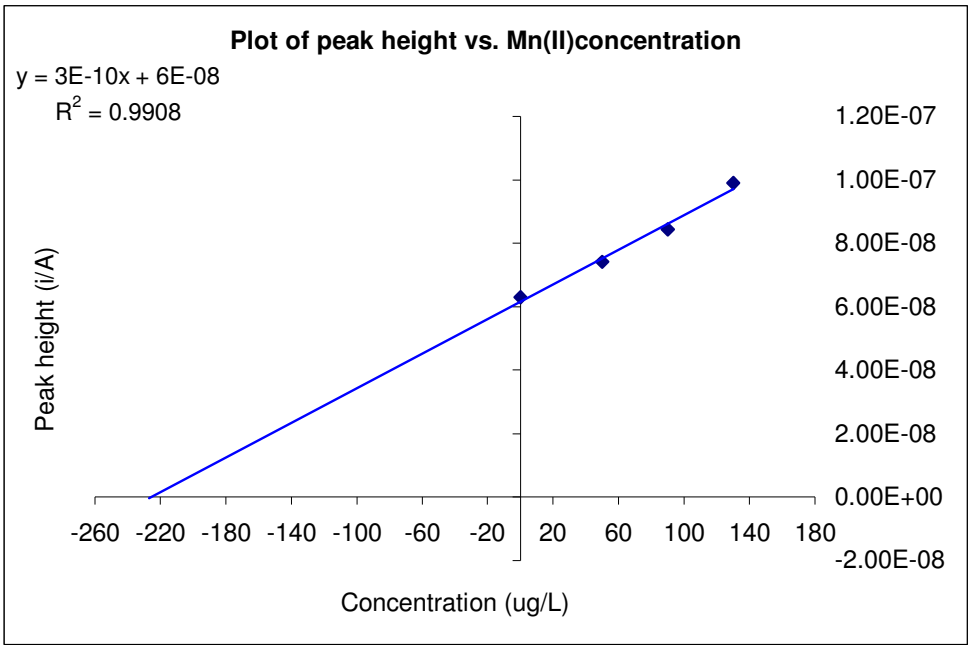

Figure 38a Extract 1 of 2000µg l^{-1} Mn(II) blood serum fraction

A concentration of Mn(II) of 1.100mg l^{-1} was found for the above extract.

Extract 2

Table 39b Extract 2 of 2000µg l^{-1} Mn(II) blood serum fraction

Mn(II) concentration /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	7.34E-8	-1.446	3.47
40	8.37E-8	-1.446	5.07
80	8.79E-8	-1.446	3.77
120	1.05E-7	-1.451	9.43
160	1.14E-7	-1.451	12.41

