SEPARATION AND DETERMINATION OF BIOMACROMOLECULAR METAL COMPLEXES IN LIVING ORGANISMS

CANLI ORGANİZMALARDAKİ BİYOMAKROMOLEKÜLER METAL KOMPLEKSLERİNİN AYRILMASI VE TAYİNİ

SERHAT DÖKER

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for degree of **Doctor of Philosophy** in **Chemistry**.

Chairman : ……
Prof. Dr. A. Rehber TÜRKER

Member
(Advisor) : ……
Prof. Dr. Mehmet DOĞAN

Member : ……
Prof. Dr. Kadir PEKMEZ

Member : ……
Prof. Dr. Elmas GÖKOĞLU

Member : ……
Prof. Dr. Emir Baki DENKBAŞ

APPROVAL

This thesis has been certified as a thesis for degree of Doctor of Philosophy by the above Examining Committee Members on …./ …./ ….

…./ …./ ….

Prof. Dr. Adil DENİZLİ
Director of the Graduate School of
Natural and Applied Sciences

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Serhat Döker

ABSTRACT

In the presented study, concentrations of chromium, copper, iron, manganese and zinc were determined in liver, kidney, brain, lung, heart and testis of mice upon intraperitoneal injection of a single dose hexavalent chromium (Cr(VI)). As results, comparing to control subject, chromium concentrations increased ca. 40-fold in liver and kidney and by a factor of 3-5 in all the other tissues. The homeostasis of Cu, Fe, Mn and Zn was also affected.

The distribution of essential trace elements between different molecular weight biomolecules was evaluated in the cytosols of the different mouse organs by size-exclusion chromatography (Superdex-75 column) with UV-VIS and ICP-MS detection. The administration of Cr(VI) resulted in differences in the elution profiles of Fe, Mn, Cu and Zn-protein complexes. Bioinduced Mn, Fe and Zn-binding proteins could be detected in some tissues, especially in liver and kidney.

Different molecular weight fractions containing chromium were heartcut and submitted to tryptic digestion prior to MALDI-TOF-MS analysis. Cr-peptide complexes could be recovered both in non-denaturing and in denaturing (in the presence of urea and DTT) conditions. They were isolated by size-exclusion chromatography with a smaller separation range (Superdex Peptide) but could not be identified. Experimental parameters in both sample preparation and analytical measurement were optimized and discussed through the analytical procedures.

Keywords: Hyphenated techniques, SEC-UV-ICP-MS, metallomics, hexavalent chromium, trace element homeostasis

Advisor: Prof. Dr. Mehmet Doğan, Hacettepe University, Department of Chemistry, Analytical Chemistry Division

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ÖZ

Sunulan çalışmada, periton içine tek dozda hekzavalan krom (Cr(VI)) çözeltisi injekte edilen deney farelerine ait karaciğer, böbrekler, beyin, akciğerler, kalp ve erbezleri dokularında krom, bakır, demir, mangan ve çinko düzeyleri tayin edilmiştir. Sonuçlar kontrol grubu deneklerine ait örneklerle karşılaştırıldığında krom içeriklerinin karaciğer ve böbreklerde yaklaşık 40 kat, diğer dokularda ise 3-5 kat arttığı; Cu, Fe, Mn and Zn dengesinin ise etkilendiği görülmüştür.

Farklı organlardan elde edilen sitosolik özütlerdeki esansiyel eser elementlerin değişik molekül ağırlıklı biyomoleküller arasında dağılımı, dedektör olarak UV-VIS ve ICP-MS kullanılan boyutça eleme kromatografisi (Superdex-75 kolonlu) hibrit sistem kullanılarak tayin edilmiştir. Cr(VI) maruziyeti Fe, Mn, Cu and Zn-protein komplekslerine ait elusyon profillerinde önemli farklılıklara neden olmuştur. Özellikle karaciğer ve böbrekler olmak üzere Mn, Fe ve Zn-bağlayıcı proteinlerin biyolojik sentezlenmelerinde tetiklenmeler veya azalmalar saptanmıştır.

MALDI-TOF-MS analizi öncesi değişik molekül ağırlığındaki krom içeren biyomoleküllere ait fraksiyonlar toplanmış ve doğal ve denature edici (üre ve DTT varlığında) şartlarda tripsin enzimi yardımıyla parçalanarak Cr-peptid kompleksleri elde edilmiştir. Daha küçük aralıktaki boyutça eleme kolonu (Superdex Peptide) kullanılarak kompleksler izole edilmiş ancak ilgili proteinlerin tanımlanması yapılamamıştır. Örnek hazırlama ve analitik ölçümlere ilişkin deneysel işlemler optimize edilmiştir.

Anahtar kelimeler: Hibrit teknikler, SEC-UV-ICP-MS, hekzavalan krom, metalomik, eser element dengesi

Danışman: Prof. Dr. Mehmet Doğan, Hacettepe Üniversitesi, Kimya Bölümü, Analitik Kimya Anabilim Dalı

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ABBREVIATIONS

AAS	Atomic Absorption Spectrometry
ACCA	α-cyano-4-hydroxy cinnamic acid
AES	Atomic Emission Spectrometry
AFS	Atomic Fluorescence Spectrometry
ATT	6-aza-2-thiothymine
amu	atomic mass unit
b.w.	body weight
CE	Capillary Electrophoresis
CRM	Certified Reference Material
CVAAS	Cold Vapour Atomic Absorption Spectrometry
CZE	Capillary Zone Electrophoresis
DNA	Deoxyribose Nucleic acid
DRC	Dynamic Reaction Cell
DTT	Dithiothreitol
EDXRF	Energy Dispersive X-Ray Fluorescence
ESI	Electrospray Ionization
ESI-MS	Electrospray Ionization Mass Spectrometry
ETAAS	Electrothermal Atomic Absorption Spectrometry
ETV	Electrothermal Vaporisation
FAAS	Flame Atomic Absorption Spectrometry
FI	Flow injection
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HGAAS	Hydride Generation Atomic Absorption Spectrometry
HPLC	High Performance Liquid Chromatography
HSAB	Hard and Soft Acids and Bases theory
ICP-OES	Inductively Coupled Plasma-Optic Emission Spectrometry
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
LA	Laser Ablation
LC-ICP-MS	Liquid Chromatography - Inductively Coupled Plasma - Mass Spectrometry
LOD	Limit Of Detection

MALDI	Matrix Assisted Laser Desorption Ionization
MIP	Microwave Induced Plasma
M	Molecular mass
MW	Molecular Weight
NAA	Neutron Activation Analysis
NIST	National Institute of Standards Technology
PAGE	PolyAcrylamide Gel Electrophoresis
PEEK	Polyetheretherkethone
PMSF	Phenylmethylsulphonylfloride
PTFE	Polytetrafluoroethylene
RNA	Ribonucleic Acid
RP	Reversed Phase
SA	Sinapinic acid
SDS	Sodium Dodecyl Sulphate
SEC	Size Exclusion Chromatography
TMAH	Tetramethylammonium hydroxide
TOF	Time-Of-Flight
TXRF	Total reflection X-Ray Fluorescence
UV-VIS	Ultra Violet-Visible

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1. INTRODUCTION

Metal ions occurring in living systems at trace levels play a vital role in the life processes. The function of many proteins critically depends on their interaction with a metal. Trace elements such as Zn, Cu, Fe and Mn have a number of functions as enzyme cofactors and structural components of proteins. Mammalian cells possess efficient uptake mechanisms to obtain ions from their environment and also intracellular delivery systems to translocate essential metal ions to specific enzymes and proteins (Guo et al., 2005). Metal-binding proteins and metalloproteins represent a large portion of the total number of proteins and it is estimated that around 40% of all proteins and enzymes contain metal ions (Garcia et al., 2006). The uptake, transport, translocation, storage and secretion of metal ions are maintained through highly regulated processes. Minor alterations in quantity, form or place of these vital elements may lead to imbalances in the essential metal ion homeostasis associated with several diseases or anomalies (Nelson, 1999; Giedroc and Arunkumar, 2007; Zheng et al., 2003). Insufficient uptake of essential metal ions causes deficiency and affects normal cell functions, but at high concentrations many metal ions even relatively low-toxicity metals can participate in undesirable redox reactions, favour the production of reactive oxygen species leading to toxic effects, or can bind inappropriately to macromolecules leading to disfunction of the biomolecules.

Heavy metal contamination arising from diverse sources but especially from anthropogenic activities such as mining wastes, industrial discharges and the use metals with many purposes is a serious problem in our age. These metals are toxic in both their chemically combined forms as well as the elemental form (Marmiroli and Maestri, 2008). When heavy metals or xenobiotics penetrate into the living organisms, the concentrations of some endogenous elements in the tissues may significantly differ. Thus, investigating the alterations in concentrations of essential trace elements in living organisms is a research need to understand the harmful actions of toxic metals and homeostasis of trace elements.

The challenges for the analytical chemists and toxicologists are many. The major ones include the need for qualitative and quantitative information of metals particularly at the tissue and cellular levels, where specific effects may occur.

Metallome which is described as the entirety of metal and metalloid species within a cell or tissue type has a growing importance thus, qualitative and/or quantitative studies of the metallome, referred as metallomics, is an emerging and growing area probing the metals and metalloids in biological samples (Williams, 2001; Szpunar, 2005). In this concept, a field of metallomics is emerging as one of the most dynamic research areas in trace element speciation analysis (Szpunar, 2004, 2005; Haraguchi, 2004, Mounicou et al,. 2009; Lobinski et al., 2010; Mounicou and Lobinski, 2008).

Information about metallobiomolecules is very important because, structural biology and genomic studies require high-throughput methods for the understanding of structure-function relation of proteins. Probing of element species in biological extracts is usually performed by combination of a high-resolution chromatographic or electrophoretic separation technique and a sensitive element-specific detection technique, referred to as hyphenated techniques (Szpunar, 2000a). SEC-ICP-MS which is primarily used hyphenated technique in our study is a very versatile tool in the analysis of biological fluids and cytosols. SEC has some advantageous over other HPLC modes such as; non-destructive nature because of the lack of interaction principally between stationary phase and metal complex, possibility of elimination of the buffer salts in mobile phase which provides the simplification of the matrix in heartcut and lyophilized fractions, and tolerance of the flow rates and compositions of the mobile phases by FAAS, ICP-OES or ICP-MS (Szpunar, 2000a). ICP-MS has not only high sensitivity and element specificity with multielement capacity but also a partner of molecular mass spectrometry such as electrospray and MALDI-MS. Coupling of SEC with ICP-MS is very versatile tool in the analysis of biological fluids and cytosols for the investigation of metallobiomolecules in complex biological matrices.

Chromium, which occurs in two common oxidation states; Cr(III) and Cr(VI), is one of the most interesting transition metals with regard to the role in biology. Not free

from controversy (Porter e al., 1999; Cronin, 2004), Cr(III) is considered as an essential element for a proper glucose and lipid metabolism (Glinsmann and Mertz, 1966; Gurson and Saner, 1971; Anderson et al., 1983; Abraham et al., 1992). On the other hand, Cr(VI) is known to be toxic, carcinogenic, and mutagenic for human and animals and one of the major chemical occupational hazards (Codd et al., 2001; Levina and Lay, 2005).

Because of interaction of trace metals in biological systems, chromium is expected to affect the concentrations of other essential trace elements (Schroeder and Nason, 1976; Li, et al., 2004; Yang and Wong, 2001), change their speciation by competitive binding to proteins, and possibly trigger off the synthesis of diverse biological ligands. Although accumulation of Cr was investigated in some target organs (mostly in kidney and liver), blood and urine of diverse laboratory animals and in occupationally exposed humans (Stearns et al., 1995; Krachler et al., 2009; Borguet et al., 1990; Horng and Lin, 1996; Raithellet al., 1998), no studies have focused on effects of the Cr exposure on the essential trace elements metallome and homeostasis. Studies of Cr speciation and fractionation in biological materials have been scarce and concerned bovine liver (Peterson et al., 2008), Cr-enriched yeast (Shoeib and Z. Mester, 2007; Kaewkhomdee et al., 2010), human and bovine serum (Lustig et al., 1999; Tkaczyk et al., 2010). When we look up to the bioinorganic chemistry of the chromium, it has not been described any Cr-binding protein with its three dimensional structure and biological function up to now. Investigation of Cr-binding biomolecules is crucial for true understanding of the biological role and functions or the mode of action of chromium. Thus, metalloproteomics approaches are necessary to clarify the points regarding bioinorganic chemistry of chromium.

The objective of this study was to carry out a comparative metallomics, describd as monitoring the changes of the metallome as a function of time and exposure to external stimuli, (Haraguchi, 2004; Szpunar, 2004) study addressing the changes of the distribution of the key essential elements (Cu, Fe, Mn and Zn) in different organs of mice exposed to Cr(VI) stress.

To the best of our knowledge, this work is the first *in-vivo* study investigating such effects either on the level of total element concentrations and their homeostasis or in terms of their binding to biomolecules as a function of the molecular size. In order for identification of Cr-binding proteins a metalloproteomics approach was followed in the study. This involves the heartcut of SEC fractions of Cr-binding proteins and their tryptic digestion prior to subsequent parallel analysis of the obtained Cr-peptide complexes by a second SEC-ICP-MS and MALDI-TOF-MS.

2. THEORETICAL

Metal ions play vital roles in living processes. Function, bioavailibity, essentiality and toxicity of trace metals in living organisms in a chemical point of view and the methods for their analytical determinations are pointed out in this section. Key essentials, Fe, Zn, Cu and Mn which were largely studied because of biological importance were briefly mentioned, while, chemistry of Cr was given in a wider concept.

2.1. Element content in the living organisms and cycles of metals in the environment

It has been shown that eleven elements appear to be more or less constant and predominant in all biological systems. In the human body, carbon, oxygen, hydrogen, and nitrogen constitute 99.0 per cent of the total number of atoms present (Table 1). The very large percentages of oxygen and hydrogen arise from the high water content of all living systems. The four elements are the basic elements of the organic structures and metabolites of living systems (Frausto da Silva and Williams, 2001).

The other seven elements, sodium, potassium, calcium, magnesium, phosphorus, sulphur, and chlorine, constitute about 0.9 per cent of the total number of atoms in the human body. These eleven elements are absolutely essential for biological systems. Therefore metal ions together with some non-metallic elements represent 0.1 per cent of the human body.

Table 2.1 Percentage of elements in the human body (adapted from Frausto da Silva and Williams, 2001)

Element Symbol	Atom (%)
H	62.8
O	25.4
C	9.4
N	1.4
Na, K, Ca, Mg, P, S, Cl	0.9
Metals and metalloids	~ 0.1

The generally accepted definition of trace elements describes them as elements which present in the organism in very small quantities, less than 0.01% (w/w).

Although metal ions exist in the living organisms at trace levels they have vital functions on life processes and essential functions. While at higher concentrations metallic species exert toxic effects even for essential metals.

The geologic and biological cycles naturally affect the redistribution of metals in the nature. Water with rain dissolves soil, rocks and ores and physically transports material to the ocean through water sources such as streams and rivers. The biological cycles bioconcentrate the metallic species by plants and animals leading to incorporation into food cycles. Human industrial activity, however, may cause to form of new compounds, and may greatly enhance worldwide distribution to land, water and also to the atmosphere. Metal contamination of the environment therefore reflects both natural sources and a contribution from industrial activity. Fig 1 demonstrates the transport of trace metals in nature.

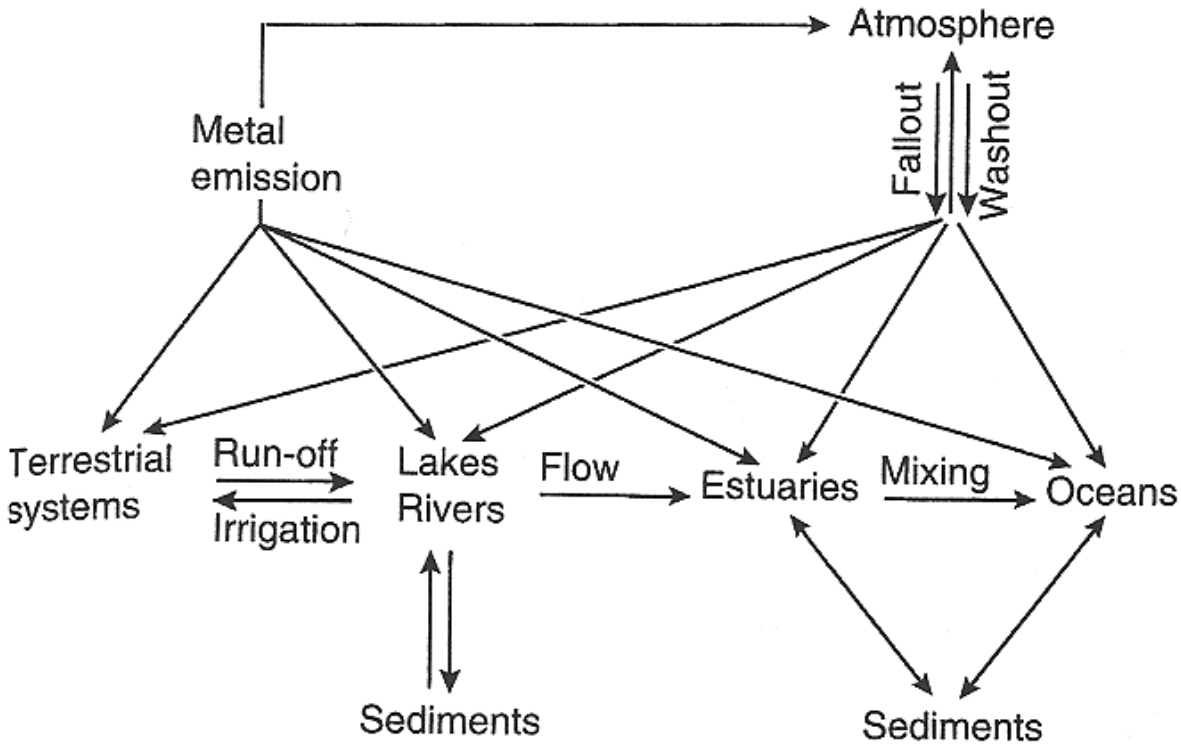

Figure 2.1. Transport of trace metals in the environment (Beijer and Jermelov, 1986)

2.2. Functions of trace metals and essentiality

Metal ions exist in living systems at mostly trace levels but play a vital role in the life processes and exert their functions as both enzyme cofactors and structural components of proteins. Trace metals serve essential roles as catalytic or structural co-factors in biochemistry for virtually all life forms. Some metals are redox active thus, co-factors for a wide variety of enzymes involved in biochemical processes. These processes such as oxidative phosphorylation, protection from oxidative stress, DNA synthesis and repair, regulatory responses to hypoxia, pigmentation, neurotransmitter biogenesis and detoxification etc. are critical to life.

Bioelements or biogenesic (bios=life and genesis=origin) elements are defined in the recent review as (Burguera and Burguera, 2009); those elements found to play a metabolic role in living organisms. The elements present in biological samples at µg/L and µg/g levels are defined as trace element; and those found in sub-fractions of these concentrations units are called ultra-trace. Trace elements are defined also by the International Union of Pure and Applied Chemistry (IUPAC) as "Any element having an average concentration of less than about 100 parts per million ppm atoms or less than 100 µg/g" (McNaught and Wilkinson, 1997).

In brief, metals involve in very different processes in biological systems by carrying ion messages (Na, K, Ca), triggering proteins (Ca, Mg), structuring (Ca, Mg), electron transporting (Mn, Fe, Cu) and catalysis (elements like Cr, Fe, Cu, Zn, Mo) (Frausto da Silva and Williams, 2001).

Whether an element is essential for life or not depends on its participation in biochemical reactions. The term *essential* is previously used in the early amino acid and protein chemistry. A better definition may be stated as; an element is "useful" to the organism and to the maintenance of health when a measurable deficit in the diet reduces the growth and vitality of humans, animals, or plants to a reproducible degree. Possibly even well-known "toxic" elements are needed in minute quantities for the normal functioning of cell metabolism (Geldmacher-von Mallinckrodt and Meissner, 1994). Another definition about essentiality criteria is; absence or deficiency of an element brings abnormalities that can be connected to

specific biochemical changes reversed by supplying the element (Marmiroli and Maestri, 2008).

The following elements have an approved essentiality for humans:
1. Essential nonmetals: H, C, N, 0, F, P, *S*
2. Essential electrolytes: Na, K, Mg, Ca, P, Cl
3. Essential trace elements: F, Si, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Mo, Sn, I

Among the essential elements are 4 light metals (Na, K, Mg, Ca) and 10 heavy metals. About 20-30 heavy metals are not yet proven to be essential but are found rather constantly in living organisms. This could be the consequence of environmental exposure. They are called “accidental” trace metals (Geldmacher-von Mallinckrodt and Meissner, 1994). However diverse classifications exist in the literature. For example in a recent review (Burguera and Burguera, 2009), 12 essential trace elements (instead of 14) are stated. Si has been classified into micro elements and Cr into toxics. The biochemistry of chromium will be discussed as a separate mini chapter.

Oxygen, carbon, hydrogen and nitrogen are classified into the most abundant elements, macroelements. These are followed by the microelements include structural elements like calcium, phosphorous, sulfur, silicon and strontium, and electrolytes: sodium, potassium, magnesium and chlorine. Some transition and post-transition elements which represent less than 1% of the cell content and are considered essentials for life like iron, copper, zinc, manganese, cobalt, nickel, molybdenum, selenium, iodine, fluorine, tin and vanadium. Few elements (arsenic, cadmium, chromium, mercury and lead), are present only because of exposure to polluted environments, while others (antimony, bismuth, lithium, gold, platinum, plutonium) are intentionally introduced into the body for special treatments such as therapeutic purpose. Aluminum, barium, boron and bromine, among others, have unknown biological functions and their essentiality for humans is under investigation (Burguera and Burguera, 2009).

2.3. Biological functions of some essential trace elements

Advances in life sciences and basic science such as chemistry and biology have brought new aspects into the biological chemistry of the elements and their functions. Especially improvements in analytical techniques give rise the deeper probing the relevance between molecular structure and function. At this concept, the number of essential trace metals differs within different classifications. However the main biological functions and properties of the some very important essential trace metals which are investigated in this study are reviewed in this section. Chromium was given primary importance and investigated.

2.3.1. Iron (Fe)

Iron is the most abundant trace element in human body. It is the structural component in heme proteins: hemoglobin, myoglobin, and cytochrome-dependent proteins. A large quantity of iron is also stored in proteins like ferritin and hemosiderin. It roles in redox reactions, by conversion of the Fe(II) and Fe(III) forms (Marmiroli and Maestri, 2008).

Interconversion between Fe^{2+} and Fe^{3+} is an essential biological property of iron as microorganisms, plants, and animals use this property for oxidative metabolism and in assimilating the metal. Ferrous iron in water is spontaneously oxidized by oxygen to highly insoluble ferric hydroxide (solubility product 10^{-39}). Thus, living organisms have developed iron-binding proteins and low molecular weight iron chelators, or siderophores to overcome this limited solubility (Alexander, 1994).

Iron deficiency is the most common nutritional deficiency worldwide, affecting older infants, young children, and women of childbearing age. The major manifestation of iron deficiency is anemia, and impaired intellectual development, decreased resistance to infection and possibly increased susceptibility to lead and cadmium toxicity are among others (Goyer and Clarkson, 2001). The best-known enhancer of iron absorption is vitamin C (ascorbic acid).

Excess iron may be carcinogenic can give some problems such as gastrointestinal problems, vomiting, and diarrhea, ending with cirrhosis for chronic exposure. The free ion would cause oxidative stress. Hemochromatosis is a genetic disease in

which iron is absorbed in excess from the diet and accumulates in different tissues with altered distribution (Griffiths, 2007).

Iron absorption is inhibited by calcium. It interacts with other essential trace metals such as copper, manganese, zinc, and chromium. On the other side, some cations such as manganese, zinc, and vanadium can bind to the iron binding proteins thereby contributing to their transport (Ballatori, 2002)

Excess exposure to iron may further increase in ferritin synthesis in hepatic parenchymal cells. The most common cause of acute iron toxicity occurs with the accidental ingestion of iron containing medicines and most often occurs in children. However, chronic iron toxicity or iron overload in adults is a more common and serious problem. It was shown that iron loading in mice can alter and damage cellular organelles in heart muscle, including mitochondria, lysosomes, and endoplasmic reticulum (Bartfay et al., 1999).

2.3.2. Zinc (Zn)

Zinc is an essential nutrient and trace metal present in all tissues of the human body. It is necessary for plants, animals and microorganisms; the second most abundant transition metal in organisms after iron and the only metal which appears in six enzyme classes, oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Zinc physiologically binds to the ligands in membranes for maintenance of the normal structural geometry of the protein and lipid components (Cousins, 1996). As calculated by genomic study, considerable part of the proteins (about 10%) has the potential for zinc binding (Andreini et al., 2006). The high abundance of zinc is due to binding preferences of zinc to biological macromolecules (Frausto da Silva and Williams, 2001). Zinc chelates with cysteine and/or histidine in a tetrahedral configuration forming looped structures, called "zinc fingers”. As a constituent of transcription factors (zinc finger domains), it involves in gene expression. Zinc is essential for the development and normal function of the nervous system. Zinc induces the synthesis of metallothionein, which is a factor in regulating the metabolism of zinc together with some other metals, including absorption and storage (Goyer and Clarkson, 2001).

Although zinc applied to soil is taken up by growing vegetables, they have lower in zinc. Sea foods, meats, whole grains, dairy products, nuts, and legumes are high in zinc content.

Zinc deficiency is rare, but insufficient intake of zinc is frequent. Deficiency therefore impacts many aspects of health in growth, reproduction, immune and behavioral systems. Acrodermatitis enteropathica is a genetic disease that leads to deficiency of zinc (Marmiroli and Maestri, 2008). Dietary inadequacies coupled with liver disease from chronic alcoholism may be associated with dermatitis, night blindness, testicular atrophy, impotence, and poor wound healing. Other chronic clinical disorders are also associated with zinc deficiency such as ulcerative colitis and the malabsorption syndromes, chronic renal disease, and hemolytic anemia. Metal chelating agents such as drugs affect zinc homeostasis (Goyer and Clarkson, 2001).

At the other extreme, excessive exposure to zinc is relatively uncommon and occurs only at very high levels and from pollution. Symptoms of acute poisoning are nausea, vomiting, diarrhea, lethargy, and fever.

Zinc and copper are antagonists, interfering for absorption in the intestine and there is a reciprocal relationship between plasma levels of zinc and copper. Zinc also influences the some other metals such as calcium and iron.

2.3.3. Copper (Cu)

Copper is widely distributed in nature and is an essential trace metal for the functioning of many metalloproteins and enzymes. It also plays a role in regulation of gene expression, required for growth, defense, bone strength, blood cells production, iron transport, and metabolism. Deficiency and toxicity are quite rare, because copper uptake and excretion are efficiently regulated in humans. Food, beverages, and drinking water are potential sources of excess exposure (Goyer and Clarkson, 2001).

The copper content of the normal human adult is 50-120 mg. The liver, brain, heart, and kidney contain the highest concentrations of copper. Copper

concentrations are found in lung, intestine, and spleen contain intermediate level. Bile content is variable since it is the main route of excretion and homeostasis is maintained in bile (Sarkar, 1994). Homeostatic mechanisms regulate the gastrointestinal absorption of copper. It is transported in serum, initially bound to albumin and later more firmly to ceruloplasmin and transcuprein.

Copper is component of all living cells and involves in many oxidative processes. It is an essential component of several metalloenzymes, including oxidases and dismutases. Dismutase enzymes scavenger superoxide radical by reducing them to hydrogen peroxide. The main proteins involve in copper metabolism are ceruloplasmin, cytochrome oxidase, superoxide dismutase, metallothionein, dopamine β-hydroxylase and tyrosinase (Sarkar, 1994). Impairment of the function of these enzymes is responsible for diverse diseases associated with copper deficiency (Chan et al., 1998).

Copper deficiency leads to anemia, neurological diseases, decrease in white blood cells, osteoporosis, and disorders of the connective tissues. It can derive from an X-linked hereditary disease, Menkes' disease, characterized by reduced intestinal absorption. Copper deficiency is uncommon in humans. Copper deficiency manifests clinically by hypochromic, microcytic anemia refractory to iron as well as susceptibility to infections and sometimes accompanied by bone abnormalities. Tissue level of copper is affected by molybdenum. Ceruloplasmin and serum copper levels, levels of low-density lipoproteins and cytochrome oxidase activity are of biomarkers of copper deficiency (Goyer and Clarkson, 2001).

Toxicity from excess copper is very rare, mainly from contaminated water, causing gastrointestinal problems. Wilson's disease and idiopathic copper toxicosis may occur in this case. In Wilson's disease, excretion in bile is reduced and copper accumulates in liver, brain, and kidney (Sadhra et al., 2007). Copper interacts with other elements, and especially with zinc. High zinc intake inhibits intake of copper by competition for the same transporters (Marmiroli and Maestri, 2008). Copper exposures in industry are to particulates in miners or to metal fumes in smelting operations, welding, and related activities.

2.3.4. Manganese (Mn)

Manganese exists in all living organisms and is an essential element and cofactor for a number of enzymatic reactions, particularly in phosphorylation, cholesterol, and fatty acids synthesis. It is necessary for connective tissues and bones, for general metabolism and reproductive functions. It could be found 11 oxidation states, from -3 to +7. The most common valence in biological systems is +2. Mn^{3+} is also important in biological systems as being in superoxide dismutase (Goyer and Clarkson, 2001). Food is the major source of intake. Spices rich in manganese are vegetables, the germinal portions of grains, fruits, nuts and tea (Keen and Zidenberg-Cherr, 1994).

Toxicity in polluted working environments leads to manganism, a neurological disease. Because manganese concentrates in mitochondria, tissues rich in these organelles including the pancreas, liver, kidneys, and intestines have the highest concentrations of manganese. Cycling between Mn^{2+} and Mn^{3+} may be potentially harmful to biological systems because it can involve the generation of free radicals. As an alternative for lead containing additives in gasoline, the manganese-containing fuel additive MMT (methylcyclopentadienyl Mn tricarbonyl) is used, therefore there is current interest in the toxicology of manganese because of potential exposure (Goyer and Clarkson, 2001). Beside, the industrial use has expanded in recent years for manganese used in the iron industry as a ferroalloy and as a component of alloys used in welding (Apostoli et al., 2000).

Manganese competes with iron for the same absorption sites and manganese deficiency leads to alterations in hair, nails, and skin (Marmiroli and Maestri, 2008). So far there are questions about whether deficiency has actually been demonstrated in humans, but in animals it is associated with bone malformations, impaired growth and disturbed reproductive function.

Manganese toxicity from oral ingestion is rare. The oral absorption of manganese is increased by iron deficiency The most common form of manganese toxicity is the result of chronic inhalation of airborne manganese (generally in the form of manganese dioxide) in the industry such as mines, steel mills, and some chemical industries. Chronic inhalation exposure to manganese dioxide, generally over a

period of more than 2 years results to neurological diseases such as manganism, symptoms similar to in Parkinson and symptoms like as irritability, difficulty in walking, speech disturbances, and compulsive behavior that may include running, fighting, singing and more (Goyer and Clarkson, 2001).

2.3.5. Chromium (Cr)

Chromium, which occurs in two common oxidation states: Cr(III) and Cr(VI), is one of the most interesting transition metals with regard to the role in biology. Not free from controversy (Porter et al., 1999; Cronin, 2004), Cr(III) is considered as an essential element for a proper glucose and lipid metabolism (Abraham et al., 1992; Anderson et al., 1983; Glinsmann et al., 1966; Gurson and Saner., 1971). On the other hand, Cr(VI) is known to be toxic, carcinogenic, and mutagenic for human and animals and one of the major chemical occupational hazards (Codd et al., 2001; Levina and Lay, 2005). As the metal has the primary importance in the context of this study, special emphasis has been given upon chromium.

2.3.5.1. Chemical properties, natural occurrence and industrial importance

Chromium is a transition element belongs to Group VI of the Periodic Table and a d^3 cation. It has an atomic weight of 52, however different isotopes exist, ^{50}Cr (4.31%); ^{52}Cr (83.76%); ^{53}Cr (9.55%) and ^{54}Cr (2.38%). Chromium can exist in any oxidation state from –2 to +6; however, oxidation states other than 0, +2, +3 and +6 are uncommon. Chromium is ubiquitous in nature, occurring in air, water, soil and biological materials over a wide range of concentrations. As all of the sources of chromium in the earth's crust are mostly in the trivalent state, hexavalent chromium (Cr^{6+}) compounds are thus considered as man-made products. Chromium compounds with oxidation states below +3 are reducing, and those above +3 are oxidising.

The trivalent is the most common and stable form and exist in biological materials. In acidic solutions Cr^{3+} is soluble, readily forming hexahedral complexes with appropriate ligands, such as oxalate and sulphate ions (Review of Chromium).

Hexavalent form (Cr^{6+}) has strong oxidizing potential and main toxic form of chromium. Chromates and dichromates are of greater industrial importance and

the principal substances for the production of all chromium chemicals. The major source of chromium is from chromite ore which is usually converted into one of several types of ferrochromium or other chromium alloys containing cobalt or nickel. Ferrochrome is used for the production of stainless steel. Chromates are produced by a smelting, roasting, and extraction process. The major uses of sodium dichromate are for the production of chrome pigments; for the production of chrome salts used for tanning leather, mordant dying, wood preservatives; and as an anticorrosive in cooking systems, boilers, and oil-drilling muds (Goyer and Clarkson, 2001).

Chromium in ambient air originates from industrial sources, particularly ferrochrome production, ore refining, chemical and refractory processing, the manufacture of cement, and fossil fuel combustion. From these sources it reaches to land and sediments by rain and water. Cobalt-chromium alloy hip replacements lead to elevated blood levels of chromium (Schaffer et al., 1999).

2.3.5.2. Essentiality of Cr(III)

Trivalent Cr is believed to be an essential trace element for mammals and considered as an essential element for a proper glucose and lipid metabolism (Abraham et al., 1992; Anderson et al., 1983; Glinsmann and Mertz, 1966; Gurson et al., 1971; Shinde et al., 2004;). There are a few reasons as evidence for the essentiality (Vincent, 2003). The most often cited evidence of Cr(III) essentiality in humans is that addition of a Cr(III) salt ($CrCl_3 \cdot 6H_2O$) to the total parenteral nutrition (TPN) solutions (Jeejeebhoy, 1999) alleviated the glucose intolerance symptoms in TPN patients. However the data accumulated in this field over the last 30 years have found to be inconclusive (Stearns, 2000; Martin, 2000; Stearns, 2007; Levina and Lay, 2008).

In the study of Levina and Lay the controversial finding on antidiabetic effects of Cr(III) have been explained by two concept. The first is hormesis (Stearns, 2007; Calabrese, 2005), which is a stimulatory or beneficial effect of subtoxic doses of a toxic chemical. This effect may be observed for Cr(III) complexes and used as a proof of Cr(III) essentiality (Mertz et al., 1961; Morris et al., 1995). The second hypothesis is that the antidiabetic activity of Cr(III) is caused by its biological

oxidation to Cr(VI/V) species (Levina and Lay, 2005; Mulyani et al., 2004; Levina et al, 2007a). That's why relatively low doses of Cr(VI) are known to decrease blood glucose levels in experimental animals (Kim and Na, 1991) and to stimulate plant growth (Pandey et al., 2005; Ortiz Castro et al,. 2007), while higher doses cause acute toxicity, and chronic exposure to Cr(VI) leads to increased incidence of cancers (Levina et al., 2003).

2.3.5.3. Chromium compounds as nutritional supplement and therapeutics

The followings have promoted the use of diverse chromium complexes as therapeutic agent or nutritional supplement:

- Very low absorption of chromium from the foods (~1%) throughout gastrointestinal system,
- Determination of lower Cr in daily diet (Anderson and Kozlovsky, 1985),
- The observed beneficial effects of Cr(III) complexes on some blood parameters and glucose and lipid metabolism (Anderson et al., 1997; Anderson, 2000; Cefalu et al., 1999; Press et al., 1990; Hopkins et al., 1968),
- The proposed beneficences such as the loss of body fat and the increase in lean body mass (Evans, 1989).

As a result, a huge budged chromium market has formed (Mirasol, 2000). A variety of Cr(III) forms, complexes with propionato, L-histidinato, D-phenylalaninato, chloride, picolinato, niacinato, nicotinato (niacinato or 3-pyridinecarboxylato) ligands etc., and as well as Cr(III)-enriched yeast (Vincent and Davis, 2001; Anderson et al., 2001; Jensen, 1989; Yang et al., 2006; Ashmead et al., 1997) are used for diverse of purported beneficial effects which are the subject of many research. Chromium picolinate ($Cr(pic)_3$) is the most popular one between commercial Cr-complexes because of its relatively higher absorption rate, 2-5 %, (Clodfelder et al., 2004).

Finally new generation synthetic molecule, $(Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3)^+$, has been described and proposed as a potential chromium therapeutics. The complex cation has been a functional biomimetic of chromodulin and proposed to stimulate insulin

receptor's tyrosine kinase ability and to lower plasma triglycerides, total cholesterol, low-density lipoprotein and insulin concentrations and to increase insulin sensitivity in healthy, obese, and type 2 diabetic rats (Sun et al., 1999, 2002) as well as to lower fasting plasma glycated hemoglobin levels in obese and type 2 diabetic rats (Clodfelder et al., 2004). It has been also reported that the cation inhibits the colorectal tumor formation in rats (Pickering et al., 2004) and does not appear to cause maternal toxicity and harmful effects to the developing offspring and no signs of in mice at the studied doses (Bailey et al., 2008). Moreover the cation is absorbed with much more greater efficiency (40–60%) when compared to $Cr(pic)_3$ (2-5 %) (Clodfelder et al., 2004) and safer than $Cr(pic)_3$, thus suggested as a potential alternative to $Cr(pic)_3$ as a nutritional supplement. Another study recently published has revealed that chromium (III) propionate complex (up to 100 mg/kg body mass/day, for 4 weeks) does not induce DNA fragmentation in rat's peripheral lymphocytes. That provides further evidence for lack of genotoxicity of this compound in rodents (Staniek et al., 2009). Very recently Kralovec and co-workers synthesized a complex which combines the Cr(III) and omega-3 fatty acids in one molecule (Kralovec et al., 2009). Exploring the synthesis and potential safely use of diverse chromium forms is emerging field for nutritionists and pharmacists.

However, the use of diverse Cr-complexes as an anti-diabetic drug, beneficial health effects, bioavailability and estimated daily intake value as well as the essentiality of Cr(III) as a nutrition were questioned recently in a variety of researches, reviews and meta-analyses (Hepburn and Vincent, 2002; Vincent, 2003,2004; Cronin, 2004; Gunton et al., 2005; Porter, 1999; Levina and Lay, 2008; Clarkson, 1997; Kreider, 1999; Lukaski, 1999; Althuis et al., 2002; Codd et al., 2001). The conclusion to a great extent is that $Cr(pic)_3$ does not exert beneficial effects neither on plasma glucose and insulin concentrations and other blood parameters nor on body composition in healthy individuals. Currently, adequate dietary intake of Cr(III) for adults was estimated as 25–35 μg/day (Trumbo et al., 2001) which was decreased from the level of 50–200 μg/day, the value in the early 1980s suggested as safe and adequate dietary intake of Cr(III) for adults. A possible reason of this re-arrangement resides on the developments in analytical techniques for accurate determination of Cr content in food. The early studies,

samples were typically contaminated with Cr from stainless steel apparatus during sample preparation (Veillon and Patterson, 1999). In addition to that emerging conflicted findings on the beneficence of chromium and the results of the recent studies indicating the potential toxicity, mutagenicity and carcinogenity of Cr(III) compounds (Mulyani et al., 2004; Levina and Lay, 2008; Codd, 2001; Whittaker et al., 2005; Lança et al., 2002) have been associated to the wide and long term use of Cr(III)-based nutritional supplements (Mirasol, 2000).

2.3.5.4. Toxicity of Cr (VI) and Cr (III)

Cr(VI), the highest oxidation state of chromium, is simply well known as toxic, carcinogenic, and mutagenic for human and animals and one of the major chemical occupational hazards (Codd et al., 2001; Levina and Lay, 2005). These hazardous effects of Cr(VI) is primarily attributed to the easily permeating the cell walls through anion channels (as chromate and dichromate). Once Cr(VI) penetrate in to the cells, it is reduced to the most stable form of Cr(III) (Cavalleri et al., 1985) by the biological enzymes (Bozcaarmutlu and Arınç, 2007; Boşgelmez and Güvendik 2004; Boşgelmez et al., 2008; Jannetto et al., 2001; Travacio et al., 2000) and some non-enzyme elements such as ascorbate, glutathione, NADH, and tocopherols (Lefebvre and Pe´zerat, 1992; Jones et al., 1991). The reduction in cells, as well as through cell membranes gives results to the formation of reactive intermediates of chromium, Cr(V/IV) (Luo et al., 1996), and ROS (Jones et al., 1991; Lefebvre and Pe´zerat, 1992; Luo et al., 1996) which lead to oxidative damage to the biological macromolecules such as proteins and DNA (Jones et al., 1991; Lefebvre and Pe´zerat 1992; Luo et al., 1996; Molyneux and Davies, 1995; Tagliari et al., 2004). Intracellular reduction of Cr(VI) ultimately results kinetically inert Cr(III)-complexes with proteins and DNA which can negatively affect the function of these biological constituents.

In fact the toxicity of chromium species is relevant to each other. It is well known that Cr(VI) is more toxic than Cr(III). On the other hand, while Cr(VI) can not form complexes with proteins or DNA, Cr(III) can. Moreover there are increasing evidences for the feasibility of biological Cr(III) oxidation to Cr(VI), accordingly formation of reactive Cr(V/IV) intermediates (Mulyani et al., 2004), therefore, antidiabetic activity of Cr(III) may involve carcinogenic Cr(VI). Further studies in

this area may provide a unified explanation to the seemingly opposite biological effects of Cr(VI) and Cr(III) complexes (Anderson, 1998).

Recent toxicological studies of Cr(III) complexes in animal or cell models are reviewed in several studies (Eastmond, 2008, Lamson and Plaza, 2002, Levina and Lay, 2008). Permissible limit of total chromium in drinking water has been determined as 50 µg/L (WHO, 2004).

2.3.5.5. Studies on Chromium Binding Proteins

Up to date it has not been described any Cr-binding protein with its three dimensional structure and biological function. Therefore in this section outstanding studies set for identification of Cr-containing biomolecules are briefly reviewed.

