NOTE TO USERS

MERCURY AND SELENIUM SPECIATION AND TOXICITY IN COMMON LOONS

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ABSTRACT

Approximately 10,000 tones of Hg are deposited annually as a result of anthropogenic activities. This increased Hg burden is known to have adverse neurological and reproductive effects on Common loons. A positive correlation between mercury (Hg) and selenium (Se) has been reported to exist in marine mammals and various species of marine and aquatic piscivorous birds. It has been hypothesized that the Hg/Se interactions may involve in the multiple mechanisms of Hg detoxification. This study focused on the suggested Hg/Se complex that forms in association with specific proteins. Specifically, this project focuses on the nature of these interactions in different tissues from wild Common loons *(Gavia immer)* that have been collected by the Canadian Wildlife Service. The Hg and Se concentrations in the various tissues were quantified using AAS. MALDI-TOF-MS and protein sequencing characterized the nature of the Hg/Se complex binding protein. Among the tissues, the liver had the highest concentrations of Hg and Se followed by kidney; muscle and brain. A strong association between Hg and Se was found in liver, kidney and eggs whereas there was no association in muscle and brain. In contrast brain and muscle had highest percentage of organic Hg suggesting that only inorganic Hg is associated with Se. Two Hg-Se binding protein complexes were found in liver both in the 15,200-15,300 Da range while one such complex in the same weight range was found in kidneys, when sequenced it was found that these proteins were the α A chain of Hemoglobin. The protein complex found in eggs was unique and although it was impossible to fully sequence it, it represents an unknown protein. The role of Se in Hg toxicity in eggs warrants further study.

RESUME

On a rapporté qu'une corrélation positive entre le mercure (Hg) et le sélénium (Se) existe dans les mammifères marins et les diverses espèces d'oiseaux piscivorous marins et aquatiques. Beaucoup d'études ont été réalisées pour déterminer la nature exacte de Hg interactions avec Se. On a proposé des mécanismes multiples de la désintoxication d'Hg en présence du Se. Cette étude s'est concentrée sur Hg suggéré Complexe de Se ce formes en association avec les proteins spécifiques. En détails, ce projet se concentre sur la nature de ces interactions dans différents tissus des Uares communs sauvages (Gavia immer) donnés par le service canadien de faune. Approximativement 10.000 tonalités d'Hg sont deposes annuellement en raison des activités anthropogènes. Les concentrations d'Hg et de Se dans les divers tissus ont été mesurées en utilisant l'AAS. MALDI-TOF-MS et l'ordonnancement de protéine ont caractérisé la nature de Hg protéine obligatoire complexe de Se. Parmi les tissus, le foie a porté le plus grand fardeau de Hg également avec la plus grande concentration du Se. Du foie a été suivi du rein ; le muscle et le cerveau ont eu les moindres concentrations élevées de Hg et du Se. Hors de Hg total, Le cerveau et le muscle ont eu le pourcentage le plus élevé de MeHg. Une association forte entre Hg et le Se a été trouvée dans le foie, rein et oeufs tandis qu'il n'y avait aucune vraie association dans le muscle et le cerveau. Deux complexes obligatoires de la protéine Hg-Se ont été trouvés dans le foie tous les deux dans la gamme de 15.200-15.300 Da tandis qu'un tel complexe dans la même gamme de poids était trouvé aux reins, une fois ordonnancé lui a été constaté que ces protéines étaient une chaîne de A d'hémoglobine. Le complexe de protéine trouvé en oeufs était unique et bien qu'il ait été impossible de l'ordonnancer entièrement, il représente une protéine inconnue.

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CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

One of the more prevalent and toxic heavy metals is mercury (Hg) and the assorted non-organic and in particular organic compounds it forms. Hg is a particularly ubiquitous pollutant that accumulates in many organisms. Methylmercury (MeHg), an organic mercury compound, has been detected in elevated non-toxic concentrations in fish (0.5-1ppm) in isolated locals far removed from any anthropogenic or natural sources (Morel et al., 1998). Various vitamins and minerals such as Vitamin C, E and selenium have established protective effects countering the negative effects produced by Hg toxicity (Chapman and Chan, 2000).

As a result of the large amount of fish consumed per body weight, Common loons are at risk of adverse behavioral and reproductive effects in regions of high mercury contamination (Scheuhammer and Blancher, 1994). Watersheds that are partially acidified or high in dissolved organic compounds (DOC) are of particular risk to piscivorous species since they favor mercury methylation leading to the production of highly toxic MeHg (Morel et al., 1998). Mercury concentrations between 0.3-0.4ppm wet weight (ww) in small (20 to 50 gram) fish are deemed to be greater than the threshold level causing negative reproductive effects in various fish eating species of birds (Scheuhammer et al., 1998).

Canada has an estimated Common loon population numbering approximately 544,000 individual birds (Scheuhammer et al., 2003). Among the eastern provinces, the population is divided as follows: 2,400 in New Brunswick (mixed breeding and wintering population), 2,800 in Nova Scotia (mixed breeding and wintering population), 120,000 in

Quebec (breeding population) and 232,000 in Ontario (mixed breeding and wintering population in southern Ontario). A serious decline of loons in their southern breeding range has been noted during the previous 100 years. This is probably due to many effects such as habitat loss, recreational use of lakes and environmental contaminants. As mentioned previously loon-breeding success is seriously compromised when fish (in the size that are preyed on) accumulate Hg concentration greater then 0.3ppm ww (Scheuhammer et al., 1998), primary exposure to such contaminated prey occurs mainly on the breeding grounds.

Studies have correlated selenium and mercury within the tissues of various organisms, particularly marine organisms (Freeman et al., 1978). As loons are top-level predators in their environment, they are ideal indicators with regards to the health of the ecosystem they inhabit, thus the general health of loons is of concern. The interactions between mercury and selenium could be an important factor affecting overall loon health.

1.2 Mercury

1.2.1 Principal Chemical and Physical Forms of Mercury

Hg has three stable oxidation states. In the ground state Hg occurs as a metallic element. When Hg has a charge of +1 it becomes the mercurous ion and is mainly associated with Cl in the compound mercurous chloride (Hg_2Cl_2) or calomel. The loss of two electrons produces the mercuric ion. The most widespread compound mercuric Hg forms is mercuric chloride ($HgCl_2$), furthermore, the mercuric ion is capable of forming various organomercercurial compounds such as dimethymercury (CH_3HgCH_3) and monomethymercury (CH_3Hg^+). Mercuric mercury's biochemistry is governed primarily by its

extremely high affinity for thiols (Cheesman et al., 1988). $MeHg^{+}$ has very similar toxicokinetics to Hg^{2+} within an organism based on its affinity for sulfhydryl groups as well. Both compounds are found in various tissues bound to the thiol groups of proteins as well as non-protein thiol containing compounds such as glutathione and cysteine.

1.2.2 Sources and Distribution of Mercury

The principal, naturally occurring sources of mercury include, the degassing of the earth's crust, volcanic emissions, forest fires, biogenic emissions of volatile and particulate compounds and finally evaporation from natural bodies of water (Suzuki et al., 1991). In the vast majority of the world, pollution contributes significantly greater quantities of mercury via long range atmospheric transport and local riverine deposits (Mason et al., Sorensen et al., 1994 from Gountner et al., 1996) than naturally occurring Hg. Exposure to elemental mercury (Hg^{0}) arises through the atmosphere. Elemental mercury occurs in both natural and anthropogenic sources (Gailer et al., 2000). Approximately 10,000 tones of Hg are annually released into the environment due to human activities (Wiken and Hintelmann, 1990). Anthropogenic sources of Hg include metal production, chlor-alkali and pulp industries, waste handling and treatment and coal, wood and peat burning. (Morel et al., 1998). In addition, mercury was used as a seed dressing in Sweden, the U.S., and other countries until it was recognized as being the cause of augmented incidences of wildlife mortality and several cases of human toxicity (Swensson, 1952; Clarkson, 1976).

Approximately 95% of the total Hg in the atmosphere is in the elemental state, there it is slowly oxidized to the mercuric ion primarily by ozone. This process is catalyzed at the

solid-liquid interface in fog and cloud droplets (Morel et al., 1998). Mercuric ions may also be re-reduced to elemental mercury via SO^{3-} or the photoreduction of $Hg(OH)_2$ (Morel et al., 1998). Due to the relatively slow rate of oxidation of elemental mercury to Hg(II), it is disseminated globally prior to returning to the planet's surface (Morel et al., 1998).

Mercury returns to the earth's surface principally through wet precipitation of solubilized mercuric mercury. An additional mechanism involves adsorption to particulate aerosols such as soot, this mechanism occurs to a greater extent over land than over bodies of water. Sixty percent of Hg(II) returns to land whereas only 40% of it returns to water, this despite the fact that land represents only approximately 30% of the globe's surface area (Mason et al., 1994). This can be attributed to the greater proximity of the anthropogenic sources to land masses as opposed to water.

1.2.3 Biotransformation and Bioaccumulation of Mercury

Due to the extensive use of mercury throughout the world a great deal of it is now present in the environment. Moreover, various species of microorganisms (e.g. strains form the Pseudomonas) (Rubenstein et al., 1978) are capable of metabolizing inorganic mercury to the substantially more toxic MeHg (MeHg) (Wood et al., 1968). Thus, as inorganic Hg accumulates throughout the environment, MeHg will correspondingly increase. In addition, certain molds in sediment can convert practically any form of Hg into MeHg (Goldwater, 1971; Grant, 1971; and Wood, 1972). It is very probable that almost any organism able to synthesize vitamin B_{12} is capable of synthesizing MeHg. One of the main problems associated with MeHg is that unlike Hg(II), Hg^0 and Me_2Hg it is bioaccumulated. Elemental Hg and

Me_2Hg are relatively unreactive in phyto- and bacterio-pico plankton, as a result they diffuse in and out at the same rate. Mercuric mercury is found naturally occurring in the environment as $HgCl_2$ whereas MeHg is found as MeHgCl, these are the species that diffuse into the microorganisms (Rubenstein et al., 1979). MeHg is transferred between a marine diatom and a copepod with a factor of four times greater efficiency than Hg(II). The greater transfer of MeHg than Hg(II) is due to the different affinities of the two mercury species. Mercuric mercury is mainly associated with the membranes of the diatoms whereas MeHg is associated with the soluble fraction of the cell. The membrane material is largely excreted by copepods whereas the soluble material is absorbed (Morel et al., 1998).

Approximately 95% of the MeHg from ingested fish is absorbed by the gastrointestinal (GI) tract (NRC, 2000). It was found that intestinal absorption of MeHg occurs mainly via specific transporters while the MeHg is complexed to non-protein sulfhydryl compounds (Urano et al., 1990). In particular when MeHg was bound to the dipeptide cysteinylglycine (MeHg-CysGly) or cysteine itself, the rate of absorption was approximately 1.5 times greater than that of a MeHg-glutathione (MeHg-GSH) complex. Thus there are multiple complexes MeHg can form that lead to its absorption and reabsorption in the intestine during its enterohepatic circulation. Furthermore, Urano et al.'s, (1990) results entertain the prospect that there is a minimum of two intestinal transport systems for MeHg-GSH: the gamma-glutamyltranspeptidase (GGT)-dependent and GGT-independent systems. However, the type of MeHg complex may also be species dependent. Urano et al., (1988) found that the concentrations of MeHg bound to non-protein sulfhydryl compounds such as those mentioned above was relatively low in rabbits and guinea pigs in bile compared to that of rats, mice and hamsters. Moreover, the principal form of MeHg in mice and hamsters was

MeHg-GSH and in guinea pigs was MeHg-CysGly. These differences reflect the different compositions of the bile in different species.

MeHg can be biotransformed in organisms to various Hg^{2+} species. Sites of demethylation are the intestinal flora and macrophages (NRC, 2000). Additionally, MeHg can be demethylated via peroxides and its own homolytic cleavage. Once demethylated it can also be metabolized back to MeHg via the actions of certain species of microorganisms found in the gut. Approximately 1% of the total burden of MeHg is excreted daily (Clarkson et al., 1988), of which 90% of it is excreted in the feces in the form of Hg^{2+} and 10% is excreted in the urine as Hg^{2+}. The half-life of MeHg in the body is dependent on several factors including species, sex and dose, notwithstanding, the general duration is 70-80 days (Nielson, 1992). Studies performed on mice have demonstrated that MeHg bound to cysteine residues may also be excreted (Yasutake et al., 1989). Most forms and oxidation states of Hg are eventually converted to Hg^{2+} in an organism (NRC, 2000).

1.2.4 Toxicokinetics of Mercury

The toxicity of MeHg is based on multiple mechanisms. The demethylation of MeHg via its own homolytic cleavage generates free radicals which cause non-specific damage, however it is still unsure as to which mechanism causes the cytotoxic death (NRC, 2000). Approximately 10% of the body's burden of MeHg is stored in the brain, a particularly sensitive organ (NRC, 2000). In addition to causing damage via the non-specific binding of MeHg to sulfhydryl groups, the generation of free radicals in this tissue causes supplementary non-specific damage. Once demethylated, Hg^{2+} also reacts nonspecifically with thiol groups;

should these sulfhydryls be cysteine residues in structural proteins or in enzymes, this could lead to the disruption of cellular processes (Clarkson, 1997). MeHg and Hg^{2+} also prevent depolymerization and repolymerization of neuronal microtubules by binding tubulin monomers, this in turn arrests such cellular processes such as cell migration and cell division (Ponce et al., 1994). In fact, microtubules, and the mitotic spindle, are especially sensitive to MeHg exposure. These mechanisms and organelles are critical for cell cycle progression. Free radical induced lipid peroxidation and the accompanying disruption of lipid membranes, has been shown to cause biochemical and ultrastructural changes in the mitochondria of rats (Denny and Atchison, 1994) in the presence of MeHg, however was not significant enough to be the main cause of toxicity. Similar results were found for neuronal membranes (Sarafian and Verity, 1991). MeHg is a potent disrupter of protein synthesis (Sugano et al., 1975), still, Hg^{2+} was found to be 10 times more a potent inhibitor of protein synthesis in rats. Although the disruption of protein synthesis has been suggested to be one of the main factors of MeHg toxicity, it remains unknown whether it is the accumulation of Hg^{2+}or MeHg itself that accounts for the greater amount of damage.