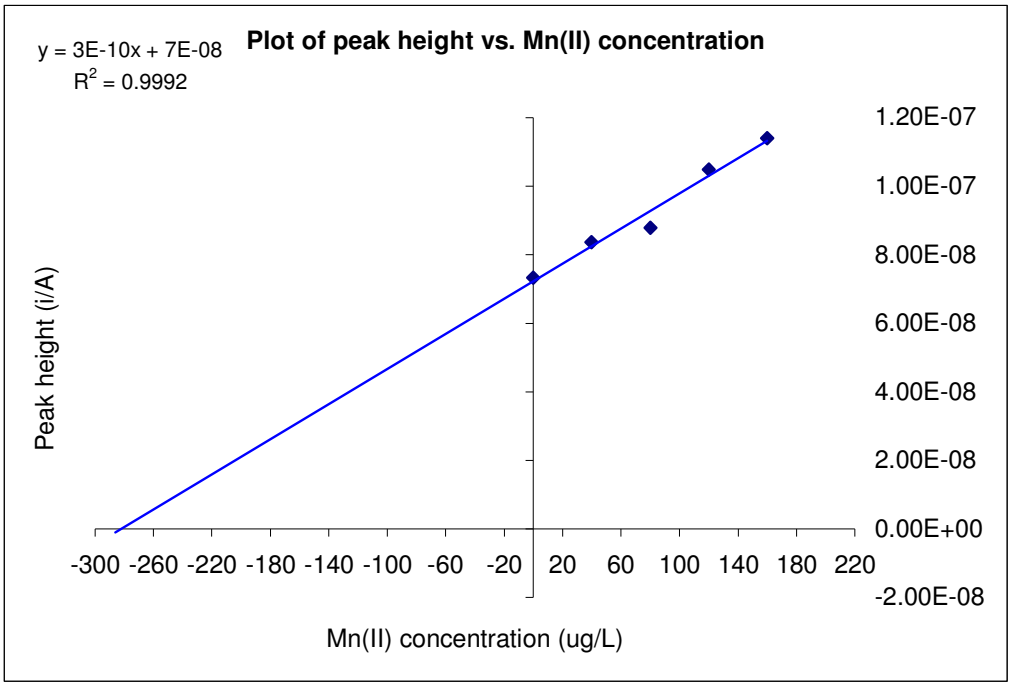

Figure 38b Extract 2 of 2000µg l^{-1} Mn(II) blood serum fraction

An intercept of 280 therefore gives the concentration is 1.400mg l^{-1}.

Extract 3

Table 39c Extract 3 of 2000µg l^{-1} Mn(II) blood serum fraction

Mn(II) concentration /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	2.30E-8	-1.446	1.23
20	2.75E-8	-1.446	2.06
40	3.14E-8	-1.446	0.901
60	3.36E-8	-1.446	1.26
80	3.72E-8	-1.446	1.14
100	3.98E-8	-1.446	0.355

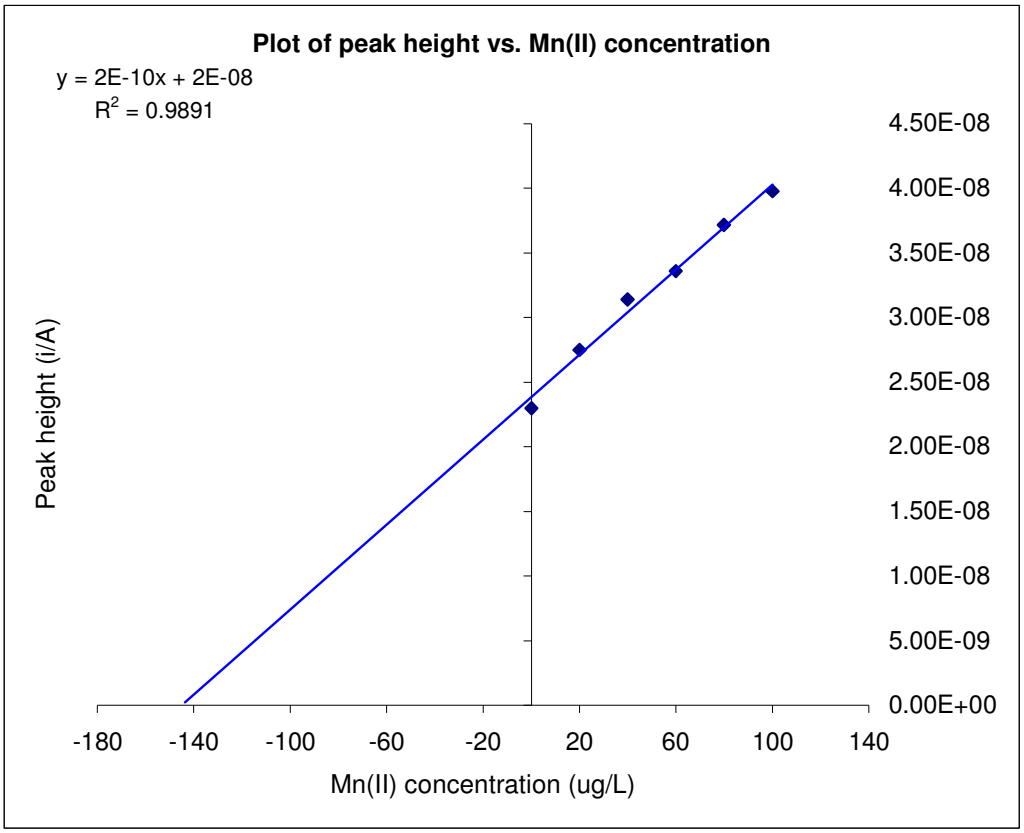

Figure 38c Extract 3 of 2000µg l^{-1} Mn(II) blood serum fraction

From the above standard addition curve the concentration of Mn(II) is 1.450mg l^{-1}.