The first suggested biological Cr(III) compound was glucose tolerance factor, GTF, (Schwarz and Mertz, 1957,1959) known an amino acid and nicotinamide complex. But it was understood that GTF is an artifact of experimental conditions (Sumrall and Vincent, 1997) and this suggestion was discarded long after the numbers of research on GTF. More recently low molecular weight chromium binding substance (LMWCr), further named as chromodulin by Vincent and co-workers, was proposed as biological form of chromium (Vincent, 2001). LMWCr was firstly described by Yamamato in 1981 (Yamamoto et al., 1981) and further isolated from a variety of mammalian source. Regardless of the exact form, molecular weight of ~1.5 kDa, LMWCr was proposed to exist in all the mammalian insulin active cells in an apo form which is considered biologically inactive. However, LMWCr has recently been isolated from non-mammalian sources, chicken and American alligator (Hatfield et al., 2006). The proposed function and received structural information of LMWCr was detailed described (Vincent, 2001). Briefly; binding of insulin activates the insulin receptor. This triggers the transport of blood Cr (possibly in the form of Cr_2-transferrin) to the apochromodulin (Sun et al., 2000). While apochromodulin is unable to bind to insulin receptor and activate its kinase activity, holochromodulin binds to the insulin receptor, and activates the receptor kinase activity by changing conformation. A drop in blood insulin levels facilitates relaxation of the conformation of insulin receptor, and the holochromodulin is

excreted from the cell into the blood. Ultimately, chromodulin is excreted in the urine (Vincent, 2001).

Beside all of these, while Vincent and co-workers proposed that LMWCr to exist in all mammalian insulin active cells in a biologically inactive form, i.e., apo form, a recent genomic informatics search contrarily suggested that LMWCr could be an acidic region in the α sub-unit of the insulin receptor and synthetic peptides derived from this sequence bind multiple Cr(III) ions (Dinakarpandian et al., 2004). In another recent study, it is claimed that chromodulin is an artifact of the isolation procedure (Levina et al., 2007). Another very recently published study (Peterson et.al., 2008) claimed that reaction of Cr(VI) with bovine liver homogenate results more than one Cr-containing biomolecule and their experimental process is non-specific for the isolation of LMWCr. Their result supports to the claim that LMWCr is an artifact of the isolation procedure. They pre-characterized a novel trivalent chromium containing protein which has a M.W. of 15.6 kDa, determined by both gel-electrophoresis and MALDI mass spectrometry.

2. 4. Interaction pathway of metals with human

Figure 2.1 shows the transport of metals in the environment. Accordingly, Figure 2.2 shows the relationship between sources of exposure, transport and distribution to various organs and excretory pathways.

The exposure ways of metals and metal compounds to the body occurs by absorption, ingestion, inhalation, and in some cases resorption through the skin. In general, human exposure to metal compounds occurs predominantly through food, drinking water and beverages, whereas in the work environment absorption following inhalation of vapor, dusts and fumes is of primary importance. Beside, accidental, suicidal and homicidal uptake in high doses also rarely occurs (Geldmacher-von Mallinckrodt and Meissner, 1994).

Species entering the body with diverse ways are transported through active or passive mechanisms. They are stored, metabolized or excreted by the functions of specific proteins. Gastrointestinal and urinary excretions represent the most important pathways. Metals not completely absorbed in the gut are eliminated with

the faeces. Metals excreted via bile may be reabsorbed in the gut (enterohepatic circulation). The other excretion routes of minor importance are excretion via saliva, sweat, lactation, and loss of hair, nails, and teeth. Exhalation only plays a role for volatile metallic species and elemental mercury (Geldmacher-von Mallinckrodt and Meissner, 1994).

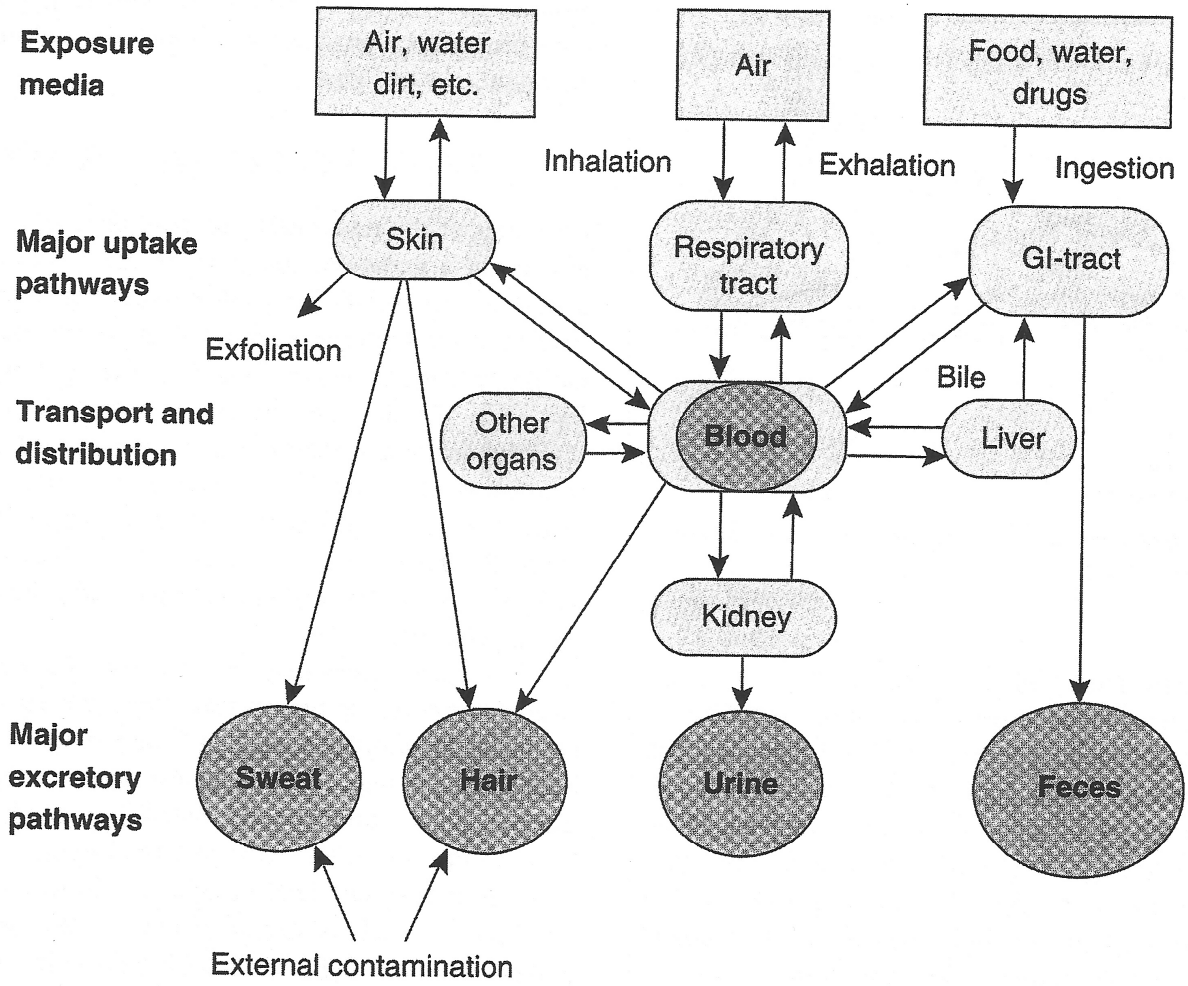

Figure 2.2. Metabolism after metal exposure via skin absorption, inhalation or ingestion. Shaded areas show the particularly useful organs for biomonitoring (Elinder et al., 1994 in Goyer and Clarkson, 2001)

2.4.1. Chemical factors on selectively binding of metals to biological ligands

Coordination chemistry defines the thermodynamic binding of cations to organic chelating agents in mononuclear complexes follows certain trends and that the different metal ions have preferences for particular donor atoms and for particular coordination geometries.

Table 2.2. Ligands preferred by different metal ions in simple coordination compounds (Frausto da Silva and Williams, 2001)

Metal ion	Ligands
Na^+, K^+	Oxygen-donor ligands, neutral or of low charge (-1)
Mg^{2+}	Carboxylate, phosphate and polyphosphate (total charge >-2), N-donation (special)
Ca^{2+}	Carboxylate, phosphate (less than Mg^{2+}), some neutral O-donors
Mn^{2+}, Fe^{2+} (thiolate)	Carboxylate, phosphate and nitrogen donors combined,
Fe^{2+} (special)	Unsaturated amines (particularly porphyrins)
Cr^{3+}, Mn^{3+}	Phenolate (e.g. tyrosine), hydroxamate, hydroxide
Fe^{3+}, Co^{3+}	Carboxylate, N-donors, polypyrroles, (thiolate)
Ni^{2+}	Thiolate (e.g. cysteine), unsaturated amines, polypyrroles
Cu^{2+}, Cu^+	Amines, ionized peptide $>N^-$, thiolate
Zn^{2+}	Amines, thiolate, carboxylate

The case of Fe^{2+} is especially complicated by spin-state changes and polymerization of mixed oxidation states.

Tables 2.3. Preferred geometries for complexes of some metal ions (Frausto da Silva and Williams, 2001)

Metal ion	Preferred geometries
Cu^{2+}, Mn^{3+}	Tetragonal > 5-coordination > tetrahedral
Cu^+	Linear, trigonal or tetrahedral
Ni^{2+}	Octahedral > others
Co^{2+}	Octahedral tetrahedral others
Zn^{2+}	Tetrahedral > octahedral > 5-coordination
Mn^{2+}	Octahedral > others
Fe^{3+}, Co^{3+}, Cr^{3+}	Octahedral > others

The ligand types and geometry preferences are summarized in Tables 2.2 and 2.3.

The stability of the complexes formed by the different types of ions (M^{n+}) are, at a great extent, independent of the ligand (L) when this ligand is built from an internally mobile organic molecule, i.e., where steric selectivity is minimal. The

binding constants of K_{ML} for monovalent, divalent and trivalent ions are expected as follows (Irving-Williams stability order);

M^+ : $Cu^+ > Na^+, K^+$

M^{2+} : $(Mg^{2+}, Ca^{2+}) < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$

M^{3+} : Co^{3+} (low- spin)$> Mn^{3+} > Fe^{3+} > Cr^{3+} > Al^{3+}$ (Frausto da Silva and Williams, 2001)

Beside the Irving-Williams stability approach, another approach is known as Hard and Soft acids and Bases theory (HSAB).

Table 2.4. Pearson's hard and soft acids and bases table (Pearson, 1963)

Hard –class a-	**Borderline**	**Soft –class b-**
Acids		
H^+, Li^+, Na^+, K^+, Be^{2+}	Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+},	Cu^+, Ag^+, Au^+, Cd^{2+},
Hg^{2+}, Mg^{2+}, Ca^{2+}, BF_3, BCl_3	Zn^{2+}, Rh^{3+}, $B(CH_3)_3$,	Pt^{2+}, Pt^{4+}, MoO_2^{2+},
$B(OR)_3$, Al^{3+}, $AlCl_3$,	R_3C^+, Pb^{2+}, Sn^{2+}	Pd^{2+}
$Al(CH_3)_3$, Sc^{3+}, Tl^{4+}, VO^{2+},		
Cr^{3+}, Fe^{3+}, Co^{3+}		
Bases		
NH_3, RNH_2, N_2H_4, H_2O,	$C_6H_5NH_2$, N_3^-, N_2, Br^-,	H^-, R^-, C_2H_4, C_6H_6,
OH^-, O^{2-}, ROH, RO^-, CO_3^{2-},	Cl^-	CO, SCN^-, R_3P, R_2S,
RSH,		CN^-,
SO_4^{2-}, ClO_4^-, F^-		RS^-, I^-

Regarding HSAB theory, we can say that soft electrophilic metals such as silver and platinum react with soft nucleophiles like sulfur in thiols, cysteinyl residues in proteins, and glutathione and sulfur in methionine (thiols and thiolates). Accordingly, hard electrophiles such as lithium and calcium react preferentially with hard nucleophiles (oxygen of purines and pyrimidines in nucleic acids phosphate oxygen in nucleic acids). On the other hand, zinc and lead fall between these two extremes and exhibit universal reactivity with all nucleophiles including nitrogen in primary and secondary amino groups of proteins, nitrogen in amino groups, and in purine bases in nucleic acids. The reactivity of an electrophile

determines which endogenous nucleophiles can react with it and become a target (Shanker, 2008).

A more detailed description is required if a deep understanding of the selectivity factors in both chemistry and biology are to be appreciated. For example stereochemical properties of ligands and preferred symmetry of sites, ligand-field effects, etc. for different metal ions are important factors.

A conclusion can be drawn that the following properties affects the selectivity between metals and biological ligands; charge, ion size, liganding donor atom, preferential co-ordination geometry, spin-pairing stabilization, binding in clusters. Beside these thermodynamic factors, biological factors such as the control of concentration of M and L and transfer coefficients from water to proteins (or membranes) (Frausto da Silva and Williams, 2001).

2.5. Toxicity of metals

Metals are different than other toxic substances that they are neither created nor destroyed by humans. Nevertheless, their utilization by humans influences the potential for health effects in at least two major ways: first, by environmental transport, that is, by human or anthropogenic contributions to air, water, soil, and food, and second, by altering the speciation or biochemical form of the elements (Beijer and Jernelov, 1986).

Although metals are probably the oldest toxins known to humans, many of the metals of toxicological concern today are only recently known to humans. About 80 of the 105 elements in the periodic table are regarded as metals, but less than 30 have been reported to produce toxicity in humans (Goyer and Clarkson, 2001). Chemical reactions that make some of trace metal ions obligatory (essential) for life are also the primary cause of their toxicity when present in surfeit. The interaction of toxic with essential metals occurs when the metabolism of a toxic metal is similar to that of the essential element. In other word, many metal ions are considered as essential for many life form but they are toxic in excess (Goyer and Clarkson, 2001).

2.5.1. Mode of action and toxicity of trace elements in general

Mode of action is defined as the sequence of key cellular and biochemical events that result in a toxic effect, while mechanism of action implies a more detailed understanding of the molecular basis of the toxic effect (Shanker, 2008). Accordingly, the strength of a toxic effect of all trace metals depends principally on the absorption, concentration, and persistence of the eventual toxicant at its location of action. The final toxicant is the metal species that reacts with the endogenous target molecules such as receptors, enzymes, DNA, protein, or lipid or critically alters the biological environment, producing structural and functional changes that result in toxic damage.

In general, the principal toxicant is the metal species to which the organism is exposed, while, in some cases a metabolite of the parent compound or reactive oxygen or nitrogen species (ROS or RNS) generated during the in vivo transformations may act as toxicant. In few cases the principal toxicant for living organism may be an endogenous biomolecule or compound synthesized as a response to primary toxicant exposure. The buildup of the definitive toxicant at its target is facilitated by various biological processes. At some cases the toxicity are eliminated by presystemic action, via distribution away from the site of action, excretion, and detoxification which work against the accumulation of the ultimate toxicant at the target molecule. The logical steps of toxicity are typically shown in Figure 2.3.

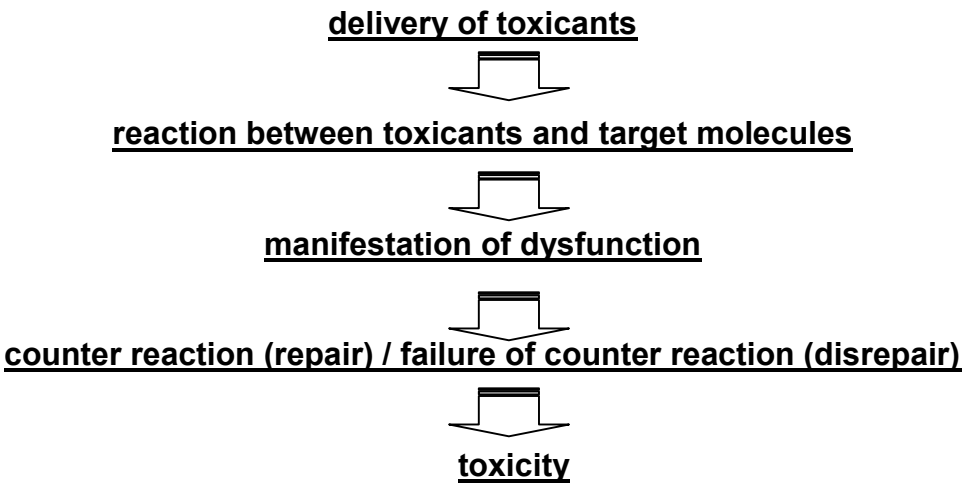

Figure 2.3. Steps in mode of action of a toxic metal (adapted from Shanker, 2008)

The reaction types after contact of the trace element and the target molecule may be two types. First; non-covalent interaction or binding by the formation of hydrogen and ionic bonds involving the interaction of some trace metals with targets such as membrane receptors, intracellular receptors, and ion channels and second; covalent bonding by most traces metals that react with nucleophilic atoms that are rich in biological macromolecules, such as amino acids, proteins, and nucleic acids. (Shanker, 2008)

2.5.2. Host factors and the toxicity of metals

Other than chemical factors, there are host factors influencing the toxicity of metals. Recognition of factors that influence toxicity of a particular level of exposure to a toxic metal is important in determining the risk of toxicity, particularly in susceptible populations. A number of factors influencing the toxicity of metals are as following (Goyer and Clarkson, 2001);

- Interactions with essential metals
- Formation of metal-protein complexes
- Age and stage of development
- Lifestyle factors
- Chemical form or speciation
- Immune status of host

In addition to that the interaction of toxic with essential metals occurs when the metabolism of a toxic metal is similar to that of the essential element (Goyer, 1995).

A typical biomolecule having the functions such as regulation, maintenance, and signalling when effectively attacked will initiate a cascade of diverse effects such as (a) changes in gene expression causing improper cell division apoptosis, (b) impaired protein synthesis, (c) changes in internal and external maintenance causing impaired ATP synthesis, and (d) altered membrane function leading to cell injury in plants and animals. Furthermore, these changes may cause unbalanced

homeostasis, bleeding, inappropriate neuromuscular activity-like tremors, convulsion, and paralysis in animals (Shanker, 2008).

The challenges for the analytical chemists and toxicologist are many. The major ones include the need for qualitative and quantitative information regarding dose and tissue levels as well as greater understanding of the metabolism of metals, particularly at the tissue and cellular levels, where specific effects may occur.

2.6. Trace Metal Homeostasis and Interaction of Metals

Mammalian cells possess efficient uptake mechanisms to obtain ions from their environment and also intracellular delivery systems to translocate essential metal ions to specific enzymes and proteins (Guo et al., 2005). The uptake, transport, translocation, storage and secretion of metal ions are maintained through highly regulated processes. They are transported into cells by specific carriers and membrane receptors then translocated to intracellular organelles to bind compartmentalized enzymes to function. Thus, the intracellular concentration of metal ions has to be tightly controlled, even for relatively low-toxicity metals. However, when some heavy metals or xenobiotics penetrate into the living organisms, the concentrations of the endogenous elements in the tissues may significantly differ. It is well known that there are interactions between essential trace metals (Giedroc and Arunkumar, 2007; Nelson, 1999; Guo et al., 2005; Rahil-Khazen et al., 2002). Interaction of trace metals in living organisms can be synergistic which means that a metal may stimulate accumulation or the function of another, or antagonistic in which case uptake or function of another metal is inhibited (Rahil-Khazen et al., 2002). Minor alterations in quantity, form or place of these vital elements may lead imbalances in the essential metal ion homeostasis. It is also known that actions of metal ions damage on DNA and proteins, their proper distribution is vital and a slight alteration in their activity could cause to severe diseases. Reverse alteration between iron and manganese in brain is a good example to the interaction of metals (Garcia et al., 2007).

Investigating the alterations in homeostasis of essential elements and their concentrations is a research need to understand the harmful actions of toxic metals on living organisms. Parallel with recent advances in basic research, many

human diseases related to micronutrients or trace elements excess or deficiency have been well characterized at the level of their molecular mechanisms and the associated defect. Some of these could be exampled as; Menke's Disease (a disease of Cu absorption), Wilson's Disease (hepatic Cu hyperaccumulation), hemachromatosis (Fe overload), aceruloplasminemia (Fe distribution defect associated with neuropathy), Parkinson's Disease, Friedrich's Ataxia (mitochondrial Fe maldistribution), acrodermatitis enteropathica (Zn absorption defect) and transient neonatal Zn deficiency (defect in Zn secretion into milk in the mammary gland).

Fundamental advances in understanding of the trace element homeostasis and mechanism have advanced the area of the medicine. Understanding of trace element homeostasis is key element for designing the followings; dietary recommendations of nutritional supplements, exploring of novel nutritional supplements with enhanced absorbance and distribution profiles, and developing diagnostic markers and tests for human dietary deficiencies or genetic diseases of trace element homeostasis.

Metallome which is described as the entirety of metal and metalloid species within a cell or tissue type has a growing importance and metallomics, qualitative and/or quantitative studies of the metallome is an emerging and growing area probing the metals and metalloids in biological samples (Williams, 2001; Szpunar, 2005).

2.7. Analysis Techniques for the determination of metals and metalloids

Commonly used techniques for trace-element analysis in biological materials are flame and electrothermal AAS, inductively coupled plasma atomic emission spectrometry (ICP-AES), inductively coupled plasma mass spectrometry (ICP-MS) and X-ray fluorescence spectrometry (XRF).

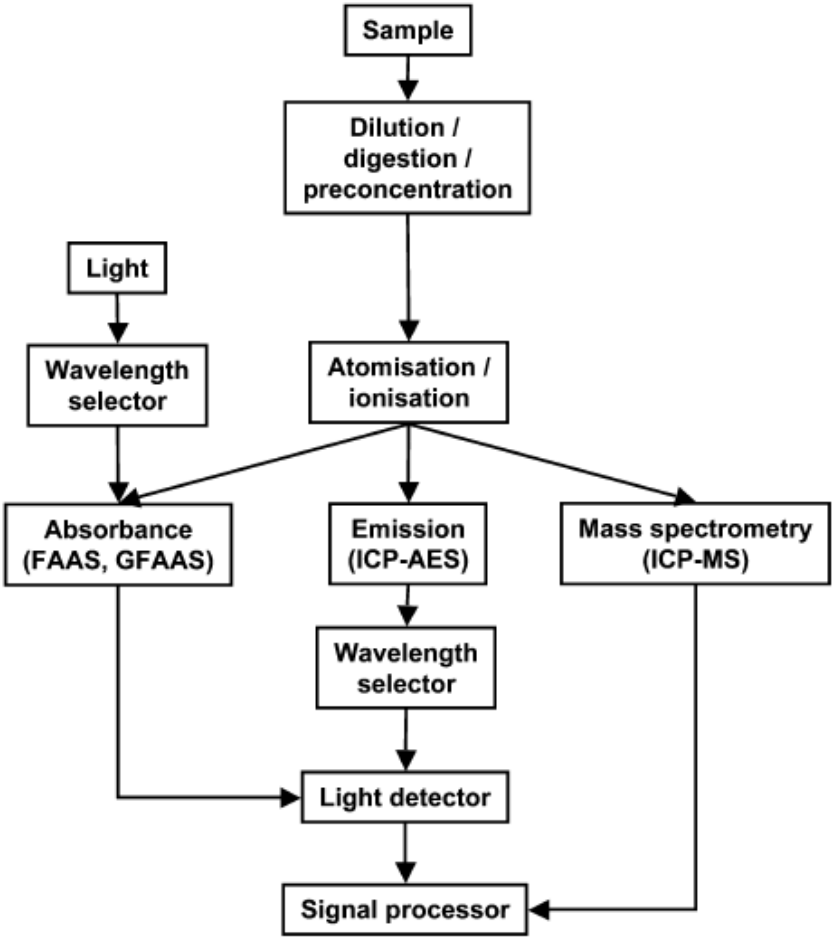

Figure 2.4. A general diagram of atomic spectroscopic techniques (Bolann et al., 2007)

The diagram in Figure 2.4 associates the tree main atomic spectrometry techniques with the atomization and detection steps. Although these techniques in general aspect are well known, advancements in both instrumentation and sampling are carried out in the field (Bings et al., 2006, 2008).

In Flame Atomic Absorption Spectrometry (FAAS) technique a flame is used to atomize a compound in solution. In general flames normally used in atomic absorption spectrometry are the air-acetylene flame and less frequently, the hotter nitrous oxide-acetylene flame. FAAS is the simplest and easiest analytical technique for element analysis. For determination of elements in the mg/L concentration range a precision of 1% relative standard deviation is typical characteristics of FAAS. Determination of single elements is fast, whereas measuring multiple elements is time-consuming, even if the method is automated. It is relatively inexpensive and it might be the method of choice if not more than

two or three different elements to determine in the samples. The major limitation in using FAAS is the large sample volume required in the analysis (several mL), which should be in an aqueous form because of the inability of FAAS to analyze solid samples directly. Another disadvantage is the rather high detection limits. Therefore, in cases where the analyte concentration is in the mg/L level, or below, precision is poor, and other techniques should be used (Bolann et al., 2007).

In Graphite Furnace Atomic Absorption Spectrometry (GFAAS) technique, differently than FAAS, the atomization takes place in a graphite tube (furnace) that is heated by an electric current. A small volume (5–100 µL) of sample, much smaller in GFAAS than in ICPAES and ICP-MS, is introduced into the furnace where the sample undergoes a series of carefully controlled heating steps that are required to dry the sample, remove a satisfactory part of the sample matrix and finally atomize the analyte element. GFAAS is a much slower technique than FAAS, so that it takes a few minutes to analyze one sample. Its main analytical advantage is low detection limits, which are about 10 to 100 times lower than the FAAS. In particular, the GFAAS technique can be used with effective background corrections. The list of advantages also includes the successful application to direct analysis of solids and slurry samples such as inorganic and organic material in powders, samples of soft or hard tissues. GFAAS is particularly favourable when limited amounts of sample are available, and when only few trace elements in a given sample are of interest and it is simpler and lower cost, either for running or purchasing, comparing with ICP-MS and ICP-AES. It has better tolerance to dissolved salts in the sample (Bolann et al., 2007).

Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) is another powerful technique used in trace element analysis. Inductively coupled plasma is an electrical conducting gaseous mixture of argon atoms and ions and electrons. Plasma is produced from a stream of argon gas, which is empowered by a high energy, radio-frequency electric field. This generates an excitation temperature of 7000–10,000 °K. The sample is introduced as an aerosol mixed with argon into the plasma for efficient desolvation, volatilization, atomization, excitation and ionization of the sample. The ICP possesses a wide linear dynamic range in the magnitude of 5 to 7 orders. Accordingly, determination of several elements

covering a wide range of concentrations in the same sample is possible. This allows the determination of several elements in a large number of samples in a short time (Bolann et al., 2007). Table 2.5 compares the main atomic techniques in terms of analytical criteria, use and cost.

Table 2.5. Comparison of main atomic spectroscopy techniques (Bolann et al., 2007)

Parameter	FAAS	Hydride generation	GFAAS	ICP-AES	ICP-MS
Detection limit (μg/L)*	0.1–100	0.01–10	<0.01–10	0.1–100	<0.001–10
Dynamic range (orders of linearity, power of 10)	2–3	2–3	2–3	5–7	5–7
Operational time	Fast	Slow	Slow	Fast	Fast
Cost	Low	Low	Intermediate	High	Highest
Automation	Yes	Yes	Yes	Yes	Yes
Element analysis	Sequential	Sequential	Sequential	Simultaneous & sequential	Simultaneous & sequential
Ease of use of instrument	User friendly	User friendly	Requires some expertise	Requires expertise	Requires high expertise
Interferences	Well characterized	Well characterized	Fairly well characterized	Well characterized	Well characterized, sometimes difficult to correct
Internal standard	Not used	Not used	Not used	Used	Used

2.7.1. Inductively coupled plasma mass spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry was introduced in mid 1980s and has been commercially available for over 20 years. The ICP-MS instruments consist of an ion source and a mass spectrometer. The MS part separates and detects the ions produced by the ICP, according to their mass to charge ratio, and measures the analyte concentration by mass fractionation for a quantitative analysis (Figure 2.5). Thus the ICP-MS combines the multielement capability of the ICP-AES with the low detection limits of the GFAAS (Bolann et al., 2007).

Developments in the field of elemental mass spectrometry are still driven by the increasing demand for high sample throughput, highest possible sensitivity, and high precision combined with fast and preferably simultaneous multi-elemental detection capabilities. ICP-MS is not universally applicable owing to its high cost and the need for trained technicians who are able to deal with the problems arising from serious interferences. However, ICP-MS performs multi-elemental analysis with excellent sensitivity and high sample throughput. It can simultaneously measure most elements in the periodic table and determine analyte concentration

down to the sub nanogram-per-liter (ng/L) or part-per trillion (ppt) levels. It can perform qualitative, semiquantitative, and quantitative analysis, and since it employs a mass analyzer, it can also measure isotopic ratios. (Agilent web site) During this time continuous development has been taking place.

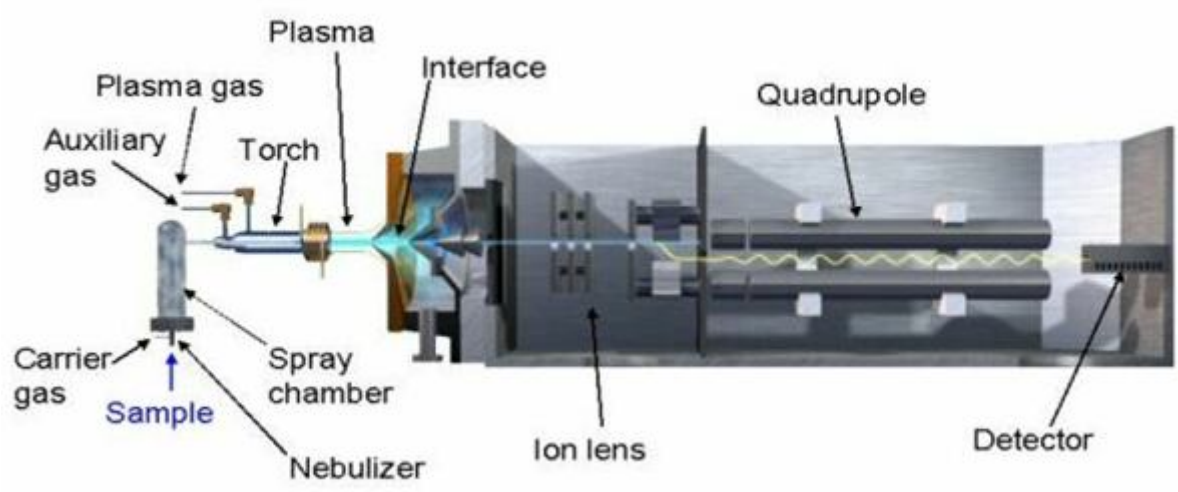

Figure 2.5. Block Diagram of an ICP-MS spectrometer (Agilent web site)

In general, liquid samples are introduced by a peristaltic pump, to the nebulizer where the sample aerosol is formed. A double-pass spray chamber ensures that a consistent aerosol is introduced into the plasma. Argon (Ar) gas is introduced through a series of concentric quartz tubes which form the ICP. The torch is located in the centre of an RF coil, through which RF energy is passed. The intense RF field causes collisions between the Ar atoms, generating a high-energy plasma. The sample aerosol is instantaneously decomposed in the plasma (plasma temperature is in the order of 6000-10000 °K) to form analyte atoms which are simultaneously ionized. The ions produced are extracted from the plasma into the mass spectrometer region which is held at high vacuum (typically 10-4 Pa). The vacuum is maintained by differential pumping: the analyte ions are extracted through a pair of orifices, known as the sampling and skimmer cones (Agilent web site). Collision/Reaction Cells (CRCs) are used in ICP-MS to remove spectral interferences that would otherwise bias results (Thomas, 2002a).

The analyte ions are then focused by a series of ion lenses into a quadrupole mass analyzer, which separates the ions based on their mass/charge ratio. The

term quadrupole is used since the mass analyzer is essentially consists of four parallel stainless steel rods to which a combination of RF and DC voltages are applied. The combination of these voltages allows the analyzer to transmit only ions of a specific mass/charge ratio (Agilent web site).

Finally, the ions are measured using an electron multiplier, and are collected by a counter for each mass number. The electron multiplier detector provides ICP-MS with its low detection limits because, at low signal levels, each individual ion that strikes the detector is counted (pulse-count mode). At higher signals, the detector switches automatically into analog mode, where the current passing down the detector is measured, rather than each individual ion. This provides another defining characteristic of ICP-MS, extremely wide dynamic range (Agilent web site).

The mass spectrum generated is extremely simple. Each elemental isotope appears at a different mass (e.g. ^{27}Al would appear at 27 amu) with a peak intensity directly proportional to the initial concentration of that isotope in the sample solution. A large number of elements ranging from Lithium (Li) at low mass to Uranium (U) at high mass are simultaneously analyzed typically within 1-3 minutes. With ICP-MS, a wide range of elements in concentration levels from ppt to ppm level can be measured in a single analysis.

2.7.2. Interferences in ICP-MS

Interferences in ICP-MS are generally classified into three major groups; spectral, matrix and physical interferences (Thomas, 2002b).

2.7.2.1. Spectral Interferences

Spectral overlaps are probably the most serious types of interferences seen in ICP-MS and known as a polyatomic or molecular spectral interference, which is produced by the combination of two or more atomic ions. They are caused by a variety of factors, but are usually associated with either the plasma and nebulizer gas used, matrix components in the solvent and sample, other analyte elements, or entrained oxygen or nitrogen from the surrounding air.

Oxides, Hydroxides, Hydrides, and Doubly Charged Species

A type of spectral interference is produced by elements in the sample combining with H, ^{16}O, or ^{16}OH from water or air. These interferences are typically produced in the cooler zones of the plasma by rare earth or refractory-type elements. Beside, doubly charged spectral interferences are associated with oxide-based spectral overlaps. The formation of oxide species and doubly charged species is related to the ionization conditions in the plasma and can usually be minimized by fine tuning of the nebulizer gas flow, rf power, and sampling position within the plasma.

Isobaric Interferences

Another class of spectral interferences is called “isobaric overlaps” produced mainly by different isotopes of other elements in the sample that create spectral interferences at the same mass as the analyte.

2.7.2.2. Ways to compensate for spectral interferences

Let us look at the different approaches used to compensate for spectral interferences. One of the very first ways used to get around severe matrix-derived spectral interferences was to remove the matrix somehow. In the early days, this has been achieved by separation and preconcentration techniques which are time consuming.

One technique to compensate for spectral interferences is **Mathematical Correction Equations**, similar to interelement corrections (IECs) in ICP-OES, works on the principle of measuring the intensity of the interfering isotope or interfering species at another mass, which ideally is free of any interferences and correcting the analytical results.

Cool/Cold Plasma Technology, another method to compensate for spectral interferences works in some conditions especially when the intensity of the interference is large, and analyte intensity is extremely low and mathematical equations are not ideally suited as a correction method. A low-temperature plasma is used to minimize the formation of certain argon-based polyatomic species. Cool plasma conditions (500–800 W rf power and 1.5–1.8 L/min nebulizer gas flow) are

applied instead of plasma conditions (typically 1000–1400 W rf power and 0.8–1.0 L/min of nebulizer gas flow) to reduce the spectral interferences such as ^{38}ArH, ^{40}Ar, and $^{40}Ar^{16}O$ in the detection of K, Ca, and Fe. However, it is often impractical for the analysis of complex samples, because of severe signal suppression caused by the matrix.

Collision/Reaction Cells; The use of cold plasma or mathematical correction equations is limited or impractical for the analysis of complex and real world samples to avoid spectral interferences. Collision/reaction cells provide enormous potential to eliminate spectral interferences and make available isotopes that were previously unavailable for quantitation. The technique uses ion–molecule collisions and reactions to cleanse the ion beam of harmful polyatomic and molecular interferences before the analyzer, shown in Figure 2.6 (Thomas, 2002a).

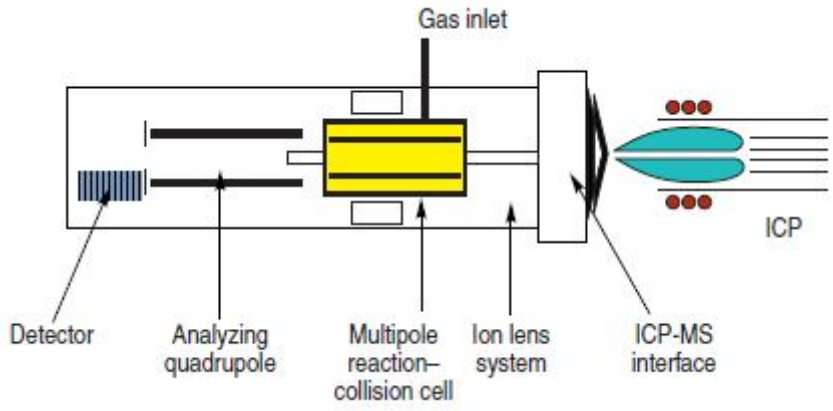

Figure 2.6. Layout of a typical collision/reaction cell

High Resolution Mass Analyzers; The best and probably most efficient way to remove spectral overlaps is to resolve them away using a high resolution-MS. Many of the problematic polyatomic and molecular interferences seen in ICP-MS, without the need to use cool plasma conditions or collision/reaction cells are prevented.

When extremely high resolution is used, there is a sacrifice in sensitivity; however, their resolving capability is far more powerful than quadrupole-based instruments.

2.7.2.3. Matrix and Physical Interferences

Another class of interference in ICP-MS is suppression of the signal by the matrix itself. These are basically two types. The one, often called a sample transport effect, is a physical suppression of the analyte signal due to the sample's impact on droplet formation in the nebulizer or droplet-size selection in the spray chamber by the matrix components. With the organic matrices, it is usually caused by a difference in sample viscosities of the solvents being aspirated. Another one, signal suppression is caused by its impact on the ionization temperature of the plasma discharge in some matrices. It occurs for example when different concentrations of acids are aspirated into a cool plasma.

The classic way is to use **internal standardization**. In this correction method some elements are spiked into the samples, calibration standards, and blank to correct for any variations in the response of the elements caused by the matrix. As the intensity of the internal standards change, the element responses are also updated every time during analysis. It is also effective to compensate for long-term signal drift produced by the some samples which have matrix components slowly blocking the sampler and skimmer cone orifices.

Space-Charge Interferences; This interference is the result of poor transmission of low mass ions through the ion optics due to large concentrations of high mass matrices which defocuses the ion beam. It leads to poor sensitivity and detection limits, especially when trace levels of low mass elements are being determined in the presence of large concentrations of high mass matrices. Unless any compensation is made, the high-mass matrix element will dominate the ion beam, pushing the lighter elements out of the way. There are a number of ways to compensate for space charge matrix suppression in ICP-MS. The use of internal standardization and applying voltages to the individual ion lens components are used to alleviate or at least reduce this effect.

2.8. Selected studies for the determination of essential trace elements in tissue samples

Knowledge of quantitative relationships between trace metals in the body is crucial for clearly understanding of trace metal metabolism and their interactions.

Table 2.6. Selected studies from literature on essential trace metal analyses

Sample	Analyte	Digestion Technique	Technique	Reference
Liver, kidney and spleen (rat and mouse)	Fe, Cu, Cr, Zn, Pb, Ni, Mn, As, Cd	Wet ashing	FAAS, ETAAS	Pereira et al,. 2006
Colon tissue (human)	Cu, Zn, Fe, Se	Microwave-assisted digestion and alkaline digestion (TMAH)	FAAS, HGAAS	Hornik et al., 2006
Fish tissue samples	Ca Mg Cu Zn Fe	Slurry sampling; Microwave-assisted digestion	FAAS	Bugallo et al,. 2007
liver, kidney, brain, muscle	Zn	Alkaline digestion	ETAAS	Muñoz-Delgado et al,. 2006
CRMs (TORT-2, DORM-2, DOLT-2, DOLT-3, LUTS-1, SRM NIST-2976, NIST-1566b)	Ag, As, Cd, Cu, Cr, Fe, Ni, and Se, Na, Ca, K, Mg, Mn	Formic acid solubilization in ultrasonic bath, microwave digestion	ETAAS, ICP–AES	Scriver et al,. 2005
Tissues in replacement of cobalt-chrome prostheses	Cr, Co, Ni, Mo and Mn	Wet ashing	ETAAS	Betts and Yau, 1989
Human thyroid tissue	Cu	microwave-assisted digestion	Slotted tube atom trap-FAAS	Yaman and Akdeniz, 2004
mussel soft tissue	As, Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn	Enzymatic hydrolysis, Microwave acid digestion	ICP–AES	Peña-Farfal et al,. 2004
Thin brain slices (human)	Cu, Zn	Direct	LA-ICP-MS	Becker et al,. 2007

Thin liver slices (human)	Fe, Cu and Zn	Direct	XRF	Osterode et al,. 2007
Bovine Muscle and Oyster Tissue	As, Se, Te, Ag, Cr, Cu, V, Ni, Mn,Co and Cd	dissolution or slurry formation with TMAH	ETV-ICP-MS	Pozebon et al,. 1998
Tumorous and non-tumorous tissues (human)	Ag, Al, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Ni, P, Pb, S, Se and Zn	Microwave-assisted digestion	ICP-MS, ICP-OES	Lavilla et al,. 2009
Fish otoliths	Al, Cd, Co, Cu, Ga, Mn, Ni, Pb, V, Zn	Wet ashing	ICP-MS	Arslan and Secor, 2008
Liver, spleen, lung, brain, testis, heart, kidney of mice	Cu	Wet ashing	ICP-MS	Chen et al,. 2007
Gills of brown trout and rainbow trout	total Zn and Zn isotope ratios	Microwave-assisted digestion	ICP-MS	Wolf et al,. 2009
NIST SRM 1566a Oyster Tissue	Sc, V, Cr, Mn, Fe, Co, Ni, Cu and Zn	Microwave-assisted digestion	Sector field ICP-MS	Townsend, 2000
Mussel tissue NIST 2976	Na, K, Ca, Mg, Cr, Mn, Co, Ni, Cu, Zn, Ge, As, Se, Rb, Sr, Zr, Ag, Cd, In, Sb, Cs, Ba, Pb and Bi	Microwave-assisted digestion	ICP-MS, ICP-OES	Krishna and Arunachalam, 2004
Human brain	Cu and Zn	Slurry sampling and Microwave digestion	TXRF and FAAS	Marco et al,. 1999

However, reliable quantitative analysis of trace metals in the different tissues is a major problem. Blood tests can only give a row indication of the total amount of a

trace metal in the body, but tell little or nothing about the distribution of the metals between the different organs. Hair, nail and sweat analysis are considerably affected from the contamination and could result to the misunderstandings.