1.2.5 Mercury and Common loons

Through bioaccumulation and biomagnification, humans and fish-eating wildlife are exposed to MeHg. The marine ecosystem is contaminated principally by MeHg via the action of anaerobic microorganisms (Martoja and Berry, 1980). Piscivorous birds, among which loons are included, are exposed predominantly to the methylated forms of Hg (Daoust et al., 1998). Hg acts on a variety of organs within an organism including the brain, liver and

kidneys. In addition, it acts on fetuses stunting growth and delaying mental development. Himeno et al., (1989) demonstrated that MeHg poisoning has a negative effect on growth of Harbor seals (Phoca vitulina). Areas in the brain particularly affected by MeHg include the visual cortex and granular layer of the cerebellum (WHO, 1990 from Vahter et al., 1993).

It has been previously determined that the neurotoxicological effects of Hg manifest themselves in loons at total Hg concentrations exceeding 15μg/g in brain tissue and a minimum of 30μg/g ww in liver or kidney tissue (Scheuhammer 1991). In a recent study performed on Common loons found dead or debilitated, none of the deaths were directly attributed to Hg poisoning. Analysis of total Hg in the liver of all loons necropsied revealed a geometric mean of 10.3μg/g ww and a range of 0.07 to 371μg/g (Stone et al., 2001). Although the geometric mean of total Hg for the loons studied was below the threshold for clinically observable neurotoxicological effects and may not have directly contributed to mortality, it has been suggested that a predatory bird such as a loon requires full coordination to feed adequately and could thus be affected by lower concentrations of total Hg (Daoust et al., 1998). This implicates that loons suffering sub lethal Hg concentrations may be malnourished and die from starvation due to the inability to hunt effectively rather than to dying from mercury poisoning.

In the study performed by Daoust et al., (1998), the majority of the total Hg found in the liver and kidneys was in the inorganic form while the majority of the Hg in the pectoral muscles was methylated (Scheuhammer et al., 1998). Cerebral and renal levels for 10 birds of substandard body condition in the study performed by Daoust did not suggest preferential redistribution of the Hg to the brain. Upon initial observation it appears beneficial that the highly toxic organic Hg accumulates in the muscle tissue as opposed to vital organs, however,

gradual muscle atrophy due to emaciation/starvation could lead to the release of significant amounts of MeHg back into the system. This greater bioavailability of MeHg exerts its effects on more vital and sensitive organs. Another possible outcome of MeHg toxicity is severely impaired immune function (Bernier et al., 1995). Therefore sub lethal levels of Hg that would typically have no direct impact on a healthy bird may lead to emaciation by limiting hunting dexterity (through subtle neurological impairment), leading to muscle atrophy via starvation ultimately leading to the greater release of MeHg internally increasing the concentration to toxic levels. In addition, the effects of mercury toxicity can interact with other pathological factors, none of which would be lethal to the bird individually however become so when combined. Another poorly studied possible behavioral effect of Hg is altered brooding behavior. Typical loon nesting behavior involves sharing egg-sitting duty but MeHg levels above a threshold (15μg/g ww in brain tissue) lead to a serious decline in this behavior (Scheuhammer et al., 1994). Evers et al. (2002) classified loons according to three behavioral categories, low risk individuals, moderate risk individuals and high-risk individuals based on Hg concentrations in available prey. Males generally spent less time than females incubating at the nest. Males and females in the low risk category spent 99% of their time incubating eggs at the nest leaving the eggs unincubated for only approximately 1% of the time (0.04 to 0.13 ppm Hg ww in prey). Males and females in the moderate risk group spent only 90% of their time incubating eggs (0.04 to 0.23 ppm Hg ww in prey). Loons within the high-risk category spent even less time incubating the eggs-86% (0.08 to 0.28 ppm Hg ww in prey). Unattended eggs have a larger chance of being preyed upon and therefore leads to decreased nesting success. A study performed by Heinz et al., (1997) on Mallards demonstrated that

birds fed a diet containing 10ppm Hg (as MeHg) produced only 1.1 young while the control group produced an average of 7.6 young.

1.3 Selenium

Selenium (Se) is classified on the periodic table as a metalloid and is thus capable of forming both anionic and cationic salts. Consequently some Se compounds are very reactive to sulfhydyl groups while others are highly reactive to heavy metal containing compounds (such as those containing Hg). Se occurs in foods predominantly as amino acid analogs primarily as selenocysteine (Shibata 1992). Se itself when present in high enough concentrations has toxic effects, e.g. in livestock excessive Se from dietary sources leads to two classically known diseases: "alkali disease" and "blind staggers". Symptoms of alkali disease include brittleness and sloughing of hooves, which is due to eating plants containing between 5-40ppm Se. Blind stagger results from diets including plants with several thousand ppm Se resulting in impaired vision, loss of appetite and hepatic necrosis (Combs and Combs 1986, Rosenfield and Beath 1964). Developmental abnormalities may occur in addition to adverse reproductive effects when Se levels are high.

In aquatic birds, elevated Se levels have been known to cause the following effects: mortality, impaired reproduction with teratogenesis, reduced growth, histopathological lesions and alterations in hepatic glutathione metabolism (Hoffman, 2002). Deaths occur when Se concentrations exceeded 20 ppm wet weight in the liver (approximately 66 ppm dry weight). High levels of Se are found in bird blood samples from interior and western Alaska, however young from these birds, in particular Emperor geese (*Chen canagica*), did not display elevated

concentrations of Se. This suggests that the geese as well as other marine birds are exposed to greater amounts of Se in their marine wintering environments than their interior breeding grounds (Franson et al., 1999). Se is also known to cause oxidative effects in Mallards, these oxidative effects were associated with teratogenesis (4.6 ppm wet weight Se in eggs), reduced growth in ducklings (15 ppm Se in liver), diminished immune function (5 ppm Se in liver) and histopathological lesions (29 ppm Se in liver) in adults (Hoffman, 2002).

Seabirds are known for the ability to tolerate elevated levels of inorganic Hg (Thompson 1996 from Stone et al., 1998) and it has been suggested that loons may also have similar capabilities.

1.4 Selenium and Mercury Interactions

Se exhibits a protective effect against systemic Hg toxicity. Parizek and Ostadalova first observed the protective effect of Se on inorganic mercury toxicity in rats in 1967. In seals, increases in hepatic Hg were accompanied by corresponding increases in Se, indicating a positive correlation between Hg and Se approximating a molar ratio of 1.0 (Koeman et al., 1973, van de Ven et al., 1979). A study performed where sodium selenite was injected into rats found that it induced a decrease in the biliary secretion of MeHg (Urano et al., 1997). This study concluded that the inhibition of the biliary secretion pathway in the presence of selenite might be influenced by the actual inhibition of the secretion of MeHg from liver to bile instead of the formation of a MeHg-Se complex.

Approximately five distinct mechanisms for Hg detoxification via Se have been suggested: (1) redistribution of Hg in the presence of Se, (2) competitive inhibition of Hg

binding by Se, (3) demethylation of the highly toxic MeHg to the less toxic inorganic Hg, (4) hindrance of oxidative damage, and (5) formation of an inert Hg/Se complex.

Redistribution

Burk et al., (1977) reported that Se induced the preferential accumulation of inorganic Hg in the kidneys of rats. Rats with Se deficiencies did not excrete ^{203}Hg via urine as did those with Se in their diet. Other studies have suggested that Se promotes the redistribution of Hg from organs such as the kidney and liver to other less sensitive tissues such as skeletal muscle (Chen et al., 1974, Sheline and Schmidt-Nielson, 1977). Negative redistribution patterns (meaning Hg was localized to more sensitive tissues rather than less sensitive ones) have also been found e.g. Stoewsand et al., in 1974 found that when Hg and Se were co administered, Hg was localized in the brain.

Competitive Inhibition

Hg and Se have a similar binding capacity and affinity with respect to sulfhydryl groups (-SH), and therefore Se may by competitive inhibition prevent the binding of Hg to proteins on gastrointestinal tract (Rubenstein et al., 1978) thus, Defecation may be one approach whereby an organism uses to excrete Hg.

Demethylation of MeHg

Recently, it has been postulated that the bioprotective role of Se may arise through the demethylation of MeHg (Caurant et al., 1996). The majority of Hg accumulated in marine mammal liver is in the inorganic form even though their main source of exposure is to dietary MeHg (Itano et al., 1984; Dietz et al., 1990, Gaskin et al., 1979; Wagemann et al., 1988). In contrast the majority of the Hg in the muscle tissue was methylated, this may indicate that the liver is the site of demethylation. No mechanisms have been elucidated for the demethylation

process via Se, however, such a mechanism would be highly beneficial to the organism since inorganic Hg has a significantly shorter biological half-life due to preferential excretion through the feces (Norsth and Clarkson, 1971).

Hindrance of Oxidative Damage

Selenium is a trace element and a component of glutathione peroxidase (GSH-px) as well as the membrane bound enzyme type I iodothyronine 5' deiodinase (Rotruck et al., 1973). Glutathione peroxidase catalyzes the following reaction: $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$. Erythrocyte homolysates taken from selenium deficient rats and treated with ascorbate and H_2O_2 exhibited serious oxidative damage, the addition of glutathione was necessary in order to prevent oxidative damage since the erythrocytes were nearly devoid of GSH-px activity (Rotruck et al., 1973). Furthermore, GSH-px is inhibited by Hg (Hirota et al., 1980), this would account for the particularly harmful effects of Hg in sensitive tissues such as the liver and nervous system since it would prevent the reduction of free radicals. Compounding this effect, is the fact that MeHg could further induce toxic effects via the formation of free radicals formed during its own homolytic breakdown (Ganther et al., 1978). In this experiment there is no direct link between Hg and Se, it merely demonstrates the importance of Se as an antioxidant and the inhibitory effect Hg has on the antioxidant enzyme GSH-px. It would be reasonable to assume these two activities would interact in an organism.

Formation of an inert Hg/Se complex

Iwata et al., 1981 (from Di Simplicio et al., 1989) suggest that the Hg/Se interaction is caused by the reduction of selenite to selenide via endogenous glutathione (GSH), this in turn promotes the formation of bis-MeHg selenide. This highly lipophilic compound is not metabolized and distributed in the same manner as inorganic Hg inside of vital tissues and

thus may account for the decreased toxicity (Masukava et al., 1982). This mechanism suggests that the formation of the complex is necessary for redistribution within tissues and may account for decreased toxicity.

More recently a study where rabbits were co-administered mercuric chloride and sodium selenite in aqueous buffered glutathione established the involvement of selenoprotein P in the detoxification process (Gailer et al., 2000). Until this study the function of selenoprotein P was unknown. According to their model selenite is taken up by erythrocytes (Suzuki et al., 1998), reduced to selenide (possibly by GSH) and expelled. Selenide then binds with albumin bound mercuric mercury to form an Hg-Se-S complex. Hg has a high affinity for thiols, and is thus likely to be bound to albumin in the plasma, which has the largest free pool of available thiol groups (Carter et al., 1994). Following this the Hg-Se-S complex binds to selenoprotein P. Suzuki et al.,(2001) suggested that up to 35 binding sites exist on selenoprotein P for the $(Hg/Se)_n$ complex. The $(Hg/Se)_n$ complex itself is composed of approximately 100 individual Hg/Se monomers (n=100).

A study performed on Pilot whales proposed that one form of Hg/Se complex exist as fossilized Tienmannite granules (Caurant et al., 1996; Martoja et al., 1980). Theses granules were found in the livers of *Ziphius cavirostris* and *Tusiops truncatus* (Martoja and Berry, 1980) and believed to be inert. Tienmannite crystals are non-biodegradable and thus imply another method for the detoxification of Hg.

1.5 Summary and Rationale

Loons are top-level predators both in fresh water and marine environments, as such they are particularly interesting to environmental scientists who see them as potential

indicator species with respect to environmental health (Pokras et al., 1998). Since common loons are fish eaters and at high risk of high Hg exposure, it is important to study the toxicity of Hg and the mechanisms of Hg-Se interactions at different organs. Marine birds maybe able to tolerate greater quantities of Hg due to the significant amounts of Se ingested in their diet. Loons winter in coastal habitats and would thus benefit from the greater bioavailability of Se in this environment as well.

Currently there are no existing studies on the existence of Hg/Se complexes in Common loons, thus necessitating the implementation of such a study.

Objective

This project was undertaken to study the interactions between mercury and selenium in different tissues of Common loons (Gavia immer) from wild populations. More specifically, the goal of this project is to characterize the complexation of Hg and Se in the various tissues.

Overall Hypothesis

The hypothesis of the study is that Hg and Se form a complex in Common loons.

CHAPTER 2. MATERIALS AND METHODS

2.1 Sample collection

Loon tissues were supplied by Dr. Tony Scheuhammer from the tissue bank of the Canadian Wildlife Service (CWS). Environment Canada. The samples were archived from dead loons across Canada and stored at -20°C. A total of 30 Loons supplied the liver, kidney, muscle, brain tissue and eggs.

2.2 Study Design

The Hg/Se protein complex was isolated and characterized by the following experiments:

1. Measurement of Hg and Se in the Loon liver, kidney muscle and brain tissue and eggs.
2. Determination of the percentage of Hg and Se in the cytosol versus the membrane fraction.
3. Isolation of the cytosolic Hg/Se complexes via microfuge and collection of the supernatant.
4. Determination of the retention time of the Hg/Se complex using size exclusion HPLC coupled to ICP-MS.
5. Isolation of the Hg/Se complex containing fractions using size exclusion HPLC.
6. Purification of the fractions and further separation by reverse phased (RP)-HPLC followed by determination of the Hg/Se containing fraction by ICP-MS.

7. Characterization of the Hg-Se complex was characterized via time of flight (TOF)-MS and amino acid sequencing.

2.2.1 Quantification of Hg and Se in different Loon tissues via AAS

Acid Digestion:

Approximately 2g of wet tissue were weighed into pre-labeled 20sm boiling tubes. Two replicates per sample were prepared. 8ml of concentrated nitric acid was added to each tube. The tubes were covered with glass reflux bulbs and the contents were allowed to soak in the acid at room temperature overnight. Following this, the tubes were then placed on a Thermolyn Dri-Bath and the temperature was increased to 120°C over a 2-3 hour interval. Once at 120°C the temperature was held there for 5 hours. The tubes were then permitted to cool to room temperature and then topped to 25.0ml with Nanopure water. The contents were transferred to a pre-labeled 25ml snap-cap polypropylene vial and stored at room temperature. The nitric acid concentration of the digests was 22% w/v.

Cold Vapor AAS Parameters for Hg Analysis:

The acid-digested sample and the reactant (10% SnCl2-20% HCl) were mixed. Hg vapor was generated and transported via the Argon carrier gas into the quartz cell of a Hitachi HFS-2 hydride formation system and determined with a Hitachi Polarized Atomic Absorption Spectrometer Z-8200 (Nissei Sangyo Canada Inc., Mississauga, Ontario). The Hg hollow

cathod lamp was operated at 6.0mA, the slit width was 1.3mm and the absorbance was measured at 253.7 nm with background correction. A Hitachi SSC-110 autosampler was used to inject samples.