Blank recovery

Table 40 Blank recovery for 2000µg l^{-1} Mn(II) blood serum fractions

Mn(II) concentration /µg l^{-1}	**Peak height /A**	**Peak position E/V**	**RSD%**
0	4.08E-10	-1.501	38.44
10	2.06E-9	-1.496	8.24
20	4.07E-9	-1.496	7.29
30	5.42E-9	-1.496	3.13
40	5.46E-9	-1.451	6.21

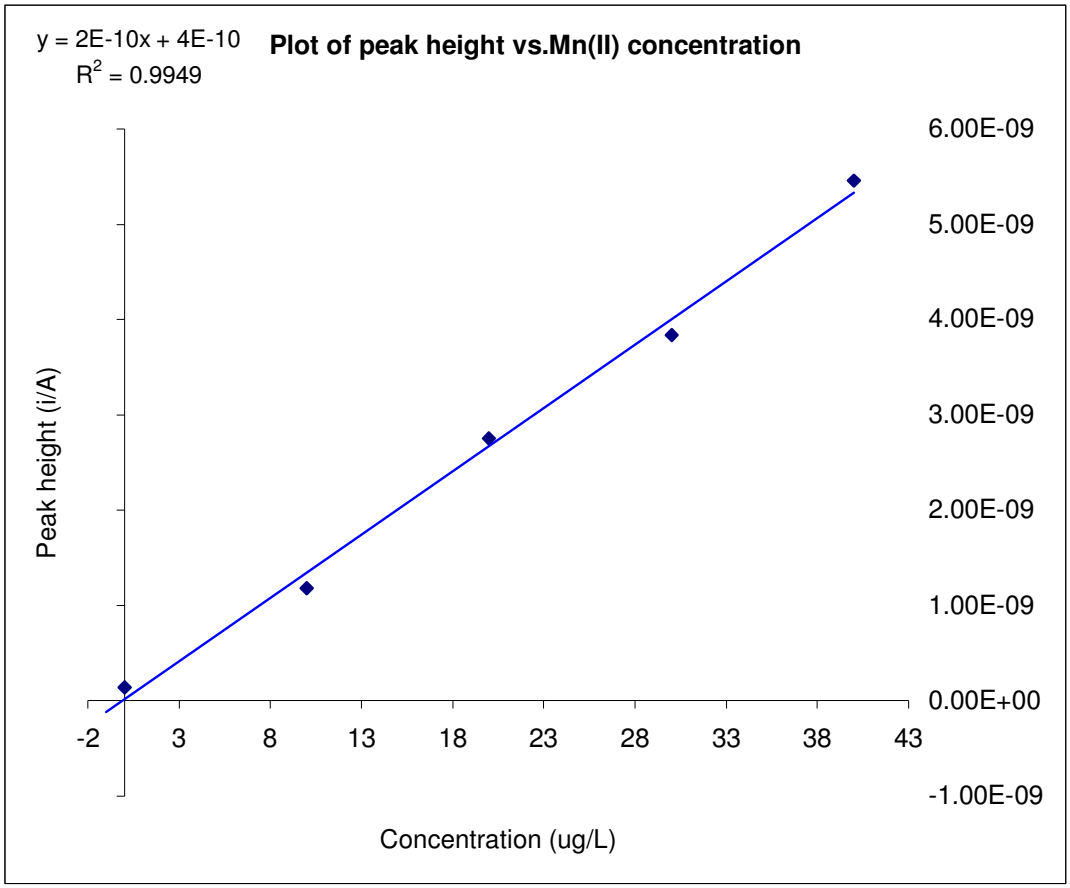

Figure 39 Blank recovery for 2000µg l^{-1} Mn(II) blood serum fractions

A blank Mn(II) concentration of 2.0µg l^{-1} was determined from the above standard addition plot with a good fit (R^2 is 0.9949) and thus was subtracted from each respective extracts Mn(II) determined.

7.4 Manganese totals

Total Mn was only determined for the 800µg l^{-1} fraction. The total concentration was determined using an ICP-OES Spectro instrument. The total Mn determined by ICP-OES was 0.726mg l^{-1} with a standard deviation of 0.198.