A few organs like kidney, liver and gut are available for biopsy, and samples from several organs can be obtained during surgical operations. The most complete analyses can only be obtained from autopsies, which are scarce, by collection of tissue samples (Rahil-Khazen, 2002). For this reason at most cases the animals are used as model organisms to investigate the interaction and homeostasis of metals.

Determination of essential trace metals whether in human or animal tissue samples is achieved generally by atomic spectroscopy. However some difficulties and interferences are faced through the analyses. These include analyte loss or contamination during transport of samples or sample preparation, and different kind of interferences from the nature of technique used. In the Table 2.6 diverse studies were given as an example to the essential trace metal determination in tissue samples by atomic spectroscopy techniques.

2.9. Metalloproteins and Metal Binding Proteins

The function of many proteins critically depends on their interaction with a metal. These are usually a transition metal, such as e.g. Cu, Fe or Zn. Most of these metals are bound to specific proteins or enzymes, and exert their effects as active or structural components of proteins. Metal-binding proteins and metalloproteins represent a large portion of the total number of proteins and it is estimated that around 40% of all proteins and enzymes contain metal ions (Garcia et al., 2006).

The difference between metalloproteins and metal–protein complexes terms rises from the strength of interaction between metal and ligand of protein. Metalloproteins were considered distinct from metal–protein complexes. The first featured high-affinity interactions that are not lost during the isolation step, the latter's interactions are lower affinity and are easily lost during sample handling.

In general metalloproteins are defined as; all the proteins which form complexes with metals which are thermodynamically stable in a given chemical environment and kinetically stable on the time scale of the analytical procedure, are referred to as metalloproteins (Szpunar, 2005). Despite the difference in the definition of Metalloproteins and Metal Binding Proteins, the terms were equivalently used through the thesis.

The major groups of metalloproteins comprehend metalloenzymes, metal-transport-proteins and metal-stress proteins.

The first group includes the enzymes which are activated and/or stabilized in the cell by the presence of trace elements, usually transition metals with small atomic radii. In metalloenzymes there are interactions by both electromagnetic as well as electrostatic attraction, making such coordinating interaction the strongest. Superoxide dismutase or catalase could be given as an example.

The second, metal-transport-proteins, comprehend the cysteine-rich proteins such as albumin or transferrin which assure the transport of heavy and essential elements in the human body. Intracellular metal trafficking proteins (metallochaperones) that carry metal ions to specific target proteins have been identified in various organisms.

The metal-stress proteins are induced when the excess amount of metals exposed to the organisms. By this way, some organisms defend themselves against to a heavy metal induced stres. The best example can be given to this group is metallothioneins which are a group of non-enzymatic low molecular mass (6–7 kDa), thermoresistant, cysteine-rich metal-binding proteins. They involve in the metabolism, homeostatic control, and detoxification of a number of essential (Zn, Cu) and toxic (Cd, Hg, As) trace elements.

2.10. Definition of some terms and concepts in bioinorganic speciation analysis

In the context of current study it may be beneficial to give the definition of some terms which can be found below (Szpunar, 2005).

Genome : The set of genes of a given organism.

Genomics : Study of the genome of an organism.

Ionome : Free metal content of a cell.

Proteome : The entire protein complement of a given genome, that is, the entirety of the proteins that are expressed by the genome.

Proteomics : Study of the proteome of an organism. The term is most commonly associated with the use of MS to identify proteins expressed in a given cell type or tissue under a given set of conditions.

Metabolome: The set of metabolites produced as a result of reactions catalyzed by certain proteins (enzymes).

Metallome : The entirety of metal and metalloid species within a cell or tissue type. It encompasses, among others, the inorganic species (ionome) and protein complexes (metalloproteome).

Metallomics : Study (qualitative and/or quantitative) of the metallome.

Metalloproteome : The entirety of metal complexes with proteins in a sample. Note that this term had originally been used in a narrower sense and concerned the proteins with enzymatic functions only.

Speciation : The distribution of an element amongst defined chemical species in a system.

Fractionation : The classification of an analyte or a group of analytes from a certain sample according to physical or chemical properties. The term often refers to low resolution chromatography where metal containing fractions are detected by AAS or ICP-AES/MS.

Speciation analysis **:** Analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample.

Comparative Metallomics **:** Monitoring the changes of the metallome as a function of time and exposure to external stimuli.

2.11. Areas of interest for metallomics

Metallomics is a transdisciplinary research area with and its areas of interest are geochemistry, clinical biology and pharmacology, plant and animal physiology and nutrition. The ultimate goal in metallomics is to provide a global and systematic understanding of the metal uptake, trafficking, role and excretion in biological systems (Mounicou et al., 2009).

Information about metallobiomolecules is very important because, structural biology and genomic studies require high-throughput methods to screen proteins for structure and function. Beside of that it is believed that the function of one-third of the existing proteins depend on their interaction with a transition metal, such as, e.g., Cu, Fe, Zn (Hasnain, 2004).

Diverse analytical approaches have been proposed for metallomics. They can be divided into three basic groups, in vivo, in vitro and in silico. In-vivo approach comprises the methods for the analysis for metal-containing biomolecules present in a biological sample. Methods based on the in vitro modification of the organism's DNA (or isolation of particular genes) and determination of the influence of this operation on the metallome. In silico approaches consist in searching for metal-binding motives (which are expected to be preserved regardless of the organism studied) in putatively expressed proteins based on genome sequences present in data banks or for other patterns (e.g. SECIS) relevant to the function of heteroelements (Mounicou et al,. 2009).

The emerging applications areas include analysis for metal binding proteins, either by virtue of being metalloproteins or having metal-binding sites. In this concept, a field of metallomics is emerging as one of the most dynamic research areas in

trace element speciation analysis (Szpunar, 2004, 2005; Haraguchi, 2004, Mounicou et al,. 2009; Lobinski et al., 2010; Mounicou and Lobinski, 2008).

2.12. Hyphenated techniques

The probing of element species in biological extracts is usually performed by the combination of a high-resolution chromatographic or electrophoretic separation technique and a sensitive element-specific detection, referred to as a hyphenated technique (Szpunar, 2000a). In our age hyphenated techniques have became very important tool in the speciation of metal and metal complexes in biological samples and understanding of the biochemical processes.

The presence of a metal bound to a biomacromolecule in a sample is necessary to use an element/species-specific detector. The research objective primarily determines the choice of the hyphenated technique. Chromatography and spectrometry can be coupled usually in on-line manner. However, when a polyacrylamide gel electrophoresis (PAGE) is selected as separation technique, the off-line detection of metal species carried out directly in the gel. Also, when the discrete atomization techniques such as ETAAS or ETV-ICP-MS are chosen, it is preferred the off-line coupling method. The choice of the detector component becomes crucial when the concentration of analyte species in the sample is very small and low limits of detection are required (Szpunar, 2000a). ICP-MS has become an attractive detector in chromatography and electrophoresis because of its high sensitivity and element specificity and also a partner of electrospray and MALDI-MS for the investigation of metallobiomolecules in complex biological matrices.

Beside of that, for a classical speciation analysis the standards of most of the anthropogenic pollutants are available, whereas for bioinorganic trace analysis the majority of species of interest have not yet been isolated in sufficient purity to be used as retention or migration time standards. Therefore, the use of a molecule-specific detector in parallel is important to establish the identity of the eluted species. Mass spectrometry with electrospray ionization (ESMS) or matrix assisted laser desorption ionization time-of-flight (MALDI-MS) modes have been the viable choices.

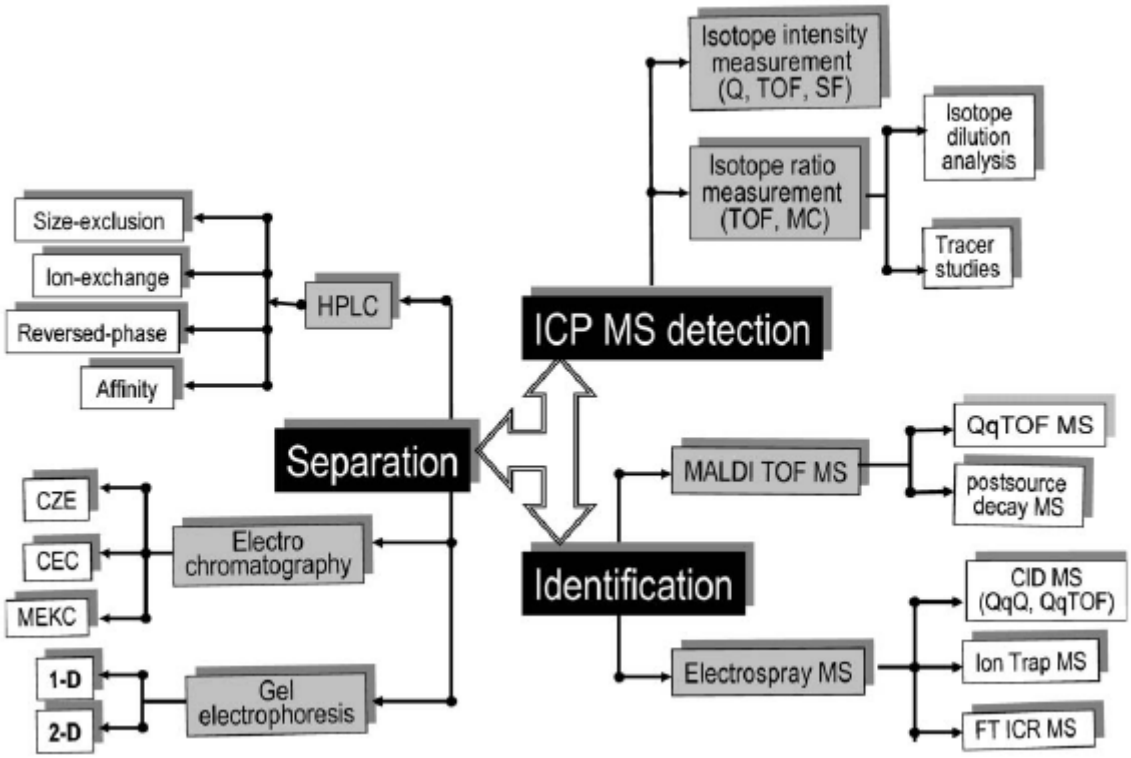

Figure 2.7. Hyphenated systems in bioinorganic speciation analysis (Szpunar, 2005a)

2.13. Liquid chromatographic methods applied to the separation of metal-binding proteins

Liquid chromatography in bioinorganic speciation analysis comprehends the High-performance liquid chromatography (HPLC), which includes the three mode size-exclusion, ion exchange and reversed-phase chromatography, and affinity chromatography (de la Calle Guntiñas et al., 2002). Size exclusion chromatography (SEC) profits of the differences in size and to a lesser extent tree dimensional configuration existing among proteins. Charge differences at a certain pH allow the use of Ion Exchange chromatography (IEC) in both cationic and anionic modes. The difference in polarity of the 20 essential amino acids causes differences in hydrophobicity which is used in reverse phase chromatography (RPC). Affinity chromatography is also used in separation of proteins and their isoforms using the existence of prosthetic groups.

Diverse of bioanalytical techniques including ultracentrifugation, dialysis and ammonium sulphate precipitation should often precede the chromatographic

separations of metal–protein complexes. The requirements and limitations concerning the interface between HPLC and atomic spectrometry vary depending on the separation mode and the detector used. The choice of the pH and ionic strength of the mobile phase are important for optimum separations.

Special emphasis was given to the SEC mode which is utilized in the study because of its non-denaturing nature and other advantageous properties.

2.13.1. Size-exclusion chromatography (SEC) in bioinorganic analysis

SEC is based on the molecular sieve effect and enables species to be separated according to their size, and to a lesser extent, shape. The average time a substance spends in the pores of stationary phase depends on its size which for a given shape, can usually be related directly to its molecular weight. SEC has some advantageous over other HPLC modes such as; the high tolerance to biological matrices; tolerance of the flow rates typically between 0.7–1.0 mL/min and compositions of the mobile phases by FAAS and ICP-OES and ICP-MS; and possibility of avoiding the buffer salts in the mobile phase, and hence the possibility of simplifying the matrix in heartcut and lyophilized fractions (Szpunar, 2000a).

The biological constituents containing metals have often fragile structures for this reason, following conditions have to be taken into consideration for their separation. Although theoretically there is no interaction in SEC between analyte and packing material, in practice, interactions between metals and buffers and chromatographic support material should be minimized. Because stationary phase surface displays charged properties, a mixed mode separation is observed. The pH of the eluent should be weakly alkaline to prevent the metals from dissociating and close to that of cytosols for the analysis of tissue samples (Makarov and Szpunar, 1998).

The two categories of packing, silica and organic polymers usually copolymeric styrene - divinylbenzene, are available for SEC. In the first, significant silanophilic effects are involved including metal losses in the presence of low ionic strength eluents. In the latter case, deposition of excess free metal ion which interacts with

the analytes, often causes severe degradation in peak resolution (High et al., 1995).

In order to assure the minimum competition between buffer and cytosolic ligands, and between these ligands and the gel, the eluent should be carefully chosen. Otherwise some undesirables take place such as structural changes, denaturation of proteins and destruction of protein-metal complexes. In practice, various aqueous mobile phases of fairly high ionic strength have been used to avoid interactions with the packing material. Dilute buffers, in general, may cause adsorption of low molecular weight proteins by the column packing. The addition of a non-complexing salt (e.g. 0.1 M NaCl) to the mobile phase was recommended to suppress the residual silanol activity of the column packing especially in the case of silica-based packings cannot be avoided. Analysis time is a function of the column size and the flow rate.

2.13.2. The coupling of size-exclusion chromatography with atomic spectroscopy techniques

The coupling of SEC to the atomic spectroscopy techniques includes AAS, AES and mass spectrometry.

The first common approach to solve the problem consisted in the separation of the metal-binding proteins and further analysis of the metals by ETAAS in an off-line manner. Another method used is hyphenation between SEC and ICP-AES, which offers the possibility of on-line coupling. When AES is chosen as a detection system the disadvantage of using is that the detection limits achieved are of the order of 10–100 ng/mL when working with transient signals, which is clearly not sufficient for speciation purposes in biological fluids (de la Calle Guntiñas et al., 2002).

In recent years atomic spectrometry has been highly substituted by mass spectrometry as detection systems and the use of SEC-ICP-MS has increased substantially. The low detection limit makes it more applicable to realistic problems. Besides the low detection limits of ICP-MS, the possibility to carry out

multi-elemental analysis in one run and the use of stable isotopes instead of radioactive ones are among other advantageous (Makarov and Szpunar, 1998).

For multielement SEC-HPLC-ICP-MS, the elements of interest should be divided into groups and replicate time, dwell time and number of sweeps per reading optimized within each group. A separate chromatographic injection is necessary for each group to ensure acceptable sensitivity (Odegard and Lund, 1997).

2.14. MALDI mass spectrometry

Matrix-assisted laser desorption/ionization-mass spectrometry, MALDI-MS, (also ESI-MS) is very sensitive mass spectrometry technique and allows the analysis of biopolymers with high molecular weight (Hop and Bakhtiar, 1997). By this technique, not only intact proteins but also non-covalent metal ion complexes with proteins and peptides can be observed because of soft ionization character of the technique (Salih et al., 1998; Woods et al., 1995).

In MALDI experiments, a droplet of a solution, containing a matrix and sample is deposited on a plate. Next, the solvent is evaporated and the plate is transferred to the vacuum system of the mass spectrometer. The function of the matrix is to isolate the sample molecules from each other and to absorb photons from a UV laser. Absorption of the laser energy causes rapid evaporation of the matrix molecules into the gas phase carrying the sample molecules with them, which are consequently protonated in the plume above the sample slide. Finally, these protonated sample molecules ($M+H^+$) are analyzed by the mass spectrometer (Figure 2.8).

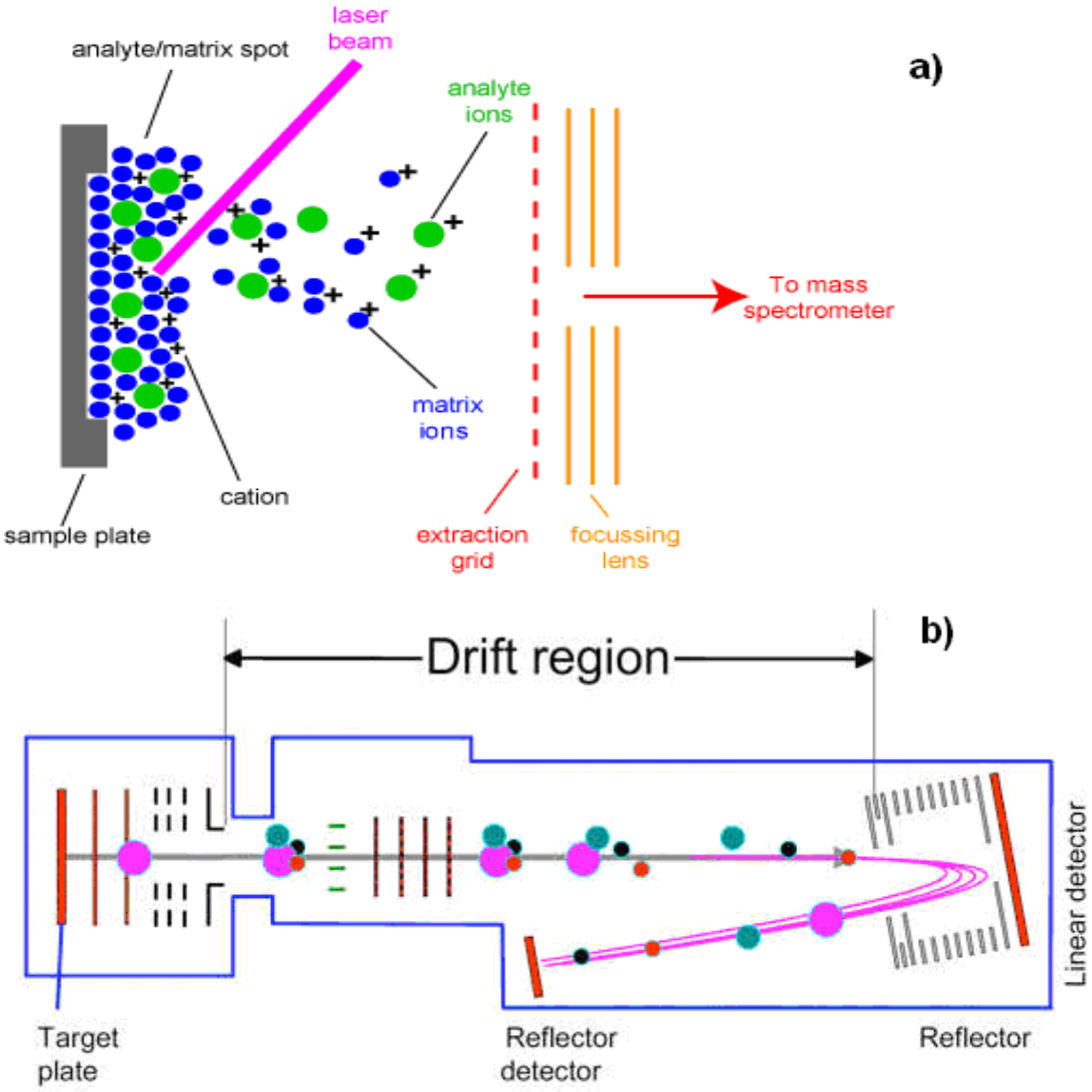

Figure 2.8. a) Schematic diagram of the MALDI mechanism (University of Bristol website); b) diagram of a MALDI-TOF-MS (giga web site)

3. EXPERIMENTAL

The experiments carried out through the study can be divided into two parts. The first group of experiments involves the administration of hexavalent chromium to mice at a single dose of 20.0 mg/kg b.w. One of the main purposes of this group of experiments was evaluation of chromium accumulation in liver and potential alteration at the concentration of two key essential elements, copper and zinc. Concentration of Cr as well as Cu and Zn in the mice liver samples were determined by GFAAS after acidic digestion. Because of importance in metabolic functions, liver was chosen as target organ. Another aim is to develop an approach to separate the Cr containing proteins in liver after mild extraction conditions by HPLC and to investigate any change in the elution profiles of protein complexes by comparing Cr-administered mice liver samples and those of control subjects. MALDI-MS analysis of samples was also performed to see the any Cr attachment to the proteins. This group of experiments was performed in Turkey, Hacettepe University Chemistry Department.

In the light of obtained data, a second group experiments were projected in details to conduct in a laboratory in Pau, France, Laboratoire de Chimie Analytique Bio-Inorganique et Environnement. Rationale to choose this laboratory is better instrumentation and sample preparation facilities. Main concern of the laboratory is investigation of macromolecular metal complexes especially by coupled instrumental techniques. The mice used in the second group of experiments were identical of those of the first group i.e., same kind of race, similar in weight and age. The dose administered was chosen as 8.0 mg Cr/kg.

3.1. Animals

Male Swiss albino mice (6-7 week old) weighing 35-40 g were maintained under standard laboratory conditions (photoperiod cycle of 12:12 h light:dark, ambient temperature at about 22 ± 2 ºC, relative humidity of 50-55 %). Standard laboratory diet and tap water were provided *ad libitum*. Laboratory acclimatized male Swiss Albino mice were randomly assigned to two treatment groups each consisting of three animals. One group mice, Cr(VI)-exposure group, were intraperitoneally (i.p.) injected with $K_2Cr_2O_7$, as Cr(VI) compound, at a single dose of 20.0 mg Cr/kg. The

other, control group, was injected by only saline solution, 0.9 % NaCl. The experimental design was approved by the Committee for Ethics of Hacettepe University and mice were cared in Hacettepe University Experimental Animals Laboratory in accordance with the Guidelines concerning the Care and Use of Laboratory Animals. At the end of 12 h after overnight fasting, mice were sacrificed by cervical dislocation and the dissected tissues were washed with ice-cold saline and stored at -80 ºC until analysis.

For the second group experiment the animals were similarly grouped into two parts each consisting of three animals and treated as described above. The only difference was the dose administered as 8.0 mg Cr/kg instead of 20.0 mg Cr/kg.

3.2. Reagents and materials

Potassium dichromate ($K_2Cr_2O_7$) was purchased from Aldrich (Darmstadt, Germany). Tris-HCl was obtained from Sigma (Buch, Switzerland) and nitric acid (HNO_3) for trace element analysis from J.T. Baker (New Jersey, USA). Trypsin and DTT (dithiothreitol) are obtained from Fluka, NH_4HCO_3 and NH_4CH_3COO from Riedel-de Haen. MALDI-MS matrices, ATT, ACCA and SA were of the highest grade available and purchased from Merck Co. (Darmstadt, Germany). All other reagents from Sigma-Aldrich (Saint-Quentin Fallavier, France) were analytical grade. A Milli-Q system (Bedford, MA, USA) was used to obtain deionized water (18 MΩ cm).

3.3. Instrumentation and Apparatus

A hand made aluminum cylindrical block, 12 cm radius, with multiple holes in the aluminum body was used for acidic decomposition of tissue samples prior to GFAAS analysis of metals. The pyrex digestion tubes with 0.5 cm inner diameter and 10 cm long were placed into the holes in aluminum block and heated on an electrical hotplate. Temperature was controlled by measuring by a thermometer. The tubes having small nodes at the bottom part to let the solution reflux were produced in Hacettepe University glass atelier and used for acidic digestion of tissues.

Measurements of total Cr, Cu and Zn levels of liver were performed by using a Perkin-Elmer AAnalyst 100 atomic absorption spectrophotometer (Boston, USA) equipped with a graphite furnace (HGA-800).

For the first group experiments, liver tissue was homogenized by a hand made homogenizer with a PTFE (polytetrafluoroethylene) piston working in a 15 mm cylindrical pyrex tube (İldam, Ankara, Turkey). A centrifuge (Merlin 506, UK) was used for separating supernatant solution after homogenization. The samples were lyophilized by a Model LP3 lyophilizer (Jouan, France).

Chromatographic separations for the first group of experiments were performed using a HPLC system (Tokyo, Japan) consisting of a high-pressure multi solvent delivery pump (Merck-Hitachi, model LaChrom L-7100), a sample injection valve (Rheodyne model 0505) with a 20 µL sample loop and an ultraviolet detector (LaChrom model L-7400). For reverse-phase separation of proteins a Teknokroma C18 nucleosil 100 analytical column (15x0.46 mm, 5 µm partical size) was used as stationary phase.

MALDI mass spectra were recorded in linear mode on a Voyager-DE PRO MALDI-MS (Applied Biosystems, USA) for the first group of experiments.

For the second group of experiments, a DigiPrep sample digestion system (SCP Science, Courtaboeuf, France) was used for mineralization of tissue samples prior to the ICP-MS analysis.

An ICP mass spectrometer (Agilent 7500c, Tokyo, Japan) equipped with a Meinhard type nebulizer (Glass Expansion, Romainmotier, Switzerland) and a collision cell used both for determination of total metal concentration and an element specific detector for chromatography.

An ultrasonic probe (Branson Ultrasonics Corporation, Danbury, USA) was used to liberate and solubilize the cell content. Cell lysates were centrifuged at 105000 g using a HimaCs 120GX ultracentrifuge (Hitachi, Tokyo, Japan).

All chromatographic separations were conducted using a Model 1100 HPLC pump (Agilent, Wilmington, DE, USA). Injections were performed using a Model 7725 valve with a 100 µL injection loop (Rheodyne, CA, USA). A lyophilizer (Jouan, France) was used for preconcentration of the fractions collected from HPLC separations. During enzymatic digestion, solutions were treated with a circulating shaker (Grant OLS-200, Keison Products, Essex, UK).

Mass spectra were acquired on a MALDI-TOF mass spectrometer (Applied Biosystems, ON, Canada) equipped with a nitrogen UV-Laser operating at 337 nm. Spectra were recorded in reflector mode.

3.4. Sample preparation

3.4.1. Experimental procedure for the analysis of liver tissues

In order to mineralize the liver tissue for the first group of experiments (20.0 mg/kg Cr(VI) administration), ca. 200 mg of wet liver tissue was accurately weighted into pyrex digestion tubes and 1 mL of concentrated HNO_3 was added.

Table 3.1. Instrumental conditions for the determination of Cr, Cu and Zn by GF-AAS

Electrothermal Atomizer:									
Step	**Temperature/^{0}C**			**Ramp time/s**			**Hold time/s**		
	Cr	Cu	Zn	Cr	Cu	Zn	Cr	Cu	Zn
Drying	130	120	120	10	10	10	45	50	50
Pyrolysis	1650	1000	700	10	10	10	25	30	30
Cooling	20	20	20	10	10	10	5	15	15
Atomizing	2500	2300	1800	0	0	0	5	5	5
Clean-out	2600	2600	2500	1	1	1	5	5	5
Spectrometer:									
	Cr			Cu			Zn		
Wavelength/nm	357.9			324.8			213.9		
Slit width/nm	0.7			0.7			0.7		
Background correction	On (deuterium lamp)								
Evaluation mode	Integrated absorbance								

After 4 hours duration time 1 mL of 65% HNO_3 and 0.5 mL of 30% H_2O_2 was added and heated in the holes of aluminum block at 110 ºC. This procedure was repeated for three times and the obtained clear solution was diluted to 5.0 mL with Milli-Q water prior to the GF-AAS analysis. Graphite furnace program and spectral details for the analysis of metals is given in Table 3.1.

For the HPLC analysis of metal-protein complexes, tissue samples were weighted into the glass tube of the homogenizer and homogenized in ice-cold media adding 4-volume (w/v) of buffer solution (pH 7.4). For the convenience of extraction solution, 50 mM solution of Tris-HCl, CH_3COONH_4 or HEPES buffers were used. The liver homogenate was centrifuged at 10000 g for 10 min and filtered through a 0.22 μm Millex Millipore filter and stored at 4 ºC until HPLC analysis. The solvent used as mobile phase in HPLC were always sonicated before use to remove the air. Chromatographic details are given in Table 3.2.

Table 3.2. Conditions for HPLC

Column	Teknokroma C18 nucleosil 100 (15x0.46 mm, 5 μm)
Mobile Phase	10 % methanol in water
Flow rate	0.7 mL/min
Wavelength	360 nm
Chart speed of integrator	5
Sensitivity	100
Injection volume	20 μL

The fractions following the HPLC analysis were collected according to chromatogram (in a one minute elution volume), frozen and lyophilized. The obtained residue was added by 0.7 mL of 0.1 M HNO_3 prior to GFAAS analysis.

Diverse MALDI matrix solutions, 2-amino-thiaamine (ATT) (20 mg/mL in acetonitrile-water in 1:1 (v/v) ratio), α-cyano-4-hydroxy cinnamic acid (ACCA) (15 mg/mL in acetonitrile-water in 1:1 (v/v) ratio) and sinapinic acid (10 mg/mL in acetonitrile-water in 1:1 (v/v) ratio), were prepared for the analysis of chromium-biomolecule complex. MALDI samples were prepared by mixing the sample extract with the matrix solution (1:10 v/v) in a 0.5 mL eppendorf® micro tubes. Finally 1 μL of this mixture was deposited on the sample plate, dried at room

temperature and then analyzed. Laser was operated at 337 nm and the spectra were recorded in linear mode with average of 500-1000 shots.

3.4.2. Experimental procedure for the analysis of other tissues of mice

3.4.2.1. Tissue homogenization

The tissues of three mice of each group (8.0 mg/kg Cr(VI) administration) were pooled and finely pulverized by means of porcelain mortar and pestle in liquid nitrogen. The tissue samples in the form of fine powder were placed into 50 mL polypropylene tubes, freeze-dried and kept in freezer.

3.4.2.2. Tissue mineralization and the determination of total metal content

Homogenized and freeze-dried tissue samples in the dry powdered form precisely weighed (~200 mg) into DigiPrep® tubes (50 mL volume). 2 mL of 65% HNO_3 was added, the caps of the tubes sealed and waited overnight, 1 mL of the HNO_3 and 1 mL of 30% H_2O_2 added into tubes and the temperature programming was applied; until 70 ºC in 30 minutes and at 70 ºC for 4 hours. The obtained clear solution was diluted to 5.0 mL with Milli-Q water. Concentrations of metals were measured by ICP-MS and tissue chromium contents were calculated. Standard addition method (three levels) was used as calibration method and yttrium was used as internal standard. The certified reference material TORT-2 (Lobster Hepatopancreas) was analyzed along with each series of samples to test the validity of the method.

3.4.2.3. Extraction of metal-protein complexes and optimization of procedure

The homogenized tissue samples weighed in 5 mL polypropylene tubes. 50 mM of ammonium acetate buffer, pH 7.4, was used for extraction. In order to extract the chromium-biomolecule complexes, two different techniques, ultrasonic probing and waterbath, were tested. The power and the quantities of the pulses applied for ultrasonic pulse technique and various durations of tissue samples in waterbath technique were examined to determine the efficiency of extraction and optimize the procedure. The probing technique was run in a manner of 1 second of pulse and 1 second of break periods, consecutively. Immediately after disrupting cell walls and taking the soluble cell content into the extraction media, concentrated solution of PMSF (phenylmethylsulphonyl floride) was added in the extraction media at a level of 1.0 µM as final concentration. Tissue digests were

ultracentrifuged at 105000 g for 30 minutes at 4 ºC. Firstly, the fat layer on the surface of the supernatant solution was discarded in order to avoid column clogging and then a clear cytosol was separated from pellet by a micro pipette and the extract, cytosol, was obtained. Chromium concentration in the extract was measured by ICP-MS and chromium extraction yield was calculated by comparing chromium contents in the extracted solution and in the whole tissue.

3.4.2.4. Chromatographic design, HPLC and ICP-MS conditions and connections

SEC of metalloproteins was performed by using two different SEC columns (Pharmacia Biotech, Uppsala, Sweden). Superdex 75 HR 10/30 with an exclusion limit of 100 kDa and an effective separation range between 3 and 70 kDa was used for the fractination of different molecular weight metal binding macromolecules. Superdex-75 column was calibrated with different protein standards as molecular weight marker.

Superdex Peptide HR 10/30 with optimum separation range of 100–7000 Da was used for the separation of peptide-metal complexes after tryptic digestion. Superdex peptide column was also calibrated with molecular weight markers. The columns were washed with 5 mM β-mercaptoethanol in 50 mM ammonium acetate buffer (pH 7.4) for 30 min to remove metal impurities when necessary and another 30 min with mobile phase to remove the chelating agent and to condition the columns. In addition, β-mercaptoethanol was frequently injected to the columns after the injection of samples which contain high metal content.

The samples were injected to the column as a volume of 100 µL solution. Elution was performed using a 50 mM ammonium acetate buffer, pH 7.4, at a flow rate of 0.7 mL/min. Protein absorbance was recorded at 280 nm by using a UV-VIS detector. ICP-MS was used as an element specific detector and ^{50}Cr, ^{52}Cr and ^{53}Cr isotopes of chromium and ^{55}Mn, ^{57}Fe, ^{63}Cu, ^{65}Cu, ^{64}Zn and ^{66}Zn isotopes of other metals were recorded. Nebulizer gas flow, rf power and lens voltages were daily optimized by using multielement solution (Li, Y and Tl). H_2 gas was used as collision gas in reaction cell at a 3 mL/min flow rate. The column exit was directly

connected to nebulizer of ICP-MS by means of polyetheretherkethone (PEEK) tubing. SEC chromatogram was constructed by using Microsoft Excel software.

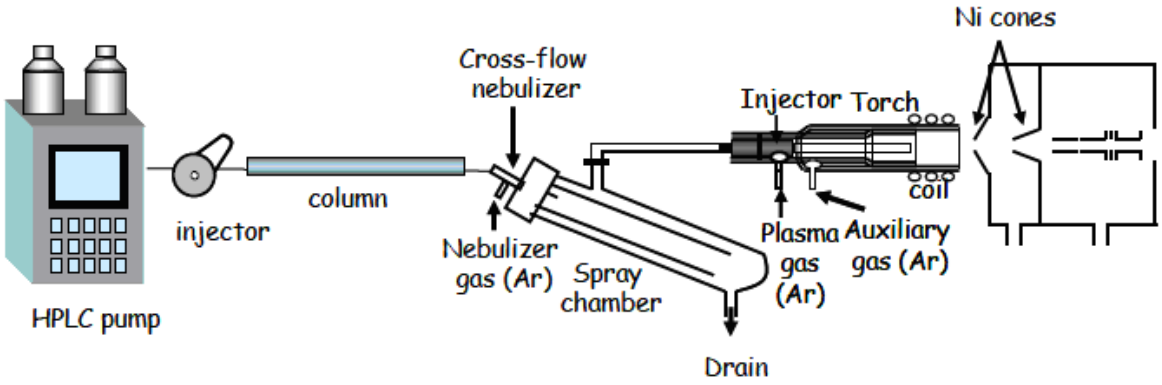

Figure 3.1. Scheme of HPLC-ICP-MS coupling

3.4.2.5. Isolation of the fractions of metal-macromolecule complexes by size-exclusion chromatography

A 100 µL supernatant solution of the different tissue samples was injected to the system, HPLC-UV-ICP-MS with a Superdex-75 column, and a size-exclusion chromatogram obtained. After disconnecting ICP-MS, chromium containing fractions were collected by following the chromium trace and lyophilized. Lyophilisates were dissolved in mobile phase and re-injected to the Superdex-75 column to examine the stability of the metal complexes. Another lyophilisate obtained in the same conditions was mineralized by acidic digestion, described in the section 3.4.2.2, and chromium content was measured by ICP-MS to calculate the column recovery.

3.4.2.6. In-vitro reaction of mice liver cytosol with Cr(III)

The control group sample of mice liver cytosol, obtained by the method described in section 3.4.2.3, was spiked with 150 and 200 ppb chromium as final concentration. The mixture was vortexed and settled down for 3 hours. A 100 µL aliquot of this solution was injected to the Superdex-75 column of HPLC-UV-ICP-MS system and the chromatogram was obtained.

3.4.2.7. Tryptic digestion of the Cr-containing fractions of mice liver sample

ICP-MS was disconnected from column (Superdex-75) exit and Cr-containing fractions were collected according to the Cr elution window. The collected fractions

were frozen and freeze-dried. The lyophilisates were treated by trypsin in native and denaturing conditions. For the native tryptic digestion; a 150 µL solution of 50 mM ammonium bicarbonate buffer, pH 7.9, and 10 µL of 1 mg/mL trypsin was treated with lyophilisate at 37 ºC for 12 hours in a circulating water bath. For denaturing tryptic digestion; a 150 µL solution of 50 mM ammonium bicarbonate buffer containing 1 M of urea and 10 mM DTT (dithiothreitol) mixed with 10 µL of 1 mg/mL trypsin and treated with lyophilisate at room temperature for 2 hours. Trypsinized fractions were lyophilized and kept at -20 ºC until use. The survival of the resulted Cr-containing peptides was tested by using size-exclusion-HPLC-UV-ICP-MS with a Superdex-Peptide column and MALDI-TOF-MS techniques.

3.4.2.8. Size-exclusion chromatography of peptides by Superdex-Peptide column

The lyophilisate obtained after tryptic digestion (both in native and in denaturing conditions) was dissolved in mobile phase, 50 mM ammonium acetate, pH 7.4. A 100 µL solution was injected to Superdex-peptide column (300x10 mm, Pharmacia, Uppsala, Sweden) with exclusion limit of 0.1-7 kDa and previously calibrated with protein standards. The separation was conducted at a flow rate of 0.7 mL/min. The connection of HPLC to ICP-MS was performed as described in section 3.4.2.4.

3.4.2.9. MALDI-TOF-MS conditions and sample preparation

Sample preparation for Experiment I, wet liver tissue samples were homogenized and centrifuged at 5000 rpm for 5 min and mixed with 6-aza-2-thiothymine (ATT) matrix solution (20 mg in 1 mL acetonitrile-water in 1:1 (v/v) ratio).

Mass calibration of TOF analyzer was performed using external standards of protein mixtures. The trypsinized lyophilizates was dissolved in two different matrix solution, the first was sinapinic acid (SA) (10 mg/mL in acetonitrile-water in 1:1 (v/v) ratio) and the other was 2-amino-thiaamine (ATT) (20 mg/mL in acetonitrile-water in 1:1 (v/v) ratio). Samples were prepared by mixing the samples with the matrix solution at changing rates. Finally 1 µL of this mixture was deposited on the sample plate, dried at room temperature and then analyzed. MALDI-MS spectra were recorded in reflectron mode with average of 1200-2200 shots.

4. RESULTS AND DISCUSSION

4.1. Analysis of liver tissue samples

4.1.1. Measurement of total metal content of liver

When total chromium content of liver samples was measured by GF-AAS for the hexavalent chromium (20 mg Cr/kg) injected and control groups, concentration of total chromium increased dramatically. It is known that liver has primary importance for the detoxification of xenobiotics and is indeed among the most vulnerable organs for chromium accumulation (Stearns et al., 1995).

Table 4.1. Liver chromium, copper, and zinc levels (µg/g) after i.p. injection of 20 mg Cr(VI)/kg b.w. of mice

	Control	Cr(VI) injected
Cr	0.28 ± 0.04	67 ± 15
Cu	12 ± 2	25 ± 4
Zn	30 ± 1	61 ± 14

*: p <0.05. Data is expressed as mean ± standard deviation, n=3.

The applied Cr(VI) dose (20.0 mg Cr/kg), administration route (i.p.) and experiment animals (male Swiss Albino mice) are identical with those in the studies of Boşgelmez and coworkers. The dose has been caused to oxidative stress and chromium accumulation in liver (nearly 72 µg/g) (Boşgelmez et al., 2008) and kidney (nearly 63 µg/g) (Boşgelmez and Güvendik, 2004) of Swiss Albino mice. This was the rationale behind the choice to inject the hexavalent chromium dose of 20.0 mg/kg.

Our result of liver Cr content following the same dose of hexavalent chromium (67 ± 15 µg/g) quite agree with that of Boşgelmez and coworker's study (Boşgelmez et al., 2008).

When we look up to the copper and zinc concentrations, it can be seen that liver levels of these elements also increased nearly 2-fold (see Table 4.1). Alteration in Cu and Zn levels indicates that administration of hexavalent chromium causes to aberration of trace element homeostasis. Another possibility is that these two

metals involves in the processes relating to the mode of action of hexavalent chromium to prevent the toxic effects of Cr(VI).

4.1.2. HPLC analysis of the liver tissue extracts

When detector was set at 360 nm, we could see different fractions in HPLC chromatogram of the liver tissue extracts for control group mice (Figure 4.1).

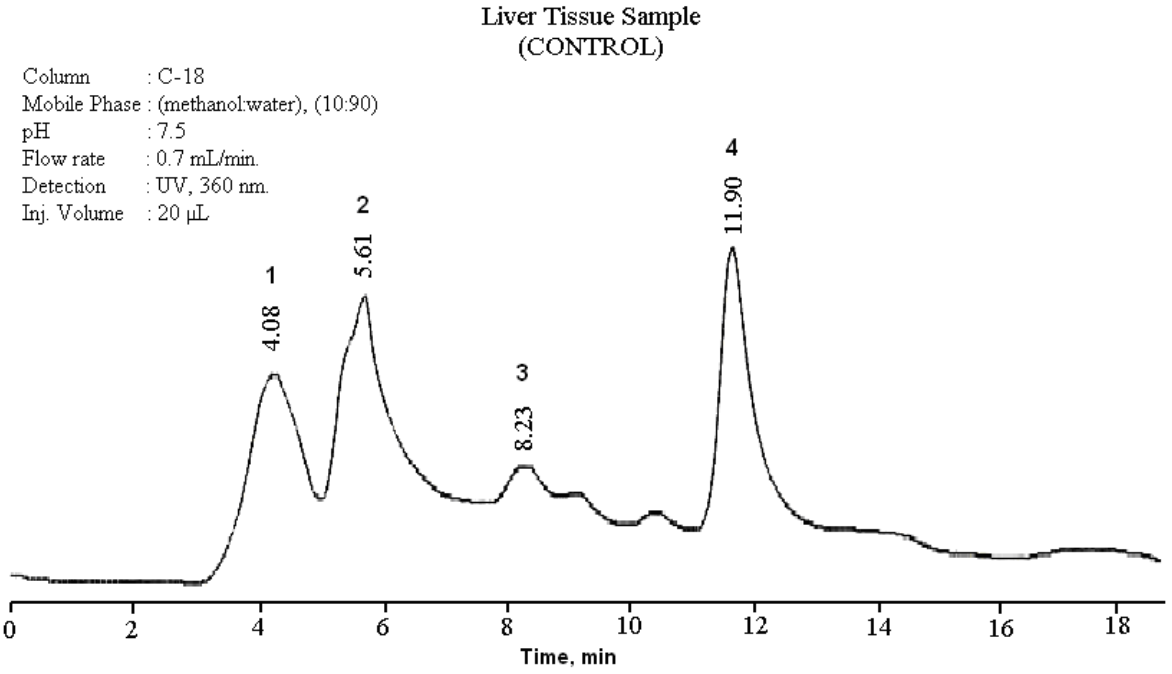

Figure 4.1. Reverse phase HPLC chromatogram of control group mice liver after extraction with 50 mM of ammonium acetate solution at pH 7.4

At this wavelength many metal complexes give absorbance relating to electronic transitions between metals and ligand. The analysis of repetitive aliquots has been given practically the same chromatogram.