Graphite Furnace AAS Parameters for Se Analysis:

Se was determined by a Hitachi Polarized Atomic Absorption Spectrometer Z-8200equipped with a Se electodeless discharge lamp (EDL) operated at 8.0mA with a slit width of 1.3nm. The absorbance was measured at 196.0nm with background correction. A Hitachi SSC-300 autosampler was used to inject samples.

Quality Assurance/Quality Control

Standard solutions of Hg and Se were prepared directly prior to analysis via serial dilutions of atomic absorption standard containing 1000ppm of Hg or Se (ACP Chemicals, St. Leonard, Quebec). The instrument was re-calibrated every ten samples.

With each batch of samples, two sample blanks were analyzed. In addition, a spiked blank was analyzed during each analysis to ensure reproducibility was maintained day to day. All of the standards and samples were measured in duplicate, the samples were re-analyzed if the standard deviation of the two measurements was greater than ±5%. Standard reference materials, dogfish liver- DOLT 2 and dogfish muscle-DORM 2, from the National Research Council of Canada (Ottawa, Ontario) were digested and analyzed with each batch of samples. The Hg and Se concentrations always fell within 1 SD of the certified values.

2.2.2 Determine the percentage of Hg and Se in cytosol

Approximately 2g of loon tissue were homogenized in 1 volume of 100mM Tris-HCl buffer (pH 7.4) using a Polytron homogenizer (kinematica, Lucerne, Switzerland). The homogenates were centrifuged at 10,000g for 30 min at 4°C in a Sorval RC 5C centrifuge (Dupont, Newtown, Connecticut). Hg and Se concentrations in the supernatant fraction (cytosol) and in the pellet were measured by AAS.

2.2.4 Use of size exclusion HPLC-ICP-MS to determine the retention time of the Hg-Se complex

1g of previously homogenized tissue was weighed into10ml conical tubes. 1ml of 10mM Tris-HCl buffer (Sigma) (pH 7.0) was added and the samples were homogenized. The samples were then placed in pre-labeled 1.5ml polypropylene Progene microtubes and centrifuged in a Sorvall MC 12C microfuge (Mississauga, Ont.) for 10 minutes at 7,500g. The supernatants were filtered using a disposable luer lock 3ml syringe coupled to a PTFE membrane syringe filter (TITAN filtration, pore size 0.45μm, filter size 13mm) into another set of prelabeled 1.5ml microtubes. Both the supernatants and the pellets were stored in -20°C until use. The supernatants were thawed on ice and applied to the head of a SEC ProgelTm –TSK G4000 PWXL (Tosoh Biosep, Supelco, particle size 10μM, Bellefonte, PA) using Tris-HCl pH 7.0 as a mobile phase at a flow rate of 0.8ml/min. The eluate was introduced directly into the nebulizer tube of an ICP-MS instrument (ELAN 6000 PE-SCIEX), operated under the following conditions: forward power 1000W, plasma gas (Ar) flow rate 15l/min, auxiliary gas

(Ar) flow rate 0.8L/min, nebulizer gas (Ar) flow rate 1.0L/min. Isotopes Se78 and Hg199 were monitored. The pump used was Shimadzu LC-6AD.

2.2.5 Collection of Hg-Se containing fractions using size exclusion HPLC

The same supernatants prepared in 2.2.4 were applied to the same column and pump using a similar mobile phase and flow rates. This time the column was coupled to a UV detector (D-star Instruments DVW-10 variable wavelength detector). The wavelength was set at 280nm. The retention time previously determined in 2.2.4 were used to collect fractions eluting from the UV detector. The fractions were collected in pre-labeled 50ml polypropylene sterile Conical Screw-Cap tubes (Ultident Scientific, St.Laurent, Qc.) and stored at -20°C.

2.2.6 Freeze-drying of Samples and RP-HPLC

The fractions were placed in -80°C for at least an hour before being placed in the freeze-drier (Flexi-dry microprocessor controlled bench top lyophilizer, FD-3-85A-MP, FTS systems, Stoneridge NY, USA). The fractions were allowed to dry completely and were removed the following morning and stored at -20°C until further use. Prior to injection the samples, the samples were re-dissolved in 300µl of Nanopure water and transferred to inserts in 2ml glass HPLC vials. An LC4 column (Supelco) was used. The mobile phase was a gradient consisting of two solvents. Solvent A was a 1% trifluroacetic acid solution and solvent B was composed of acetonitrile:H_2O, 60:40 with 0.08% trifluroacetic acid. The flow rate was 1.3ml/min.

Protein peaks were monitored via a UV detector at both 210 and 280nm. The composition of the gradient was as follows.

Time (min)	Rate (ml/min)	Solution A (% flow rate)	Solution B (%flow rate)
0.10	1.3	100	0
60		0	100
65		100	0
70		100	0

Separate fractions were collected according to the UV response of the eluate. The HPLC system was a Beckman HPLC System Gold. The injections from the gel-phase fractions were not sufficiently concentrated to produce large enough peaks for the following protocol.

2.2.7 Determination of the Hg-Se containing fraction via ICP-MS

The fractions collected via RP-HPLC were freeze dried via the same protocol used in 2.2.6 and redissolved in 1ml of ddH_2O prior to analysis in the ICP-MS (Elan 6000 PE-SCIEX). Unfortunately, the fractions were insufficiently concentrated to allow for the detection of Hg and Se.

2.2.8 RP-HPLC of Initial Tissue Extracts

Tissue samples that were particularly high in protein (determined from 2.2.5) were selected based on high UV absorbance. 100µl of the original extract were injected into the Beckman

system using the same C4 column. Again, separate fractions were collected according to the UV response of the eluate. The same protocol used in 2.2.7 was repeated and the fractions containing high Hg and Se concentrations were determined. Five 200µl injections of the same tissue extract were run through the HPLC consecutively and the peak containing the elevated Hg and Se was collected in 15ml polypropylene sterile Conical Screw-Cap tubes (Ultident Scientific, St.Laurent, Qc.) and stored at -20°C.

2.2.9 Isolation of Individual Hg-Se Binding proteins

Fractions from 2.2.8 were freeze dried according to the same protocol used in 2.2.6 and redissolved in 300µl of ddH_2O. 20µl of sample were injected into the same HPLC setup used in 2.2.8 under the following gradient:

Time (min)	Rate (ml/min)	Solution A (% flow rate)	Solution B (%flow rate)
0.10	1.3	50	50
60		0	100
65		50	50
70		50	50

Once the exact point in the gradient where the protein eluted was determined, for example 32%A and 68%B, 100µl of the fraction would be injected according to the following gradient:

Time (min)	Rate (ml/min)	Solution A (% flow rate)	Solution B (%flow rate)
0.10	1.3	34	66
60		30	70
65		34	66
70		34	66

Separate fractions were collected according to the UV response of the elute. The majority of the peaks were collected in 15ml polypropylene sterile Conical Screw-Cap tubes (Ultident Scientific, St.Laurent, Qc.) and stored at -20°C. The uppermost portion of the peaks were collected in 1.5ml polypropylene Progene microtubes, approximately 9-10drops were collected. If multiple peaks had been collected the portion of the fraction contained in the 15ml tubes were be freeze dried and analysed on ICP-MS in order to determine which peak contained the Hg and the Se. The corresponding 1.5 ml tube were applied to the following protocol.

2.2.10 Characterization of Hg-Se complex binding protein using MALDI TOF-MS

This analysis was performed at the Sheldon Biotechnology Center. 0.5µl of the secondary HPLC fraction were applied to a microplate to which 0.5µl of matrix solution was added, the plate was inserted and the approximate molecular weight was determined. The running conditions were as follows: Accelerating Voltage 15,000, Grid Voltage 92,000%, Guide Wire Voltage 0.300%, Delay: 700 ON, Laser intensity 2032, Scans Averaged 12, Pressure: 1.95e-07, Low Mass Gate: 2000.0, Negative Ions: OFF.

2.2.11 Sequencing of Hg-Se complex binding protein

The proteins were sequenced in the McGill proteomics department via an automated protein sequncer. Automated Edman chemistry was performed on the protein or peptide sample in nanomolar to picomolar quantities.

MeHg concentrations in the extract were determined by a protocol taken from Schintu et al. (1992):

Extraction of MeHg

1. Approximately 0.5g of wet tissue were homogenized in 9 volumes of 50mM Tris-HCl in 30ml Sorvall polypropylene centrifuge tubes for 45 sec. At high speed. The homogenizer shaft was rinsed with Nan-pure water in between samples.
2. 0.25ml of 2mg/ml protease was added to all tubes. The tubes were capped, vortexed for 10 sec. And incubated at 50 C in a water bath for 1 hour.
3. After removal from the bath, 1.25ml 40% NaOH and 0.5ml 1% cysteine were added immediately. The tubes were capped, vortexed and placed in a shaker for 5 min. at 200.
4. 0.5ml of 0.5M cupric sulfate was added and then 5mls acidic NaBr. The tubes were capped , vortexed and placed in a shaker for 5min. at 200.
5. 2.5ml toluene was added and the tubes were capped, vortexed for a second and shaken for 2 minutes at 200. The tubes were then centrifuged in the Sorvall RC5C for 10 minutes at 6,800 RPM in the SLA-1000 rotor (6,600g).

6. The toluene (top) layer was removed with a disposible glass pipette and was placed in another pre-labeled plastic test tube. Step 5 was repeated. The second removed toluene layer was added to the first.
7. 4mls of the toluene layer was transferred into a 15ml disposible glass centrifuge tube. (If a different volume is used, record the new volume)
8. 1ml 5mM sodium thiosulfate was added and vortexed for 10 sec. Then it was centrifuged at 4,000g for 4 min.
9. The aqueous (bottom) layer was removed with a pipette and placed in a glass acid-washed pre-labeled test tube (Ensure the removal of any toluene transferred simultaneously by accident).
10. Steps 8 and 9 were repeated. The aqueous layers from both steps were combined.
11. 1.5ml of the aqueous layer was transferred into 50ml glass acid-washed digestion tube (If a different volume is used record the new volume).
12. DORM standard reference materials were used for quality assurance.

Acid digestion:

Nano-pure water 0.5ml and nitric acid 0.75ml were added to each sample which was subsequently capped loosely. The samples were heated to 70 C in a dri-bath fro 1-2 hours (with periodic monitoring for faming). The samples were removed from heat and permitted to cool a few minutes. When sufficiently cooled, 0.75ml of sulfuric acid was added to each tube. Each tube was shaken again very gently to mix (take care to avoid violent reactions). Samples were heated again at 70° C in a dri-bath for 2-3 hours. Samples were then cooled at room

temperature overnight. The digestion products were transferred to a 10ml acid washed glass graduated cylinder. The test tube and lid was rinsed with 2mM potassium dichromate-3% HCl using a glass pipette and the contents were added to the same graduated cylinder. The total volume was diluted to 10mls. After mixing, the acid digested sample was decanted into a 40ml vial. Then 9.9 ml 1.5%HCL and 100µl octanol were added respectively (the volume of the digest was 20mls). The samples were capped tightly and set-aside until Hg determination.

2.3 Statistical Analysis

Correlation analyses were performed using SAS Vs 6.11 (SAS Institute Inc., Cary, NC). A *p*-value of <0.05 was considered to be significant in all statistical analysis.

CHAPTER 3. RESULTS

3.1 Hg and Se Concentrations in Loon Tissues

Hg and Se concentrations were measured in four different Loon tissues from 27 different Loons. The tissues tested were brain, liver, kidney and muscle. Approximately 89 % of Hg was found to be associated with the membrane whereas 11% was associated with the cytosol (Table 1). For Se, approximately 77% of Se was found to be associated with the membrane whereas 23% was associated with the cytosol (Table 2). Among the tissues, the liver carried the greatest burden of Hg correspondingly with the greatest concentration of Se. Liver was followed by kidney; muscle and brain had the lowest concentrations of Hg and Se. Correlations between Hg and Se concentrations in various tissues can be seen in Table 3 and 4. There was a significant correlation (R^2=0.9540, $p<0.05$) between Hg and Se concentrations in liver tissue and in kidney tissue (R^2=0.7940, $p<0.05$), in contrast muscle and brain did not exhibit strong correlations (R^2=0.4416, $p<0.05$ and R^2=0.3081, $p<0.05$ respectively).

3.2 Gel Phase HPLC Coupled with ICP-MS

100μL of tissue extract supernatants were applied directly to the head of an SEC ProgelTm – TSK G4000 PWXL column coupled to an ICP-MS instrument (ELAN 6000 PE-SCIEX). The profiles of the chromatograms and the relationship of Hg and Se were characteristics of the tissue type. Molecular weight markers were run under identical HPLC conditions however were coupled to a UV detector (D-star Instruments DVW-10 variable wavelength detector) set

at 280nm. The protein peaks of Aprotinin (MW 6,500), Carbonic Anhydrase (MW 29,000), Albumin (MW 66,000) and Aproferritin (443,000) were eluted at 12.00, 11.06, 10.55 and 9.36, minutes respectively. Retention time and molecular weight (log MW) demonstrated a linear relationship with an R^2=0.9947. All of the tissue types demonstrated a common Hg and Se peak at approximately 630 seconds corresponding to protein of MW 72,000. In egg, both Hg and Se appear to exist in a single bound complex with MW 72,000 Da. There was also a close association between Hg and Se in liver and kidney. Both tissues had multiple complexes. The liver an additional peak of Hg and Se corresponding to the approximate molecular weight of 37,200 Da (Figure 1). The kidney had three peaks of Hg and Se corresponding to approximate molecular weights of 72,000 Da, 37,200 Da and <1000 Da (Figure 2). The associations between Hg and Se in brain (Figure 4) and muscle (Figure 3) tissues were minimal whereas the association for egg (Figure 5) was the strongest.

3.3 Collection of Fractions from Gel Phase HPLC

100µL of tissue extract supernatants were applied directly to the head of an SEC ProgelTm – TSK G4000 PWXL column coupled to a UV detector (D-star Instruments DVW-10 variable wavelength detector) set at 280nm. Retention times of the corresponding Se and Hg peaks found using the ICP-MS were used for each individual tissue type and sample (Figure 6). The collected fractions volumes varied depending on the length of the Hg and Se peak, these fractions were then freeze dried. Insufficient volume was collected to provide a noticeable precipitate; nevertheless, fractions were redissolved in 300µL and injected into a reverse phase C4 column under the gradient conditions described in 2.2.6. Peaks produced by this

injection proved insufficiently large enough to contain a sufficient amount of Hg and Se to be detected via direct application to ICP-MS.