CHAPTER 8

Conclusion and final discussion

8.1 SLM extraction methods and optimisation

An extensive evaluation of Supported Liquid Membrane extraction methodology and parameter optimisation has been carried out and proved to be successful in other applied biological speciation applications. The optimisation was performed in stages by which water was initially used as the sample matrix type then in order to mimic a more complexed matrix as that found typically in biological fluids of the human body, fresh fat free milk was used.

The effect of various matrix parameters was looked at such as time of Mn(II) spiked in the matrix, concentration of spiked sample, and a number of other extraction parameters. The new extraction/preconcerntration method was used for sample pre-treatment in the preconcerntration and extraction of Mn(II) first in whole unfractionated blood serum and secondly in two different spiked concentrations of fractionated blood serum all of which was obtained freshly from the NCOH (National Centre for Occupational Health).

The results from the voltammetric analysis of water showed that the procedure is reproducible and effective for the trace analysis of Mn(II) in water. Furthermore, by

comparing the extraction results of: milk and milk-aging 48 hours, a considerable number of parameters of importance were identified and then optimised. It was determined that by modifying the general SLM set up (flat spiral disk), better optimal results were obtained indicating that there is a large potential in the exploration of optimal miniaturized SLM procedures and experimental configurations. Two different SLM designs were investigated for their feasibility in biological trace analysis. It was found when comparing the SLM flat spiral disc vs. extraction using a SLM probe that the probe produced superior results in terms of higher extraction efficiencies. Experimentally it also proved advantageous, especially regarding simplicity and shorter extraction times. One of the main advantages the membrane probe produced was that small sample volumes could be extracted from which in biological trace analysis is important as sample sizes are often volumes below 10ml.

8.2 Adsorptive Stripping Voltammetry-method optimisation

It was decided to optimize the Adsorptive Stripping Voltammetry determination method of Mn(II) further by using correlation matrix experiments and the relevant statistical software for data analysis.

Initially optimization was carried out on a 2d level (single factor response), then, 2-factor response experiments were performed which optimized two parameters simultaneously. The deposition potential and pH were optimized, then deposition time and deposition potential, equilibrium time, concentration of manganese(II) complexing ligand (ascorbic acid). Other parameters optimized included stirring during deposition, stirring speed and the elimination of one of the background electrolyte components found to cause peak current suppression i.e. gelatin which is believed to decompose in certain biological matrix. Although one of the gelatin's

functions was initially as a maxima current fluctuation suppressor (Pletcher, 1991; Crow, 1969; Wang et al., 1993) this function seemed to be performed by perhaps some of the organic components in the biological matrix itself as no maxima current fluctuation was visible. Throughout the optimization stages blank recovery runs to determine the concentration of Mn(II) in the background solution (residual contamination) were carried out and the concentrations of Mn(II) determined were very low.

This allowed for the advancement of SLM methodology to be applied to different types of biological sample speciation of manganese in fractionated vs. unfractionated blood serum. Sample preparation and determination was also carried out to investigate the effect of aging on extraction efficiency and thus on the binding of Mn(II) to blood proteins. It was determined that after a 24 hour period of the sample being spiked to contain 50µg l^{-1} prior to extraction, the extraction efficiency of Mn(II) decreased by approximately 4.5 times. This shows the immediate effect blood proteins have on the binding of free Mn(II) with time. The implications of this could give an accurate indication as to the time of exposure to Mn(II) an individuals blood needs to be tested.

Finally two differently spiked blood fraction were extracted and analysed. Using the optimized SLM probe and AdSV determination method, four extracts were analysed and the concentrations of Mn(II) determined for the 2000µg l^{-1} Mn(II) spiked fraction were found to have better reproducibility than the 800µg l^{-1} Mn(II) spiked fractions. The lower spiked Mn(II) concentration achieved overall higher extraction efficiencies relative to their spiked concentration. This could indicate that fractions containing lower spiked concentrations could (with a reproducibility improvement) be used for further speciation studies of Mn(II) in fractionated blood serum.

In order to obtain the fractionated blood serum Size Exclusion Chromatography was used as a separation method. The speciation of Mn(II) in biological matrix is difficult if not impossible without the use of analytical sample preparation methods like SLM and SEC which enabled for the determination of Mn(II) in blood serum and fractionated blood serum at trace levels.