The chromatogram of hexavalent chromium injected mice liver samples is shown in Figure 4.2. When the two chromatograms are compared to each other, the intensity of peak eluted at 4.08 increased with Cr(VI) administration, accordingly some peaks were disappeared in chromatogram of Figure 4.2. This is probably due to the reason that this biomolecule selectively binds chromium. One indicator supporting this possibility is Cr content of the fraction of regarding peak. Cr content of the fractions collected for a one minute of elution time was measured by GFAAS.

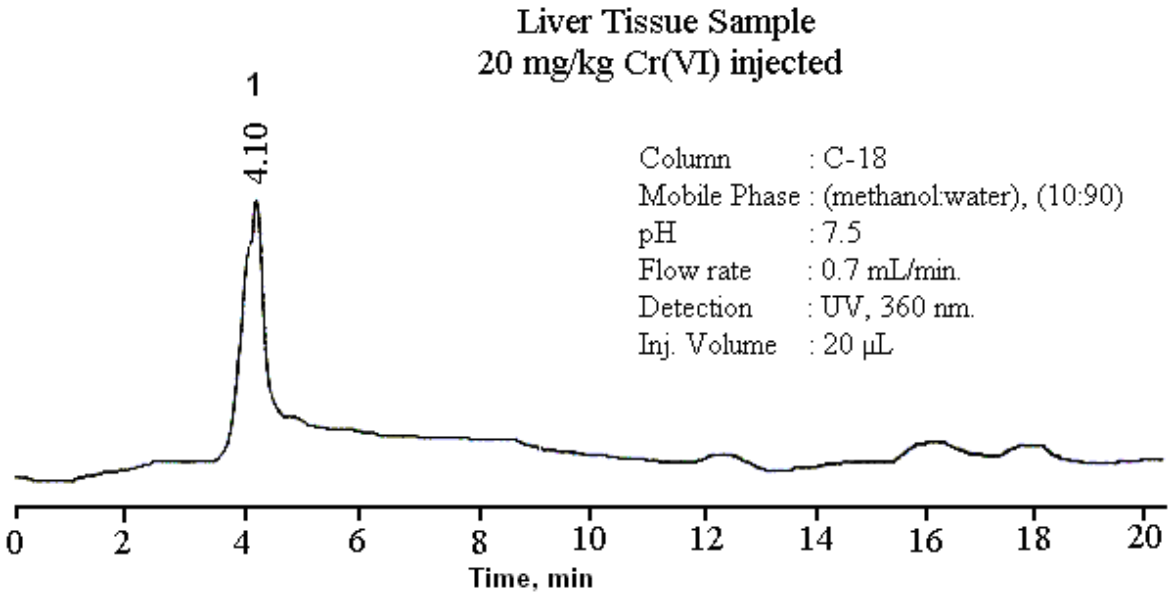

Figure 4.2. Reverse phase HPLC chromatogram of hexavalent chromium injected mice liver after extraction with 50 mM of ammonium acetate solution at pH 7.4

The mean of three measurements has been given in Table 4.2. Cr concentration of the first fraction (numbered as 1 in the chromatograms) has been found considerably higher in the hexavalent chromium injected mice liver samples (see Table 4.2).

Table 4.2. Cr contents of the collected HPLC fractions

Control group		**Cr(VI) injected group**	
Fraction	**Cr concentration (ng/mL)**	**Fraction**	**Cr concentration (ng/mL)**
1	0.6	**1**	5.5
2	1.7	**2**	1.8
3	0.7	**3**	0.6
4	0.8	**4**	0.8

The chromatograms in Figure 4.3 and 4.4 were obtained with the detection at 215 nm, reflecting the absorbance of the peptide bonds of proteins. When the two chromatograms of liver sample were compared to each other, some apparent alterations can be seen. Because, not only the intensity of some peaks, especially belongs to the more hydrophilic (more quickly eluted) ones, but also their elution

profiles as a result of change in hydrophilicity seems to change after the single dose Cr(VI) administration.

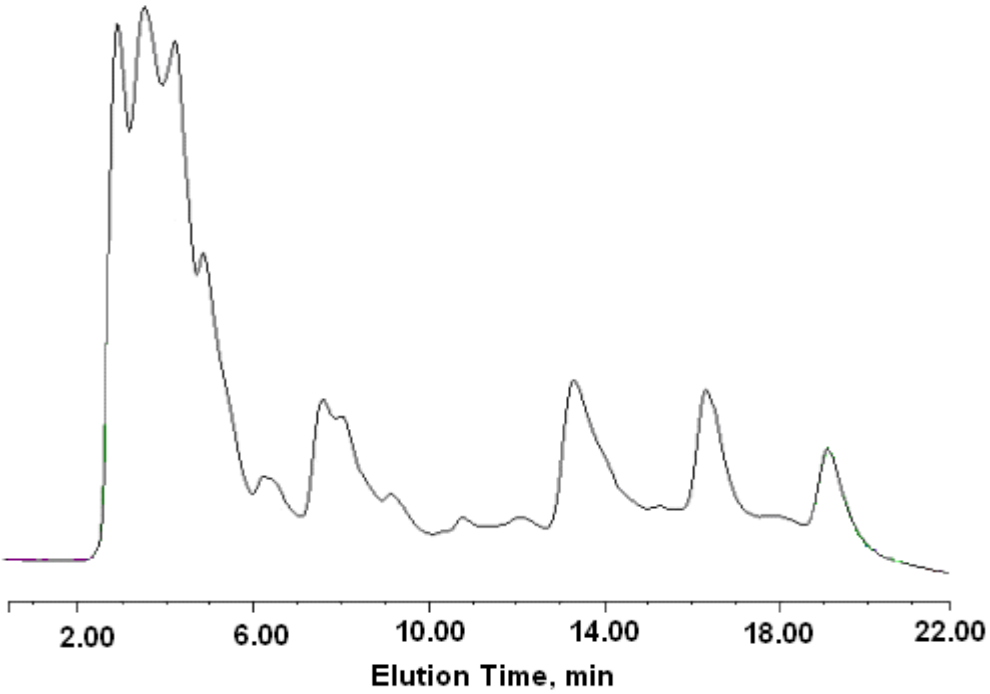

Figure 4.3. RP-HPLC chromatogram of liver sample for control group mice at 215 nm UV detection

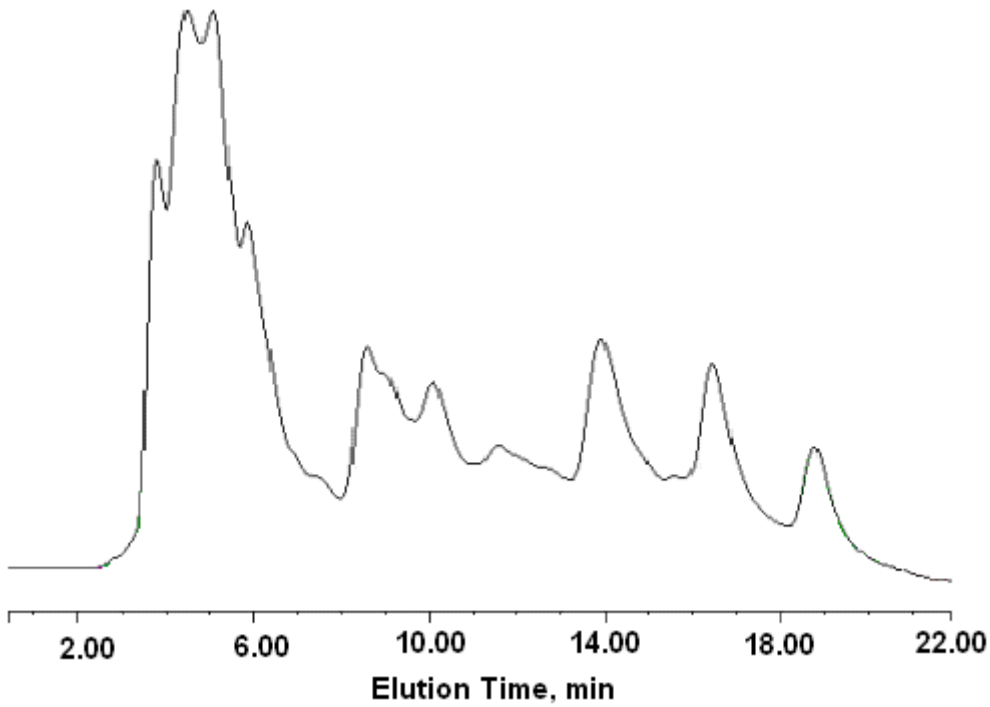

Figure 4.4. RP-HPLC chromatogram of liver sample for hexavalent chromium injected group of mice at 215 nm UV detection

Alteration in the elution profile of the proteins may be associated by chelation of metals by protein or polypeptides accordingly a change in the conformational or two dimensional form which affects their disturbance between mobile and stationary phase.

On the other side, it is clear that chromatograms obtained at 215 nm or 360 nm detections are quite different to each other.

4.1.3. MALDI-TOF-MS analysis of liver tissue extracts

When we look at MALDI-MS spectrum of the hexavalent chromium injected mice liver samples, we could see numbers of biopolymers in Figure 4.5.

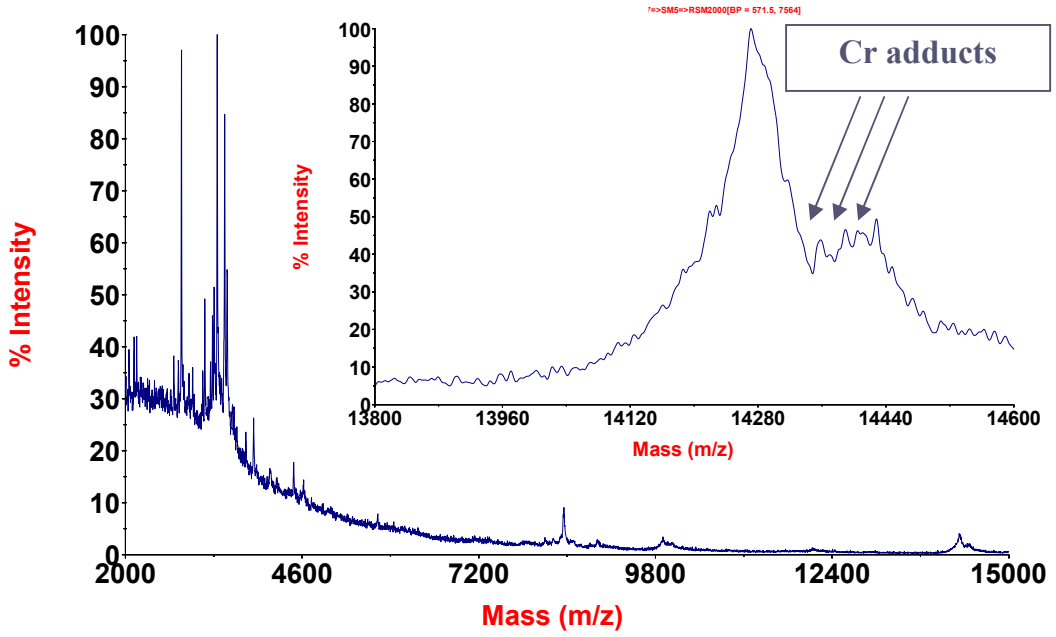

Figure 4.5. MALDI-MS spectrum of the hexavalent chromium injected mice liver sample homogenized in 50 mM Tris-HCl buffer at pH=7.4. Inset shows the near projection of 14000 amu region

Chromium adducts are shown on the protein peaks (inset in Figure 4.5). These adducts have 52 mass difference from each other, which represents atomic weight of chromium. It was seen that the soft ionization mechanism in MALDI allows the observation of non-covalent Cr-protein complexes.

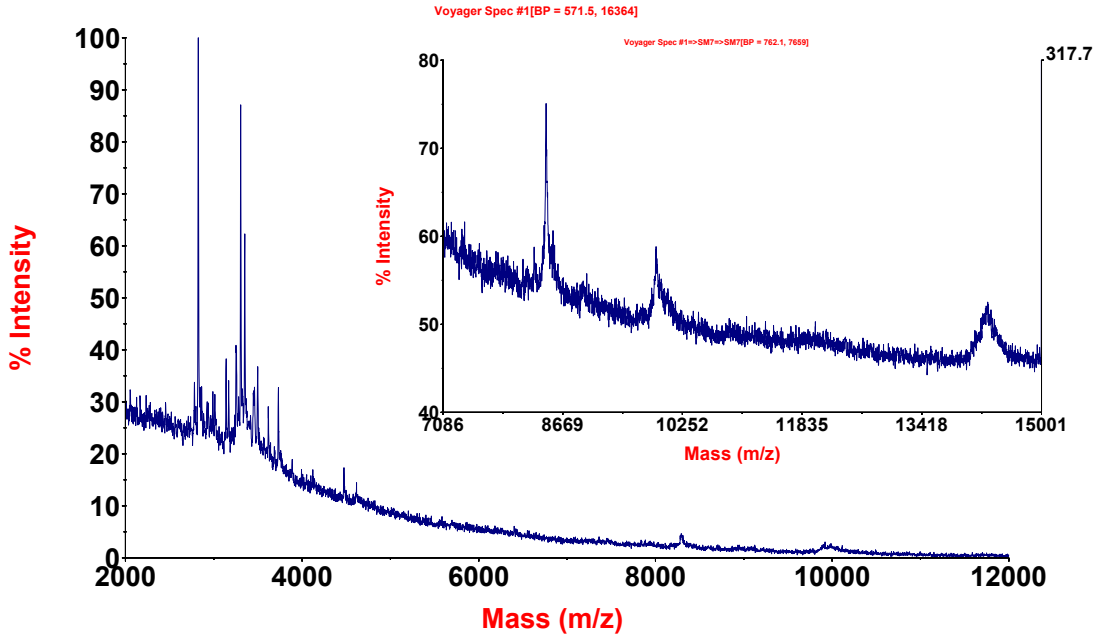

Figure 4.6. MALDI-MS spectrum of control group mice liver sample homogenized in 50 mM Tris-HCl buffer at pH=7.4. The mass region between 7000-15000 amu is shown at the inset

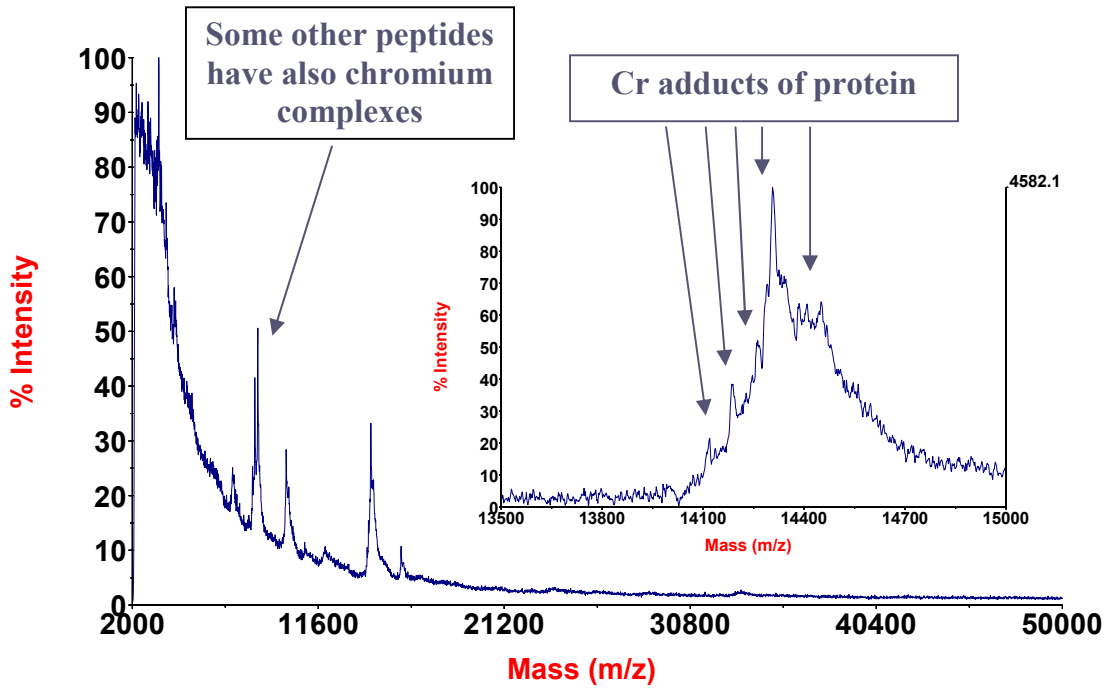

Figure 4.7. MALDI-MS spectrum of 20 mg Cr(VI) /kg of b.w. over-loaded mice liver sample after extracted into 50 mM of ammonium acetate solution. Deconvoluted form of the spectrum at around 14000 amu for Cr-complexes is shown at the inset.

When looking to the MALDI spectrum of the no chromium injected (control group) mice liver samples, it does not seem any chromium adducts on the protein peaks and the intensity of some protein complex peaks is really lower (Figure 4.6) compared to chromium over-loaded mice samples (Figure 4.5).

It is shown that some other polypeptides have also chromium complexes and chromium adducts of polypeptides are present when 50 mM of ammonium acetate was used for extraction of metal complexes from liver samples. It is noteworthy that when ammonium acetate solution was used for the extraction of metal-complexes, more intense peaks were observed in MALDI-MS spectrum (see Figure 4.7). Beside, the use of ATT which is a neutral type MALDI matrix was found quite appropriate for the efficiency of ionisation of Cr-protein complexes.

In the experiments of 20 mg/kg hexavalent chromium injected mice liver samples, determination of metals by GFAAS showed that single dose hexavalent chromium exposure affects not also tissue content of Cr, but also Cu and Zn content. Indeed excess amount of an element, especially a toxic metal, can alter the concentration of other elements in living organisms. This effect was observed in the case of chromium (VI) exposure to mice.

More interestingly investigation of proteins by HPLC showed that introduction of a xenobiotic into body can affect the binding of proteins with metals leading to toxic effects. In order to preserve the native form of non-covalent metal-protein complexes, both extraction and analysis should be maintained in a media which have highly similar conditions with protein's native environment (Szpunar, 2000a). For this reason extraction was carried out in ice-cold media by a buffer at physiological pH.

Analysis of samples by MALDI-TOF-MS showed that chromium can bind to different biological macromolecules in liver tissue which plays primary role in protein synthesis and other metabolic processes such as detoxification.

The outputs on the liver studies have been presented at diverse national and international symphosiums (Döker et al., 2004; Doğan et al., 2006).

4.2. Analysis of other tissues of mice and essential trace metal homeostasis

In order to wider and deeper investigation of both essential trace metal homeostasis and their binding with biological macromolecules, further experiments were enlarged to the other mice tissues namely liver, kidney, brain, lung, heart and testicles. Following a moderate dose, 8.0 mg/kg of Cr(VI) to mice, a wider range of essential trace metals, Cr, Cu, Zn, Fe and Mn have been investigated in the tissues.

The experiments carried out in this group involve both total measurement of tissue metal content by ICP-MS and distribution of metals between different molecular weight proteins by SEC-UV-ICP-MS.

Regarding the choice of the dose (8.0 mg Cr/kg), it has been considered that the dose should be high enough to arise oxidative stress, chromium accumulation and histopathological effects, but not too high to bind non-specifically to the proteins to be investigated. Indeed, it has been shown that a low dose, 10 mg $K_2Cr_2O_7$/kg (~3.5 mg Cr/kg) subcutaneous injection of $K_2Cr_2O_7$ to rats is unable to induce nephrotoxicity suggesting a threshold of this compound to induce renal damage which induced at a dose of 20 mg $K_2Cr_2O_7$/kg (~7 mg Cr/kg) (Gumbleton and Nicholls, 1988).

On the other side Chmielnicka and coworkers revealed that histopathological changes in the kidneys of rats exposed to toxic metals occur when the toxic metal concentration in the tissue is between 3-30 μg/g (Chmielnicka et al., 2002). Therefore, an acute dose of 8.0 mg Cr/kg, chosen in our study, corresponds to renal Cr content of 14.5 μg/g. Indeed, Cr(VI) being nephrotoxic, kidney is target organ to cope with the Cr(VI) exposure.

4.2.1. Determination of trace metals in the tissues

The concentrations of essential trace metals of Zn, Cu, Fe and Mn as well as Cr were determined in diverse tissues of mice by ICP-MS.

4.2.1.1. Optimization of instrumental and experimental conditions

Instrumental conditions for ICP-MS measurements were briefly given in Table 4.3.

Table 4.3. Instrumental conditions for ICP-TOF-MS

Rf power	1500 W
Carrier gas flow rate	1.1 L/min
Sampling Depth	8 mm
Quadrupole bias	-17 V
Octopole bias	-18 V
Isotopes detected	^{50}Cr, ^{52}Cr, ^{53}Cr, ^{64}Zn, ^{66}Zn, ^{63}Cu, ^{65}Cu, ^{57}Fe, ^{55}Mn.
Integration time	0.1 s
Collision gas and flow rate	H_2 (3 mL/min)

Four different calibration methods were tested for accurate determination of metal concentrations.

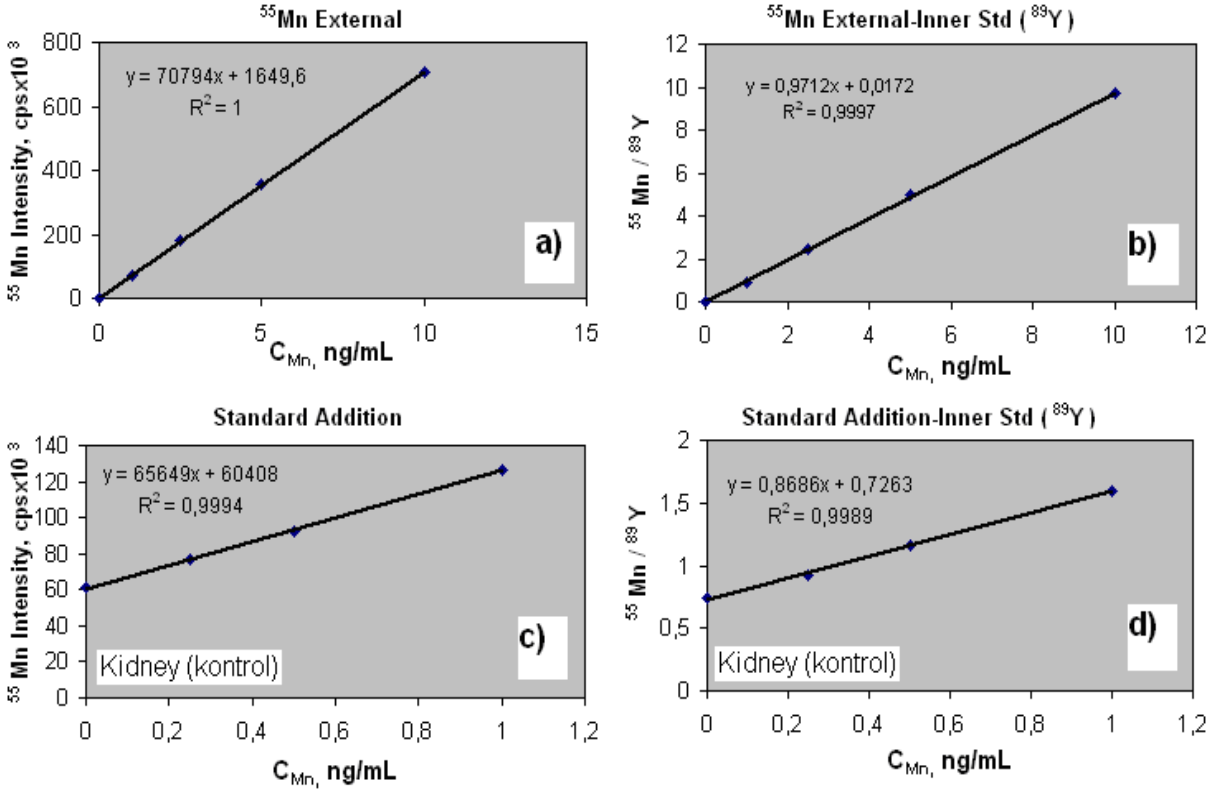

Figure 4.8. Calibration graphs for the estimation of metal content in tissue samples. a) External calibration graph of ^{55}Mn, b) External calibration graph of ^{55}Mn in which ^{89}Y was used as inner standard, c) calibration graph of ^{55}Mn in which standard addition method was applied for kidney sample of control group, d) calibration graph of ^{55}Mn in which standard addition method was applied and ^{89}Y used as inner standard for kidney sample of control group.

These are external calibration and standard addition methods with and without internal standard. Yttrium (^{89}Y) was added as internal standard to the solutions to be measured.

Figure 4.8 shows the calibration techniques for the determination of Mn in the kidney sample of mice. The use of an internal standard to eliminate the possible effects from instability of atomization source (plasma or flame) is generally applied in qualitative and quantitative analysis. ^{89}Y was a commonly preferred internal standard because of its characteristics such as rare occurrence in nature and the lack of interference with other elements at mass 89 amu. On the other hand, standard addition method helps to eliminate the errors coming from matrix components. Although the four calibration techniques successfully work in quantitation, standard addition method without use of ^{89}Y (internal standard) signal intensity has given the most accurate results in the analysis of CRM. Thus, this method has been chosen as calibration method (Fig. 4.8c).

4.2.1.2. Method Validation

In the present study, certified tissue sample, TORT-2 (Lobster Hepatopancreas) was parallelly analyzed with tissue samples and the obtained results were compared with the certified values of the metals (Table 4.4). The obtained values of metals were found highly agree with the certified values for the analyzed CRM.

Table 4.4. Metal concentration values of certified reference material and the values obtained in the present study (µg/g dry tissue)

Metal	TORT-2 (Lobster Hepatopancreas)	
	*Certified	*Obtained
Cr	0.77 ± 0.15	0.73 ± 0.06
Mn	13.6 ± 1.2	14 ± 1
Cu	106 ± 10	101 ± 8
Fe	105 ± 13	101 ± 8
Zn	180 ± 6	183 ± 20

* both data (certified and obtained) expressed as mean ± ts / $N^{1/2}$ at 95% confidence level, n=3.

It worth to mention that, total metal content of the biological samples especially in tissue samples considerably differ from each other in the literary data. One difficulty to compare the data is that expression of the tissue metal contents in different units, i.e., metal amount per weight of wet or dry tissue. Variability of water content of tissues in diverse storage conditions (i.e., time until analysis, temperature etc.) leads to differentiation in tissue contents of metals. The absence of certified reference materials to test the accuracy of the measured values and analysis method in many studies is another difficulty. Accordingly, contradiction in the analytical data was enhanced by various solubilizing procedures and analyzing methods used.

4.2.2. Metal content of mice tissues

Screening a range of trace elements in one sample can reveal the interactions between elements. The data analysis of the analytical results may be useful in such investigations. Table 4.5 shows the concentrations of metals in the control and Cr-injected mice tissue samples which prepared for the analysis as described in section 3.4.2.2.

Three aliquots pertaining to pooled tissue samples were parallelly analyzed for total metal content and ICP-MS measurements were also triplicated. All results are expressed as mean ± standard deviation. Data were analyzed for difference using independent samples T test by using SPSS statistics program. Differences between groups were judged to be statistically significant when the *p*-value was <0.05. Alteration in metal contents as percentage and statistically significant alterations ($p<0.05$) in metal content of tissues were indicated with an asterisk on the table.

Table 4.5. Total metal contents of tissues for control and Cr-administered group mice, (µg/g dry tissue).

		Control	Cr-injected	Alteration, %	*p*
Liver	Cr	0.63 ± 0.03	25 ± 2	3870	*0.000
	Mn	1.08 ± 0.02	0.92 ± 0.07	-15	*0.017
	Cu	3.6 ± 0.2	3.8 ± 0.2	6	0.227
	Fe	56 ± 2	85 ± 2	54	*0.000
	Zn	22.5 ± 0.9	21 ± 0.2	-6	0.076
Kidney	Cr	0.37 ± 0.02	14.5 ± 0.2	3824	*0.000
	Mn	1.04 ± 0.03	1.09 ± 0.07	5	0.345
	Cu	3.6 ± 0.2	3 ± 0.3	-18	*0.021
	Fe	69 ± 3	65 ± 3	-6	0.195
	Zn	15.5 ± 0.5	14.0 ± 0.9	-10	0.074
Brain	Cr	0.24 ± 0.03	1.4 ± 0.5	483	*0.015
	Mn	0.39 ± 0.01	0.65 ± 0.06	67	*0.003
	Cu	2.52 ± 0.09	2.01 ± 0.05	-20	*0.006
	Fe	18 ± 2	7.2 ± 0.7	-59	*0.006
	Zn	13.3 ± 0.8	9.9 ± 0.2	-26	*0.002
Lung	Cr	0.40 ± 0.04	2.2 ± 0.4	438	*0.002
	Mn	0.25 ± 0.06	0.30 ± 0.02	20	0.454
	Cu	1.8 ± 0.1	1.4 ± 0.1	-24	*0.018
	Fe	86 ± 4	81 ± 3	-6	0.306
	Zn	20 ± 2	13 ± 2	-37	*0.020
Heart	Cr	0.70 ± 0.07	1.9 ± 0.3	177	*0.002
	Mn	0.55 ± 0.06	0.48 ± 0.02	-13	0.246
	Cu	3.6 ± 0.2	3.88 ± 0.06	9	0.081
	Fe	246 ± 16	179 ± 5	-27	*0.012
	Zn	13.6 ± 0.5	16 ± 2	15	0.126
Testicle	Cr	0.80 ± 0.04	2.9 ± 0.5	266	*0.001
	Mn	0.31 ± 0.05	0.57 ± 0.04	84	*0.002
	Cu	0.78 ± 0.04	0.96 ± 0.01	23	0.057
	Fe	8.1 ± 0.4	11.3 ± 0.3	40	*0.000
	Zn	13.1 ± 0.2	14 ± 2	6	0.431

* : $p < 0.05$. Data is expressed as mean ± standard deviation, n=3.

4.2.2.1. Cr levels

Once hexavalent chromium was administered to mice, considerable increase was remarked in the Cr concentration of all tissues, as expected. The highest chromium accumulation was observed in the liver and kidney. Chromium enrichment factors of kidney and liver revealed to be as 37 and 36, respectively,

when hexavalent chromium was injected to mice at a dose of 8.0 mg Cr(VI)/kg. Chromium enrichment factors of lung, brain, testis and heart were found as 9, 5, 4, and 3, respectively. However, when the concentration value is considered, distribution of chromium to the examined mice organs is in the decreasing order of;

Liver > kidney > testis > lung > heart > brain

Many studies exist in literature investigating the accumulation of Cr in different mammalian tissues. However, in these researches chromium with various chemical forms and oxidation states have been administered to diverse animal subjects by different administration routes; i.e., dietary, oral gavage, intraperitoneal injection, intravenous injection etc. For these reasons comparison of the data regarding Cr distribution of tissues is highly difficult.

Many studies have investigated accumulation of Cr(III) in laboratory animals such as rats, mice, and rabbits as well as in human tissues related to occupational exposure. Liver and kidney have been reported as the most vulnerable organs for Cr retention and accumulation. Diverse researches regarding chromium retention in tissues has been exampled in the study of Stearns et al. (Stearns et al., 1995). They have analyzed the accumulation of chromium in some mammalian animal models after both Cr(III) and Cr(VI) administration as well as in human tissues related to occupational exposure. Quantitative estimates based on Cr(III) pharmacokinetic models has revealed that; since chromium is slowly excreted from cells and tissues, increased absorption of chromium due to dietary supplementation may lead to Cr accumulation in tissues and poorly understood consequences.

The concentration of essential trace elements such as Zn, Cu, Fe, Mn in diverse tissues and alteration in their levels followed by Cr(VI) exposure has not been clearly investigated yet. On the other hand, some target organs such as kidney and liver were partially investigated in literature, in terms of Cr retention. It was reported that single intraperitoneal potassium dichromate treatment (20 mgCr/kg), as Cr(VI) compound, to the mice has resulted significant chromium accumulation

(nearly 63 µg/g) in kidney (Boşgelmez and Güvendik, 2004) and (nearly 72 µg/g) in liver (Boşgelmez et al., 2008). Higher kidney Cr levels found in this study may be a result of the higher Cr(VI) dose, 20 mg Cr/kg, while the Cr(VI) dose in our study was 8.0 mg Cr/kg. Both in Bosgelmez's study and in the present study, potassium dichromate, as Cr(VI) compound, was intraperitoneally administered and the tissues were collected at the end of 12 h, after injection. Pedraza-Chaverri et al., administered Cr(VI) to male Wistar rats as single subcutaneous injection at a dose of 15 mg $K_2Cr_2O_7$/kg (~5 mg Cr/kg) and observed that this exposure elevated the kidney Cr content to approximate value of 200 µg/g (Pedraza-Chaverri et al., 2005). Although the chromium dose administered to rats in this study is lower than the dose in our study, kidney Cr content was approximately fourteen times higher than our finding (14.5 ± 0.2 µg/g). This difference may be a result of utilization of different species as experimental animals (rat vs. mouse) as well as different exposure routes (subcutaneous vs. intraperitoneal) in the experimental design. However, there is any expression about Cr determination method.

As a response to hexavalent chromium exposure to the mice, it was observed not only rise in Cr levels of all mice tissues, but also remarkable alterations were shown in the concentration of biologically important trace metals, such as Zn, Cu, Fe and Mn which are cofactors of many enzymes and structural components of proteins. To the best of our knowledge, this is the first report investigating the alteration of trace metal homeostasis by using ICP-MS in mice organs, after Cr(VI) administration.

When the alteration was considered in organ base, one of the most important changes was observed in the trace metal levels of brain. Interestingly, a single dose of Cr(VI) resulted in a considerable decrease in the brain Fe, Zn and Cu levels whereas increase in the Cr and Mn levels ($p<0.05$). Metal ion transport from the blood to the brain necessitates the crossing of the blood–brain barrier (BBB) which protects the central nervous system (CNS) against chemical insults, by different complementary mechanisms (Zheng, 2003). Regulation of blood–brain tissue exchange plays a pivotal role in CNS homeostasis and is accomplished by individual endothelial cells, which are continuously linked by tight junctions.

Essential trace elements are needed to maintain proper brain functions as catalysts, second messengers, and gene expression regulators. They activate and stabilize enzymes, such as superoxide dismutase, metalloproteases, protein kinases, and transcriptional factors containing zinc finger proteins. For this reason metals must be supplied to the CNS at an optimal level, because both deficiency and excess can result in aberrant CNS functions. Aberrations in brain metal homeostasis are generally associated with diverse neurological diseases (Burdo and Connor, 2003) such as Parkinson's disease (Gorell, et al., 1997) and aceruloplasminemia (Xu et al., 2004).

4.2.2.2. Fe and Mn Levels

Determination of metal content in tissues showed that, one of the most important changes was observed in the Fe and Mn levels. Single dose Cr(VI) administration resulted in decreased Fe levels in brain and heart, whereas increased Fe levels in liver and testis ($p<0.05$). Mn levels in brain and testis were considerably increased; beside of that, slight increase was observed in lung.

Both Fe^{2+} and Mn^{2+} are grouped into redox active metals, Fe^{2+}, Cu^{2+}, Co^{2+} and to a lesser extent Mn^{2+}. The redox-active ions normally function in enzymes that participate in redox reactions. As a component of heme group, iron serves critical roles in metabolic functions. Cytochrome b_5, a heme (iron) containing protein, is a common mediator of electron transfer to Cr(VI) and involved in reduction of Cr(VI) in hepatic and lung microsomes (Myers and Myers, 1998; Myers et al., 2000; Borthiry, 2007).

According to our results; hexavalent chromium exposure to the mice at the administered dose (8.0 mg Cr/kg) resulted in a decrease in brain Fe levels which antagonistically affected brain Mn levels. Garcia et al., revealed that Fe deficiency can enhance brain Mn accumulation even in the absence of excess Mn in the environment or the diet (Garcia et al., 2007). The results of the study confirm that there is homeostatic relationship among several essential metals in the brain and not simply between Fe and Mn. Our results revealed that the reverse alteration between Fe and Mn levels is consistent not only in brain but also in liver, kidney and lung as well. Our finding, inverse relation between Fe and Mn levels in brain,

was obtained in other diverse surveys as well (Garcia et al., 2007; Malecki et al., 1999a,b; Roth and Garrick, 2003). Indeed the alterations in the tissue content of both metals (Fe and Mn) occur at the similar amounts but in opposite way (see Figure 4.9 and Table 4.5).

Iron transport is mediated in mammalians predominantly by the transferrin system, involves the adsorption of Fe(III) onto transferrin, followed by the binding of the Fe–transferrin to its receptor (TfR), internalization of transferrin-bound receptors into endosomes and releasing the bound iron by the low pH generated by V-ATPase. The iron released is transported into the cytoplasm by DMT1 (Nelson, 1999). The abnormal elevation in brain Cr levels accompanied by the sharp decrease in Fe levels following Cr(VI) administration to mice is shown in Table 3. This rise may be potentially caused by the binding of excess Cr(III), instead of Fe(III), to transferrin and internalization by TfR into cell.

Fe and Mn share similar chemical characteristics; both have oxidation states of (II) and (III) and their ionic radius values are not very different from each other as adjacent elements in the first row transition metals. Cr is a next-door to Mn in periodic table as well. Similarity in bioinorganic preferences of the ternary (Cr, Fe and Mn) reflects their interactions with biological molecules. A series of active or receptor-mediated transport systems serve to control the transport of these metals into the brain. But the reverse alteration between Fe and Mn may possibly result from that both metals compete for the same carrier transport systems, transferin receptor (TfR) and divalent metal transporter-1 (DMT1/DCT1/Nramp2) which regulate the metals influx into the brain.

Fe circulates in the plasma predominantly as the trivalent state complexed with transferrin, a glycoprotein with an approximate molecular weight of 80 kDa. Physiological delivery of Fe into the brain is most prevalently mediated by TfR, a disulfide-linked, integral membrane glycoprotein with an approximate molecular weight of 180 kDa, and its endocytosis. Another predominant iron transporter is DMT1 which found in a location able to influence iron transport into and out of the brain, namely ependymal cells that line the ventricles, blood vessel endothelial cells and astrocytes associated with these vessels (Burdo and Connor, 2003). Iron

is a critical component of the CNS that must be tightly regulated; iron deficiency can result in energy insufficiency while excess iron can result in oxidative stress. The brain uptake and homeostatic mechanisms of iron has been reviewed by Burdo and Connor (Burdo and Connor, 2003). Other proteins responsible from iron homeostatic mechanism are; Melanotransferrin (P97, a homologue of transferrin); Hfe (a membrane protein that can influence cellular iron uptake); Metal Transport Protein 1 (MTP-1, also known as Ireg1 and ferroportin); Ferritin (classically been known as an iron storage protein in many types of cells, including those of the brain); and Lactoferrin (Lf, an iron binding glycoprotein similar to transferrin). In conclusion, Fe and Mn influx and efflux of brain maintained by different specialized metal transport mechanisms, but TfR and DMT-1 are common pumps to influx Fe and Mn inside brain. On the other hand, transferrin has a metal binding capability not only with iron but also with other metal ions including chromium and manganese (Aisen et al., 1969; Harris and Messori, 2002; Sun et al., 2000). That is why hexavalent chromium exposure (chromium in trivalent state, Cr^{3+}, is final product of hexavalent chromium, Cr^{6+}) led to a decrease in brain Fe levels which caused an increase in brain Mn levels. Brain is particularly susceptible to excess Mn and accumulation can cause a neurodegenerative disorders such as manganism. Neurotoxicity of manganese has been subjected in (Dobson et al., 2004).

In addition, iron is the classic metal associated with hydroxyl radical generation by Fenton reactions (ferrous iron plus H_2O_2) and these type of reactions have been shown involving in the reduction of Cr(VI) in human cell lines and animal models *in vivo* and *in vitro* (Chen et al., 1997; Shi et al., 1994; Kadiiska et al., 1998; Hodges et al., 2001). Therefore, the decrease in brain Fe levels may be a metal homeostatic response in CNS to prevent the radical generation stimulated by hexavalent chromium exposure. Furthermore, iron is known to stimulate the rate of Cr(VI) reduction by human microsomes (Myers and Myers, 1998; Myers et al., 2000) and Cr(VI) reduction is exerted also by iron containing reductive enzymes such as SOD and CAT (Bosgelmez and Guvendik, 2004; Bosgelmez et al., 2008), cytochrome P450 (Mikalsen et al., 1995; Jannetto et al., 2001) and cytochrome b_5 (Borthiry et al., 2007; Jannetto et al., 2001).

The decreases in Fe levels in brain and heart seems to be related with the increase observed in liver and testicles ($p<0.05$, see Table 3). This response may be due to Fe release from brain to blood and transport of blood Fe by transferrin to liver. On the other hand, remarkable increase in brain Mn levels might have possibly been compensated by the decrease in liver and heart. The decrease in liver Mn levels is not as much as the increase in brain, but when relatively bigger mass of liver is taken into consideration it is possible to compensate the increase in brain Mn levels by a slight decrease in liver Mn levels.

Another biological relevance between Cr and Fe is cell apoptosis. Apoptosis is a programmed cell death mechanism to control cell number in tissues and to eliminate individual cells that may lead to disease states. ROS generated through Cr(VI) reduction is responsible to the early stage of apoptosis, whereas p53 contributes to the late stage of apoptosis and is responsible for the enhancement of Cr(VI)-induced apoptosis at this stage to prevent the effects of ROS (Ye et al., 1999). In the study by Ye and coworkers showed that Cr(VI)-induced ROS generation occurred within a few minutes after Cr(VI) treatment of the human lung epithelial cells (A549). After 5 h (p53 induction takes place) following Cr(VI) exposure, the level of the apoptosis has been found higher in p53-positive cells than in p53-negative cells (Ye et al., 1999). It has been also shown that p53 gene play a role in the toxicity of xenobiotics including Cr(VI) in mice (Bagchi et al., 2000). Beside of that, it has been hypothesized that upregulation of hepcidin, an iron-regulatory protein, by p53 is part of a defense mechanism against cancer, through iron deprivation (Weizer-Stern et al., 2007). On the other hand, it has been revealed that a multitude of cell cycle control molecules are regulated by Fe. These include p53, $p27^{Kip1}$, cyclin D1 and cyclin-dependent kinase 2(cdk2) (Yu et al., 2007). The findings clearly showed that iron is associated with cell apoptosis mechanisms which prevents the cells from ROS generated through Cr(VI) reduction.