3.4 Collection of Fractions from RP-HPLC and application to ICP-MS

Due to the failure to produce positive results on the ICP-MS regarding Hg and Se detection, 100μL of tissue extract supernatants were injected directly into the C4 column under the aforementioned solvent gradient. Absorbance was monitored at 210 and 280nm. This produced similar chromatograms to those generated by the Gel-Phase fractions with a nominal increase in the number of peaks while producing a significant increase in the absorbance of the corresponding peaks. Fractions of individual peaks were collected as they eluted. These peaks were freeze dried and redissolved in 1ml whereupon they were applied directly to the ICP-MS machine. Liver (Figure 7), kidney (Figure 8) and egg were the only tissues injected that produced peaks at approximately 50.6minutes containing high levels of Hg and Se. As can be seen the peaks generated were not singular and thus had multiple protein components. A white precipitate was collected from the freeze drying protocol following the consecutive injections and fraction collections. This precipitate was redissolved in 250μL of DDH_2O and 50μL was injected into the gradient conditions described between 2.2.8 and 2.2.9. For liver tissues this produced a chromatogram having between two and four peaks (Figure 10). These peaks were collected individually for both the purpose of ICP-MS and MALDI-TOF-MS. The second and third peak contained elevated Hg and Se (Figure 12) in liver and were thus the fractions applied to MALDI-TOF-MS (Figure 15 and 16). The second RP-HPLC run of kidney tissue produced generally only one peak (Figure 11), when

tested on ICP-MS it did indeed contain elevated Hg (Figure 13). Only the egg extracts were put through the RP-HPLC once as the peak collected at 50.6 minutes (Figure 9) was deemed pure enough for direct application to the ICP-MS (Figure 14) and MALDI-TOF-MS (Figure 18).

3.5 MALDI-TOF-MS

Liver tissue produced two Hg-Se binding complexes, each within the 15,200-15,300Da range (Figure 15 and 16) whereas kidney produced only one such complex, this complex too was within the 15,200-15,300Da range (Figure 17). When the egg fraction was applied to the MALDI-TOF-MS it produced no detectable protein peaks (Figure 18). Since both peptide and protein matricies were used, it is possible that the fractions collected were too dilute for detection for MALDI-TOF-MS analysis.

3.6 Hg-Se Protein Complex Sequencing

The final fractions from the liver and kidney tissue extract yielded a protein whose first 47 amino acids had the following sequence:

VALSAJDKTN VKGVFSKIGG HAEEYGAETL RMFITYPXXE TYAXLKV

When this sequence was compared with a database of existing proteins it was found to have its closest homology with Mallard Hemoglobin with the exception of a few point mutations. This is indicative that the protein purified was most likely loon hemoglobin. kidney tissue

extracts produced similar results. Egg tissue extract however produced a unique protein with the following sequence:

XSIGAASTEF

Table 1. Hg Concentrations in Cytosol and Bound to Membrane in Common loon Tissue (mean ± SD) n=8

Loon Tissue	Hg (μg/g wet weight)	Cytosol (%)	Membrane Bound (%)
Liver	138.18 ± 120.39	13.0 ± 10.47	87.0 ± 10.46
Kidney	50.33 ± 41.52	10.83 ± 11.21	88.83 ± 10.99
Muscle	6.21 ± 8.21	7.66 ± 9.97	92.33 ± 9.97

Table 2. Se Concentrations in Cytosol and Bound to Membrane in Common loon Tissue (mean ± SD) n=8

Loon Tissue	Se (μg/g wet weight)	Cytosol (%)	Membrane Bound (%)
Liver	40.70 ± 44.77	19.45 ± 15.10	80.55 ± 15.07
Kidney	24.31 ± 16.02	20.02 ± 9.49	79.80 ± 9.49
Muscle	1.48 ± 1.35	28.26 ± 22.87	71.73 ± 22.87

Table 3. Hg Distribution among Common loon tissues

Sample Identification	Total Mercury content per g (µg)	Methyl Mercury content per g (µg)	%
Muscles			
48707	4.1	3.1	75.8
L94-68604	8.0	-	-
L94-68605	2.8	-	-
L95-69356	2.4	-	-
L95-70033	11.9	2.2	18.7
L95-70038	20.3	15.1	74.4
L95-70039	28.5	21.1	74.0
L95-70040	1.3	1.0	76.8
Livers			
48707	141.0	3.7	2.6
L94-68604	278.0	16.9	6.1
L94-68605	5.7	3.3	58.5
L95-69356	23.6	2.2	9.2
L95-70033	274.0	5.1	1.9
L95-70038	123.0	23.8	19.3
L95-70039	129.0	32.1	24.9
L95-70040	19.4	2.2	11.1
Kidneys			
48707	79.1	3.5	4.5
L94-68604	112.0	10.6	9.5
L94-68605	3.0	2.4	81.0
L95-69356	11.0	1.7	15.3
L95-70033	240.0	4.1	1.7
L95-70038	129.0	18.9	14.7
L95-70039	146.0	25.7	17.6
L95-70040	7.1	1.2	17.4
Brains			
L95-70039	13.6	9.8	72.0
L95-70040	1.0	0.7	68.6

Table 4. Se Distribution among Common loon tissues

Sample Identification	Total Selenium content per g (µg)
Muscles	
48707	4.29
L94-68604	6.84
L94-68605	3.38
L95-69356	1.95
L95-70033	11.3
L95-70038	3.13
L95-70039	6.83
L95-70040	2.35
Livers	
48707	42.9
L94-68604	79.4
L94-68605	8.44
L95-69356	11.4
L95-70033	70.9
L95-70038	15.7
L95-70039	39.7
L95-70040	13.3
Kidneys	
48707	50.2
L94-68604	44.9
L94-68605	6.70
L95-69356	10.6
L95-70033	66.1
L95-70038	40.5
L95-70039	38.6
L95-70040	11.9
Brains	
L95-70039	4.67
L95-70040	2.25

Figure 1. Gel-Phase HPLC coupled to ICP-MS Chromatogram for Liver tissue extract

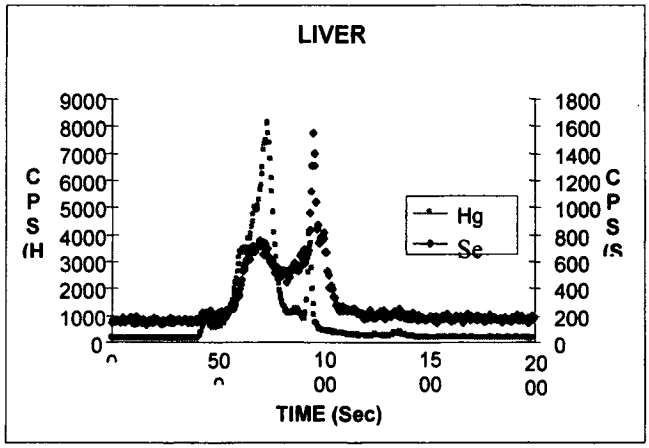


Figure 2. Gel-Phase HPLC coupled to ICP-MS Chromatogram for Kidney tissue extract

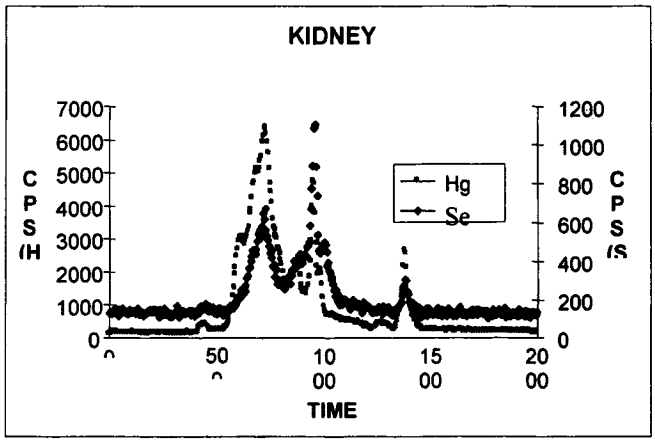


Figure 3. Gel-Phase HPLC coupled to ICP-MS Chromatogram for Muscle tissue extract

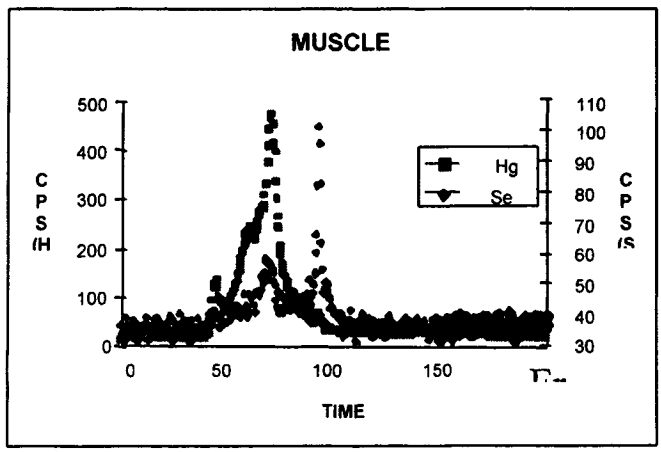


Figure 4. Gel-Phase HPLC coupled to ICP-MS Chromatogram for Brain tissue extract

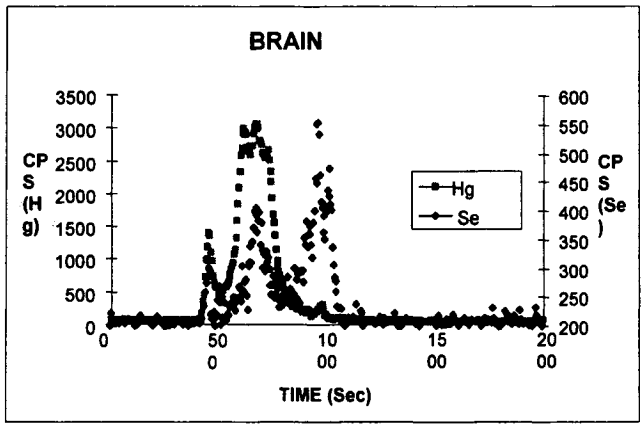


Figure 5. Gel-Phase HPLC coupled to ICP-MS Chromatogram for Egg extract

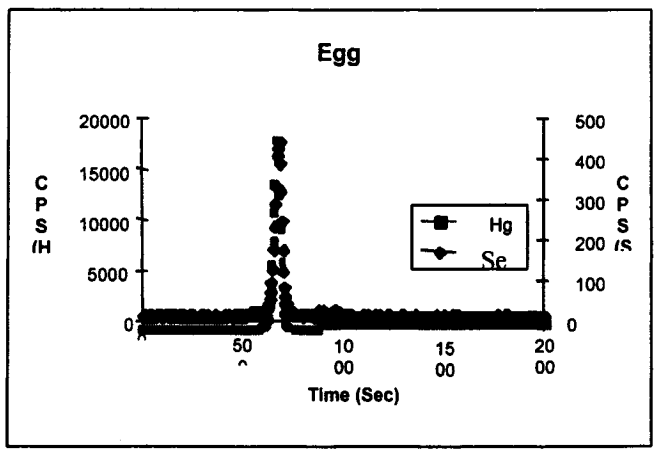


Figure 6. Example of Chromatogram (liver extract) of fraction collection times from Gel phase column coupled to UV detector at 280nm

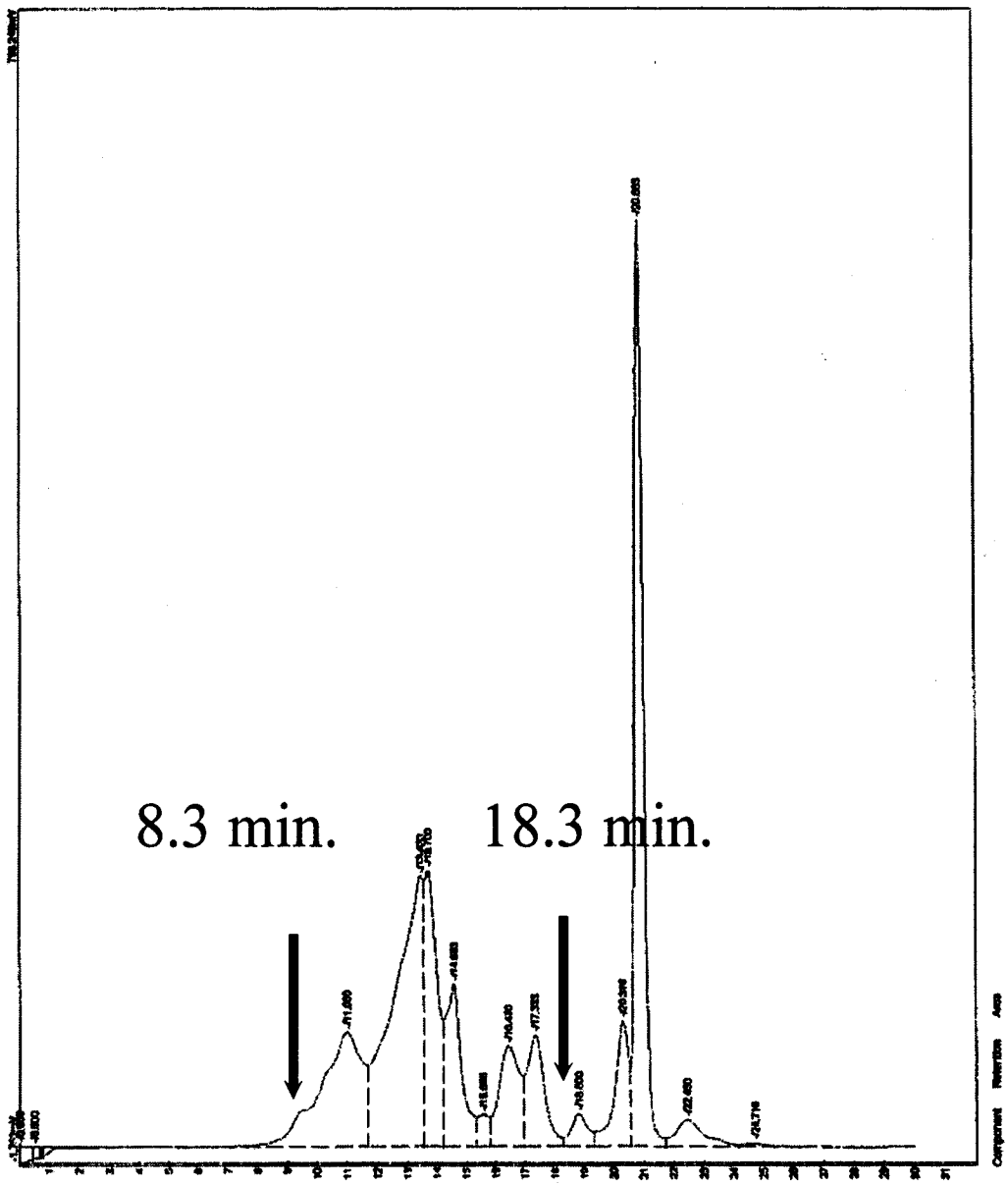


Figure 7. RP-HPLC coupled to UV detector set at 210nm and 280 nm Chromatogram of a liver extract