Future research should be focused in the following areas:

- The determination of Mn(II) concentrations in six different blood serum fractions using Adsorptive Stripping Voltammetry. The use of Size Exclusion Chromatography as a sample preparation method to obtain the different blood serum fractions.
- More detailed focus on the binding characteristics of Mn(II) to the different blood proteins and possible methods that can be used for analysing these binding sites.
- The further miniaturization of the SLM (membrane probe) technique for use in micro extraction scale sample preparation and analysis (sample size<2 ml).

Publications

Soko, L., Chimuka, L., Cukrowska, E., Pole, S., Analytica Chimica Acta 485, (2003) 25-35. (See Appendix 4)

Other publication (manuscript submitted to S.A.J.S (South African Journal of Science), 23 August 2004)

Cukrowska, E.M., McCarthy, T.S., Pole, S., Backwell, L., and Steininger, C., The chemical removal of manganese dioxide coatings from fossil bones from the Cradle of Humankind, South African Journal of Science.

This research describes a novel method developed for the removal of manganese dioxide coatings from fossils that does not damage the bone. Many fossils found in dolomitic cave sites possess a dark brown-black coating on their surfaces. This layer obscures fine detail, preventing identification and interpretation of surface modifications. Here a description of the procedure used was provided and evidence was shown of early human behavior that was obscured prior to chemical treatment.

Presentations

1) S. Pole, E.M. Cukrowska, T.S. McCarthy, The chemical removal and speciation of manganese dioxide from bones originating from South African caves – SACI 2004 37th Convention, 4 –9 July 2004 (Pretoria)

2) S. Pole, E.M. Cukrowska, T.S. McCarthy, L. Backwell, and C. Steininger, The chemical removal of manganese dioxide coatings from fossil bones from the Cradle of Humankind – Geosciences Africa 2004 Convention, 12 – 16 July 2004, School of Geosciences, University of the Witwatersrand (Johannesburg).

3) Environmental chemistry workshops on 8th September 2004 at the University of the Witwatersrand Johannesburg (Also for Metrohm and Swiss Lab). A demonstration and introductory lecture on a Computrace 757 VA instrument regarding the basic principles of use for the trace determination of metals and the potential scope of the software applications and hardware.

4) Demonstrations on a VA Computrace on Adsorptive Stripping Voltammetry and Anodic Stripping Voltammetry. Methods and applications. Presented to 2003 and 2004 honours students of Rand Afrikaans University Johannesburg.

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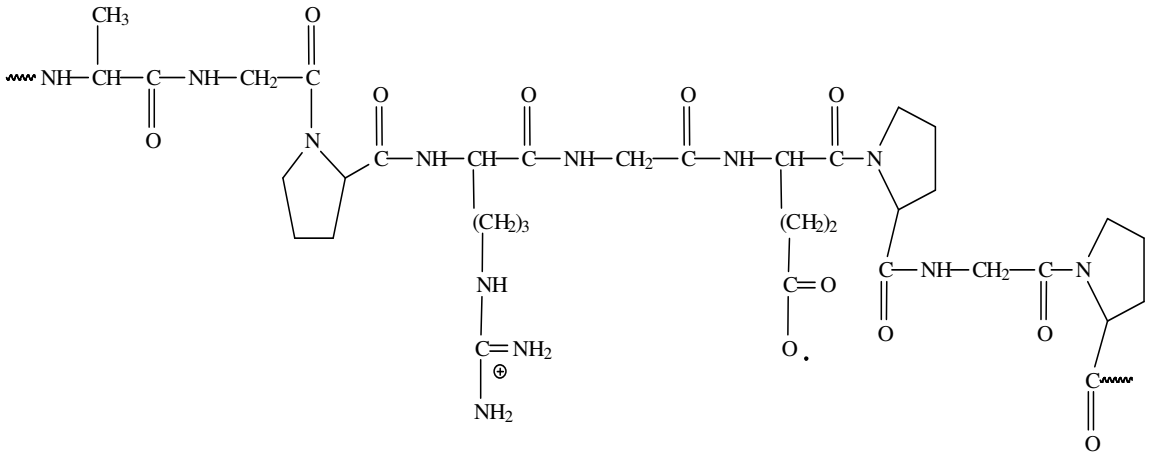
Appendix 1