Administration of hexavalent chromium also alters the tissue Mn levels. The alterations occur in the same organs and in the very similar manner to those of Mn administration to mice. Jursa and Smith have used aceruloplasminemic versus wild-type mice which treated with $MnCl_2$ as well as ^{54}Mn as radiotracer. It has been

revealed that ceruloplasmin affects the retention of manganese in blood and its distribution to tissues, most notably kidney, brain and lung (Jursa and Smith, 2009). The parallel Mn retention was observed in our finding that kidney, brain and lung Mn levels have been increased by Cr(VI) exposure.

In conclusion; considerable alterations were found in the Fe and Mn levels of brain and other tissues with Cr(VI) administration. Inverse alteration in the tissue concentration of the ternary (Cr, Fe and Mn) is potentially raised from the common transportation by transferrin and internalization by TfR and DMT-1.

4.2.2.3. Cu and Zn levels

Chromium is known to produce nephrotoxicity (Chmielnicka et al., 2002; Pedraza-Chaverrí et al., 2005). The cells of the proximal renal tubule have an important role in maintaining essential metal ion homeostasis. For this reason, kidney is target organ for studying exposure to Cr(VI). However, the studies investigating the changes in essential trace metal levels in kidney are very scarce. In the study by Chmielnicka and coworkers, when the rats were intraperitoneally injected with $K_2Cr_2O_7$ at a dose of 5 mg Cr/kg (a near dose to ours, 8.0 mg Cr/kg), kidney Cu content was moderately decreased and Zn content was slightly decreased. Very high agreement was obtained in our study; as decreases in the kidney levels of both Cu and Zn. Cu levels of kidney, brain and lung were moderately decreased by Cr(VI) administration, whereas; liver, heart and testis Cu levels were slightly increased. On the other hand, decrease in brain Cu level (and also decrease in brain Fe level) might be a link to the increase in liver Cu level (and also increase in brain Fe level).

Decreases in kidney Cu level, to a lesser extend in Zn and Fe levels, in our results could be associated with the decrease in kidney activity of superoxide dismutase and catalase enzymes. These enzymes involved in antioxidant defense mechanism and exert their actions with metal cofactors. Catalase is a common enzyme found in nearly all living organisms which are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen. It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. Superoxide dismutases are a class of enzymes

that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Inhibition in Cu/Zn-SOD activity with Cr(VI) administration have been shown previously. Boşgelmez and coworkers have shown that single intraperitoneal potassium dichromate treatment (20 mgCr/kg), as Cr(VI) compound, significantly elevated the level of lipid peroxidation in kidney and liver of mice as compared with controls. This was accompanied by significant decreases in nonprotein sulfhydryls (NPSH) level, superoxide dismutase (SOD) and catalase (CAT) enzyme activities (Boşgelmez and Güvendik, 2004, Boşgelmez et. al., 2008). Pedraza-Chaverrí and coworkers have also revealed that Cu/Zn-SOD activity and SOD content as well as CAT enzyme activity decreased after a single subcutaneous $K_2Cr_2O_7$ injection at a dose of 15 mg $K_2Cr_2O_7$/kg (equal to ~5 mg Cr/kg) decreased after two days from Cr(VI) exposure in male rats (Pedraza-Chaverrí et al., 2005).

Finally remarkable diminishing at Zn levels ($p<0.05$) were observed in brain and lung as a result of Cr(VI) administration. Decreased zinc content in these tissues may probably be due to active role of zinc in most enzymatic processes. Zinc is an essential trace element, necessary for plants, animals and microorganisms; the second most abundant transition metal in organisms after iron and the only metal which appears in all enzyme classes.

4.2.3. Interaction of Metals and Trace Metal Homeostasis

Considering all the alterations in the tissue metal contents in our study, administration of hexavalent chromium seems to result in imbalances in metal ion homeostasis in tissues. Figure 4.9 shows the percent alteration values in essential trace element concentrations in the six mice tissues with Cr(VI) administration. In some cases, interaction of a metal with another occurs in synergetic way; alterations in Fe and Cu levels can be given as an example to this; except heart in the all studied mice organs parallel alterations in Fe and Cu levels were observed. Whereas in some cases the altered levels of metals occurs in antagonistic way; reversely altered Fe and Mn levels in brain can be given as an example to this, as revealed by many other researchers as well (Garcia et al., 2007; Malecki et al., 1999a,b; Roth and Garrick, 2003). Fe-Mn antagonism is consistent in other mice tissues, except testicle.

Unbalanced metal ion homeostasis probably leads to aberrations in living processes which require the defined amounts of specific metal ions at the right position and in a timely fashion. Further research is necessary to elucidate the metal-metal interactions and their mechanisms in livings.

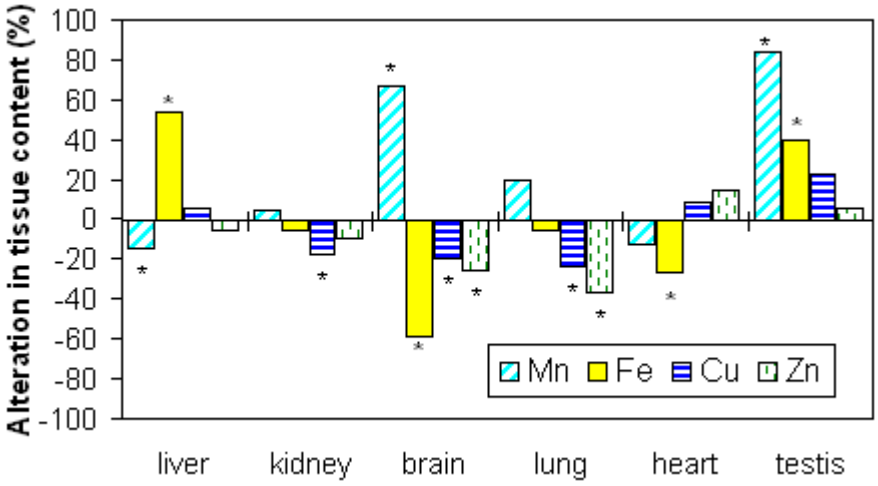

Figure 4.9. Alterations in the total contents of Mn, Cu, Fe and Zn in mice tissues after 8.0 mg Cr/kg hexavalent Cr administration. Bars marked with an asterisk (*) refer to $p < 0.05$

4.2.4. Analysis of metal-protein complexes by HPLC-ICP-MS

Hybridization of HPLC with ICP-MS is a very versatile method for species selective analysis of biomolecules. Although different separation modes such as reverse phase, ion exchange or size exclusion have been applied for this purpose, SEC is the most convenient mode when the analysis of non-covalent metal complexes is targeted. In SEC, there is not interaction between analyte and stationary phase principally, samples with complex matrix are well tolerable and the aqueous mobile phase is compatible with the plasma of ICP.

4.2.4.1. Estimation of Molecular Weight of Macromolecular Metal Complexes

The average time a substance spends in the pores of stationary phase depends on its size which for a given shape, can usually be related directly to its molecular weight. Molecular weight of a protein can be calculated by using the elution time of the compounds.

Table 4.6. Molecular masses of different compounds and their retention times on Superdex-75 column

Protein standard	M, kDa
thyroglobulin	670
transferrin	81
bovine serum albumin	66
chicken albumin	44
Cu/Zn SOD	32
myoglobin	17
metallothionein	7
hydroxocobalamin	1.3

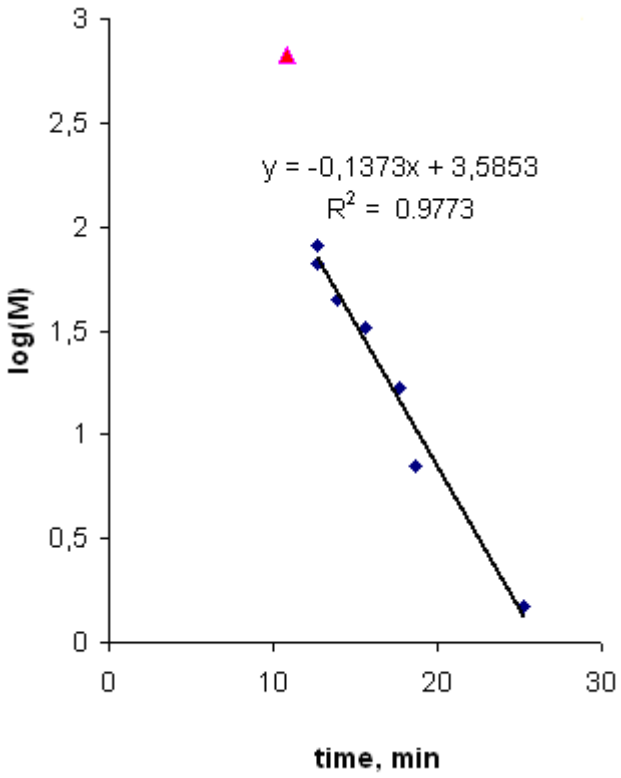

Figure 4.10. Calibration curve for Superdex-75 column

4.2.4.2. Optimization of extraction method for metal-protein complexes

The extraction method was optimized in order to recover metalloproteins into the aqueous phase and preserve the native metal composition of the metalloproteins. For this purpose the extraction was conducted in mild conditions; i.e, 50 mM ammonium acetate buffer with a pH of 7.4, similar to that of physiological medium,

was used for extraction. Ammonium acetate was used as extract solution because of its convenience as mobile phase in HPLC and agreement with ICP mass spectrometry and it is not problematic in freeze-drying. All procedure was maintained at 4°C in a clean cold room. PMSF was added into extraction media in order to inhibit the protease activity. Two different methods, waterbath and ultrasonic probe, were applied for the extraction of metalloproteins. The liver sample of the Cr-injected mice was chosen as model tissue, because firstly; liver is the first organ to which Cr is transported after absorption, secondly; liver is organ where protein synthesis mainly takes place and finally the highest Cr concentration was determined in liver. The size exclusion elution profile of chromium was used to optimize the extraction parameters. The efficiency of the extraction has been illustrated in Figure 4.11.

In the general point of view, three minor Cr peaks and one broad, major Cr peak can be seen in the obtained SE-HPLC-ICP-MS chromatogram. The three minor peaks are relatively higher molecular weight, less intense, well separated, and sharper as compared to the broad, major Cr peak which is more intense, lower molecular weight. In the sense of poor resolution of the size-exclusion principle, it is highly likely that the broad peak belongs to more than one Cr-containing species, because; some shoulders emerge on the peak.

Figure 4.11 illustrates the optimization steps of the extraction procedure. Figure 4.11-a and –b show the chromatogram of Cr-binding proteins which was extracted in waterbath applying 10 and 20 min sonication time, respectively. In the case of waterbath extraction, when the sonication time was applied during 10 min, the intensities of peaks in both high MW (>7 kDa) and low MW (<7 kDa) regions are higher than that of the 20 min. It can be said that; when the extraction time was increased from 10 min to 20 min, the efficiency of the extraction decreased. That is probably because of decomposition of some Cr-protein complexes due to the long sonication time in waterbath.

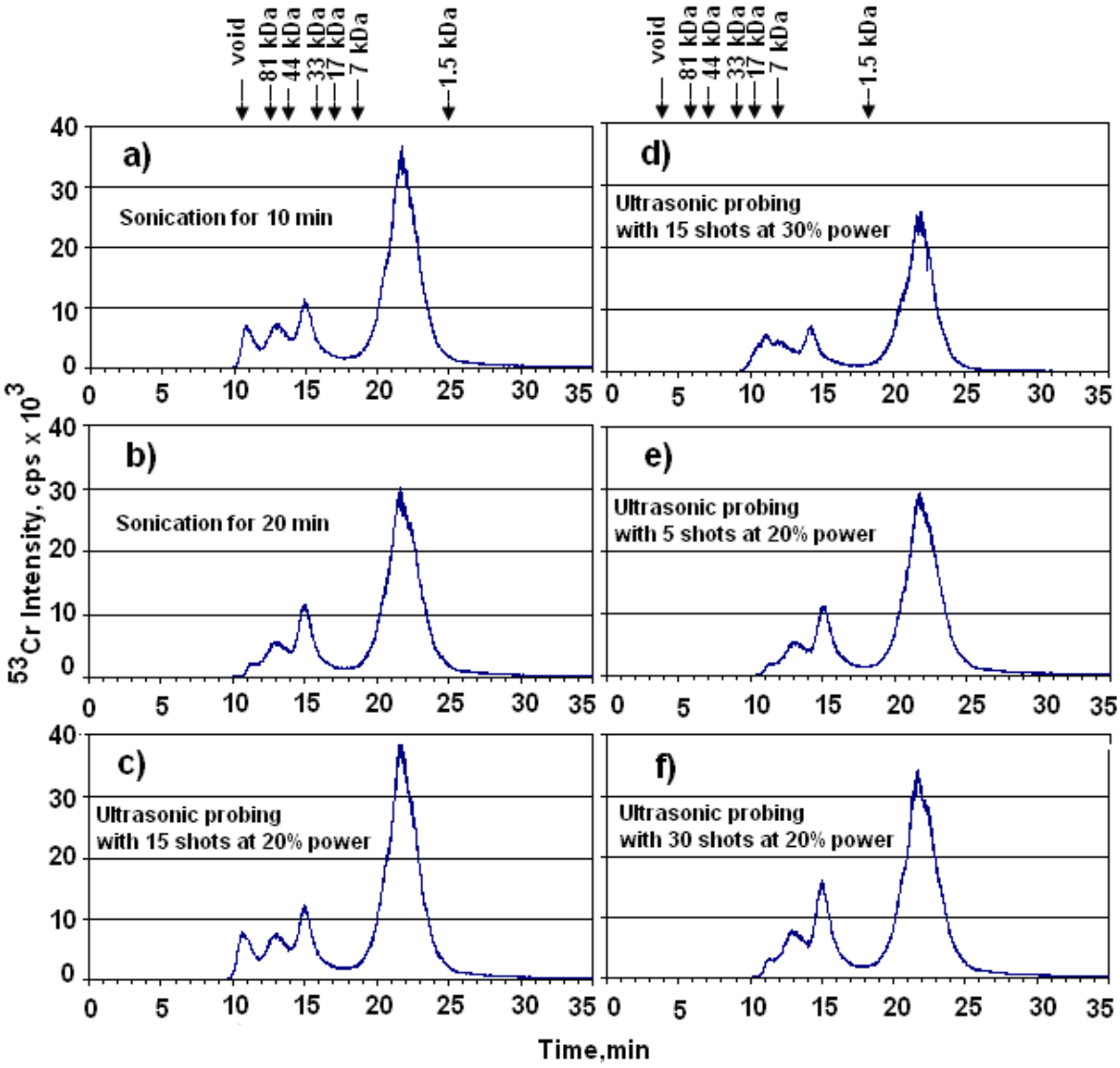

Figure 4.11. Optimization steps for the extraction of Cr-biomolecule complexes from mice liver and the chromatograms which obtained after extraction with sonication in waterbath for 10 min (a); sonication in waterbath for 20 min (b); ultrasonic probing with a 15 shots serie with 20% power (c); ultrasonic probing with a 15 shots serie with 30% power (d); ultrasonic probing with a 5 shots serie with 20% power (e); ultrasonic probing with a 30 shots serie with 20% power (f).

Another extraction method was ultrasonic probing and at the first step; the effect of the power of the generated ultrasonic waves on the extraction efficiency was examined. The instrument software allows 20% of the generated power as minimum power to be applied. So the effect of ultrasonic power on the extraction was tested for 20 and 30% of the generated power output. The chromatogram in Figure 4.11-c was obtained in 20% generated power of ultrasonic probe with 15 pulses (number of shots). As it was seen from Figure 4.11-c and 4.11-d, when the power was elevated from 20% to 30%, the peak intensities of the all Cr-containing

proteins decreased in the chromatogram. The reason of this attenuation at the peak intensities is probably the low stability constant of some Cr-protein complexes that collapsed with the intensified ultrasonic waves. It is noteworthy that, there are differences in the elution time of complexes when the power was elevated to 30%. A possible reason of shift in elution volume is oxidation of complexes as a result of contact with air. Therefore, all operations were conducted at 20% of the maximum power of the instrument throughout the study. Another parameter tested was the number of the ultrasonic pulses. Each pulse was consecutively radiated during 1 second and followed by a 1 second break time. At this step of the optimization, diverse numbers of the pulses were applied to the tissue homogenate. The chromatograms obtained at the shot numbers of 5, 15 and 30 were depicted in Figure 4.11-c-f. Insufficient extraction stands out in Figure 4.11-e in the case of 5 pulses; the peaks with quite low intensity are noticeable. When the number of pulses was raised from 5 to 15 (Figure 4.11-c), intensity of peaks increased regarding both high- and low-MW Cr-species and improvement in peak resolution became apparent as well. But when the number of pulses was increased from 15 to 30, the intensities of peaks in the both high-MW (>7 kDa) and low-MW (<7 kDa) regions decreased, except the peak centered at fifteenth min (Figure 4.11-f). It has to be noted that the intensity of the third peak (centered at ~15 min) has risen, while the intensity of all other peaks decreased when the shot number was increased from 15 to 30 shots. It may be due to the collapse of the complexes between chromium and ligands with low affinity and further exchange of chromium between different ligands which show different affinity to chromium. Another possibility is transformation/decomposition of the higher MW complexes to lower ones.

It can be concluded that optimum conditions for the extraction of Cr-containing species from tissue samples obtained with using ultrasonic probe technique by applying 15 ultrasonic pulses at 20% power of generated waves of ultrasonic probe in the presence of 50 mM ammonium acetate buffer, pH 7.4, at 4 °C (Figure 4.11-c). The most intense peaks for Cr-complexes was obtained by ultrasonic probing technique, however, the extraction in waterbath by applying 10 min sonication (Figure 4.11-a) could be applied as an alternative method for the extraction as well.

The extraction yield of chromium was calculated for the studied tissues of the Cr-injected mice. The efficiency of extraction could be ordered to be;

kidney (49%) ≈ liver (48%) > testis (45%) ≈ brain (44%) > lung (40%) >heart (31%).

Thus; in terms of the extraction yield of the tissues for Cr-species, kidney and liver could be classified as high; testis, brain and lung as medium; and heart as low in the studied conditions. Tissual similarity of kidney and liver resulted in almost equal and high extraction yield; this is probably due to the soft character of the kidney and liver that allowed the effective extraction of Cr-biomolecule complexes. Adipose tissue of brain and insolubility of fatty material in aqueous media; and presence of some capillary vessels in the lungs are likely reasons to classify these tissues in the class of middle range extractability. The possible reason of relatively low extractability of Cr-containing biomolecules from heart is the rigid nature and the abundance of blood vessels in this organ.

4.2.4.3. Selection of separation conditions

In order to isolate and partially separate the macromolecular complexes of Cr, Zn, Cu, Fe and Mn, SEC with on-line UV-VIS spectrophotometric and ICP-MS detection was applied to cytosol of mice tissues. Since extraction of metalloproteins was performed in aqueous ammonium acetate buffer, extracted macromolecules with hydrophilic character fitted well with the aqueous mobile phase of SEC. On the other side, reverse phase chromatography requires the partially organic phase and ion exchange chromatography necessitates the concentrated buffers as mobile phase which would adversely effect the ICP-MS detection. Besides, in SEC principle the lack of interaction of the analytes with the stationary phase, at least theoretically, preserves the analyte molecules from structural changes, denaturation of proteins and decomposition of protein-metal complexes. The high tolerance to biological matrices is the advantageous of SEC over the other HPLC technique. Another potential advantage of size exclusion-HPLC is the possibility of avoiding the buffer salts in the mobile phase that leads to simplify the sample matrix. However, further investigation of Cr-binding proteins through the use of other chromatographic principles (such as ion exchange and

reverse phase) should be examined to obtain purer species by preventing non-covalent metal complexes. On the other hand UV-VIS spectrophotometric detection at 280 nm allowed the detection of different molecular weight fractions of proteins and peptides eluted from the column. In some cases the UV-VIS detector was set at 570 nm wavelength to visualize the Cr(III)-protein complexes (Peterson et al., 2008). Figure 4.13 shows the chromatogram of mouse liver extract. Multiple detections were carried out by UV-VIS detector at 280 and 570 nm and also by ICP-MS at line 53 amu. It seems that the signal at 570 nm is highly in agreement with the ICP-MS signal. But, because of the small extinction coefficient for d-d transition of Cr(III) at 570 nm, Cr detection in this wavelength allows to get a poor quality chromatogram (Figure 4.13). The dead time, the time between UV-VIS and ICP-MS detections, was estimated as 0.3 minute (~18-20 second).

The two SEC column, Superdex-75 HR 10/30 and Superdex peptide HR 10/30, used in this study have the identical material as stationary phase; cross-linked agarose–dextran which is preferable to silica based packings responsible for metal losses in trace metal speciation using low ionic strength eluents (Szpunar, 2000a), but they have different effective separation ranges. The Superdex 75 column has been proven very suitable for many bioinorganic speciation analyses (Szpunar, 2000a) such as the separation of metal complexes in tea samples (Odegard and Lund, 1997), Cd speciation with metallothioneins (Vacchina et al., 1999) and the Superdex peptide for speciation of Cd-phytochelatine complexes in plants (Vacchina et al., 1999).

The pH of the media in both extraction of metal complexes from tissues and in chromatography was maintained at pH: 7.4 by ammonium acetate buffer. This pH value has been selected in a variety of work to mimic the physiological conditions and to maintain the proteins' native form. The pH is an effective parameter on the three dimensional shape of proteins. Accordingly the molecular shape affects the retention of macromolecules by stationary phase in SEC. Figure 4.10 shows the calibration graphics of Superdex-75 HR 10/30 and Superdex peptide HR 10/30 columns which constructed by using UV absorbance (280 nm) of molecular weight markers described in Table 4.6.

The concentration of the buffer in the mobile phase does not play an important role in SEC (Vacchina et al., 1999) provided that it is not lower than 10 mM, thus the concentration of ammonium acetate was selected to be 50 mM. The mobile phase was freshly prepared and ultrasonically degassed before use. The chosen conditions assured the narrowest and most intense peaks.

As previously recommended (Vacchina et al., 1999), β-mercaptoethanol solution was frequently injected between samples (Figure 5a). In addition to the general column cleaning procedure, the columns were washed with mobile phase containing 5 mM β-mercaptoethanol for 30 min when necessary and subsequently conditioned with mobile phase. By this way, especially Cu and Zn were considerably cleaned from the sample loop and column. This treatment prevents the accumulation of metals in the head of the column which may cause some undesirable results such as memory effect, the exchange of metals on other biological macromolecules, deteriorated peak shape and poor reproducibility.

Collision/reaction cell technology allows the elimination of polyatomic spectral interferences by minimizing formation of interfering species before they enter the mass analyzer. H_2 gas was used as collision gas and a flow rate 3 ml min^{-1} was found efficient to achieve this.

4.2.4.4. The effect of time and temperature on the stability of Cr-complexes

It is known that the stability of metal complexes in biological samples is affected by storage conditions such as temperature, air oxygen and time. For these reasons, stability of Cr-complexes was tested in different storage conditions and the obtained SEC-ICP-MS chromatograms were compared. Overlapped chromatograms in Figure 4.12 belong to freshly prepared mouse liver extract (blue line), the extract obtained from the sample stored at -20°C for 1 month (red line) and the extract stored at +4°C for 7 days (doted pink line). The signal intensity of ^{53}Cr for the extract which was stored at -20°C (blue) was considerably diminished after one month storage, compared with the chromatogram of freshly prepared extract. Cr-complexes but especially in high molecular weight region were adversely affected with time even stored at -20°C. It is worth to mention that in samples stored at either -20 °C or +4 °C, the peak intensities of low molecular

weight Cr-complexes do not seem to be diminished. The likely reason of such a behavior is the existence of the small Cr-peptide complexes which were possibly decomposed from high-MW complexes and contributed to the signal intensity of Cr associated to small peptides. The peak of low molecular weight complexes includes possibly a few Cr-containing species, that's why the peak is highly broad. On the other side when the extract was stored at +4°C for 7 days (doted pink line), the quantity of complexes decreased (similarly in the case of storage at -20°C) but more importantly the elution time of complexes especially in the high molecular weight region were shifted. The possible reason of this shift may be formation of different complexes which differently retained through size exclusion column or the oxidation of complex at relatively high temperature (+4°C).

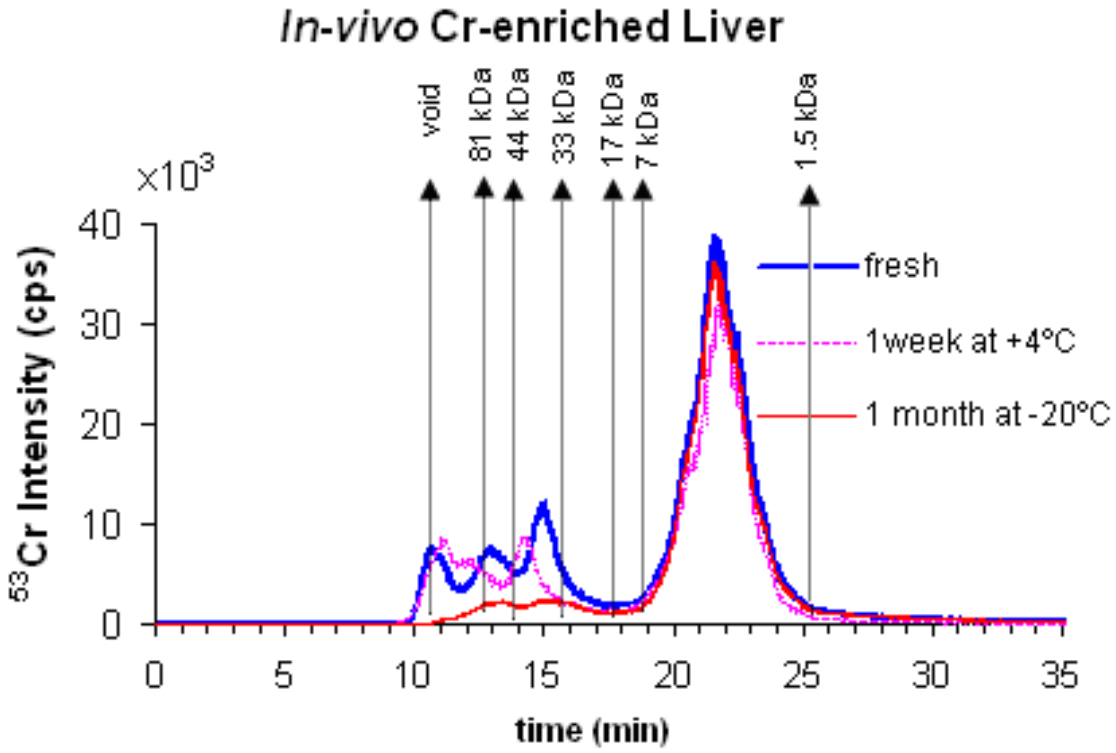

Figure 4.12. Chromatograms obtained from freshly prepared extract of Cr-administered mouse liver (blue trace), the extract obtained from the sample stored at -20°C for 1 month (red) and the extract stored at +4°C for 7 days (yellow). Column: Superdex 75 HR, mobile phase: 50 mM NH_4CH_3COO, flowrate: 0.7 ml min^{-1}, mass detected: ^{53}Cr.

In summary, it seems that; the time during storage to a great extent affects the intensity of Cr-complexes and relatively high temperature (+4°C) possibly results in the formation of different complexes or oxidation.

4.2.5. Distribution of essential metals (Cr, Zn, Cu, Fe and Mn) between different molecular weight macromolecules in mice tissues and alterations in SEC-UV-ICP-MS elution profiles with Cr(VI) stress

Size-exclusion chromatography coupled to on-line with inductively coupled plasma-mass spectrometry, SE-HPLC-ICP-MS, has been widely applied to the speciation of trace/ultra trace metals in the various biological samples. This approach combines the advantageous of both techniques. In contrary to other liquid chromatographic modes, in SEC there should not be interaction between the solute and the surface of the stationary phase and the molecules are separated based on their effective size and shape (mass) in solution. On the other side, ICP-MS has become a very popular and versatile tool because of its multi-element capability, low detection limits and the possibility of on-line isotope dilution (Szpunar, 2000a). The flow rates and compositions of the mobile phases in SEC are readily tolerated by ICP mass spectrometers.

Metal ion homeostasis carried out by metalloregulatory proteins which control the expression of genes that allow organisms to quickly adapt to chronic toxicity or deprivation of both biologically essential metal ions and heavy metal pollutants found in their microenvironment (Giedroc and Arunkumar, 2007). Understanding of biological mechanisms necessitates elucidating the structures of macromolecules involved in living processes. Relevance between molecular structure and function is the key point to elucidate the nature of biological systems. In order to clarify the nature and structures of Cr-binding proteins, stepwise examining the nature of these macromolecules and their chromium binding properties needs to be performed. In this point of view SE-HPLC-UV-ICP-MS is useful for determination of the distribution of elemental species between different molecular weight biomolecules. By this way, not only distribution of Cr, Zn, Cu, Fe, and Mn between different molecular weight biological ligands but also differences in the elution profiles of metals with the exposure of Cr(VI) were investigated. To the best of our knowledge, this is the first attempt as speciation of essential trace elements bound to biological macromolecules, in mice organs, after *in-vivo* Cr(VI) administration by size exclusion-HPLC with on-line UV and ICP-MS detection.

In this part of the study, we exploited the coupling of SE-HPLC with ICP-MS for characterization of metalloproteins in the tissue cytosols of different organs of mice which injected with a hexavalent chromium at a dose of 8.0 mg Cr(VI) / kg bw. A UV detector set up at 280 nm was positioned to the column exit. SE column was calibrated with molecular weight markers given in Table 4.6.

The SE-HPLC-UV-ICP-MS chromatograms were obtained for liver, kidney, brain, lung, heart and testis of mice. Beside of Cr profile, Zn, Cu, Fe and Mn elution profiles were recorded and discussed. The obtained chromatograms for Cr-stressed mice tissue samples and their control subjects were compared and alterations in the elution profiles were discussed. Beside of that, the total metal levels of the studied tissue samples were evaluated together with the obtained chromatograms of metal containing species.

4.2.5.1. Chromium Complexes

Figure 4.13-c and -d show the ^{53}Cr chromatograms for liver cytosols of control group mice and Cr-injected group mice respectively. Apparent differences are shown between the two chromatograms reflecting the binding of chromium to different molecular weight biomolecules. The detailed discussion on the elution profile of Cr-binding macromolecules exists in section 4.2.4.2 (Optimization of extraction method for metal-protein complexes). Additionally a UV trace at 570 was added to the chromatogram in Figure 4.13.

When looked at the chromatogram of control subject (upper one in Fig. 4.13) it seems different MW Cr-species. MW distribution of these complexes is very similar to that of in-vivo Cr enriched sample. However they are at quite low amount. UV traces are also differ to each other indicating that Cr(VI) administration bring about alterations on the proteins pattern in-vivo. With Cr(VI) exposure to mice induction of Cr-binding proteins take places in the liver samples. It has to be noted that the most intense Cr peaks were seen in liver chromatograms. Paralelly, highest total Cr amount was determined in liver (Table 4.5). Indeed liver is known as the tissue where the most of the of proteins biosynthesize. The red line indicates the elution time of inorganic Cr(III) and Cr(VI) which Superdex-75 column has not ability to separate the two inorganic species.

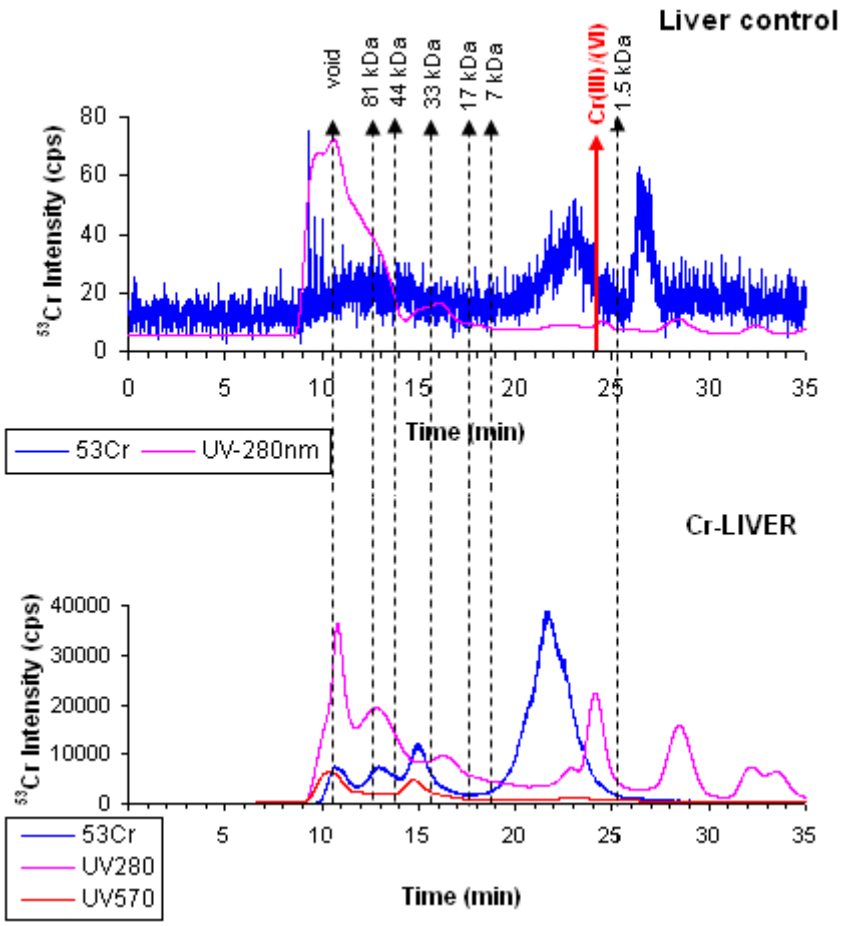

Figure 4.13. ^{53}Cr chromatograms for liver cytosols of control group mice (upper) and in-vivo Cr enriched mice group (below)

Figure 4.14 shows the Cr elution profiles in kidney samples for in-vivo Cr enriched mice and their control subjects. Similarly Cr species are at minute amounts in control subject but quite high in in-vivo Cr enriched sample. Similarly to liver sample, nearly one third of the Cr species constitute the high MW species which compose of four clusters. The other two third of the Cr species constitute the low MW species which consist one intense major peak.

The UV pattern also changed with Cr administration, it seems that the amount of HMW proteins rises while LMW protein amounts to decrease. Kidney is known as the tissue has a vital role in Cr metabolism and detoxification of metals and one of the critical tissue where the metal ion homeostasis balanced.

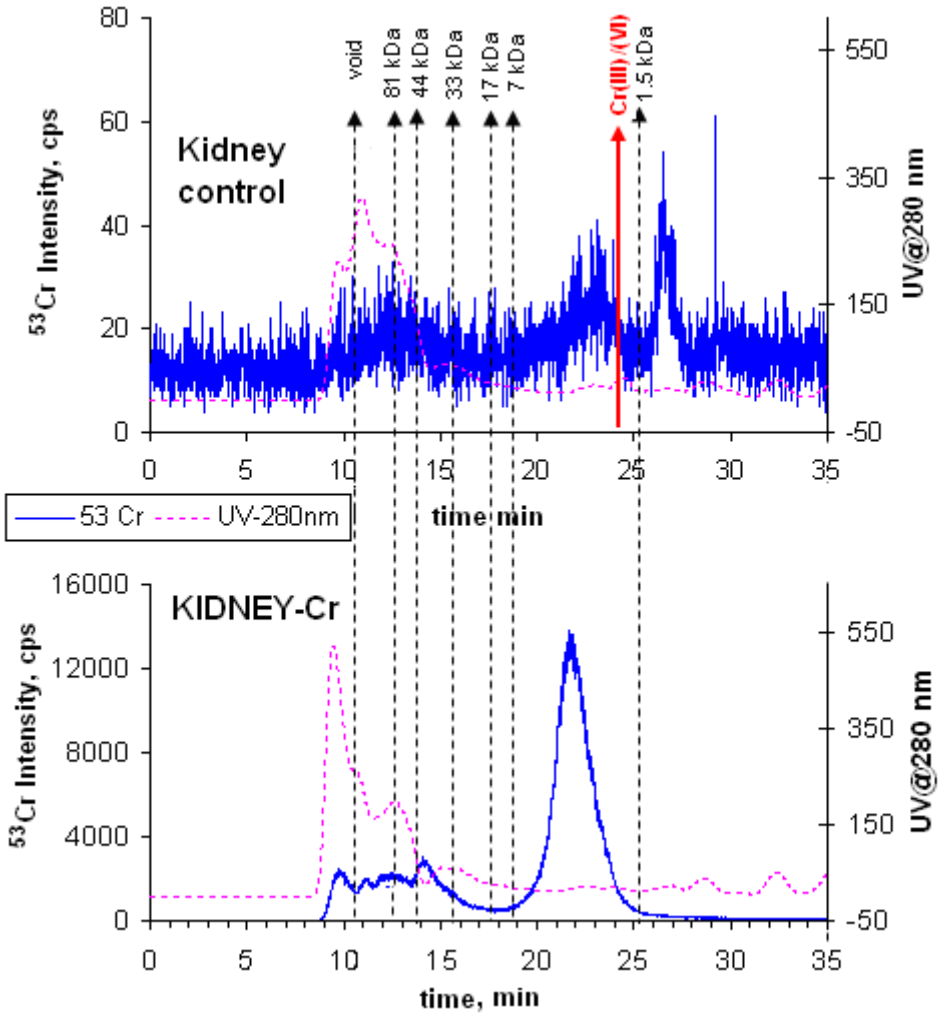

Figure 4.14. ^{53}Cr chromatograms for kidney cytosols of control group mice (upper) and in-vivo Cr enriched mice group (below)

It has been shown that acute subcutaneous potassium dichromate exposure to mice at a dose of 20 mg/kg (~ 7 mg Cr/kg) results in adverse effect such as cellular degeneration in the kidney (Oliveira et al., 2006).

Extensive renal damage of potassium dichromate has been observed in rats with elevations in the urinary excretion rates of the brush border enzymes, 7-glutamyltransferase, alkaline phosphatase and leucine aminopeptidase, the cytosolic enzymes, aspartate aminotransferase and lactate dehydrogenase and the lysosomal enzyme, N-acetyl-/3-n-glucosaminidase (Gumbleton and Nicholls, 1988).

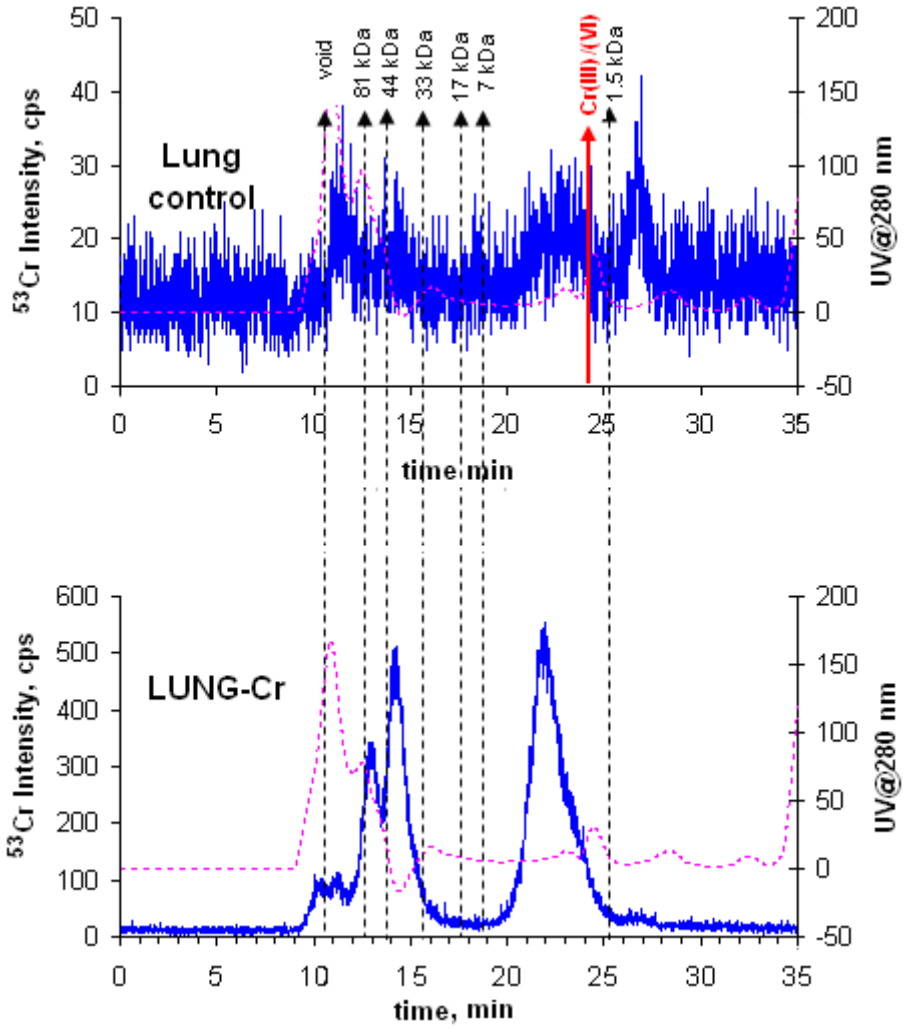

Figure 4.15. ^{53}Cr chromatograms for lung cytosols of control group mice (upper) and in-vivo Cr enriched mice group (below)

It can be seen the chromatogram in Figure 4.15 that lung tissue concentrates the Cr with the in-vivo Cr exposure but the amount of Cr is not high as being in liver and kidney tissue. This result can be taken from both SEC-ICP-MS chromatogram and total Cr amounts of tissues (Table 4.6). Another apparent different in the Cr elution profile with the liver and kidney samples, HMW Cr species comprise approximately more than a half of total amount. We can conclude then Cr affinity to HMW proteins is higher in lung. These comprise of four species have MW of bigger than 30 kDa. It is known that inhalation of chromic species result to the cancer.