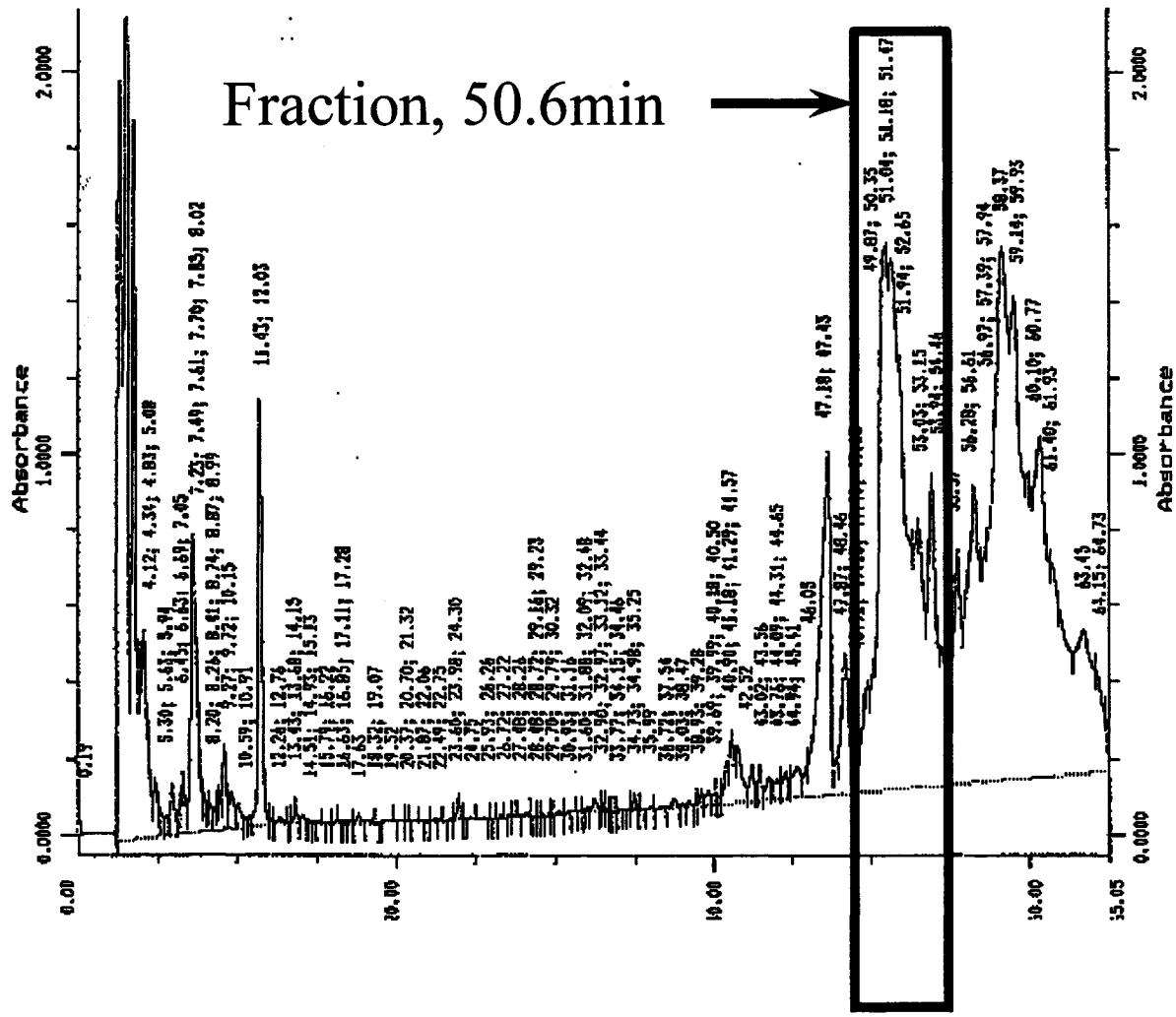


Figure 8. RP-HPLC coupled to UV detector set at 210nm and 280 nm Chromatogram of a Kidney extract

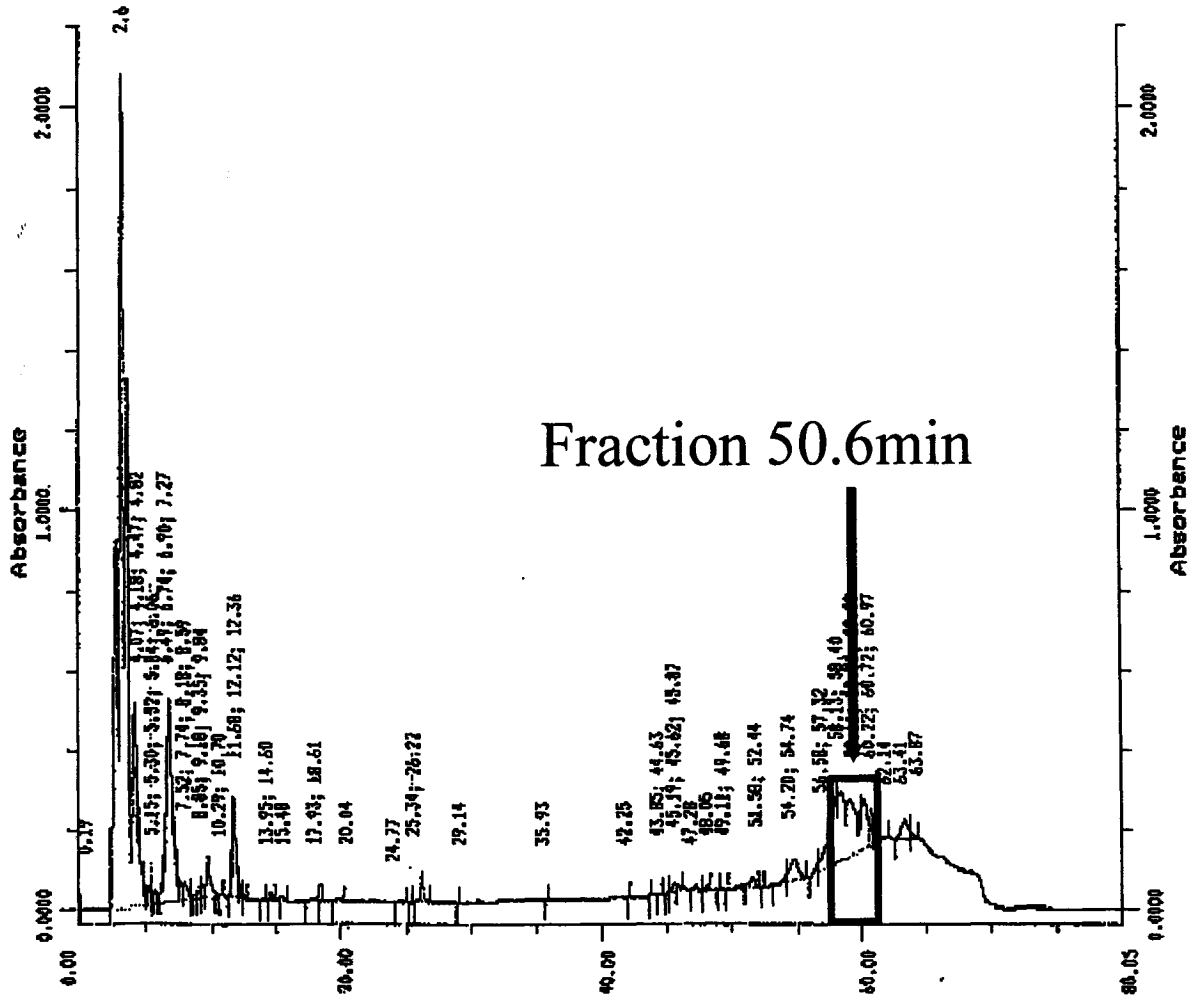


Figure 9. RP-HPLC coupled to UV detector set at 210nm and 280 nm Chromatogram of an Egg extract

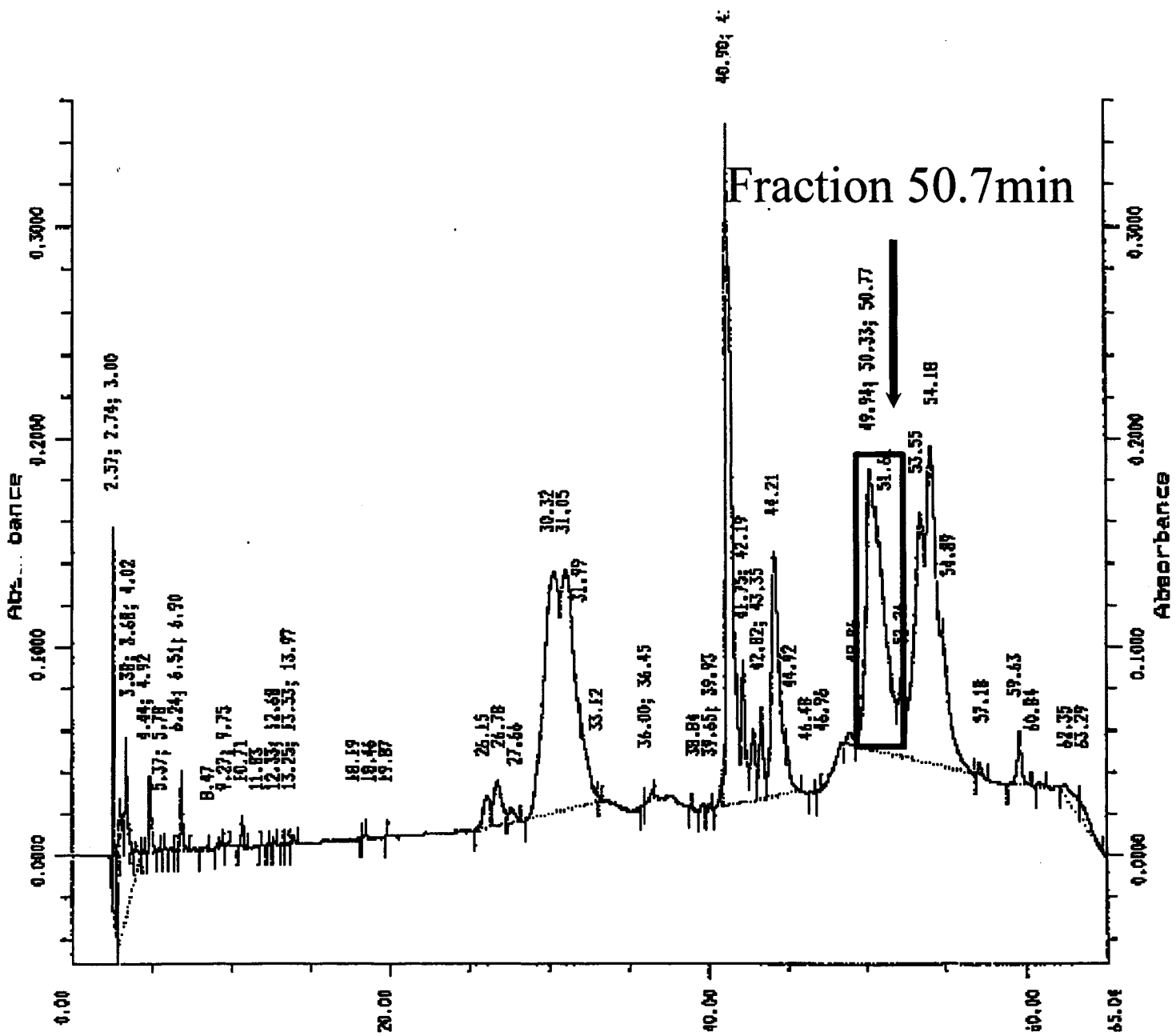


Figure 10. Second RP-HPLC coupled to UV detector set at 210nm and 280 nm Chromatogram of a Liver extract fraction

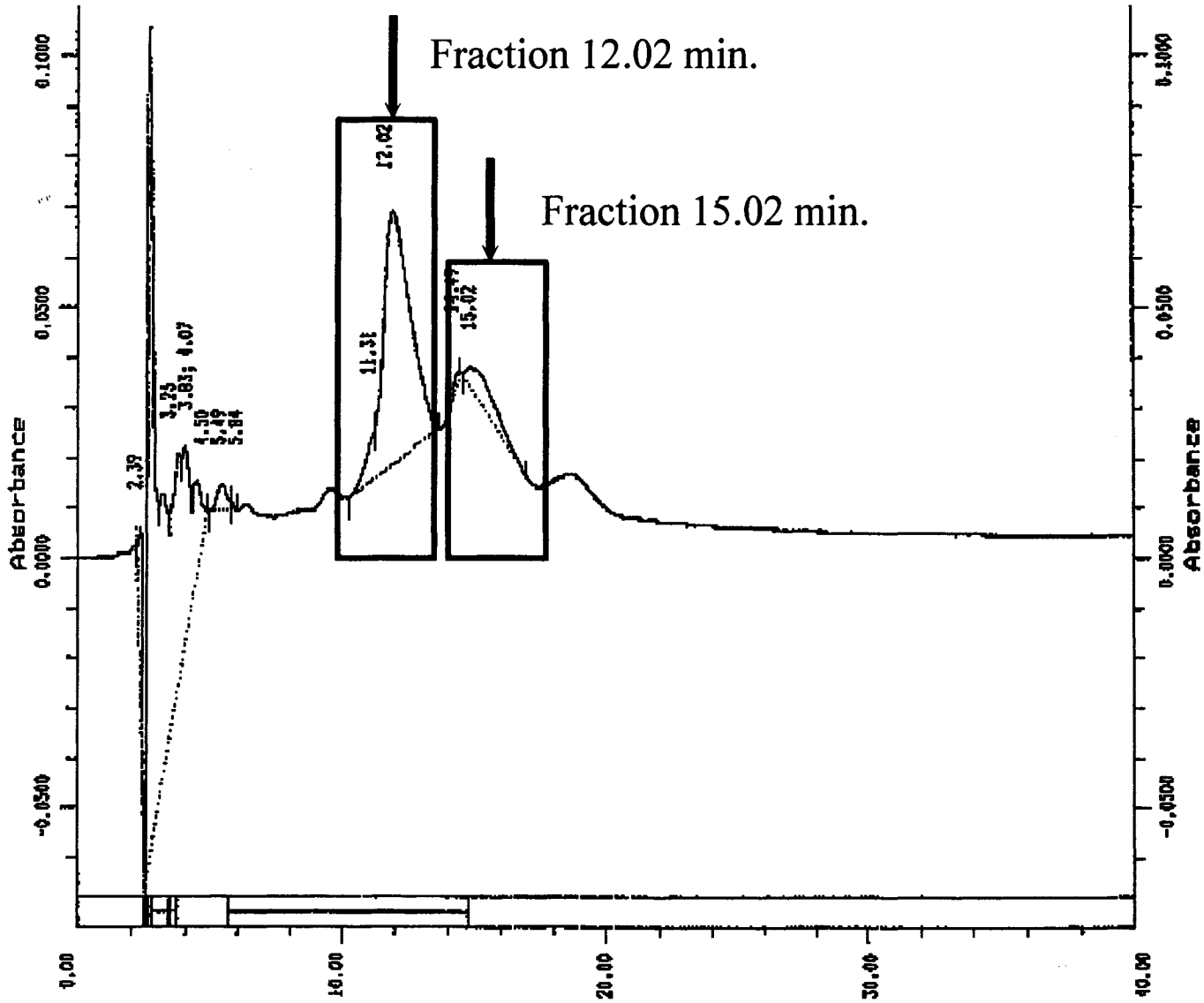


Figure 11. Second RP-HPLC coupled to UV detector set at 210nm and 280 nm Chromatogram of a Kidney extract fraction