Structures of gelatin and ascorbic acid

Gelatin

Gelatin is a product of the structural and chemical degradation of collagen. (Sokolov, 1937; Zaides, 1960). It has some individual importance as a polymeric product which is widely utilized in the manufacture of various articles and materials (Titov, 1952; Tucker, 1965). In most cases, except for the food industry, gelatin is used in the solid state.Gelatin contains a large number of glycine (almost 1 in 3 residues, arranged every third residue), proline and 4-hydroxyproline residues. A typical structure is: Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro-.

Gelatin structure

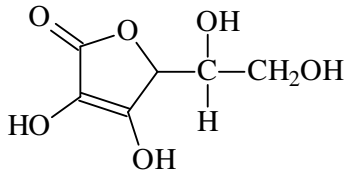


There are many different uses for gelatin in modern food production. This is because of its unique ability to react under heat. Gelatin melts at 37°C and becomes firm again when it cools down. Its pleasant eating properties make it irreplaceable for the food

sector. The food industry uses gelatin to make Jell-o, desserts, sweets, meat and fish sauces, and various drinks (http: // www. niroinc.com / html / chemical / Blood_Gelatin .html).

Ascorbic acid

Ascorbic acid structure



(http://www.people.virginia.edu/~rjh9u/vitac.html)

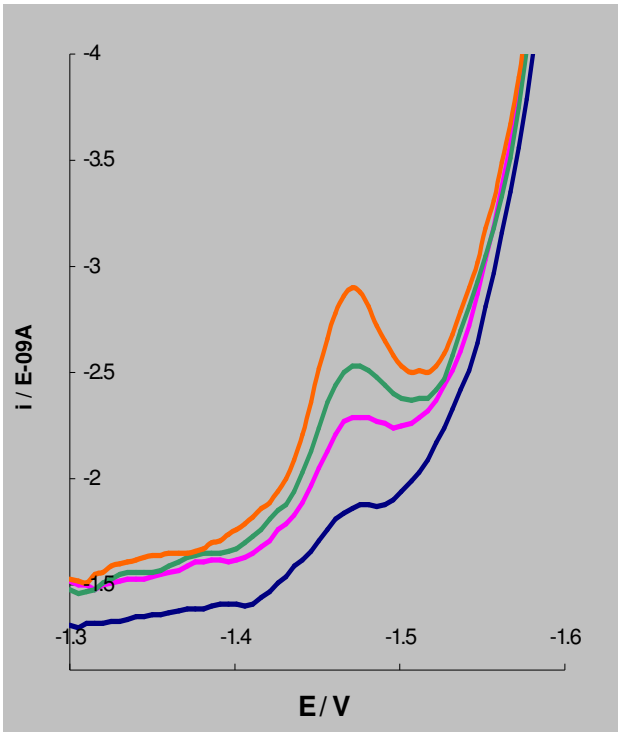
Ascorbic acid is an essential component in the diet of humans and a small range of other mammals. It has been associated with fertility for many years and may have evolutionary significance (Millar, 1992). A range of body tissues accumulates the native vitamin and its fully active, oxidized form, dehydroascorbic acid. Tissues vary widely in content but the highest concentrations occur in the pituitary, adrenal gland, and gonads (Davis et al., 1991; Das et al., 1993; Chinoy, 1972)

Appendix 2

Summary of analytical parameters initially used in AdSV procedure for the determination of Mn(II)

Parameter	Value
Pretreatment	
N_2(g) purge time(s)	120-300
Conditioning potential (V)	0
Duration(s)	0
Deposition Potential	-1.4
Duration(s)	60 /120
Equilibrium time(s)	15
Measurement	
Cell off after measurement	Yes
Modulation time	0.05
Interval time	0.25
Potentials	
Initial Potential(V)	-1.3
End Potential(V)	-1.6
Step Potential(V)	0.0051
Modulation amplitude(V)	0.02505
Standby potential(V)	0

Appendix 3



Typical voltammograms of Extraction of 40µg l^{-1}Mn(II) in milk with each respective voltammograms representative of additions of 20µg l^{-1}Mn(II);

Appendix 4