It has been shown that many workers hired to work in a chromate plant diagnosed with lung cancer. 66 workers with lung cancer were found in the total death of 283, constituting the 23.3% of total deaths and 64.7% of all cancers (Mancuso 1997a).

Continuation of the study with the autopsy data with comprehensive chemical analyses showed histological demonstration of insoluble chromium in the lung (Mancuso 1997b).

There is slight shift in UV pattern at 280 nm. The UV absorbance of HMW proteins give rise compared to lower ones.

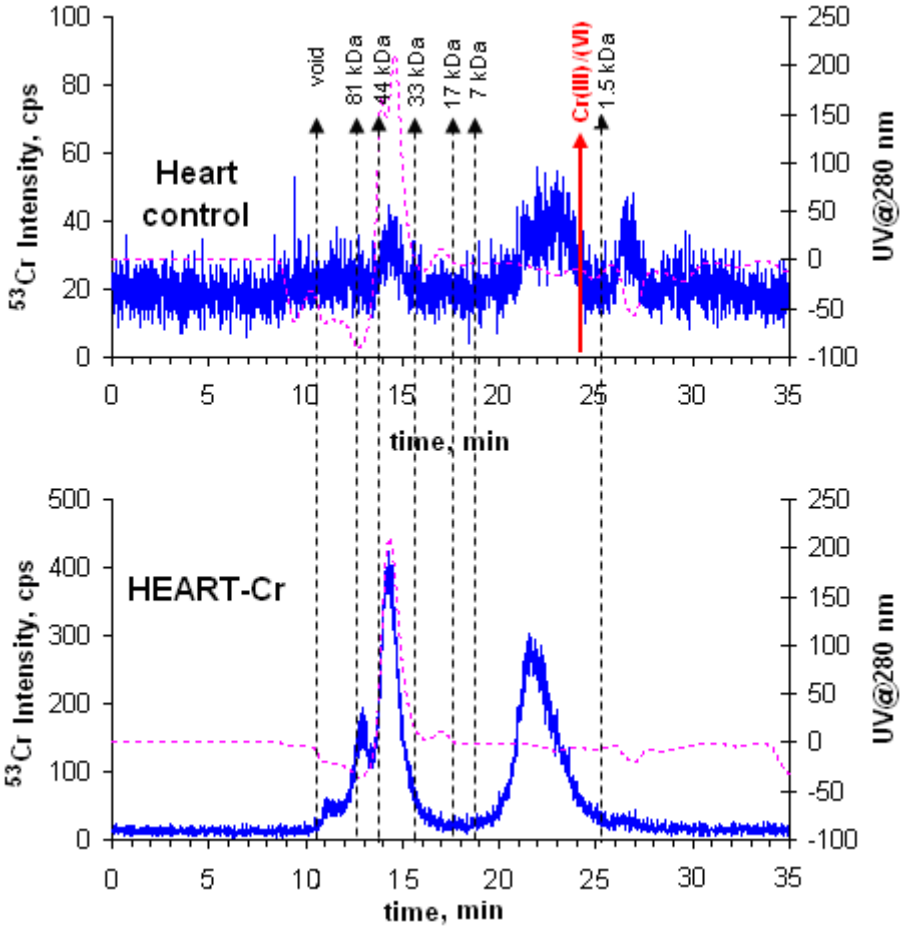

Figure 4.16. ^{53}Cr chromatograms for heart cytosols of control group mice (upper) and in-vivo Cr enriched mice group (below)

Heart Cr species are also lower amount than that of liver and kidney and distribution pattern is similar to lung sample (Figure 4.15) i.e., more than a half of the Cr species composed of HMW species which include three different size macromolecules with higher than 30 kDa (Figure 4.16).

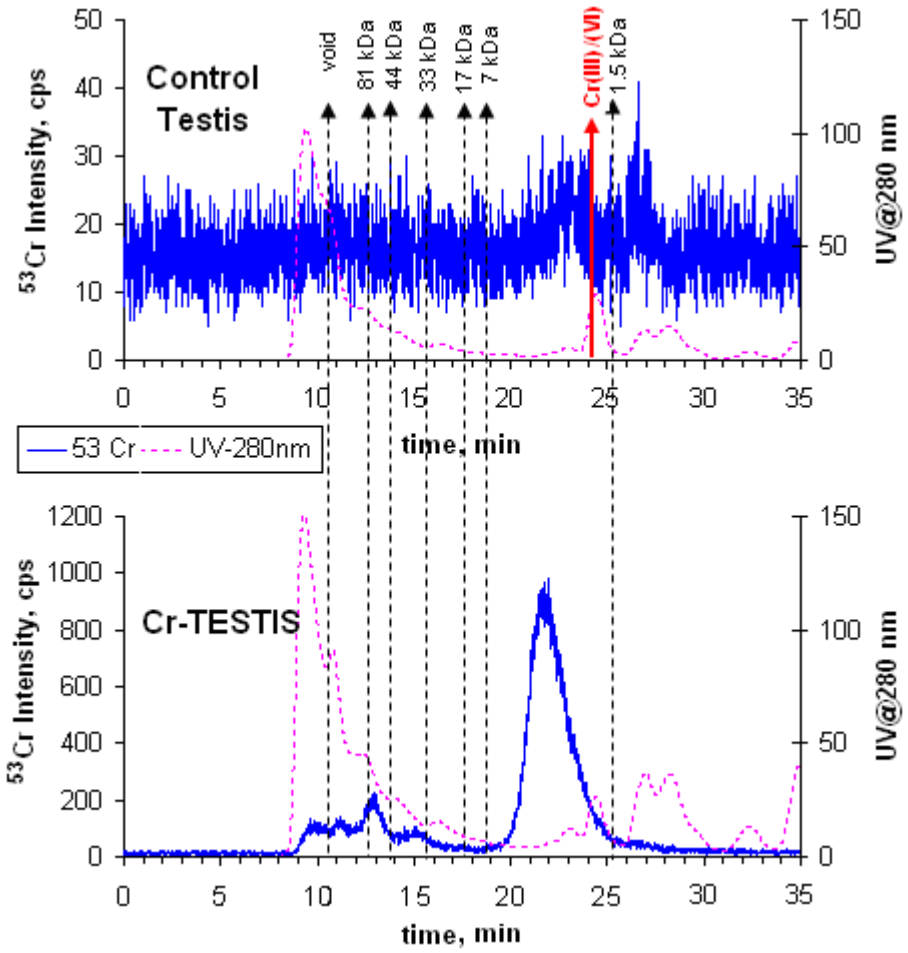

Figure 4.17. ^{53}Cr chromatograms for testis cytosols of control group mice (upper) and in-vivo Cr enriched mice group (below)

^{53}Cr intensity in the chromatogram of control group sample for testicles are quite poor as being in other samples of control.

Regarding in-vivo Cr enriched mice tissue samples, at least four species with HMW at lower amount and one big broad, intense peak composed of LMW species stand out as Cr elution profile of tissue cytosol of in-vivo enriched sample. LMW complexes are dominant in testis sample and consist of nearly two third of total Cr. The distribution pattern likes liver and kidney samples but not lung and heart samples.

An intensity increment is shown in the UV signal of the chromatogram for in-vivo Cr enriched sample.

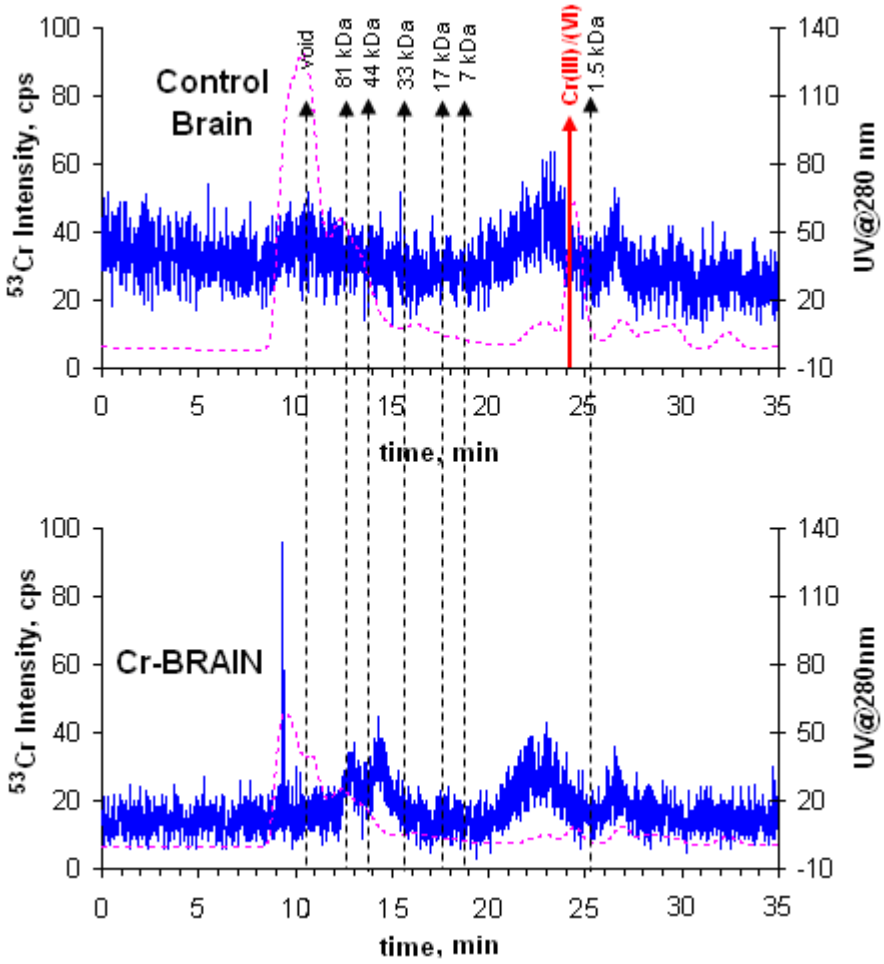

Figure 4.18. ^{53}Cr chromatograms for brain cytosols of control group mice (upper) and in-vivo Cr enriched mice group (below)

Brain Cr elution profile for in-vivo Cr enriched and control subject is not so different compared to each other (Figure 4.18). This is most probably raised from the protecting function of blood brain barrier (BBB). BBB protects the central nervous system (CNS) against xenobiotics by highly selective transport mechanisms. Blood–brain tissue exchange plays a pivotal role in CNS homeostasis and is accomplished by individual endothelial cells, which are continuously linked by tight junctions. Hydrophobicity is an important factor to across through BBB into brain (Zheng et al., 2003).

Although the lowest total Cr has been determined in brain tissue, the increase in the brain total Cr levels has been found statistically significant. Some more hydrophobic Cr complexes may exist in brain tissue, because extraction with ammonium acetate mostly extracts the hydrophilic proteins. This may be a reason

that the chromatogram of Cr-enriched sample does not consist a considerable amount of Cr-complex. Only two proteins within a 40-80 kDa region are appearing in the chromatogram.

In conclusion, the hexavalent chromium exposure to mice not only gives rise to affect the concentrations of other essential trace elements,[19-21] change their speciation by competitive binding to proteins, and trigger off the synthesis of diverse biological ligands. Non-denaturing nature of SEC and sensitive and multielement analysis property of ICP-MS make the combined technique (SEC-UV-ICP-MS) a powerful tool in the analysis of biological samples. Collision cell technology eliminates the spectral interferences faced in elemental mass spectroscopy and makes possible the correct detection of metals. By the method the physiological distribution of Cr-biomolecule complexes could be determined in mice tissues after Cr(VI) exposure.

4.2.5.2. Manganese Complexes

Differentiation in the Mn-elution profile of liver sample is very interesting. Firstly; emerging of a new Mn-containing biomolecule which eluted at, ca. MW of ≈ 5 kDa are shown in the chromatogram of in-vivo Cr enriched sample however, it is absent in the chromatogram of control sample for liver (Figure 4.19). Moreover; it should be noted that the new Mn-complex coeluted with the major, broad Cr-band (eluted between 19 and 24 minutes). Coelution of Cr- and Mn-complexes resulted in two possibilities in questionable. One is induction of the synthesis of new Mn-binding biomolecule possibly involving in chromium metabolism in order to eliminate the toxic effect. The other possibility is binding of Mn to the already existing Cr-binding apoprotein which biosynthesized as a result of Cr(VI) exposure to mice. The latter is highly probable because of similarity in chemical preferences of manganese and chromium. It seems in both cases that; a Mn-specific biomolecule involves in Cr-metabolism.

Mn is speculated as an Cr(VI) reductant which involves in the reduction mechanism of Cr(VI) (Pourahmad and O'Brien, 2001). Pourahmad and O'Brien has shown that Cr(VI)-induced cytotoxicity and ROS formation, lipid peroxidation,

mitochondrial membrane potential collapse and lysosomal membrane damage were also inhibited by Mn(II) in isolated hepatocytes.

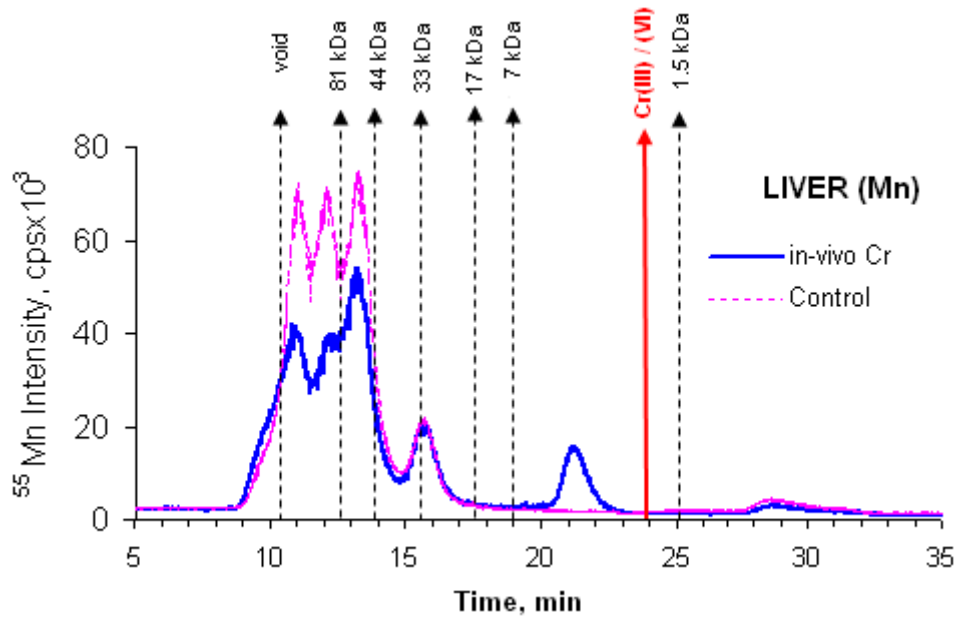

Figure 4.19. Mn elution profiles of liver sample cytosol

In addition to that; a difference in the intensities of the already existing Mn peaks were observed as a result of Cr-administration. A decrease in the peak intensities of Mn-species are shown in Figure 4.19. However, the intensity of the Mn peak eluted at 13 min, ca. MW of ≈80 kDa, are still high as compared to the intensity of nearby Mn-complexes. Beside of Mn-complex, Fe and Cr-complexes also exist in this MW region. It might be transferrin which is reported as a metal ion transporter not only for Fe but also for Cr (Clodfelder et al, 2001; Aisen et al., 1969), Mn (Frausto da Silva and Williams, 2001) as well as other transition metals (Harris and Messori, 2002). Mn(II) can be oxidized on binding to transferrin in the bloodstream of animals and then, as Mn(III) transferrin, it may be transferred into cells, only to revert to Mn(II) on release. It is then again pumped into animal cell vehicles and organelles (Frausto da Silva and Williams, 2001). Cr and Fe are known to bind to transferrin at trivalent oxidation state and chromic ions are transported in blood to the tissues by transferrin. In tissue cells chromium is bound to purported biomolecule chromodulin, and then is expelled from cells, transferred from the blood to the urine and ultimately lost in the urine as bound to chromodulin (Clodfelder et al, 2001).

It worth to mention that it is observed the low intensity Mn species at the out of effective separation range of the SE column, at 28-30 minutes, in both Cr-stressed and control mice liver samples (Figure 4.19). These might be Mn compounds with small size or Mn-peptide complexes. It is denoted in (Frausto da Silva and Williams, 2001), abundance of the free Mn(II), i.e., unbound transferrin, in vacuoles of animal cell.

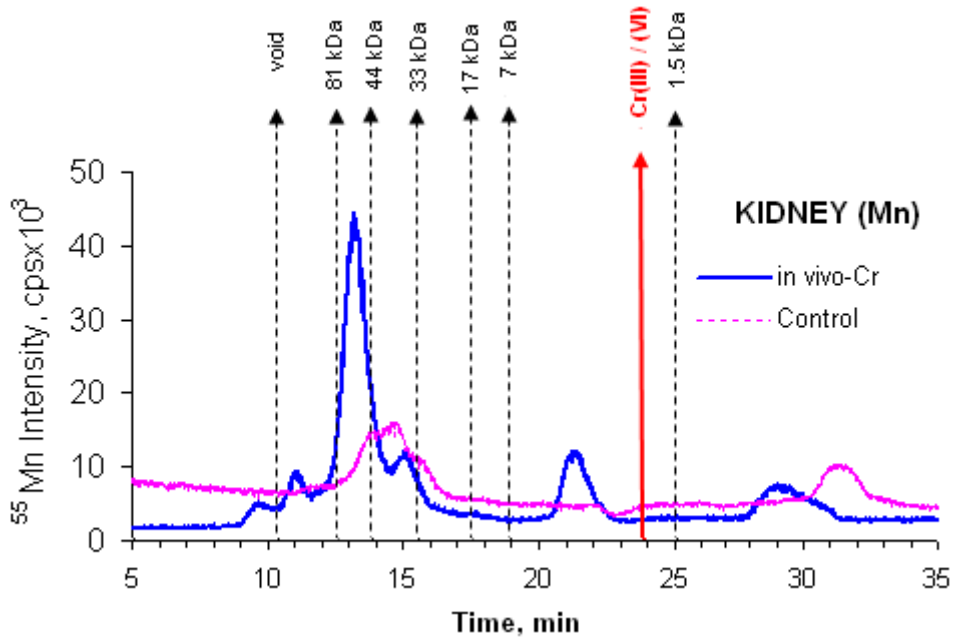

Figure 4.20. Mn elution profiles of kidney sample cytosol

When looked into Mn-elution profile of kidney for control and in-vivo Cr-enriched mice, the same bioinduced Mn species at elution time ~21.2 min are shown in the chromatogram. Likewise kidney sample this ~5 kDa Mn protein does not seem in control samples chromatogram. Indeed, liver and kidney have primary importance for maintaining trace element balance and detoxifying the heavy metals.

Other than the ~5 kDa Mn protein, two HMW proteins (>80 kDa) has been emerged in the in-vivo Cr- enriched mice kidney sample. These two HMW biomolecules also does not exist in control samples chromatogram.

Additionally, it is apparently shown that the biosynthesis of the peak which has the highest intensity in the chromatogram considerably increased with Cr(VI) exposure.

Finally it is worth to mention that the elution time of the smallest Mn-species are not matching, probably due to difference in their molecular weights.

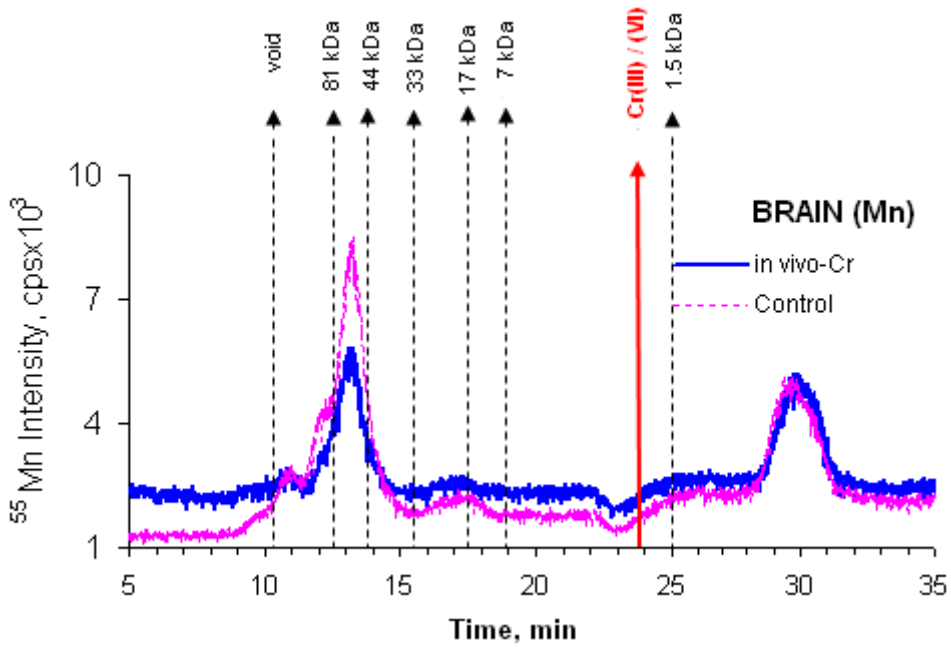

Figure 4.21. Mn elution profiles of brain sample cytosol

The brain Mn-elution patterns for control and in-vivo Cr-enriched mice do not seem so different to each other. Likewise other tissues there are peaks of HMW proteins and similar elution profile are shown in both case. Brain samples also consist of small Cr-containing species (<1.5 kDa). However on the contrary to liver and kidney the molecular weight distribution of Mn is more or less equal between high and low MW biomolecules.

Toxicity in polluted working environments leads to manganism, a neurological disease has symptoms similar to in Parkinson desease (Goyer and Clarkson, 2001). The use of manganese containing fuel additive MMT (methylcyclopentadienyl Mn tricarbonyl) (Goyer and Clarkson, 2001) in gasoline and the expanded use in the industry such as iron industry (as a ferroalloy and as

a component of alloys used in welding) (Apostoli et al., 2000) make manganese a potential toxicant for health.

On he other hand, manganese competes with iron for the same absorption sites and manganese deficiency leads to alterations in hair, nails, and skin (Marmiroli and Maestri, 2008).

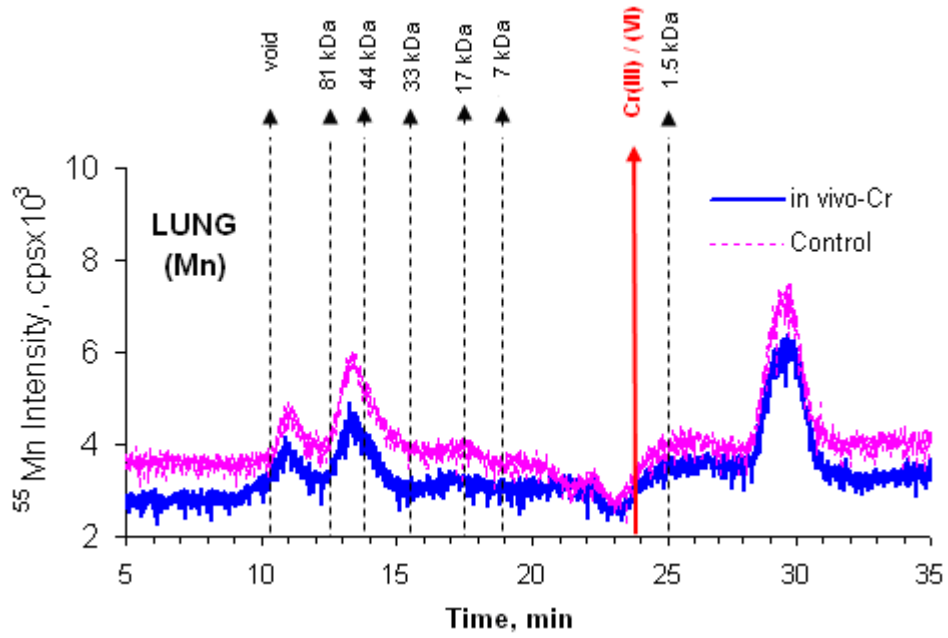

Figure 4.22. Mn elution profiles of lung sample cytosol

Figure 4.22 shows the Mn-binding proteins in lung samples for control and in-vivo Cr-enriched mice. It seems there is not considerable difference between the two samples. Mn distribution between high and low MW species seems in the favor of LMW species.

Mn elution profile in lung sample is worth to investigate further because it is known that chronic inhalation exposure to manganese dioxide, generally over a period of more than 2 years results to neurological diseases such as manganism.

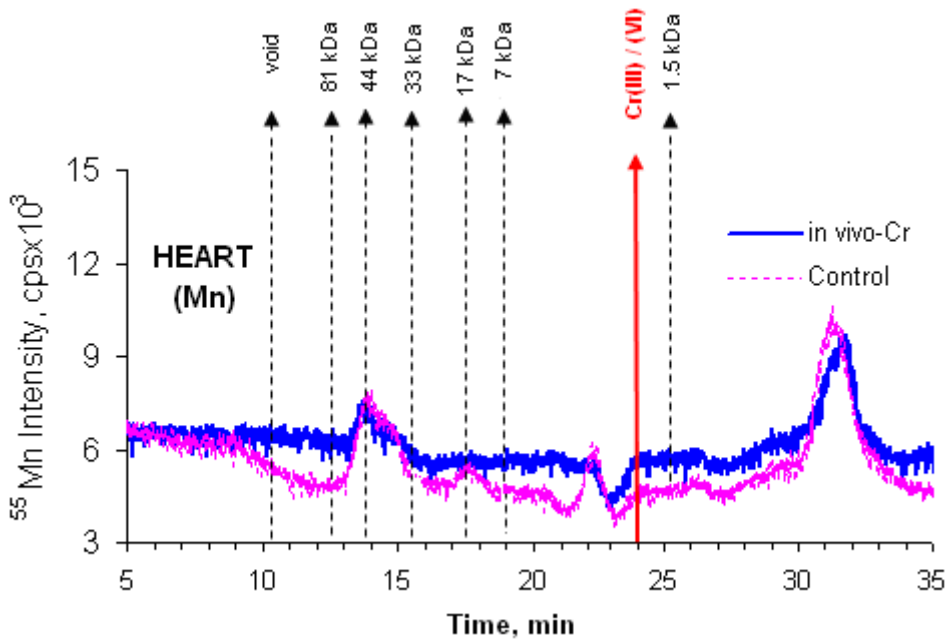

Figure 4.23. Mn elution profiles of heart sample cytosol

As shown in Figure 4.23 there is not considerable difference in Mn-elution patterns between control and in-vivo Cr-enriched mice heart samples. Almost no Mn species eluted in the HMW region while very small Mn-species are shown in the chromatogram.

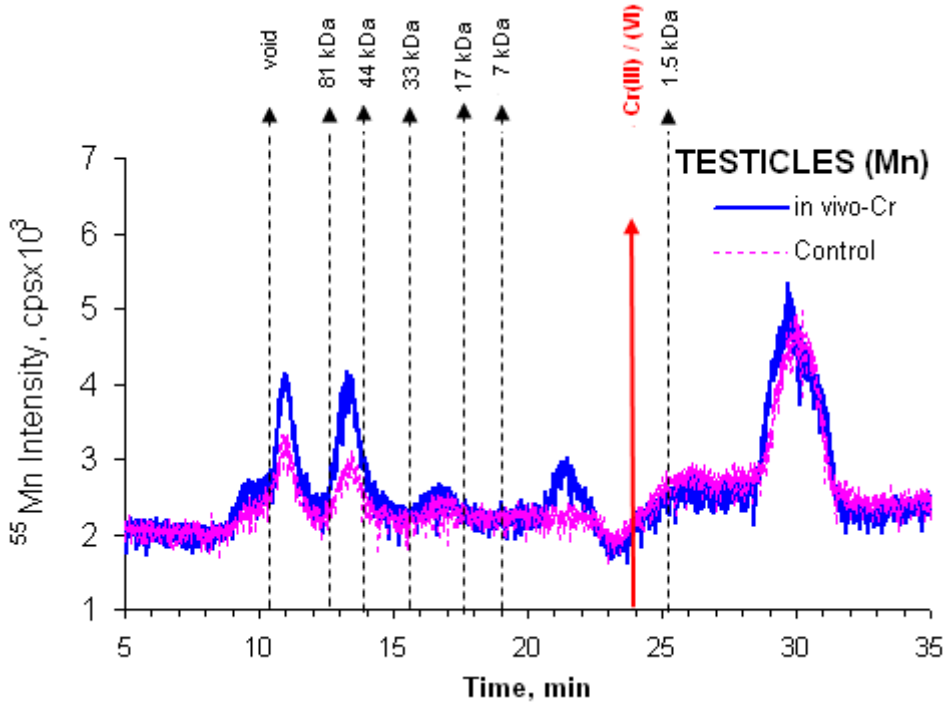

Figure 4.24. Mn elution profiles of sample cytosol for testicles

In Figure 4.24 Mn elution profiles are shown in the testicles for control and in-vivo Cr-enriched mice. The two early eluted HMW species (~tr : 11 and 13 min) and one relatively small (~tr : 16.5 min) and one LMW Mn-bound species seems in the chromatogram of testicles sample for control group mice. However the chromatogram of in-vivo Cr-enriched mice' sample, a Mn-bound protein (~tr : 21.2 min) are shown in in-vivo Cr-enriched mice sample. This is highly likely that the same protein which bioinduced in liver and kidney with Cr(VI) exposure.

Beside it seems that the biosynthesis of the two highest MW species increased with the Cr(VI) exposure.

Another important point considering the all tissue samples is that the elution time of LMW Mn-bound species differs to each other for tissue by tissue. However all of these LMW species elute between the ranges of tr: 19-35 min. These are probably Mn compounds with small molecules or Mn-peptide complexes abundant in vacuoles of animal cell (Frausto da Silva and Williams, 2001).

4.2.5.3. Iron Complexes

As being the most abundant trace element in human body iron is the structural component in heme proteins and potential cofactor of many enzymes. Redox active property of iron determines the interaction with bioligands.

When the HMW region in the chromatogram of basal mice liver for iron is investigated, three main Fe-complexes, eluted at 11.2, 13.6 and 15.2 minutes are shown; approximate MW of 163, 112 and 39 kDa respectively. In the LMW region, two low-intensity Fe-complex peaks exist, eluted at ~19.1 and 22 minutes; approximate MW of 9 and 4 kDa, respectively. It can be easily noticed that the intensities of the highest MW Fe-containing biomolecules increased ca. five times indicating the increased biosynthesis of this protein with the Cr-stress.

Increase in the amounts of Fe-binding macromolecule could be associated with the excess Cr(III) in cells. Because of similarity in chemical preferences and coordination characteristics of Cr and Fe, Cr-binding macromolecules of which

syntheses were dramatically increased may bind to Fe. Binding of Cr and Fe to the same MW macromolecules (tr: 11.2 min) supports this possibility which should be investigated in further.

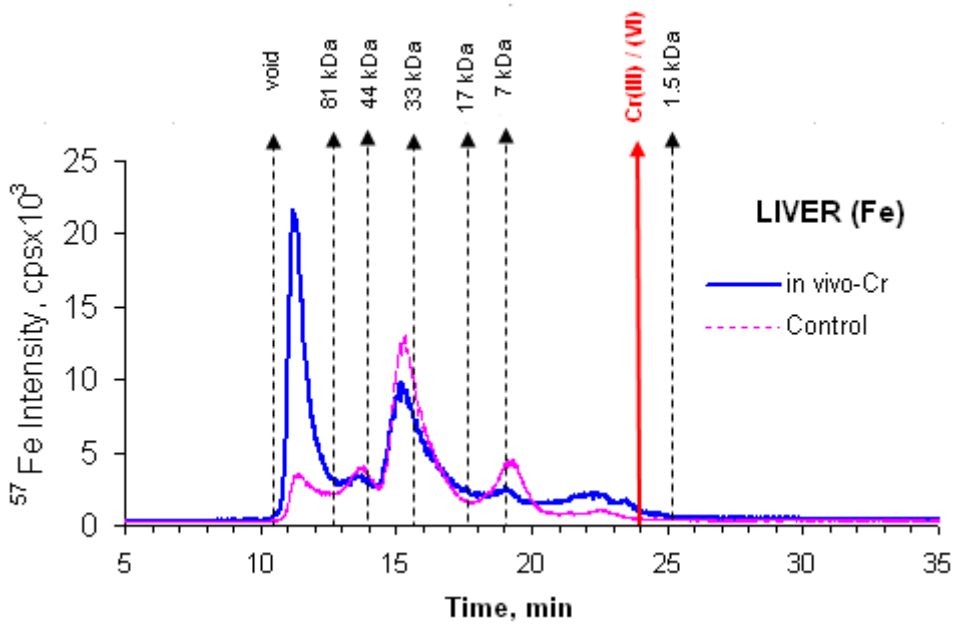

Figure 4.25. Fe elution profiles of liver sample cytosol

On the other side, the intensity of wide peak eluted at ~22 min increased with Cr(VI) exposure. Whereas the intensity of peaks eluted at ~15.2 and 19.1 min were observed to be decreased following Cr(VI) administration.

It was reported that cytochrome b_5, a heme (iron) containing protein, is a common mediator of electron transfer to Cr(VI) and involved in reduction process of Cr(VI) in hepatic and lung microsomes (Myers and Myers, 1998; Myers et al., 2000; Borthiry, 2007). TfR and DMT-1 are common pumps for iron transport into and out of the brain (Burdo and Connor, 2003).

When we look at total hepatic Fe levels there is an increase with Cr(VI) administration (Table 4.5).

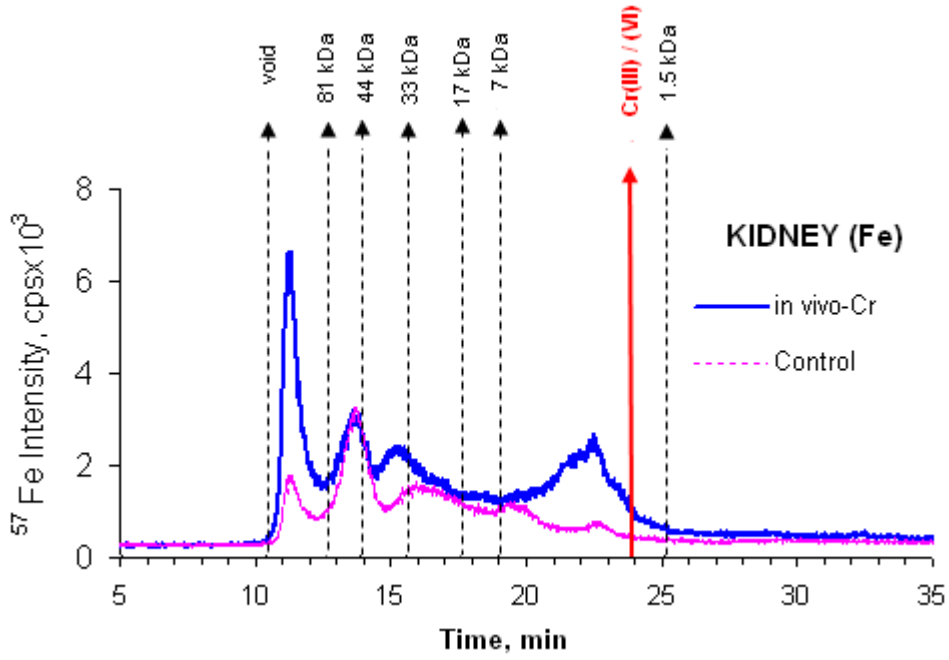

Figure 4.26. Fe elution profiles of kidney sample cytosol

Figure 4.26 shows the Fe-elution profile of kidney samples for control and in-vivo Cr-enriched mice. Likewise liver sample, the increased intensity of the same peaks (tr: ~11.2 and 19.1 min) is prominent in kidney samples chromatogram.

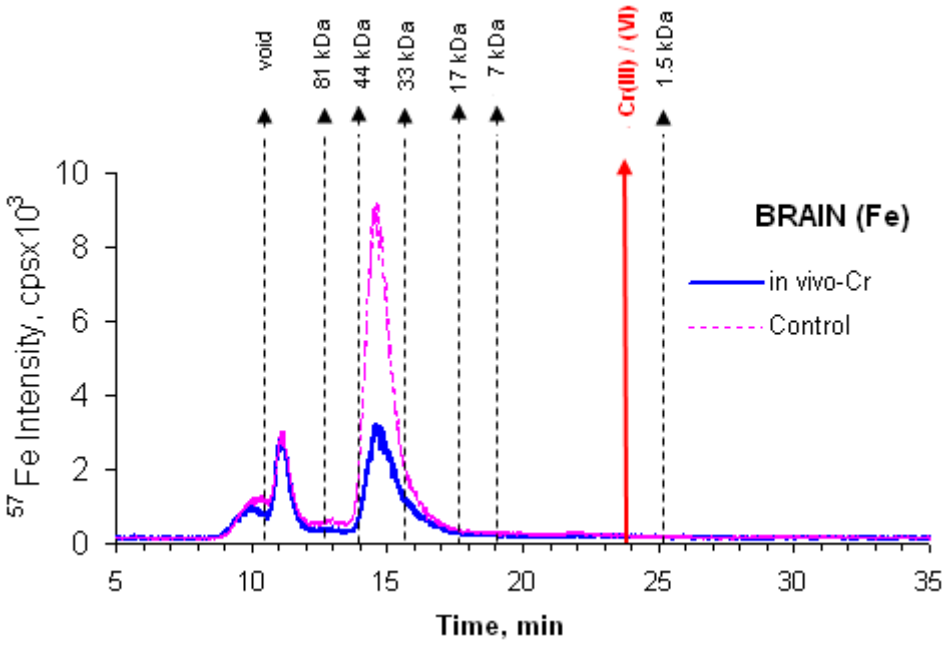

Figure 4.27. Fe elution profiles of brain sample cytosol

There are three main peaks stand out in the chromatogram of Fe for brain tissue. Elution times of ~10, 11 and 14.5 min match the MWs of ~163, 119 and 39 kDa. The peak intensity of peak at ~14.5 min considerably decreased with the hexavalent chromium administration. This was found parallel to the decrease in the total Cr level in brain tissue.

Actually iron is distributed in a heterogeneous fashion among the different regions and cells of the brain. Dietary iron deficiency, associated with delayed development in early life, can rapidly deplete brain iron concentrations but repletion is able to normalize it (Beard and Connor, 2003). Brain iron concentrations increase with age and in many diseases and decrease when iron is deficient in the diet. Fe iron imbalance has been associated with neurodegenerative diseases (Pinero and Connor, 2000)

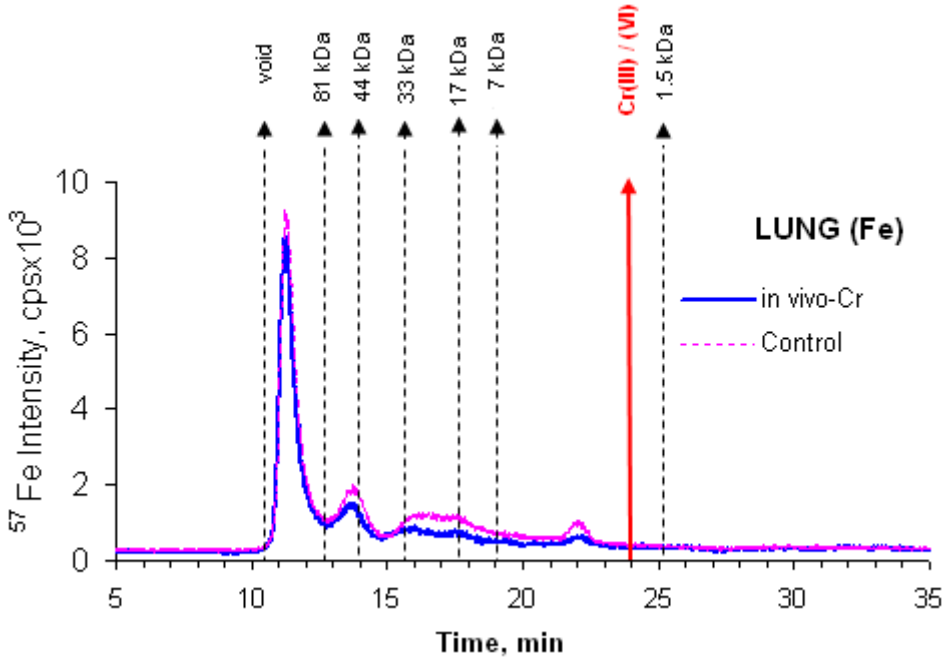

Figure 4.28. Fe elution profiles of lung sample cytosol.

Fe-elution profile of lung is shown in Figure 4.28 for control and in-vivo Cr-enriched mice. With regard to the total Fe levels, similar to lung tissue, there is not statistically significant alteration in lung Fe levels. SEC-ICP-MS chromatogram is not so different to each other.

It is clearly shown that Fe distribution between different size bioligands is in the favor of HMW species.

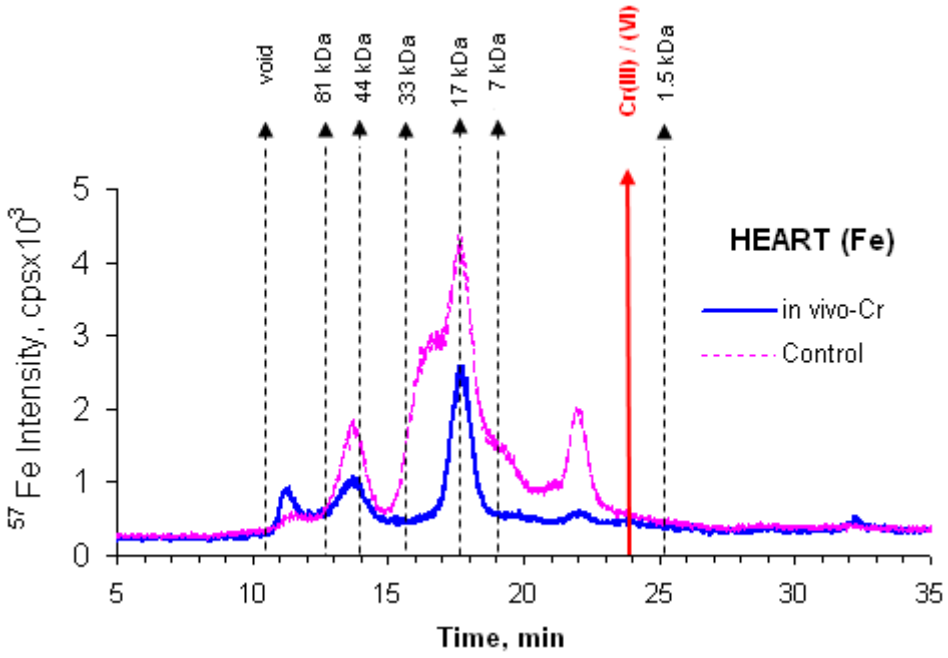

Figure 4.29. Fe elution profiles of heart sample cytosol

The Fe elution profile for heart samples clearly shows that there is a decrease in the intensity of Fe-bound species in the chromatogram (Figure 4.29). This diminishing in the amounts of protein bound Fe is coherent with the decrease in the total Fe levels of the heart samples.