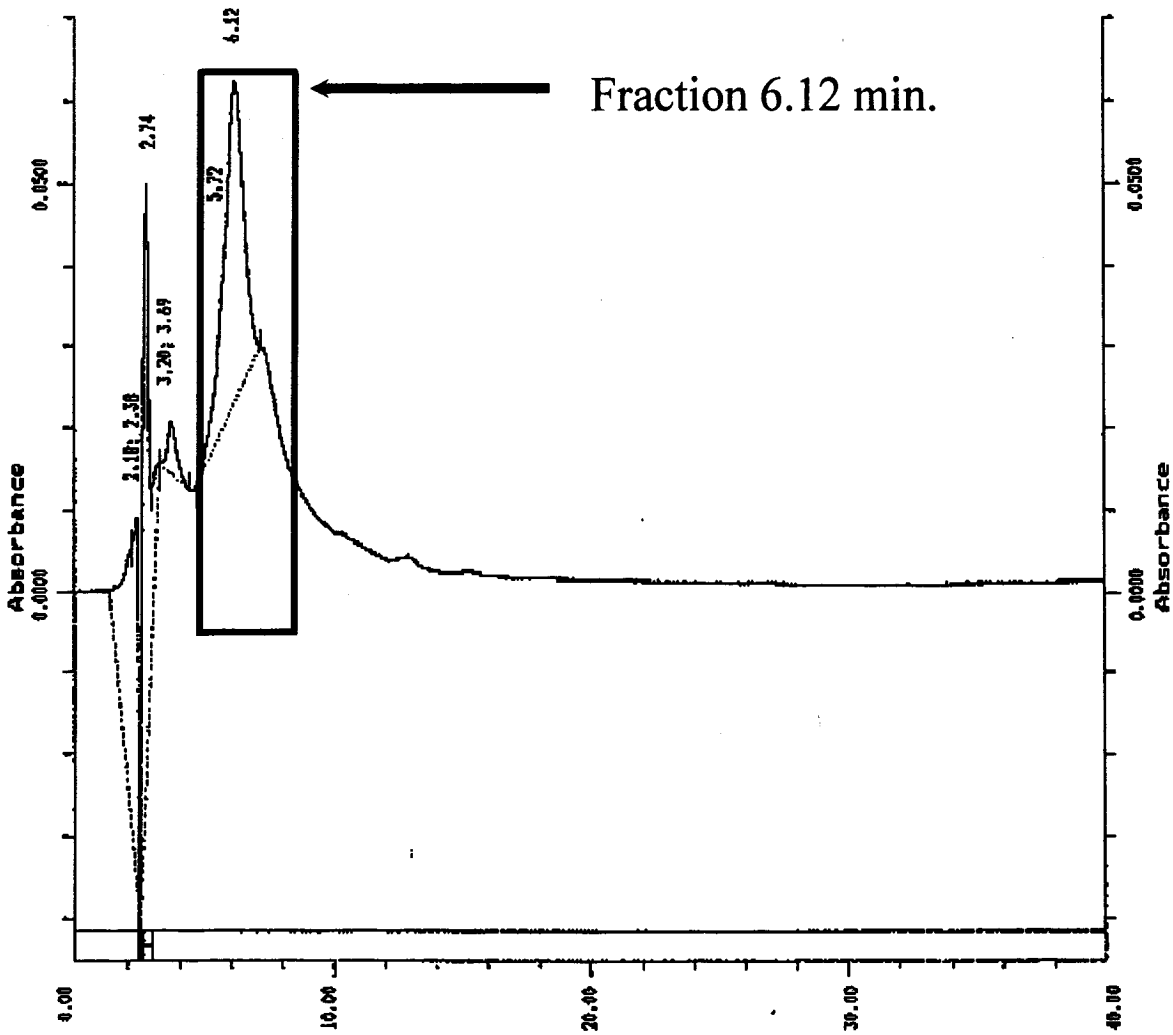
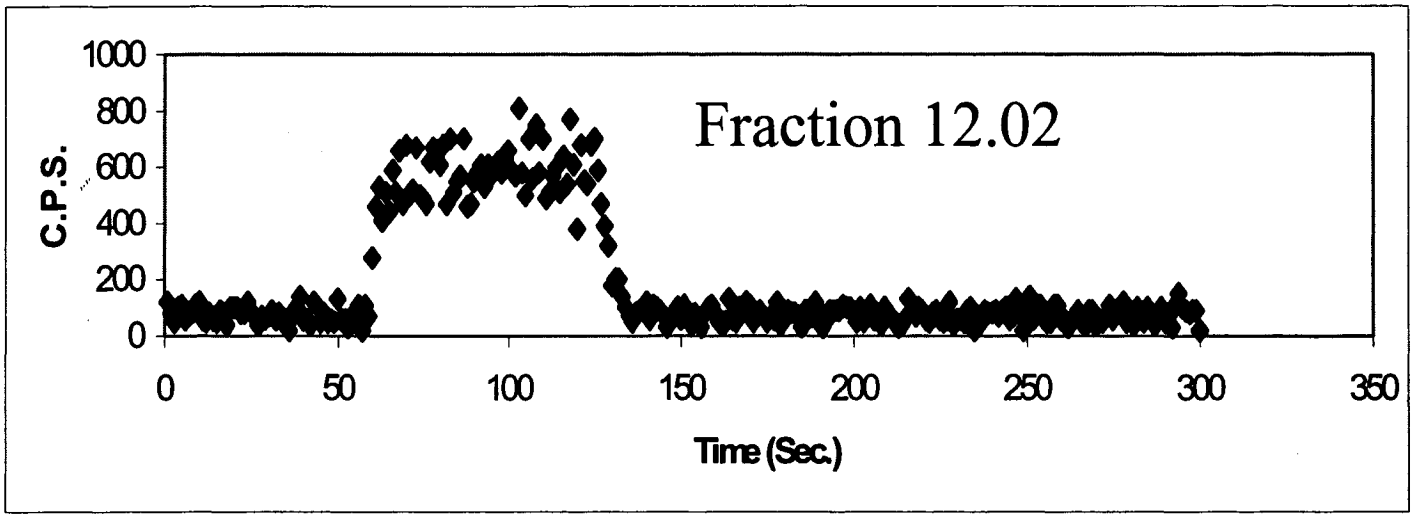


Figure 12. ICP-MS Chromatogram of RP-HPLC fractions from second run of Liver extract (Hg)



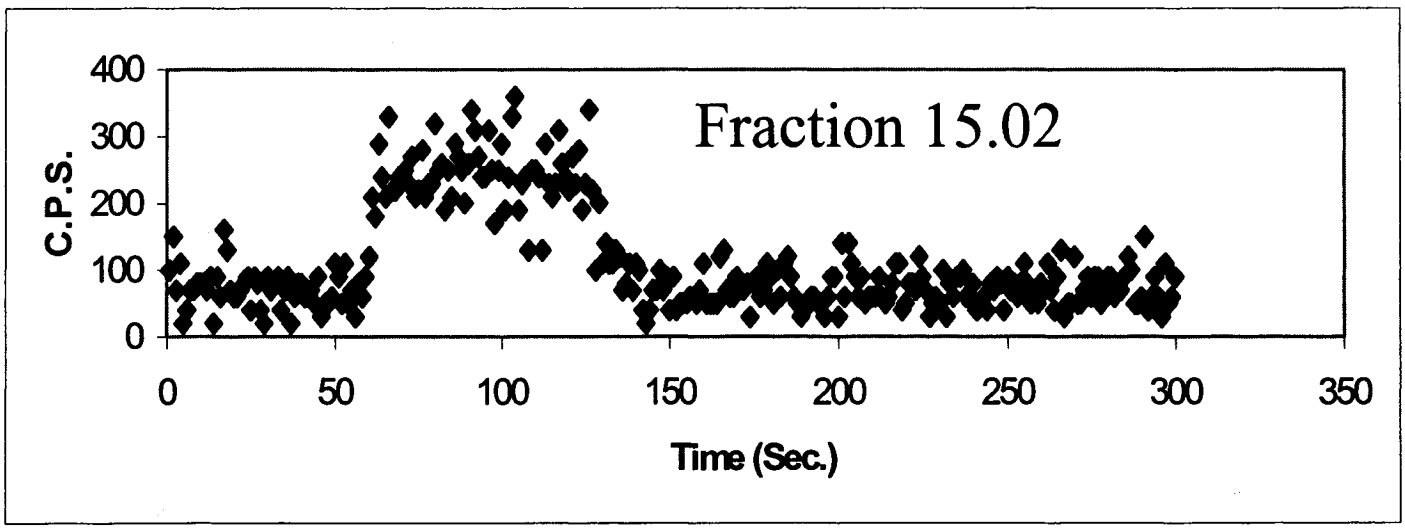


Figure 13. ICP-MS Chromatogram of RP-HPLC fractions from second run of Kidney extract (Hg)

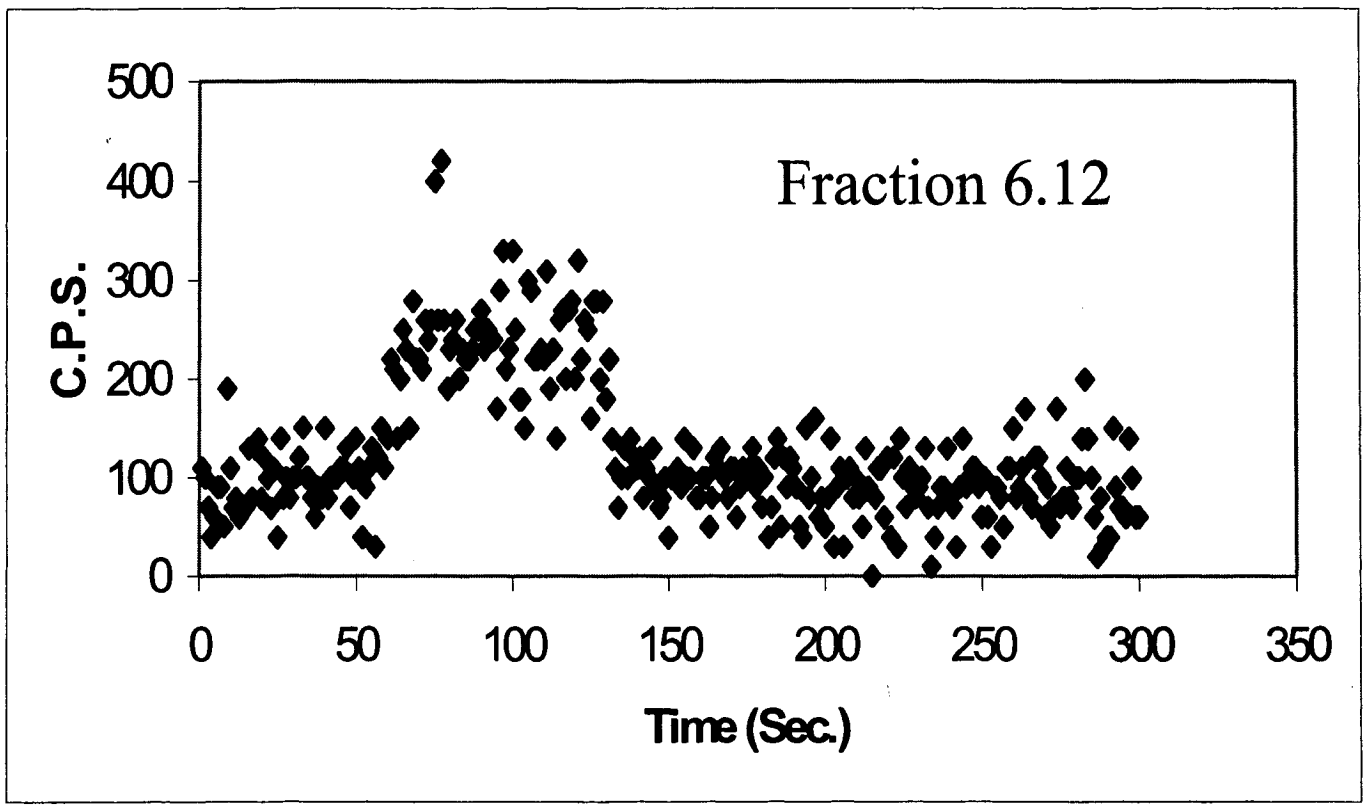


Figure 14. ICP-MS Chromatogram of RP-HPLC fractions from first run of Egg extract (Hg)

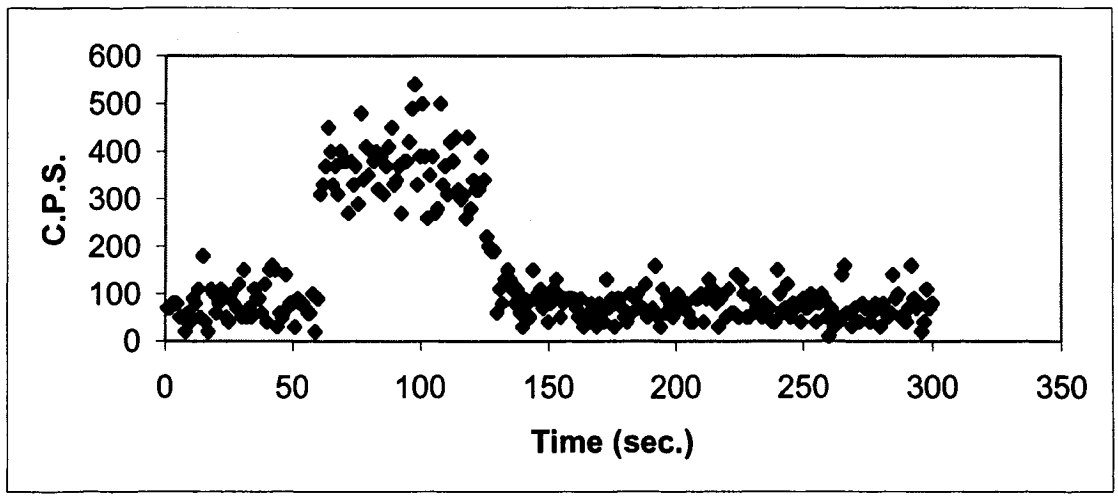


Figure 15. MALDI-TOF-MS of Hg and Se containing Liver fractions

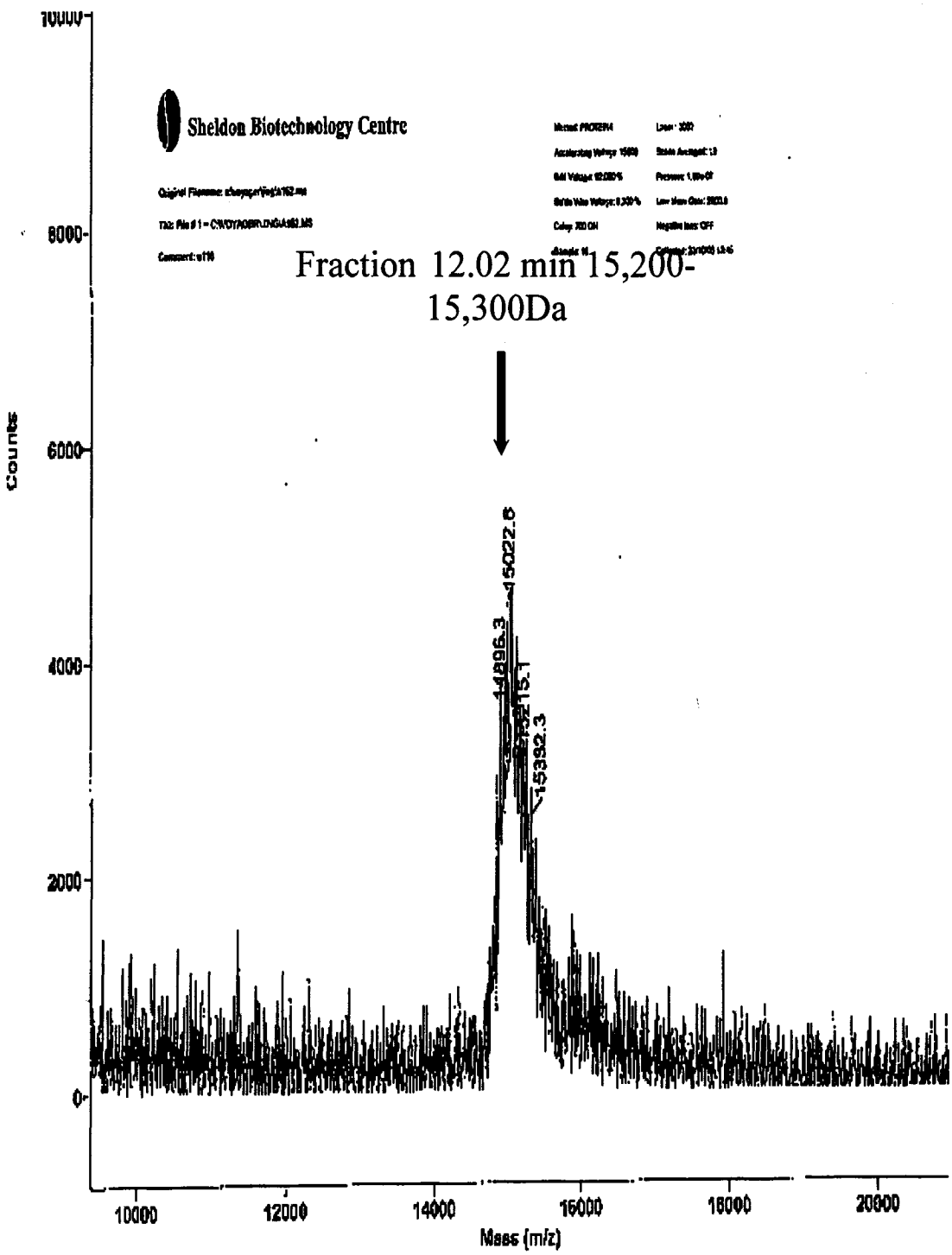


Figure 16. MALDI-TOF-MS of Hg and Se containing Liver fractions

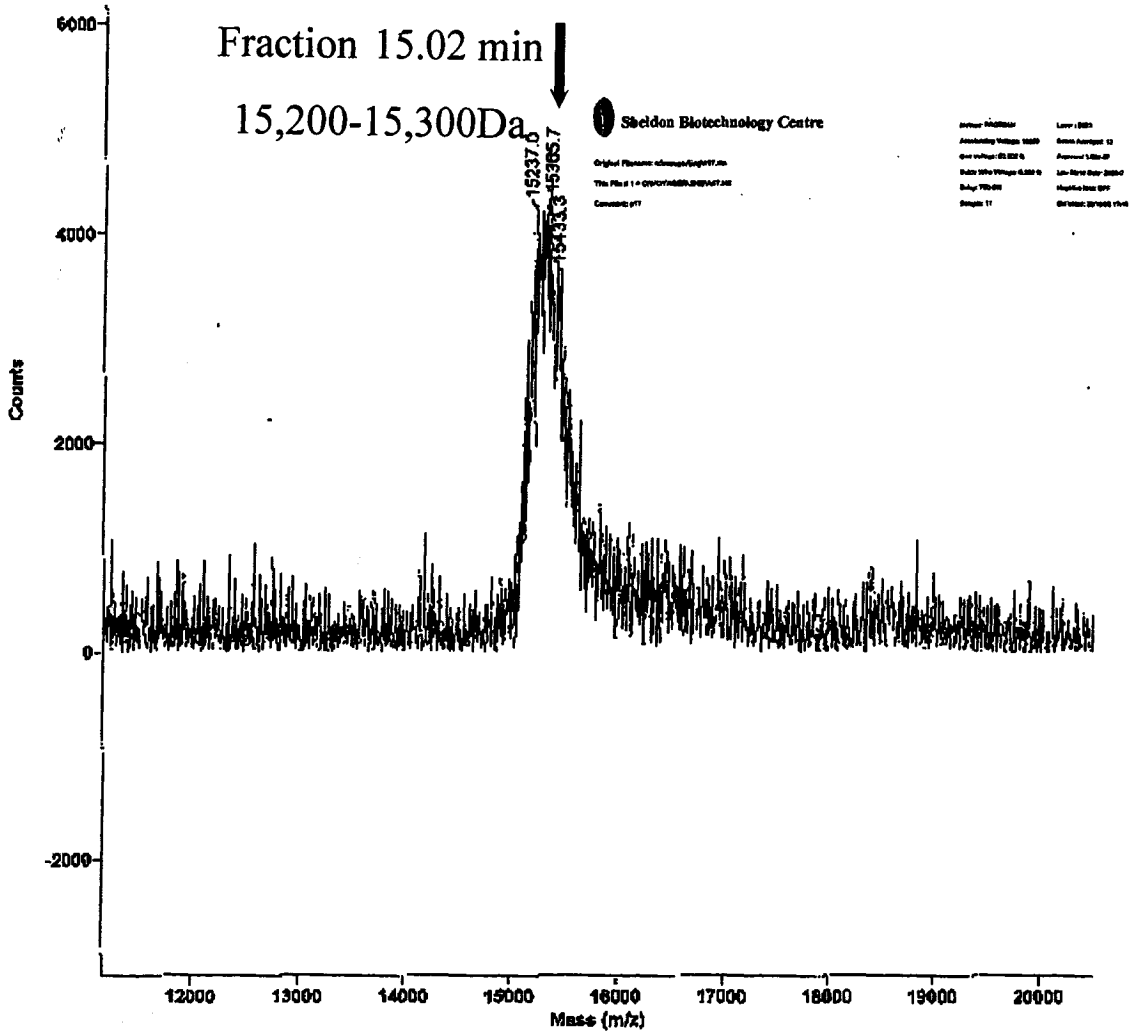


Figure 17. MALDI-TOF-MS of Hg and Se containing Kidney fractions

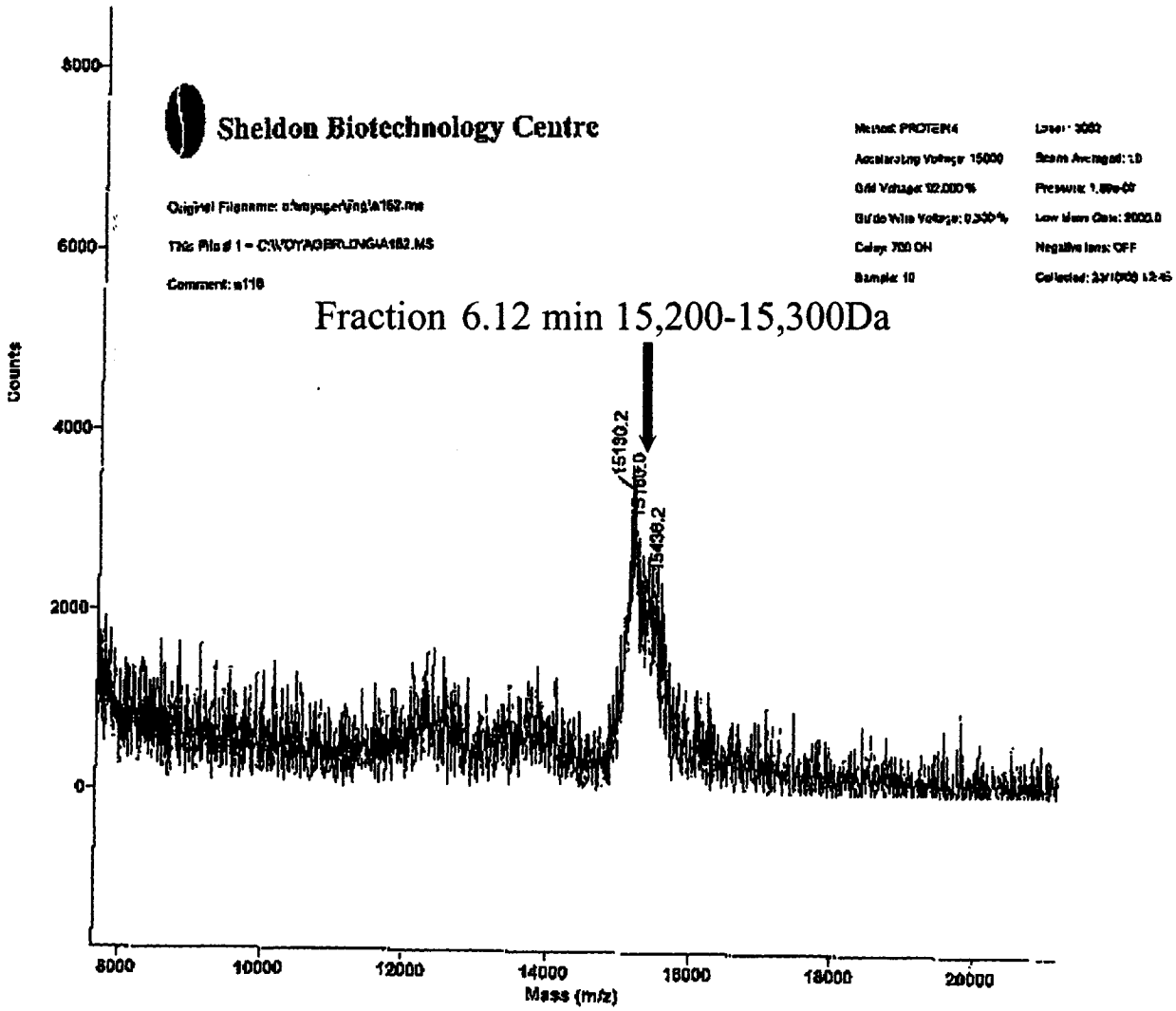
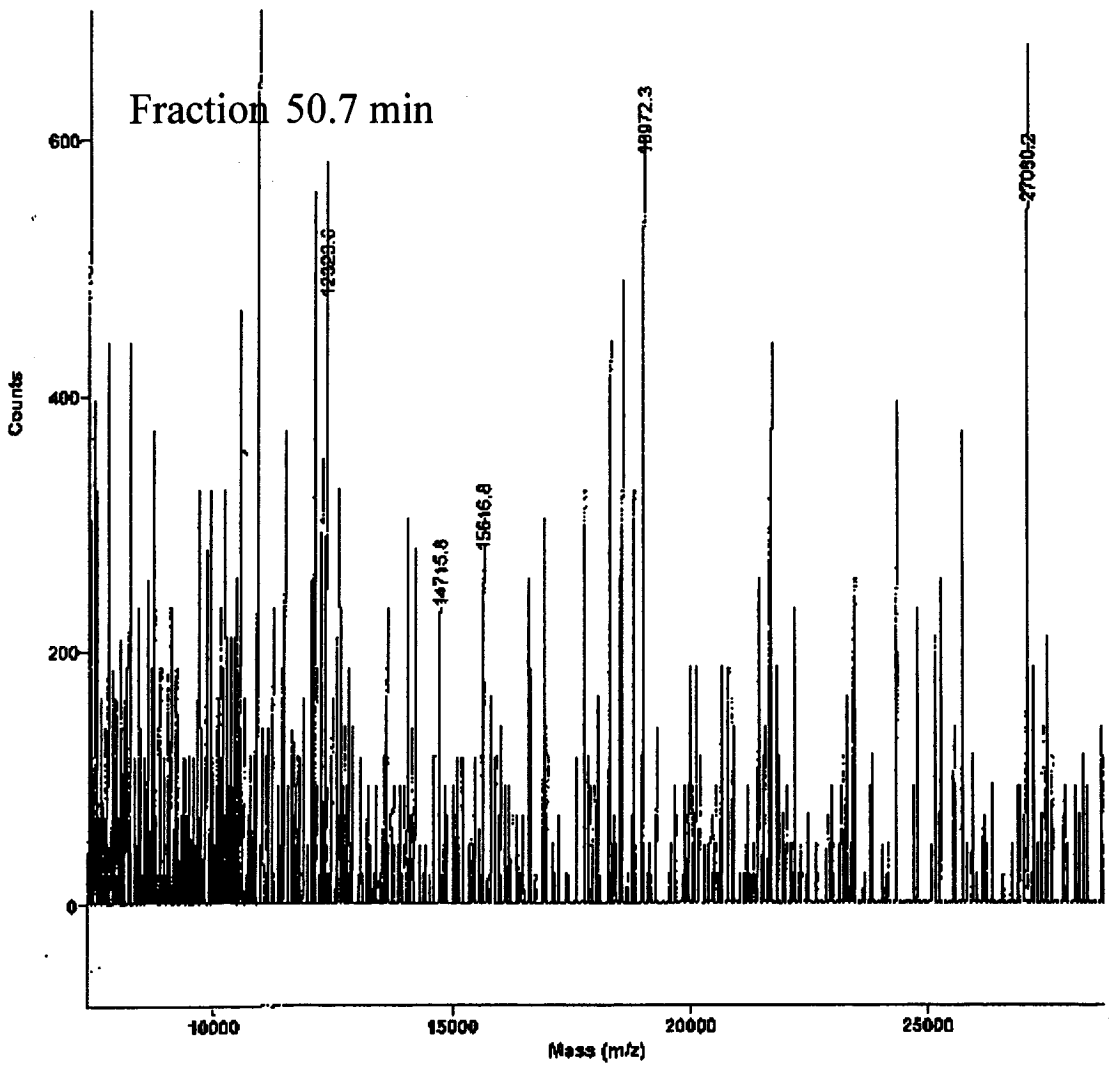