The intensity of all peaks decreased while the peak intensity of the highest MW Fe-bound biomolecule was increased. It should be noted that the peak seen in the basal heart sample at tr: ~16.5 min completely disappeared with the hexavalent chromium administration to mice. Additionally the peak of Fe-complex eluted at tr: ~22 min almost was annihilated.

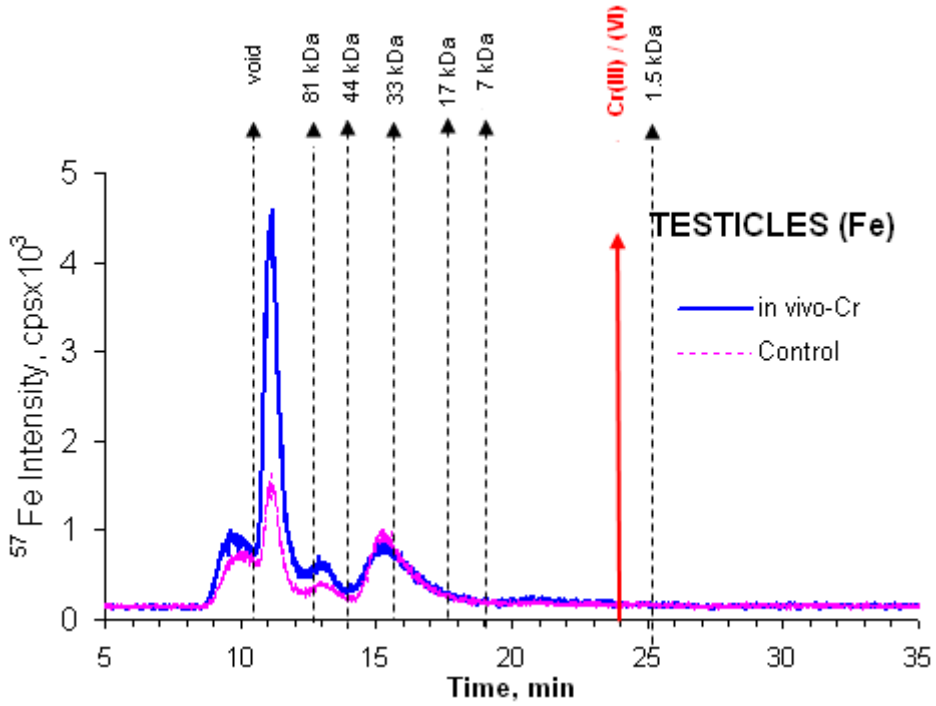

Figure 4.30. Fe elution profiles of sample cytosol for testicles

When looked to the testicles Fe elution profile, four Fe-bound macromolecules are shown in the chromatogram (Figure 4.30). It is clearly shown that Fe is distributed mostly between HMW macromolecules in testicles.

Considerable increase in the peak intensity of the bioligand eluted at 11 min is apparent indicating the induction of the biosynthesis of this ligand. Beside increase in the intensity of peaks eluted at ~9.5 and 13 min is noticeable. Looking to the total Fe levels in Figure 4.9 (percent alteration), increased Fe levels with the Cr(VI) exposure to mice can be seen. This finding shows that total Fe level data obtained by ICP-MS agree with the SEC-ICP-MS data.

4.2.5.4. Copper Complexes

Cu trace drew four main cupric biomolecule peaks on the chromatogram for both Cr-stressed mice liver samples and their control subject. Peak apexes centered at ~10, 12, 15.5(base) and 19 minutes match the approximate MW of 163, 87, 29 and 10 kDa respectively.

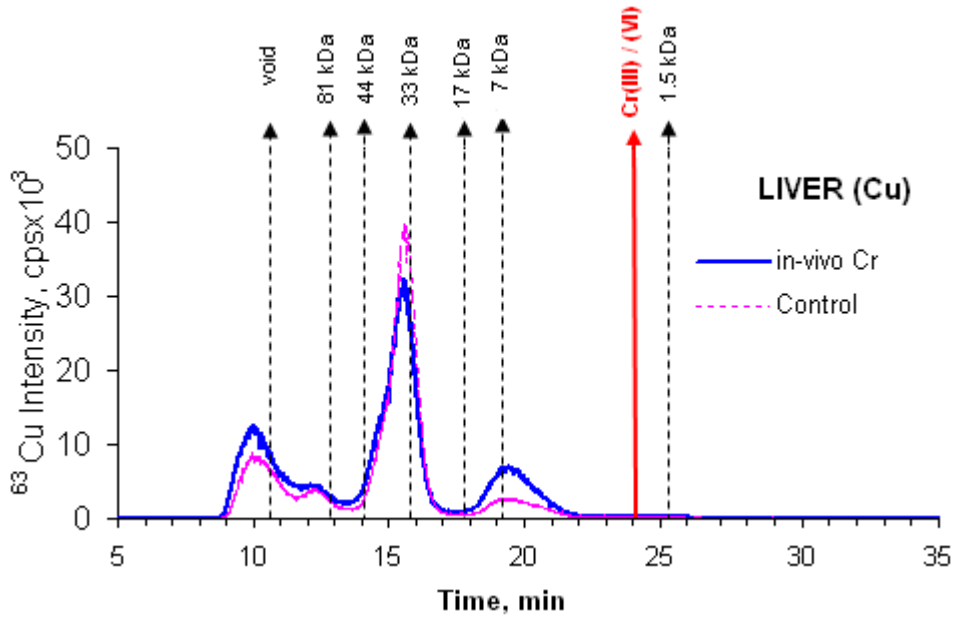

Figure 4.31. Cu elution profiles of liver sample cytosol

It is known that many enzymes contain copper in their structures or bind to copper specifically. A good example is ceruloplasmin which is suggested to be affecting the tissue distribution of manganese which leads to oxidative stress in the brain of mice (Jursa and Smith, 2009).

It is noticeable that the intensity of the most intense Cu-peak (t_r: 15.5) decreased in response to the Cr-stress compared to the control sample. On the contrary to the decrease in the intensity of peak at ~t_r: 15.5, the other peaks intensities slightly elevated.

According to calibration graph for Superdex-75 SE-column, calculated MW of the peak, eluted at 19 min, gives a molecular mass of 10 kDa, but when the elution time (t_r: 18.6) for MT-1 standard is considered, this complex possibly may be a metallothionein (MT) isoform. MTs are a family of cysteine-rich, low molecular weight (3500 to 14000 Da) proteins. MTs have the capacity to bind both physiological (such as Zn, Cu, Se) and xenobiotic (such as Cd, Hg, Ag, As) metals by the thiol group of its cysteine residues, which represents nearly the 30% of its amino acid residues.

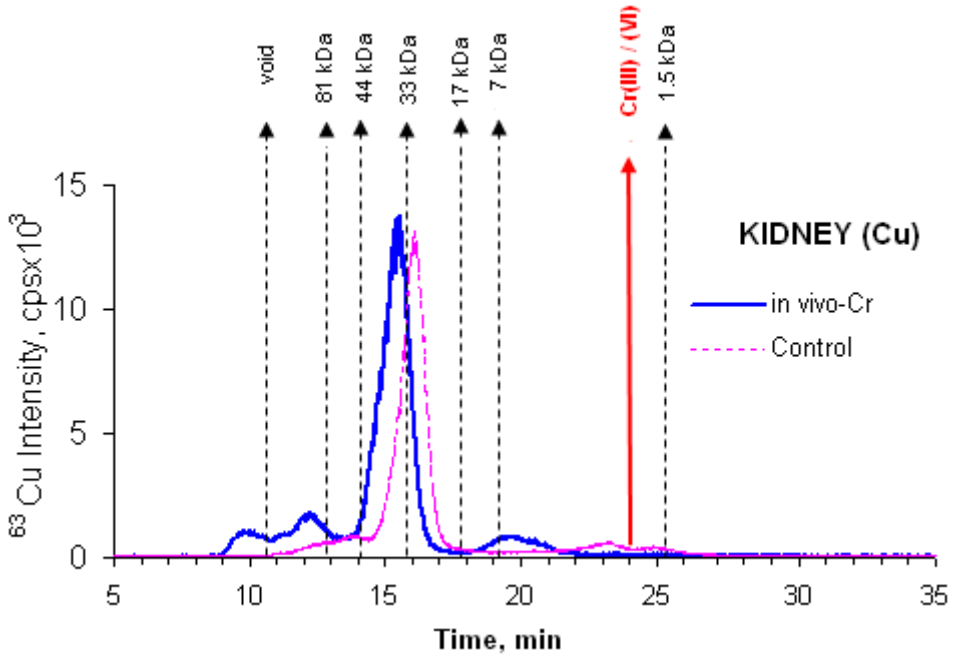

Figure 4.32. Cu elution profiles of kdney sample cytosol

The control group mice Cu-elution profile for kidney tissue shows that copper at great extent binds to the biomolecule which has a tr: ~16 min and approximate MW of 25 kDa. However elution of the fraction shifted to the higher MW region in the chromatogram. A similar shift was observed for the fraction eluted at ~23 min of control sample. The reason of the shifts may be combination of Cu-binding biomolecule with another factor as a result of Cr(VI) exposure. As Cr(VI) exposure is expected to trigger the synthesis of some biochemical process, copper binding to diverse of bioligands may be effected.

Copper exist in all living cells and involves in many biological function including oxidative processes. Oxidases and dismutases are among Cu containing enzymes. Superoxide radicals produced after an entry of oxidant such as Cr(VI) into body are scavengered by dismutase enzymes reducing them to hydrogen peroxide. The main proteins involve in copper metabolism are ceruloplasmin, cytochrome oxidase, superoxide dismutase, metallothionein, dopamine β-hydroxylase and tyrosinase (Sarkar, 1994).

On the other hand it seems that biosynthesis of the ligand eluted at ~12 min was increased and a new Cu-binding ligand eluted at ~9.5 min was emerged. This

indicates that hexavalent Cr exposure gives rise to alterations on the structure of Cu-binding ligands and induction of new Cu-binding ligands.

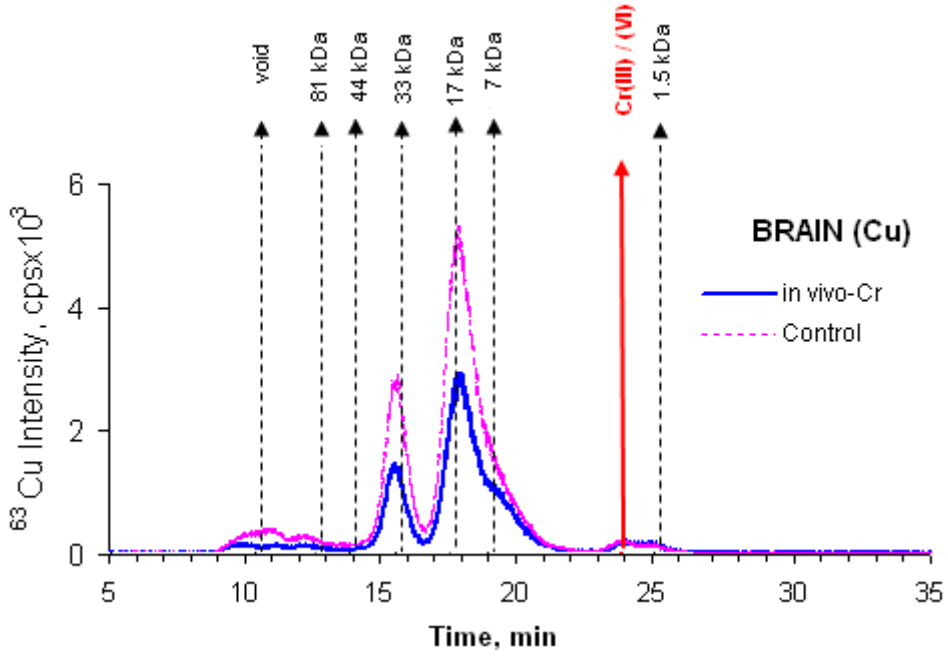

Figure 4.33. Cu elution profiles of brain sample cytosol

Cu elution profile of brain for control and in-vivo Cr-enriched mice are shown in Figure 4.33. Cu-binding bioligands appears in the chromatogram as five different MW clusters. The two more intense peaks, representing the Cu-biomolecule complexes with approximate molecular weight of 35 and 17 kDa, carry the most of total copper. Intensities of these two peaks decreased with the hexavalent chromium exposure.

The decrease in the total Cu levels of brain by Cr(VI) administration to mice fits well with the decrease in the intensity of Cu-binding ligands.

As copper may play an important role in the brain in aging and neurodegenerative diseases quantitative Cu analysis in brain is very important. In the very recent study by Wang and coworkers the active Cu uptake, Cu-containing enzyme levels, and total Cu distribution in the brains of young and aging mice were investigated by autoradiography, laser ablation inductively coupled plasma mass spectrometry and immunohistochemistry techniques (Wang et al., 2010). The study illustrates

the importance of a multi-modality approach in studying the biodistribution and homeostasis of Cu in the brain.

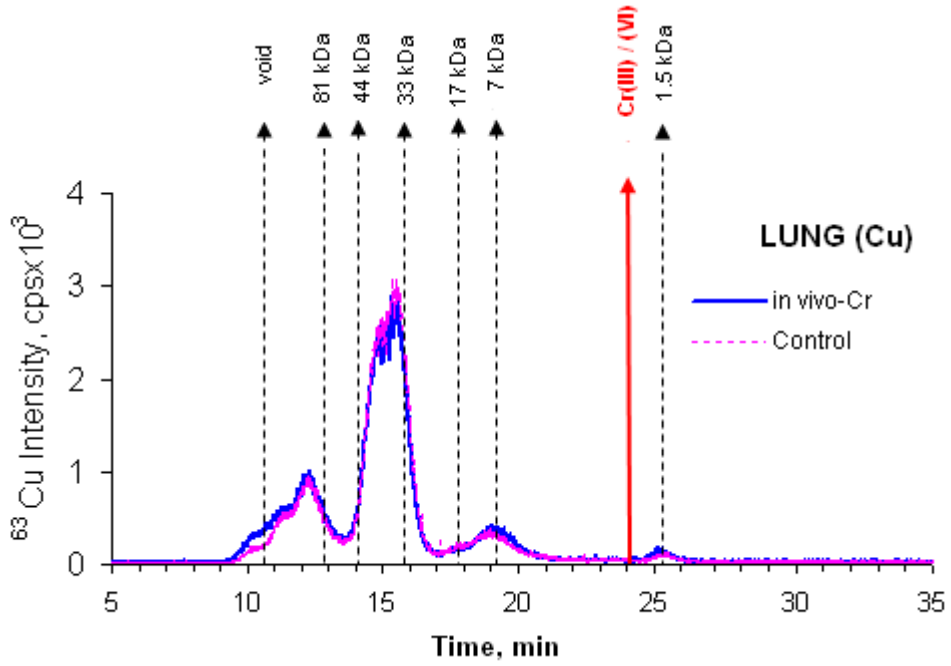

Figure 4.34. Cu elution profiles of lung sample cytosol.

Figure 4.34 shows the Cu-elution profile of lung samples for control and in-vivo Cr-enriched mice. Although the amounts of total copper levels were decreased by hexavalent Cr exposure (Figure 4.9), it seems there is not a considerable alteration in the intensity of Cu-protein complexes (Figure 4.34).

There are four Cu-containing clusters in the chromatogram of lung sample. However when the limited resolving power of SEC is considered, it is apparent that the each cluster composed of sub-clusters. That is clear from the shoulders on the broad peaks.

Copper exposures to air particulates in miners or to metal fumes in smelting operations, welding, and related activities in industry are main roads of direct copper exposure to the lungs.

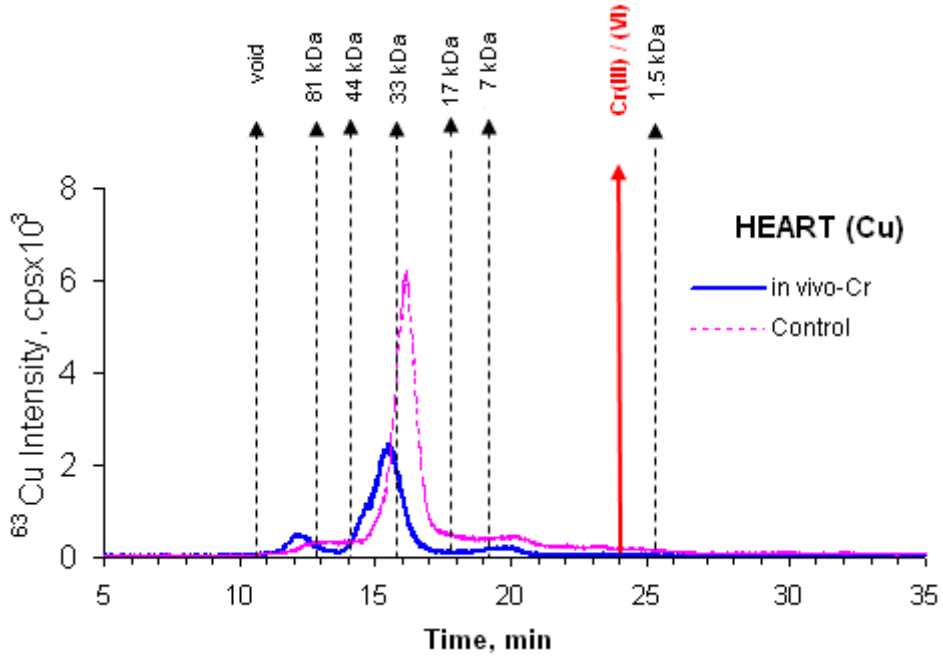

Figure 4.35. Cu elution profiles of heart sample cytosol

Cu elution profile of heart for control and in-vivo Cr-enriched mice are shown in Figure 4.35. Similar to the Cu elution profile of kidney, there is a shift in the elution time of Cu peak to the HMW region. This shift makes probable of a possible attachment of a factor which possibly play roles in detoxification processes to the Cu binding bioligand.

On the other hand the induced biosynthesis of the highest MW Cu-binding macromolecule is appearing in the chromatogram or a similary this HMW protein complex binds to another molecule.

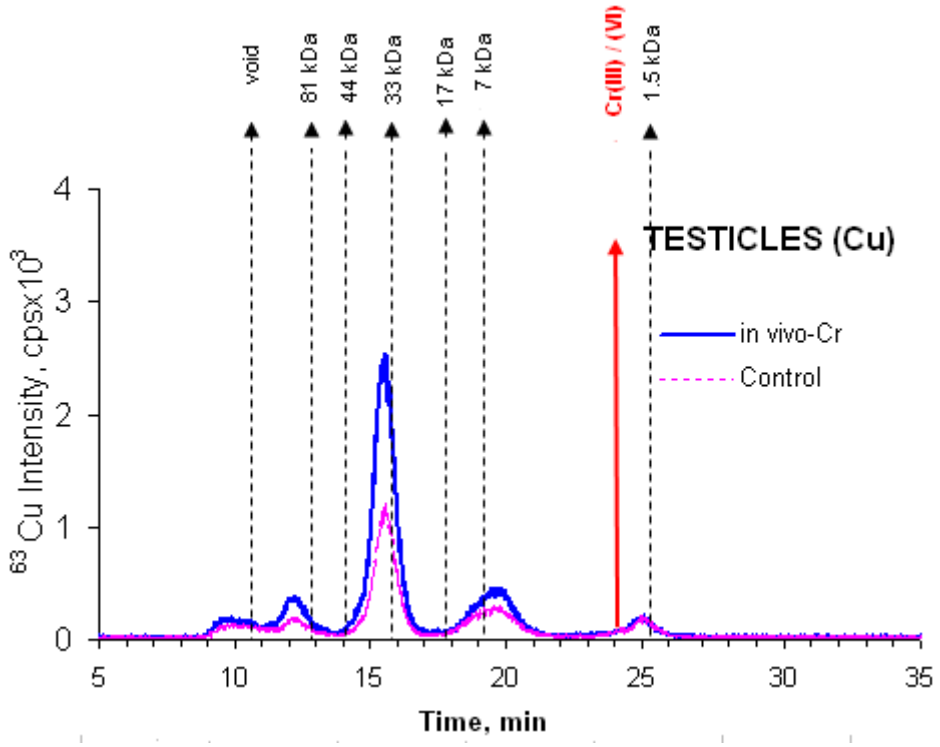

Figure 4.36. Cu elution profiles of sample cytosol for testicles

Figure 4.36 shows the Cu-elution profile of testis samples for control and in-vivo Cr-enriched mice. Five different molecular weight Cu-bound macromolecules appear in the SEC-ICP-MS chromatogram. the intensity of Cu containing species peaks increased in general with the Cr(VI) stress to mice, as found in the total copper levels of the testicles.

Considerable increase in the relative intensity of the ligand has a MW of 35 kDa (eluted at 15.5 min) indicates the bioinduction of this protein.

4.2.5.5. Zinc Complexes

Quite high amount of zinc associated with macromolecules distributed through the chromatogram are noticed in the SEC-ICP-MS chromatogram (Figure 4.37) and the elution of the species are in broad bands rather than peaks. This should not be surprising because, zinc is a constituent in more than about 200 enzymes and proteins (Garcia et al., 2006). There are some reasons of this, i.e., why zinc is highly valuable in biological systems relative to other metals: Availability of zinc is higher than other transition metals such as nickel, cadmium, iron, copper, it is strongly retained in living organisms; Fast ligand exchange capability that

reactions must be easily carried by metal ions which can take up and release molecules from their coordination spheres rapidly; No redox reaction; Flexible coordination geometry and importantly a good Lewis acid preference (only copper amongst divalent ions is better) (Frausto da Silva and Williams, 2001). As being common in some cells, zinc may well be a link between control and regulation. One very obvious distinction of zinc is its highly concentrated charge because of its small ion radius (0.65 Á). All these properties make zinc biologically important and selective metal (good binding and selective recognition). Besides its structural role, zinc is a good trigger ion and catalyst of systems, because it binds well and exchanges rapidly.

Zn-complexes are seen as 3 main bands (eluted between 9 and 18 minutes) in the cytosolic hepatochromatogram and middle range MW Zn-species (tr: 18 and 23 minutes) appear following the tail of the broad peak at 15-18 minutes. Low intensity Zn species with low MW (< 1.5 kDa) also seem in the chromatogram.

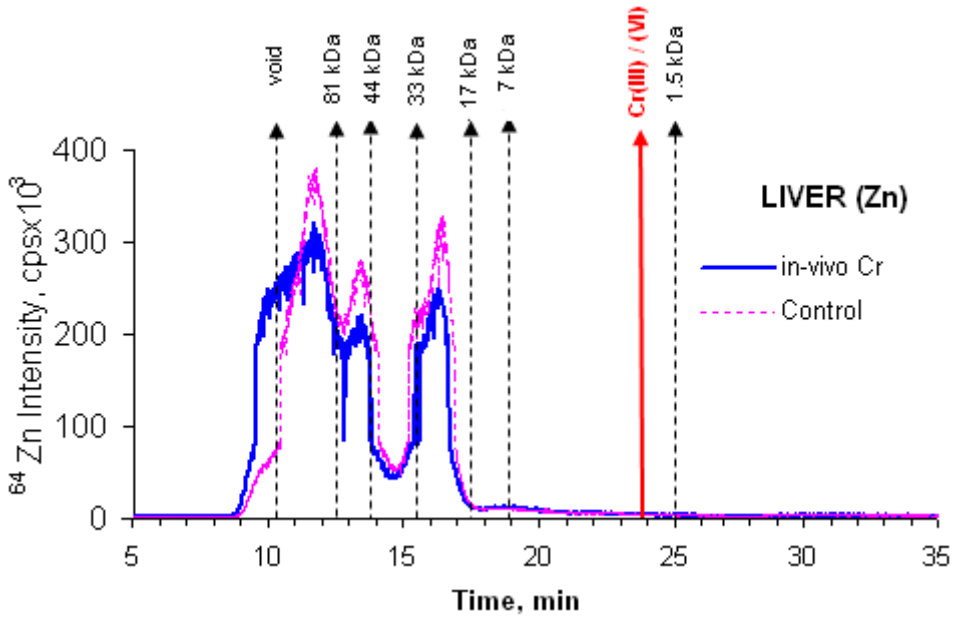

Figure 4.37. Zn elution profiles of liver sample cytosol

It is known that zinc binding in enzymes is strong with binding constant $>10^{11}$ M^{-1}. There are also some non-enzyme proteins that bind zinc, a well-known example is insulin complexes, where zinc and insulin are retained in a vesicle. These complexes have weak character and the concentration of zinc must be high. Other

non-enzymatic but very important protein group is metallothioneins; a random coil protein without binding to metal ions, only on zinc, cadmium or copper binding does form a series of turns. The structure of this metal scavenger protein bound by different metals is not the same but the essential core structure is fixed. While metallothioneins which may transport zinc in cytoplasm there are also membrane located zinc pumps called CPx-type ATPases and DCT 1. Another extremely important zinc protein group, as non-enzymatic, is zinc fingers. Most of them bind to the DNA but others exist in enzymes with a structural or regulatory function (Frausto da Silva and Williams, 2001).

The two obvious alterations are shown in the elution profile of zinc for mice liver cytosol by Cr(VI) administration. The first is, in general, attenuation at the peak intensities of Zn-complexes (Figure 4.37). The decreases in concentrations of the Zn-bound biomolecules in liver comply with the decrease in the total Zn level of liver (see Table 4.5 and Figure 4.9). The other is highly observable increase at the intensity of Zn peak which belongs to the highest MW zinc-species and eluted just before 10^{th} minute. This peak is shown as a pre-shoulder of the first zinc band in the chromatogram of control group of mice liver sample, while the peak intensity of this species increased and associated to the broad zinc band in the chromatogram of Cr(VI) administration group of mice liver samples.

Cr(VI) administration seems to have affected the amount of zinc-species. It can be said that altered metal concentrations in living systems affect the levels of other metal ions, their distribution and forms. These may lead to undesirable events such as inhibition of biological processes, structural damages to the biological macromolecules such as protein or DNA, tissue damages and toxicity which results in some disorders/diseases.

Ceruloplasmin, 151 kDa protein which is synthesized in liver, possibly plays an important role in maintaining the dual Cu-Fe homeostasis. Because, ceruloplasmin has been proposed to exhibit a copper-dependent oxidase activity (Davidsson et al., 1989; Gibbons et al., 1976); like iron, manganese in plasma is oxidized from the (II) to the (III) oxidation state by the oxidase protein ceruloplasmin for loading onto transferrin, which can only carry iron in the ferric state, and transport to

tissues as well as cellular iron efflux. But a very recently published study (Jursa and Smith, 2009) has suggested that ceruloplasmin does not play a role in the loading of manganese onto plasma transferrin *in-vivo*.

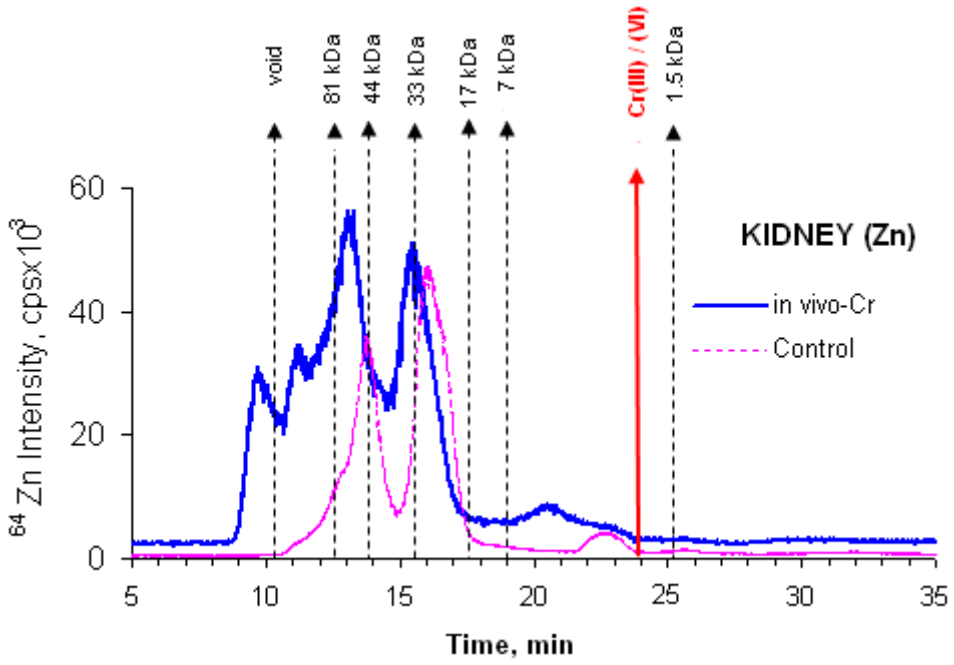

Figure 4.38. Zn elution profiles of kidney sample cytosol

Similar to liver, very high level of bioligand associated to zinc are shown in the kidney chromatogram of control and in-vivo Cr-enriched mice (Figure 4.38). The peak at tr: 9.5 min is clearly shown in the chromatogram of in-vivo Cr-enriched mice kidney while does not exist that of control subject that indicates the biologically synthesis of the ligand. Beside of that, biosynthesis of a few Zn-binding ligands is apparent in the kidney. Intensity of the peaks eluted at 11, 13, 15 and 20.5 min were also increased as a result of Cr(VI) exposure.

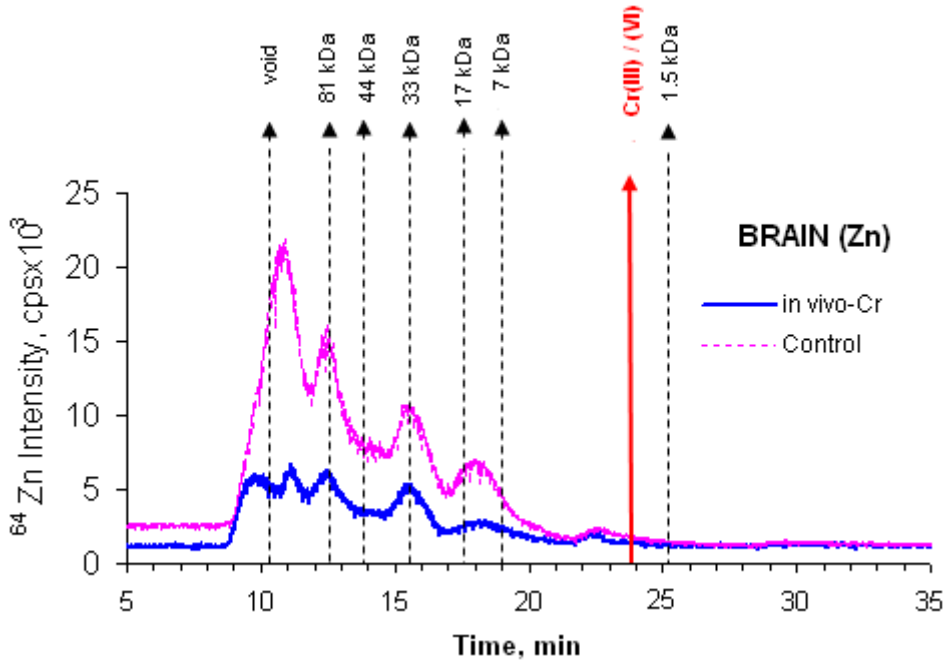

Figure 4.39. Zn elution profiles of mice brain cytosol

Figure 4.39 shows the Zn-elution profile of brain samples for control and in-vivo Cr-enriched mice. Five peaks are shown in the chromatogram of control sample. However, six peaks exist in the chromatogram of in-vivo Cr-enriched mice samples. The emerged peak eluted at 11.2 min probably due to the effect of concentration on peak resolution. The decrease in the peak intensities of Zn-species through all peaks is coherent with the decrease in total levels of Zn in brain.

Interestingly in the very recent study of Matusch and coworkers, an imaging LA-ICP-MS technique for Fe, Cu, Zn, and Mn was developed to produce large series of quantitative element maps in native brain sections of mice (Matusch et al., 2010). The methodology is very incentive for further pursuing the metallomic approach in research of and drug development for neurodegenerative diseases.

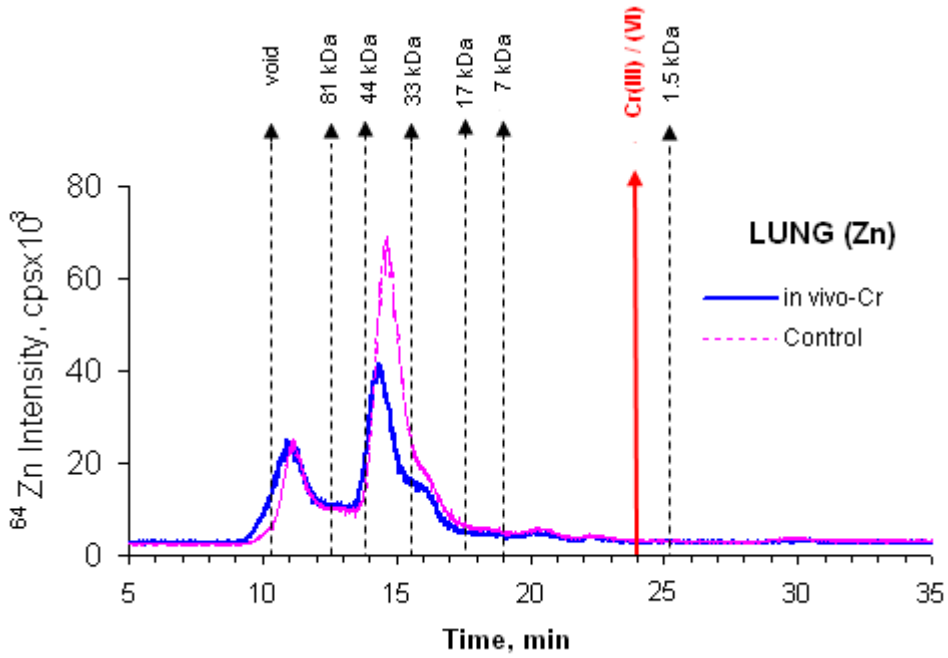

Figure 4.40. Zn elution profiles for lung cytosol of mice

Lung tissue Zn binding macromolecules exist mostly in two large peaks in which species' molecular weight is higher than 30 kDa. There are also very less intense and LMW (>7kDa) species which appear in the chromatogram after elution time 19 min. Although intensity of the peaks does not change so much with the Cr(VI) exposure, intensity of the peak at 14.5 min decreased.

This behaviour, diminishing in the intensity of zinc binding protein (Figure 4.9), obeys the statistically significant decrease in the total zinc content of lung tissue (Table 4.5).

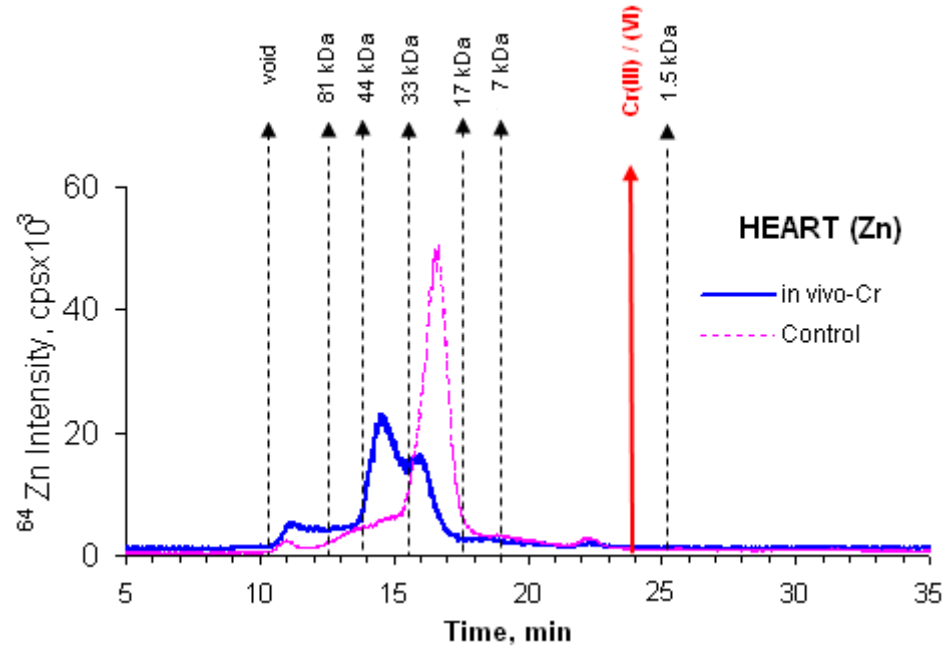

Figure 4.41. Zn elution profiles for heart cytosol of mice

Zinc elution pattern of heart samples are quite different to each other. The first two peaks intensity considerably high in the in-vivo Cr(VI) administered mice heart sample, while the intensity of the peak at 16.2 min is lower.

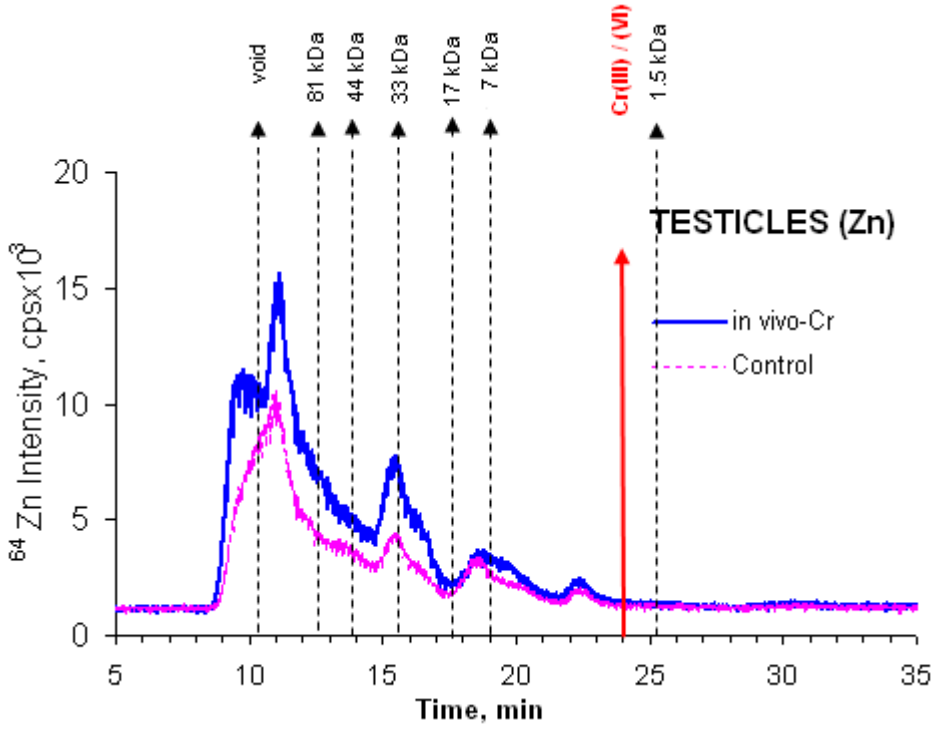

Figure 4.42. Zn elution profiles for cytosol of testicles

Zinc complexes distributed through all the chromatogram for testis samples of mice. There is slight increase in the intensity of peaks in general with the hexavalent chromium stress. Parallel data has been obtained in the total Zn level data of testis samples.

In conclusion, the hexavalent chromium exposure to mice gives rise to affect the concentrations of other essential trace elements (Schroeder and Nason, 1976; Li et al., 2004; Yang and Wong, 2001), change their speciation by competitive binding to proteins, and trigger off the synthesis of diverse biological ligands. The data of the elements associated to biological ligands showed that external exposure of a toxic metal cause to not only non-specific binding to biological ligands but also affects the amount and composition of the other metal complexes in organism.

Beside, screening the chemical changes inside an organism following an uptake of chromium is critical for the understanding of the biological mechanisms involving this element.

4.2.6. In-vitro Cr(III) binding to the macromolecules in mice liver cytosol

In order to probe the selectivity of chromium towards biological ligands, 150 and 200 ppb chromium, as final concentration, was added to the tissue cytosols of control group of mice. In Figure 4.43, three chromatograms were combined to compare chromium elution profiles. The chromatogram with blue color belongs to the *in-vivo* Cr(VI)-administered group mice liver cytosol; the red one belongs to the control group mice liver cytosol which was spiked with 200 ppb chromium; and the turquoise color chromatogram belongs to the control group mice liver cytosol which was spiked with 150 ppb chromium. The red line in the Figures indicates both the elution time of trivalent and hexavalent inorganic chromium which can not be separated on Superdex-75 column.

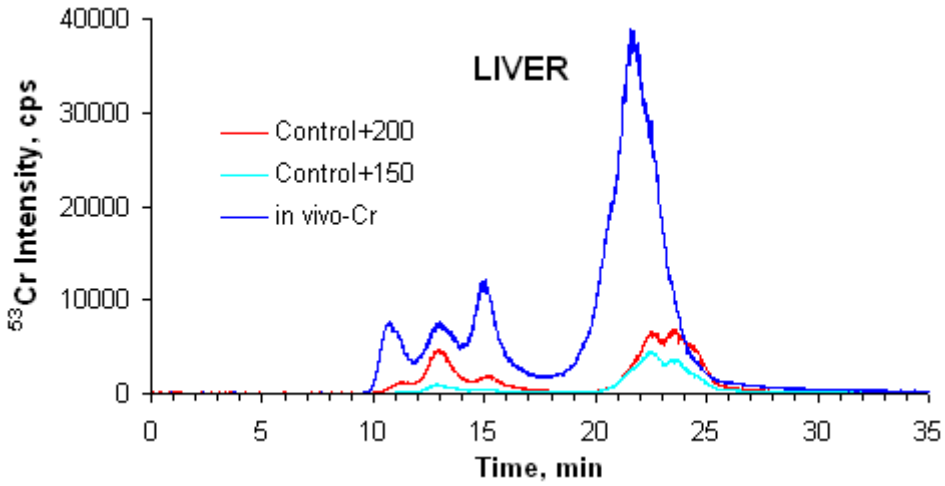

Figure 4.43. Distribution of chromium between different MW biological ligands in mouse liver after *in-vivo* and *in-vitro* chromium administration

When chromatograms were compared, it can be seen that the macromolecules in mice liver cytosol differently bind chromium *in vivo* and *in vitro.* When Cr(VI) was intraperitoneally injected to the mice at a single dose of 8.0 mg/kg b.w., intracellular or extracellular reduction of Cr(VI) to Cr(III) is considered and the complexed form of chromium is in trivalent state. Evident judged by UV-Vis absorbance at 570 nm, which reflects the d-d transitions of Cr(III), highly agrees with the ICP-MS signal of ^{53}Cr (see red trace in Figure 4.13).