Figure 18. MALDI-TOF-MS of Hg and Se containing Egg fractions



CHAPTER 4. DISCUSSION AND CONCLUSION

Hg and Se concentrations and speciations within the loon tissues displayed a notable amount of variation. In addition Hg and Se concentrations between individual loons displayed significant variation as well, this is likely a reflection of the different locales and geographic areas the loons were collected from. Loons with higher concentrations of Hg in one of their tissues displayed high concentrations of Hg within their other tested tissue as well, conversely loons with low concentrations displayed low concentrations of Hg in all other tissues. Daoust et al., 1998 found similar results in their necropsy study. Studies confirm that loon populations in the eastern portion of their range (particularly Maritime provinces) are exposed to greater quantities of MeHg and as a result have higher concentrations within their tissues when compared to loons in the western portion of their range (Evers et al., 1998).

In this study, liver generally had the highest concentration of Hg (138.18 ± 120.39 μg/g wet weight), followed by kidney (50.33 ± 41.52 μg/g wet weight) and finally by muscle tissue (6.21 ± 8.21 μg/g wet weight). Similar results were found in another study by Scheuhammer et al., (1997), liver exhibited the highest concentration (19 ± 15 μg/g dry weight), followed by kidney (15 ± 15 μg/g dry weight) and then breast muscle (2.9 ± 1.1 μg/g dry weight). Scheuhammer et al.,'s study displayed somewhat parallel results to this one when Se distribution in the tissues was compared. As per Hg, the liver displayed the highest concentration followed by the kidney and muscle in this study (40.70 ± 44.77 μg/g wet weight, 24.31 ± 16.02 μg/g wet weight and 1.48 ± 1.35 μg/g wet weight respectively) correspondingly Scheuhammer et al., found 15 ± 12 μg/g dry weight in the liver, 11 ± 6.2 μg/g dry weight in the kidney and 3.0 ± 0.2 μg/g dry weight in the breast muscle.

The only tissues where a significant correlation between Hg and Se were seen were liver, kidney and egg, Scheuhammer et al., (1997) found similar results for liver and kidney however did not test eggs. In regard to eggs, of those analyzed MeHg accounted for 87% of the total and organic Hg concentrations in eggs (Scheuhammer et al., 2001). A total of 125 eggs were analyzed for Hg and Se of that 9 eggs had Hg concentrations higher than the level associated with reproductive impairment in birds generally 1 microg g(-1) ww; (Thompson, 1996). None had higher Se levels than those associated with negative reproductive effects. In this present study, egg Hg levels were not accurately quantified due to insufficient sample size Another study on loon eggs found eastern concentrations higher than their western counterparts, egg Hg concentrations ranged from 0.07 to 4.42 μg/g (wet weight) which is lower than the Hg levels found in the other tissues analyzed in this study (Evers et al., 2003). Liver and kidney exhibited the lowest percentage of MeHg out of total Hg (16.7% and 21.2% respectively) whereas muscle and brain had elevated percentages of MeHg out of total Hg (63.9% and 70.3% respectively). Gaskin et al., (1979) found in a study on Harbor Porpoises that the majority of Hg in the liver was inorganic and only about 17% was methylated in contrast with muscle where virtually all of the Hg was methylated. Generally, in both liver and kidney as the total Hg increased the percentage of MeHg decreased, conversely the total Se increased concurrently with total Hg. Percentages of MeHg out of total Hg remained fairly constant in muscle as did total Se, again these results are paralleled by Scheuhammer et al., (1997). This is indicative that liver and kidney are possible tissue sites of MeHg demethylation and Hg sequestering via Se. As mentioned in the introduction MeHg inhibits protein synthesis while Hg^{2+} is an even more potent inhibitor. The inhibition of protein synthesis may be one of the principal causes of Hg (both organic and inorganic) toxicity; in

rats, inorganic Hg was 10x more a potent inhibitor of protein synthesis than organic Hg (Sugano et al., 1975) The inhibition of protein synthesis is believed to be the main cause of neurotoxic death (Sugano et al., 1975). MeHg is also known to induce peroxidation and cause oxidative damage, Sarafian and Verity (1991) demonstrated that MeHg causes membrane peroxidation in nerve cells, however according to another study this peroxidation does not seem be the critical mechanism for cell death (Atchison and Hare, 1994). The increased burden of having the more potent inhibitor of protein synthesis-inorganic Hg, is offset its relatively short biological half-life when compared to MeHg due to its preferential excretion through the feces and urine (Norsth and Clarkson, 1971), this is one pathway an organism might use to excrete Hg. Another possibility is that MeHg is demethylated elsewhere in the body (Tissue macrophages, intestinal flora) complexed with Se and sequestered in the liver and kidney, in this respect inorganic Hg has a relatively long half-life, however when complexed with Se is unable to exert negative effects. This conceivably could be a mechanism an organism uses to remove Hg from being bioavailable and therefore able to cause toxicity. However, muscle and brain displayed a high percentage of MeHg out of total Hg. This suggests that there is no mechanism for demethylation within these tissues. The main mechanism of MeHg toxicity in muscle tissue is by disturbing the mitochondrial energy metabolism in skeletal muscle via the decrease in mitochondrial enzyme activity (Usaki et al., 1998). MeHg gradually lead to the loss of muscle strength and weakness in rats (Usaki et al., 1998). The brain is generally accepted as the critical organ for MeHg toxicity (NRC 2000). It is well known that MeHg crosses the Blood Brain Barrier via the L-system (Leucine preferring) amino acid carrier when bound to cysteine (Kerper et al., 1992). Once in the brain MeHg accumulates and slowly degrades to Hg^{2+}, most likely through its own homolytic

cleavage. As mentioned earlier the main cause currently accepted as the mechanism for cytotoxicity is via the disruption of protein synthesis. Whether this accumulation and lack of demethylation is due to the deficiency of Se in the brain and muscle is unknown. It has been suggested that the brain may have its own system of generating peroxides in order to demethylate MeHg, this system has not been elucidated and would appear operate at a relatively low kinetic rate since brain displays such a high percentage of MeHg out of total Hg.

Hg-Se complexes were found only in liver and kidney tissues, via MALDI-TOF, the complexes isolated from RP-HPLC from eggs were not detected for reasons that remain unknown, it is possible that the peptides were too small to be detected via the particular matrix used (although peptide specific matrices were applied). The lack of the ability to characterize this complex in eggs in this study demonstrates the need for further analysis. The first protein complex known to bind Hg and Se to be characterized was done by Gailer et al., in 2000, it was isolated by injecting rabbits simultaneously with mercuric chloride and sodium selenite buffered in glutathione. The complex (Selenoprotein P) was isolated out of the plasma and was found to be approximately 57,000Da. In addition it was found to be extensively glycosylated. The is one of the first Hg-Se-protein complexes to be characterized, however, it should be noted that the conditions under which the rabbits were administered Hg and Se are totally different than those found in the ingestion of piscivorous birds and most wildlife in general (which bioaccumulate MeHg and Se from food sources). In the present study, two Hg-Se binding complexes were isolated from liver, both in the 15,200 to 15,300Da range, one protein complex of this weight range was isolated from the kidney. Although both peaks from the liver yielded the same species of protein, they eluted separately on RP-HPLC.

This might be explained by the fact that they may have been slightly fragmented differently and thus have to some extent different retention times due to minor differences in hydrophobicity.

It was impossible based on the methodology of this study to determine that the Hg and Se were chemically bound to one another, only an association could be determined. Suzuki et al, (1998) using X-ray crystallography were able to prove that Hg and Se were bound and did indeed form polymers which were bound to Selenoprotein P in the plasma. In loons, there are currently no studies quantifying the ratios of Se bound Hg to free Hg. Using a combination of AAS, NMR, ICP-MS and X-ray crystallograpghy it would technically be possible to determine this.

The formation of an inert Hg-Se-protein complex has long been suggested as a method an organism utilizes to protect itself against Hg toxicity (both organic and inorganic). How this complex behaves in an organism and its effect on various tissues remains unknown. Studies performed as early as 1977 by Nomiyama and Foulkes seem to disqualify the possibility of heavy metals bound to proteins being eliminated via urine. Indeed, mainly only inorganic ionic forms can be eliminated via urine excretion (Magos and Butler, 1976). When sequenced it was found that these proteins were most likely the α A chain of hemoglobin since they were virtually identical to the α A chain of the Common mallard (Anas platyrhynchos) hemoglobin. In addition, the weight of the α A chain of the Common mallard is 15,248Da which is very close to the weight of the protein isolated in this project (15,200-15,300Da). It should be known that the tissues analyzed were not fresh, and other Hg and Se binding proteins and enzymes may have degraded prior to analysis. A study confirms that more than 90% of the MeHg in human blood is bound to hemoglobin in red blood cells,

specifically to the α chain on cysteine residue number 104, it also binds the β chain at cysteine residues 93 and 112, (however no β chain was not isolated in this project) (Kershaw et al., 1980). Residues 104 and 112 are in the contact junction of hemoglobin and are not as easily bound as residue 93, which lies on the external face of hemoglobin. The fact that the isolated protein was hemoglobin however, does not explain why kidney and liver had such a strong correlation between Hg and Se and why brain and muscle tissue did not. It is possible that MeHg is demethylated in the presence of Se via mechanisms specific to these tissues and sequestered there. Complexes found in the blood such as Selenoprotein P may transfer these complexes back to Hemoglobin, this is highly speculative and should be another path for further research. Egg tissue had a unique protein. Eggs had the highest correlation between Hg and Se, one might speculate that this protein may exert protective effects on the developing chick against Hg toxic effects allowing it to develop normally, however studies performed on Mallards indicate that when Hg and Se were administered together, reproductive success was significantly lower than when Hg or Se were administered individually (in addition to being substantially lower than controls) (Heinz and Hoffman, 1998). The protein was not completely sequenced, nor is its specific molecular weight known, an estimated weight of 72,000 Da was determined via HPLC-ICP-MS. Further protein must be isolated and a complete sequence elucidated in order to determine if any homologous proteins exist and if the binding to the Hg-Se complex is their sole function. Since the complete sequence was not established it is impossible to say whether the protein isolated by Gailer et al., (2000) is homologous to the protein found in this study. Clearly there is a substantial weight difference between the two Hg-Se binding complexes; therefore plasma may have a specific protein for binding Hg and Se. With regards to the weight discrepancies

between the gel-phase HPLC and the weights ultimately determined via MALDI-TOF-MS, the Hg-Se containing proteins may have been complexed with other cellular proteins causing a distortion in the retention times. As mentioned earlier the conditions under which Gailer's study was performed and this one were very different, therefore there is no reason to suggest that there is a possibility the two complexes found have any homology. Unfortunately, loon blood was not analyzed for such complexes; this may be an avenue for future studies to investigate.

In response to the hypothesis, it was determined that Hg and Se did form complexes and that in fact these complexes were bound to the α A chain of Hemoglobin. In addition the location of these entire complexes appeared to be tissue specific. As to whether the formation of these complexes afforded a form of protection against the toxicological effects of Hg is difficult to ascertain based on the results of this study. Thus this would be another area requiring further research. Future studies may also want to look at geographical variation between the occurrences of complexes if any do indeed exist.

General Conclusion

Hg and Se concentrations were measured in various Common loon tissues; brain, muscle, liver and kidney were included. The highest concentrations of Hg and Se measured were in the liver, liver also demonstrated the highest correlation between Hg and Se (R^2=0.9540, $p<0.95$). Multiple runs of RP-HPLC were used in conjunction with ICP-MS in order to isolate and characterize the Hg-Se complexes, and once isolated the purified complexes were sequenced. Upon extraction it was found that the majority of the Hg and Se in the liver, kidney and muscle tissues was bound to the cell membrane. The results from gel-phased HPLC coupled to ICP-MS indicated that for liver cystosol, two complexes may exist, a complex of 72,000 Da and a complex of 37,200 Da. Kidney had the same two complexes and an additional third of <1000 Da. Egg had a single complex of 72,000 Da. There were not significant correlations between Hg and Se in muscle and brain. RP-HPLC yielded proteins in the 15,200 – 15,300 Da range for liver and kidney, upon sequencing these were found to share homology with Mallard hemoglobin and were thus most likely loon hemoglobin. It is possible, that when put through the gel-phase column coupled to the ICP-MS, and they remained complexed with other proteins yielding higher molecular weights and multiple complexes. For reasons unknown, neither peptide nor protein matricies allowed approximate molecular weights determination on the egg extracts via MALDI-TOF-MS. Thus, the only approximation of the complex's weight comes from the gel-phased HPLC (72,000 Da). The complete sequence of the egg complex is an avenue for future research, as well as the search for other reasons and mechanisms of why fish-eating birds demonstrate such a high tolerance to MeHg.

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