HMW region of the chromatogram consist a series of Cr-ligands. At this region three to four Cr-species with various sizes exist. In the LMW region of the chromatogram, there is a broad Cr peak which possibly consists different molecular weight Cr-species. When the chromatograms of *in-vivo* and *in-vitro* Cr-administered samples were compared, it could be shown at the high molecular weight region, peak apexes are superimposing to a great extent at same elution time, which means that they have the same or very similar size or molecular weight.

One basic difference between the chromatograms of *in-vivo* and *in-vitro* Cr-administered samples is the splitting of broad Cr-peak in the case of *in-vitro* spiking. This may possibly rise from the effect of concentration on resolution of the peaks. Finally when looked at the differences at intensities of chromium signal in

each chromatogram; it can be said that Cr-binding ligands differ either in terms of specificity (affinity to Cr(III)) and the chromium binding capacity.

The change in proportion of the intensity of the 40 kDa peak which is proportionally higher in the *in-vivo* than in the *in-vitro* chromatogram indicates the bioinduction of this protein. Note that the large Cr peak in the LMW range could not be obtained by spiking the cytosol *in-vitro* which indicates an *in-vivo* synthesis of unknown ligands.

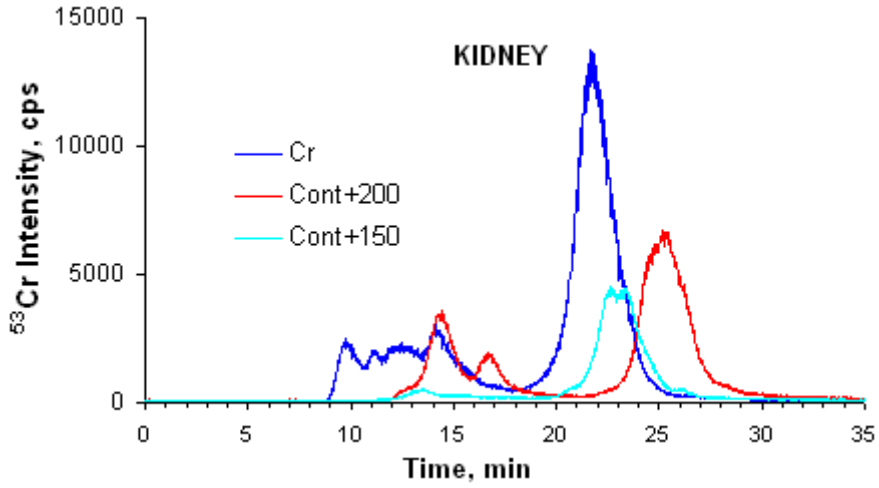

Figure 4.44. Distribution of chromium between different MW biological ligands in mouse kidney after *in-vivo* and *in-vitro* chromium administration

The in-vitro spiking experiments were carried out for the control group kidney tissue cytosol as well. The chromatogram of the *in-vivo* interaction of Cr(VI) with the control group kidney cytosol (blue trace) and *in-vitro* Cr spiking chromatograms (red trace for 150 ppb and turquoise trace for 200 ppb) clearly differ to each other. The first two peaks of highest MW Cr-binding species in the *in-vivo* chromatogram are absent in *in-vitro* chromatograms. This indicates the biological induction of these proteins. The large Cr peak of *in-vivo* chromatogram in the LMW range could not be obtained by spiking the cytosol *in-vitro* which indicates an *in-vivo* synthesis of unknown ligands. On the other hand in the *in-vitro* chromatograms LMW Cr-binding species eluted at different times for an unknown reason.

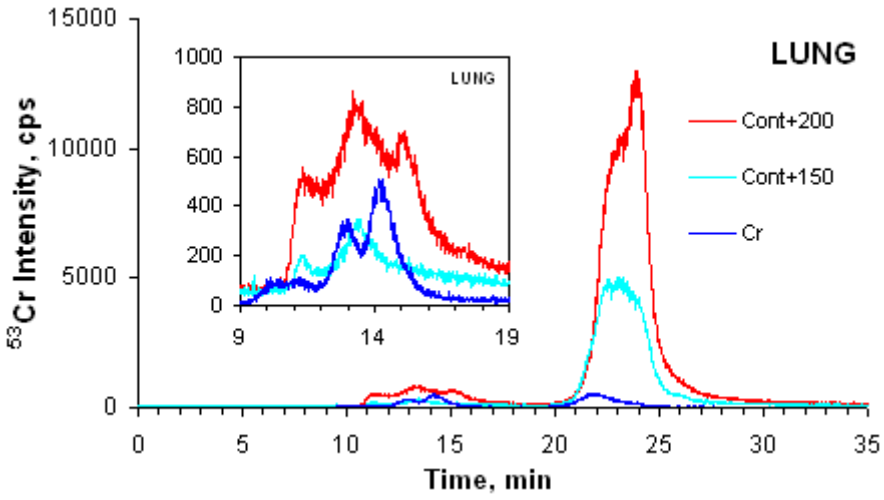

Figure 4.45. Distribution of chromium between different MW biological ligands in mouse lung after *in-vivo* and *in-vitro* chromium administration

The first apparent difference in the chromatograms of lung samples (Figure 4.45) is that contrary to liver and kidney samples, the intensity of the *in-vivo* chromatogram is not higher than that of *in-vitro* which a result of higher Cr uptake of liver and kidney *in-vivo* than that of lung.

Regarding LMW Cr-species in lung samples, the large peak of *in-vivo* chromatogram in the LMW range could not be obtained by spiking the cytosol *in-vitro* which indicates an *in-vivo* synthesis of unknown ligands, as being in liver and kidney samples. Note that elution volume of large peak in the *in-vivo* chromatogram was shifted in comparison with the standard solution which indicates interaction with LMW ligands in the sample.

On the other hand, lung sample chromatograms are different than the liver, kidney and heart chromatograms in terms of the distribution of Cr between different MW species. Because HMW complexes are lower in quantity than LMW complexes. The HMW range of the chromatogram was magnified at the inset in Figure 4.45. The intensity of the peak eluted at 14.1 min (~ 40 kDa) is proportionally higher in the *in-vivo* than in the *in-vitro* chromatogram which indicates the bioinduction of this protein.

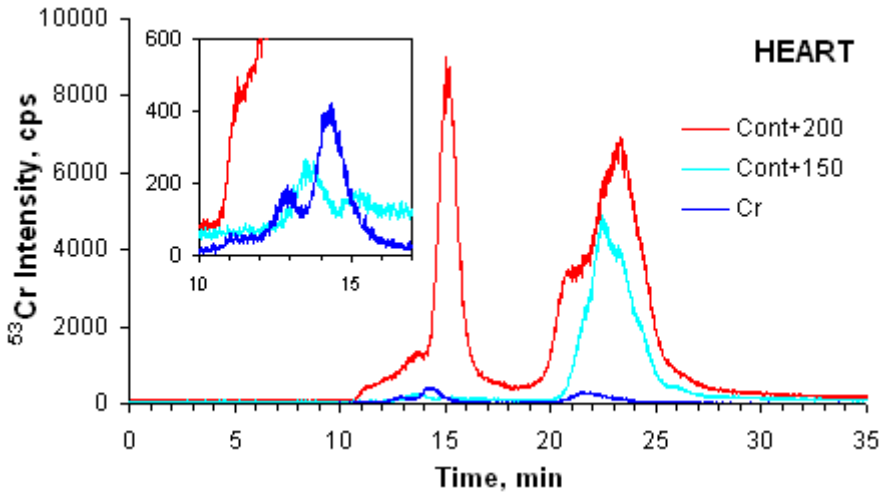

Figure 4.46. Distribution of chromium between different MW biological ligands in mouse heart after *in-vivo* and *in-vitro* chromium administration

Heart sample Cr-binding distribution is shown in Figure 4.46. It should be noted that when the spike concentration was raised, the peaks in LMW region became broader. This behaviour indicates that increase in the concentration of external spiking brings about the non-specific interaction of Cr and proteins. Similar to lung sample, the increased proportion in the intensity of the peak eluted at 14.1 kDa is an indicator of bioinduction of this protein (see the inset in Fig. 4.46).

4.2.7. Tryptic digestion of Cr-containing proteins

A Dual (Size Exclusion- Size Exclusion) HPLC-UV-ICP-MS system

In order to test the survival of Cr-containing peptides, the fractions collected from Superdex-75 HR 10/30 column were digested with trypsin in non-denaturing and denaturing conditions and injected to a second SE-HPLC-UV-ICP-MS system with Superdex-Peptide HR 10/30 column.

Table 4.7. Molecular masses of different compounds and their retention times on Superdex peptide column

Protein standard	M, kDa
myoglobin	17
metallothionein	7
hydroxocobalamin	1.3
SeCys	0.2

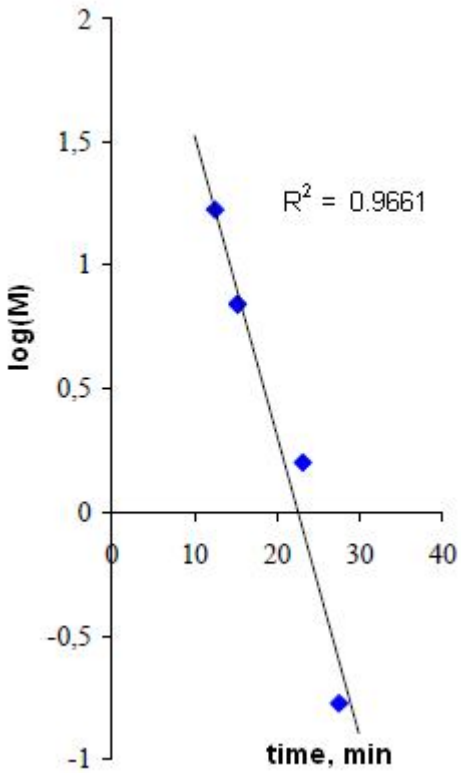

Figure 4.47. Calibration curve for Superdex-Peptide HR 10/30 column

Superdex-Peptide HR 10/30 column was previously calibrated with protein standards given in Table 4.7 and the calibration curve are shown in Figure 4.47.

The liver sample of the Cr-injected mice was chosen as model tissue to probe, because firstly; liver is the first organ to which Cr is transported after absorption, secondly; liver is organ where protein synthesis mainly takes place and finally the highest Cr concentration was determined in liver. The fractions (shaded areas) collected for the extract of *in-vivo* Cr-administered mice liver samples were named as F1 to F5 depending on the elution order of Cr-species. The methodology is illustrated in Figure 4.48 which shows the size exclusion chromatograms of Cr-containing peptide fragments after specific cleavage of proteins in the collected SE fractions (from F1 to F5) by trypsin in non-denaturing and denaturing conditions.

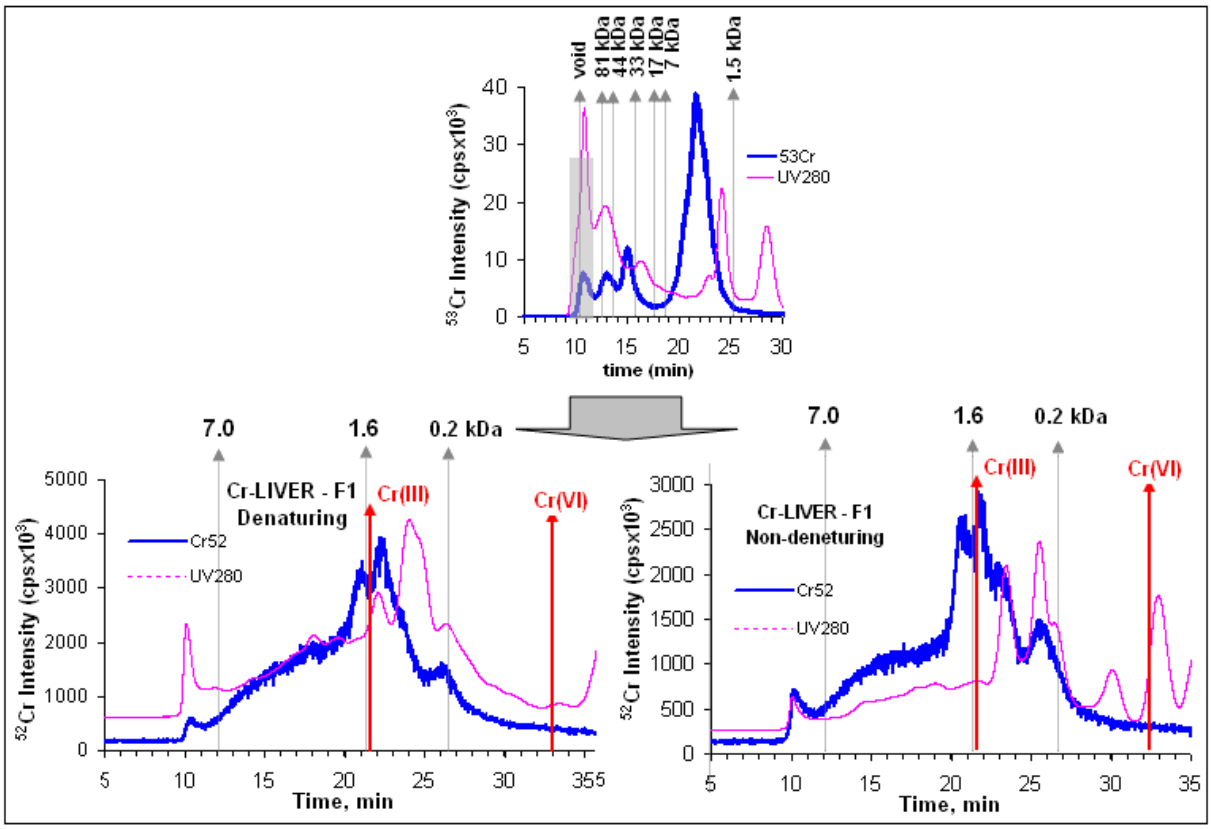

Figure 4.48. SEC-UV-ICP-MS (using Superdex-75 column) chromatogram and the heatcut fraction (shaded and called F1) of in-vivo Cr-administered group mice liver cytosol (upper). SEC-UV-ICP-MS chromatograms (using Superdex peptide column) of fraction after tryptic digestion in denaturing conditions (left); and non-denaturing conditions (right). The thin lines show the elution volume of molecular weight markers. Red lines indicate the elution volume of trivalent and hexavalent chromium standards.

When the chromatogram of fractions of *in-vivo* Cr-administered mice' liver was examined, tryptic digestion of both in non-denaturing and denaturing conditions seem to be successful. It is noticeable that digestion of the first three fractions, F1, F2 and F3 in Figures 4.48-50 respectively, by trypsin has been performed more efficiently than that of F4 and F5 in Figures 4.51-52 respectively. Likely reason of this result is that; F4 and F5 are already composed of lower molecular weight species.

According to the mass calibration of Superdex-75 HR 10/30 column (Figure 4.10), there were high molecular weight chromic species in the fractions (especially in F1-F3). But, when these fractions were digested with trypsin and separated through Superdex-Peptide HR 10/30 column, it was seen different size peptide fragments. Moreover peptides still contain chromium as proven by UV absorbance

at 280 nm and ICP-MS chromium signal. MALDI-MS spectra of peptides have also showed the Cr adducts of peptides (see Figure 4.53). This suggests that tryptic digestion of the Cr-protein complexes with different molecular weights could successfully be achieved and the remaining peptides as Cr-complexes are still stable enough through the experimental procedure and in chromatography conditions.

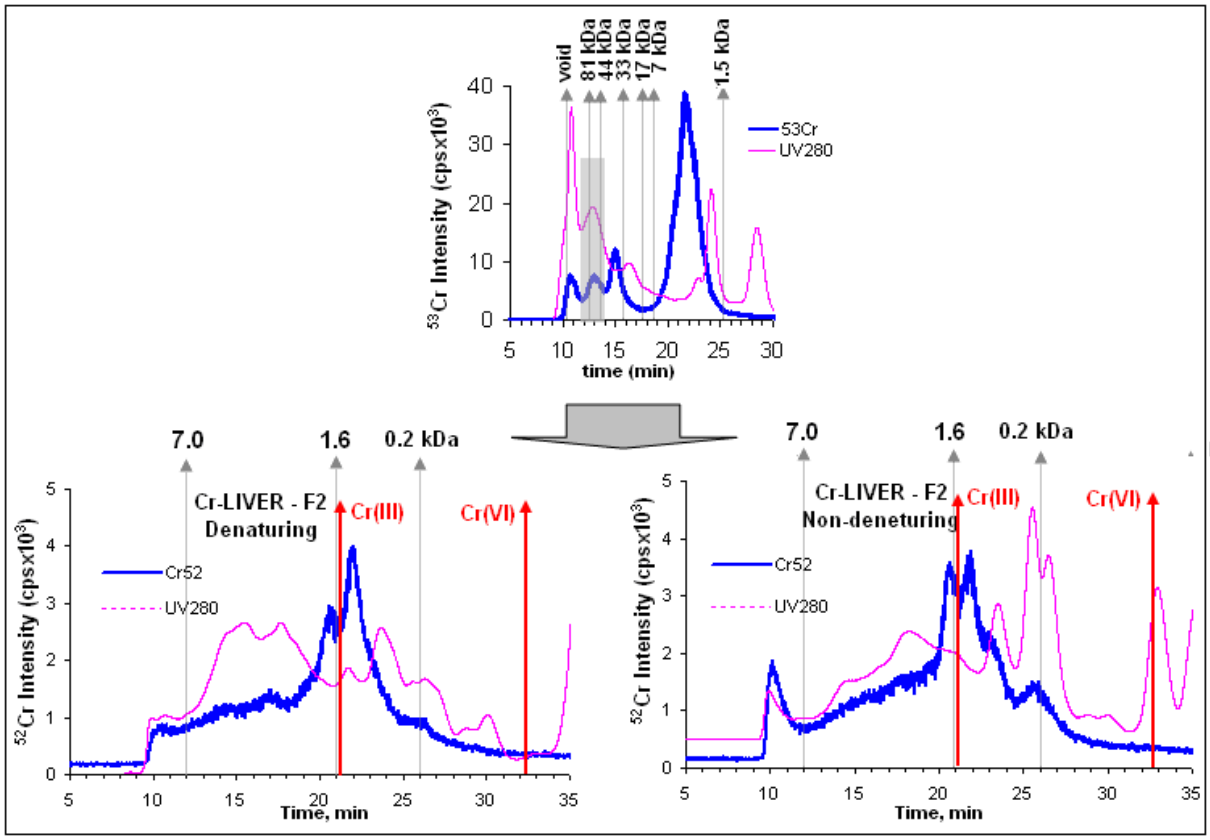

Figure 4.49. SEC-UV-ICP MS (using Superdex-75 column) chromatogram and the heatcut fraction (shaded and called F2) of in-vivo Cr-administered group mice liver cytosol (upper). SEC-UV-ICP-MS chromatograms (Superdex peptide column) of fraction after tryptic digestion in denaturing conditions (left); and non-denaturing conditions (right).

The methodology used by sequencial (using Superdex-75 and Superdex peptide columns) SEC-UV-ICP-MS system was applied to the other fraction of Cr-containing biomolecules. Figure 4.49 shows the chromatogram of the peptide fragments after tryptic digestion of the second fraction (F2) in denaturing and non-denaturing conditions.

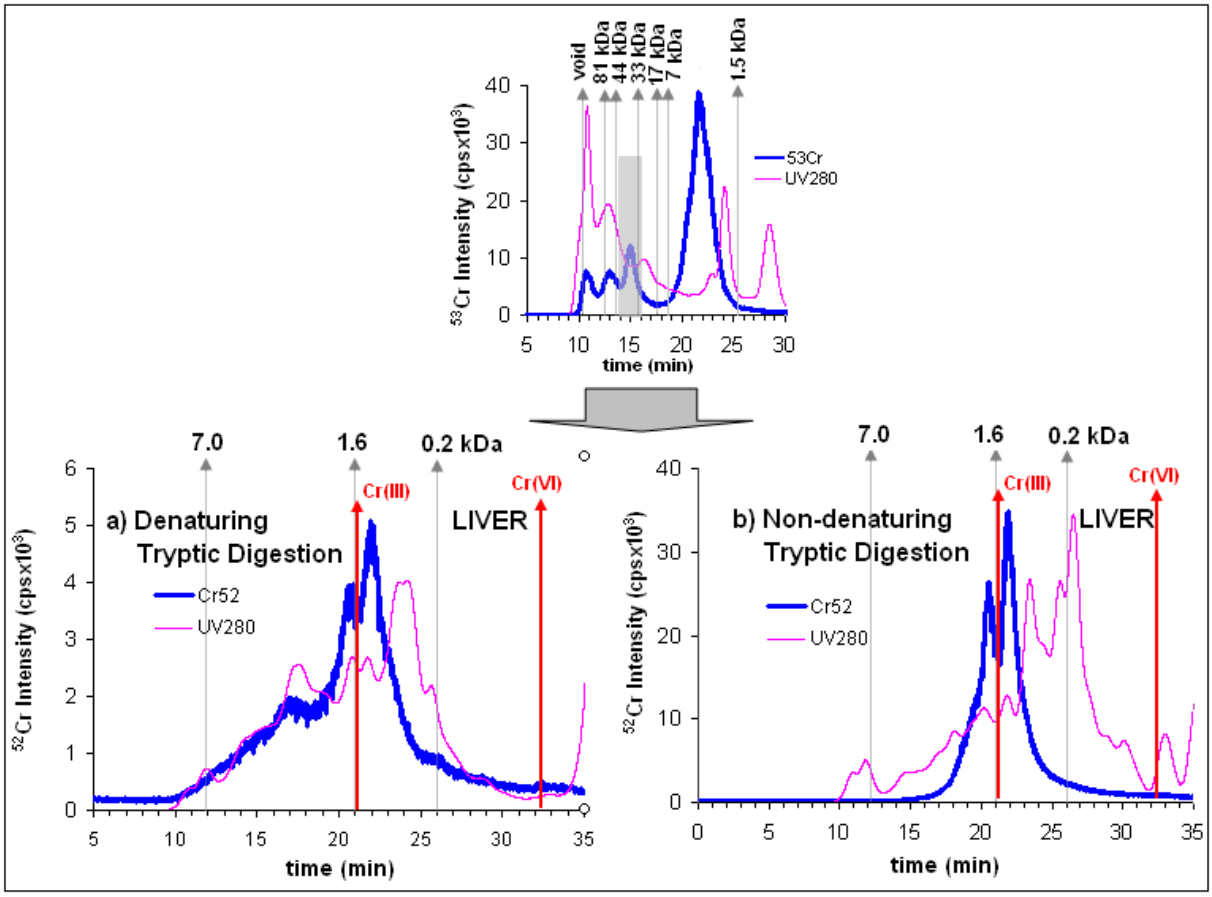

Figure 4.50. SEC-UV-ICP MS (using Superdex-75 column) chromatogram and the heatcut fraction (shaded and called F3) of in-vivo Cr-administered group mice liver cytosol (upper). SEC-UV-ICP-MS chromatograms (Superdex peptide column) of fraction after tryptic digestion in denaturing conditions (left); and non-denaturing conditions (right).

Identification of the Cr-binding protein has an approximate MW of 40 kDa in F3 is especially important as its synthesis is biologically induced by hexavalent chromium exposure to mice (Figure 4.50). The Superdex Peptide SEC-UV-ICP-MS chromatograms obtained after the tryptic digestion in denaturing and in non-denaturing conditions validate both approaches. Although the Cr-binding was sufficiently strong to preserve Cr-peptide complexes in denaturing conditions, the digestion in non-denaturing conditions is much more efficient leading to a mixture of Cr-binding peptides in the molecular mass range of 0.1-7 kDa.

The fractions F4 and F5 and their chromatograms following tryptic digestion are shown in Figure 4.51 and 4.52, respectively. When the chromatograms of the peptide-Cr complexes were compared to each other for the collected fractions of F1-F4, higher intensity peaks were obtained in non-denaturing conditions. This finding indicates that denaturing effect of DTT and urea results to the partial loss of

Cr coordination in the proteins. However, the intensity for digestion of F1 in the favor of denaturing conditions. In this case denaturation may facilitates the task of trypsin by increasing accessible cutting points in the 3D structure of protein and preventing steric hindrance.

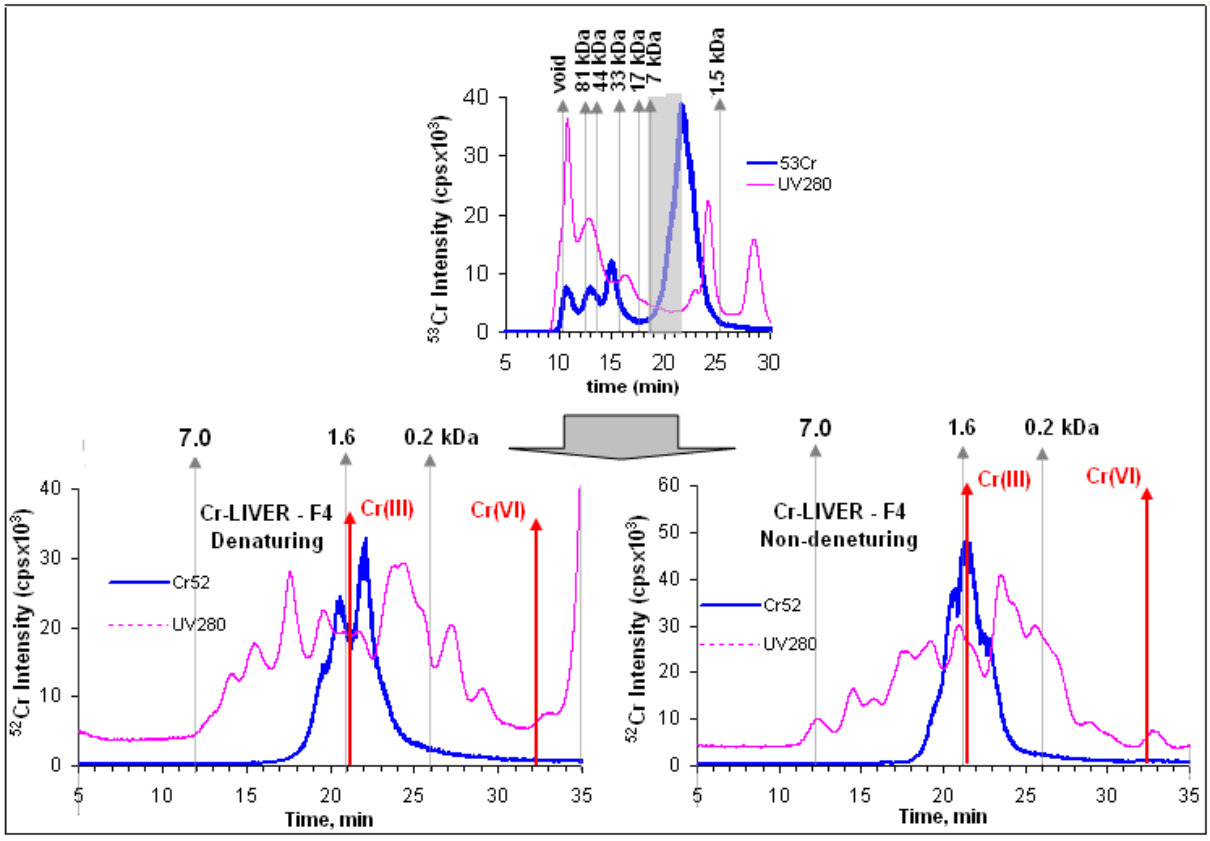

Figure 4.51. SEC-UV-ICP MS (using Superdex-75 column) chromatogram and the heatcut fraction (shaded and called F4) of in-vivo Cr-administered group mice liver cytosol (upper). SEC-UV-ICP-MS chromatograms (using Superdex peptide column) of fraction after tryptic digestion in denaturing conditions (left); and non-denaturing conditions (right).

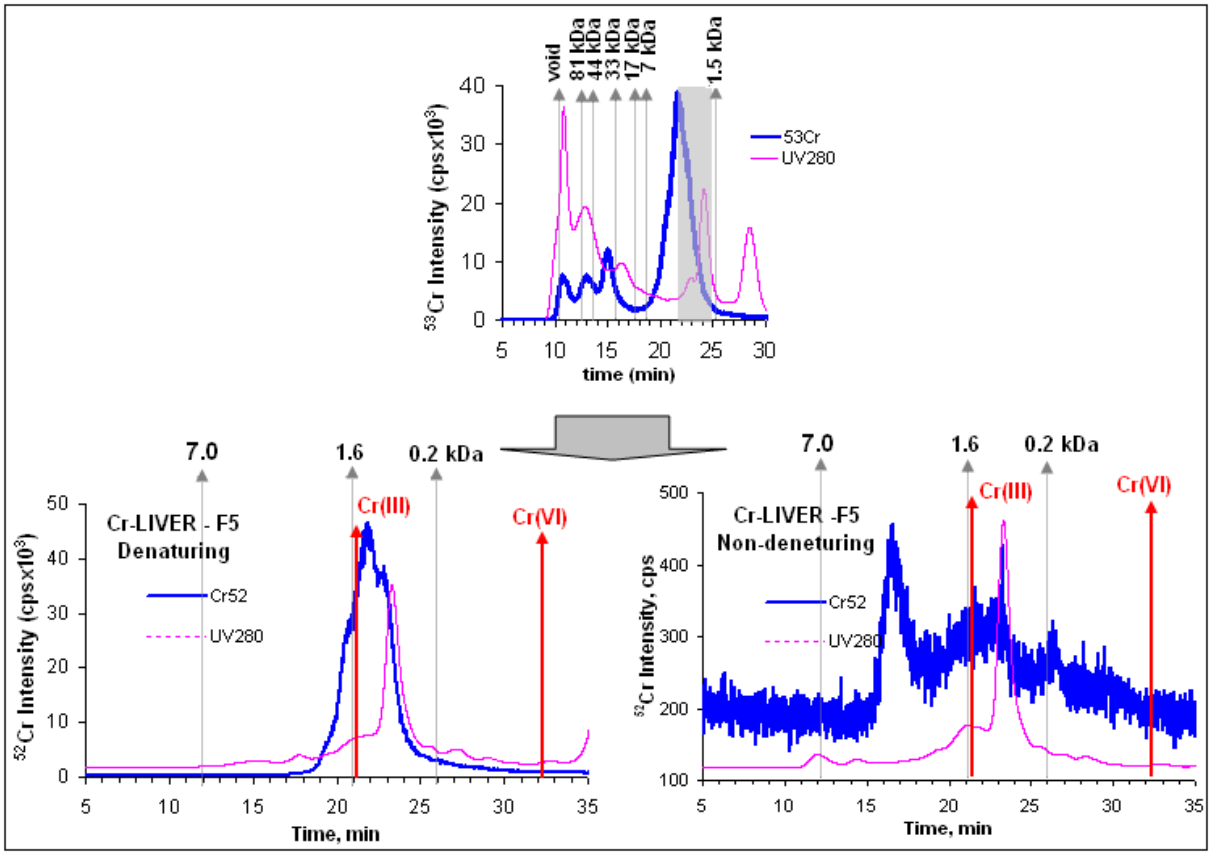

Figure 4.52. SEC-UV-ICP MS (using Superdex-75 column) chromatogram and the heatcut fraction (shaded and called F5) of in-vivo Cr-administered group mice liver cytosol (upper). SEC-UV-ICP-MS chromatograms (using Superdex peptide column) of fraction after tryptic digestion in denaturing conditions (left); and non-denaturing conditions (right).

It can be said that our experimental procedure allows the detection of non-covalent Cr-complexes and analysis of biological samples by dual SEC-ICP-MS hybride system. The recovery of Cr from the column (ratio of total Cr concentration in the raw extract and in the obtained lyophilizate after chromatographic running with Superdex-75 column) was ca. 50% (49.7% for liver and 55.8% for lung tissue). The survival of the weak non-covalent Cr-complexes is attributed to the extraction procedure which optimized by simulating the physiological conditions and non-destructive nature of size exclusion chromatography. This is the key point for further separation of metal complexed species by other chromatographic mechanisms such as ion exchange or reverse phase and to deeply investigate of Cr-containing proteins at a molecular level by state of the art techniques.

Except Cr, essentiality of the all first-row transition elements from V to Zn has been shown for at least one form of life. Beside of that, a biomolecule containing

each of transition metals has been crystallized and its three-dimensional structure determined (Vincent, 2004). The controversy in chromium biochemistry and the lack of any completely defined chromium specific biomolecule necessitate the further investigation of chromium in biological samples. To the best of our knowledge, this is the first attempt to examine the stability of Cr-containing peptides after specific cleavage of Cr-binding proteins by tryptic digestion in mice liver by SE-HPLC-UV-ICP-MS after *in-vivo* Cr(VI) administration.

4.2.8. Towards identification of Cr-binding proteins and MALDI-TOF-MS analysis

Cr-peptide complexes in the heartcut fractions were simultaneously analyzed by MALDI-TOF-MS following tryptic digestion. Matrix molecules are very important for ionization of analyte without any harm to the target molecule. A neutral type ATT matrix was found more successful for the analysis of Cr-peptide complexes.

MALDI-TOF-MS analysis of the fractions collected at the peak apexes (Fig. 5c) failed to identify the Cr-binding peptides although many peaks were observed in the mass spectra (not-shown). The main reason was that the high abundance of the major Cr isotope (^{52}Cr 83.8%) did not allow the recognition of the Cr isotopic pattern and this to discriminate the Cr-peptide complexes from other species. The mass accuracy (ca. 25-50 ppm) was insufficient to detect the Cr complexes on the basis of the molecular mass either. It seems that identification would only be possible with high mass accuracy mass spectrometers allowing the determination of the empiric formula of the species producing peaks and thus screening them for the presence of chromium.

Nevertheless, the data obtained give clues for the necessity of further study with high resolution mass spectrometers. Despite the fact that insufficient mass accuracy and low resolution in MALDI-TOF-MS spectra, many m/z values were scanned in the spectra of peptides to discriminate the peaks from the trypsin autocatalysis fragments both theoretical and experimental (blank).

When the selected peaks were carefully evaluated, Cr isotopic patterns in some spectra exist. In Figure 4.53 and 54 possible Cr-peptide complexes are shown for F1 and F3.

Considering the isotopes of chromium (^{50}Cr (4.31%); ^{52}Cr (83.76%); ^{53}Cr (9.55%) and ^{54}Cr (2.38%)), MALDI mass spectrum of F1 most probably contains a Cr-peptide peak; because the m/z values of 939, 941 and 942 match the Cr-complex of a peptide with Cr isotopes of $(M+^{50}Cr)^+$, $(M+^{52}Cr)^+$ and $(M+^{53}Cr)^+$ (Figure 4.53).

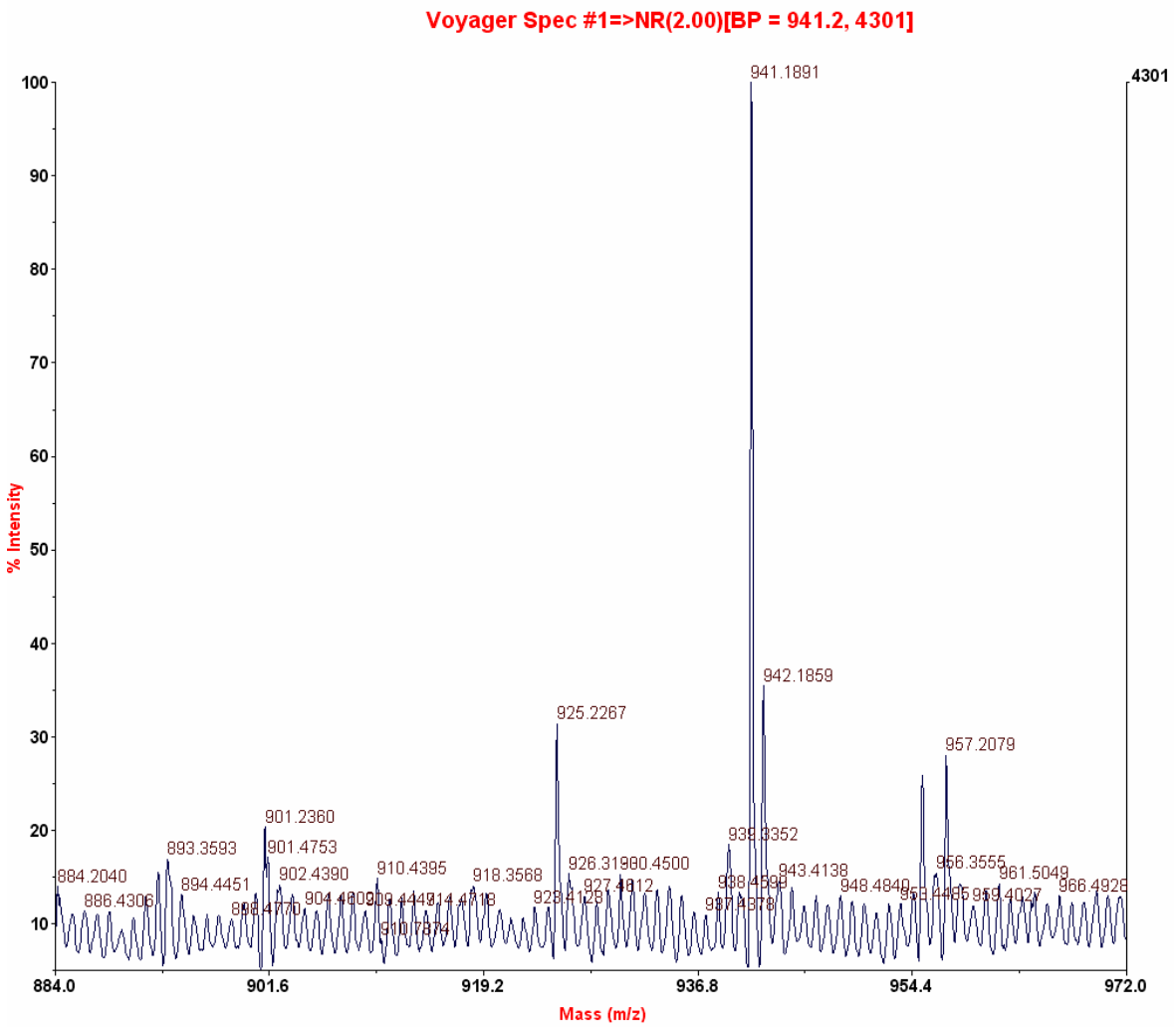

Figure 4.53. MALDI-TOF-MS spectrum (reflector mode) of peptides in F1 after tryptic digestion in native conditions; Matrix used: ATT

Depending on the stochiometry of complex (number of Cr) isotopic disturbance of pattern is expected to change. Thus, the patterns regarding to the complexes bearing more than one Cr ion are quite difficult to determine for Cr existence.

5. CONCLUSION

The administration of Cr(VI) to mice resulted in the accumulation of chromium in the examined organs but mainly in the liver and kidney. Beside of increase in Cr levels of all tissues, statistically significant alterations in Mn, Cu, Fe and Zn levels in many tissues were observed by determining total concentrations of metals by GFAAS or ICP-MS. The obtained results revealed that an exposure to toxic heavy metal affects not also concentration of chromium but also levels of key essential trace elements in mice tissues.

An experimental procedure for the recovery of biomacromolecular metal complexes was developed and applied for obtaining the tissue cytosol of mice. The procedure includes the homogenisation of the tissue samples in liguid nitrogene, releasing the cell content by applying ultrasonic probing and ultracentrifugation of the extract to obtain tissue cytosol. 50 mM of ammonium acetate solution at pH 7.4 was found the most convenient for both as extracting buffer and as mobile phase in the SEC.

Screening of biomacromolecular metal complexes was achieved by injecting the obtained cytosol to the native liguid chromatograpy, i.e., SEC. Detection was performed by a non-destroying UV-VIS detector at the chosen wavelengths following by an ICP-MS as a sensitive species selective detector. The hyphenated technique, HPLC-UV-ICP-MS, has been applied for the fractination of metallic species bound to the different size bioligands. The method allowed the demonstration of the bioinduction of some Cr, Mn, Fe and Zn-binding proteins in different tissues, especially in liver and kidney as a result of Cr(VI) stress.

The obtained data showed that beside of the changes in the biosyntheses of metal-protein complexes, in some cases, hexavalent chromium exposure gives result to the combination of the metal complexes with the other factors which possibly play roles in detoxification processes.

Cr-protein complexes with different molecuar weight have been obtained by heartcut of fractions by following the obtained SEC-UV-ICP-MS chromatogram.

The Cr-peptide complexes could also be recovered after the tryptic digestion of the Cr-protein complexes in denaturing and non-deneturing conditions. The chromatography of obtained Cr-peptide fragments by a second SEC-UV-ICP-MS method indicated the survival of Cr-complexes even after tryptic digestion, but their identification failed because of the impossibility to distinguish Cr complexes from other peaks in MALDI-TOF mass spectra. Higher resolution MS allowing the identification of the presence of Cr and observation of mass fingerprinting on the basis of the accurate molecular mass determination is necessary.

The outputs of this study give evidence to changes in essential elements concentrations provoked by an exposure to heavy metal, Cr(VI), and supply insight into the changes in the molecular size elemental distribution. This is clearly an element of novelty of the thesis which can probably be interpreted by physiologists who may also have an idea of further research in this direction.

Controversy on chromium in terms of biochemistry and essentiality and the lack of any completely defined biological molecule for chromium necessitates the characterization of Cr-binding proteins in livings to elucidate the biological role of chromium. Both very minute abundance of chromium in the tissues and weak characters of non-covalent Cr-complexes make the task very challenging.

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CURRICULUM VITAE

Name, Surname : Serhat Döker

Place of Birth : Turhal

Date of Birth : 25.03.1978

Marital Status : Married

Educational and Academic Position

High School : 1992-1995 Pazar High School, Tokat

B. Sc. : 1995-2000 Department of Chemistry, Anadolu University Eskişehir

M.Sc : 2000-2004 Institute of Sciences, Hacettepe University Ankara

Foreign Language

English

Work Exprience

2002- Onwards : Research Assistant at Hacettepe University, Department of Chemistry