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DEVELOPMENT AND APPLICATIONS OF NEW
ANALYTICAL METHODOLOGIES BASED ON
HPLC-ICP-MS FOR TRACE SPECIATION ANALYSIS
OF SELENIUM IN BIOLOGICAL SAMPLES

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*“The most important
is what I discovered about myself”.*

OVERVIEW

Selenium is an essential trace element for human health. Despite its very low optimum level in the organism, it plays an extremely important and unique role among the (semi)metal elements, because it is the only for which incorporation into proteins is genetically encoded, as constitutive part of the 21th amino acid seleno-cysteine. About 25 seleno-proteins have been identified in the human proteome. Some of them have still unknown biological functions, but others showed to be involved in key processes including antioxidant defence, redox state regulation and a wide variety of specific metabolic pathways. In relation to these actions, the seleno-proteins emerged in recent years as possible biomarkers for several diseases with large occurrence, such as diabetes and many forms of cancer.

Up to the last 90ies, the studies to investigate the association between selenium and human health, moved following two independent directions. Qualitative or semi-quantitative researches were carried out adopting methods proper of biology, biochemistry, immunochemistry and genetics, in order to elucidate the metabolic action of seleno-proteins, and to search for possible associations between their expression levels and altered physiological conditions. On the other side, analytical chemistry methods were used in quantitative studies to correlate the levels of total selenium in the organism to the disease of interest and its clinical parameters.

In the last decade, elemental speciation analysis emerged as a new approach able to extract both qualitative and quantitative information. That method exploits the high selectivity, specificity and sensitivity of high performance liquid chromatography (HPLC) - mass spectrometry (MS) hyphenated techniques to individually determine in a unique analytical run a pool of seleno-proteins. Even if great work has been done for the development of that new analytical methods for speciation of selenium in biological samples, most of the studies focused until now on low molecular weight compounds, including inorganic species, seleno-amino acids and light seleno-metabolites. Extraction of the proteic species and their preservation during sample preparation, storage and analysis, as well as technical problems related to extremely low concentration and matrix effects, strongly limited the improvement of methods for intact seleno-proteins determination. Even less works were carried out to transfer the developed methods to real studies of epidemiological interest.

This Ph.D. project sets in this context, with the double aim of developing new analytical methods for the speciation analysis of intact seleno-proteins in animal samples, but also applying some of them in real pilot studies for epidemiology.

The first part of the research focused on the speciation analysis of human plasma/serum glutathione peroxidase, selenoprotein P and seleno-albumin. Instrumental apparatus based on double affinity-HPLC were explored with the goal of separation system miniaturization, in order to reduce sample amount and time for analysis in respect to the state of the art of the method. Hyphenation to inductively-coupled plasma-mass spectrometry (ICP-MS) was realized to test for the first time the potentiality of detectors such as double focusing sector field-mass spectrometer and quadrupole mass spectrometer equipped with octopole reaction system. A deep optimization and characterization of the methods were carried out. Particular attention was dedicated to the study of species-unspecific isotope dilution analysis as quantification strategy. This approach was investigated in relation to one of the most critical issues in seleno-proteins determination, which is method validation. The almost total lack of pure standards and “speciated” certified reference materials constitutes a key aspect that was addressed in our study by a interlaboratory comparison. The work allowed to provide for the first time the indicative values of individual seleno-proteins concentration in the commercially available BCR-637 human serum.

The experience acquired in plasma/serum seleno-proteins speciation was then successfully applied to two pilot epidemiological studies, where the pattern of these species was investigated in patients affected by type II diabetes and colorectal cancer. The results showed promising correlations between individual seleno-proteins level in serum and presence of the diseases, as well as some clinical parameters. This marks the importance of quantification approaches in epidemiology, and at the same time the urgent need to consider the species individually to bring to light information concerning their specific metabolic action.

In the last part of the project, we moved from plasma/serum samples to the more complex matrix of colon tissue. A new method was developed to speciate selenium in rat colon tissues, optimizing all analytical steps, from proteins extraction and preservation to their separation by HPLC systems coupled to ICP-MS detection. The method allowed to isolate five species of Se, among which glutathione peroxidase types 1 and 2, and thioredoxin reductase type 1 were potentially identified. The

method was finally transferred, with appropriate modifications, to human samples. Promising preliminary results were obtained, showing high potentialities of HPLC-ICP-MS methods for the determination of individual seleno-proteins level also in human colon tissues, which is particularly important for studies regarding the diagnosis, progression and care of colorectal cancer.

LIST OF ABBREVIATIONS

AAS	atomic absorption spectrometry
AcN	acetonitrile
ACR	albumin-to-creatinine ratio
AD	Alzheimer's disease
AE	anion-exchange
AEBSF	-(2-aminoethyl)benzenesulfonylfluoride
AES	atomic emission spectrophotometry
AE-SPE	anion exchange-solid-phase extraction
AF	affinity
ASF	atomic fluorescence spectrometry
AmAc	ammonium acetate
ANCOVA	analysis of covariance
ANOVA	analysis of variance
APCI-MS	atmospheric pressure chemical ionization-mass spectrometry
BEC	background equivalent
BME	β -mercaptoethanol
BMI	body mass index
BSA	bovine serum albumin
CA	correspondence analysis
CAD	coronary artery disease
CC	case-control
CCH	case-cohort
CE	cation-exchange
CHD	coronary hearth disease
CID	collisional induced dissociation
CL	confidence limits
CRC	colorectal cancer
CRF	chronic renal failure
CRM	certified reference material

CS	cross-sectional
CVD	cerebral perfusion disease
CZE	capillary zone electrophoresis
DAD	diode-array detector
DMSe	dimethylselenide
DSHEA	Dietary Supplement Health and Education Act
DTT	dithiothreitol
EAR	Estimated Average Requirement
EC	external calibration
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
EFA	exploratory factor analysis
ELISA	enzyme-linked immunosorbent assay
ESI-MS	electrospray ionization-mass spectrometry
ESI-Q-TOF-MS	electrospray ionization-quadrupole-time of flight-mass spectrometry
ET-AAS	electrothermal - atomic absorption spectrometry
FA	factor analysis
FPG	fasting plasma glucose
GC	gas chromatography
GE	gel electrophoresis
GF-AAS	graphite furnace - atomic absorption spectrometry
GGMSC	γ -glutamyl-Se-methylselenocysteine
GPx	glutathione peroxidase
GSH	reduced glutathione
GS-Se-SG	selenodiglutathione
GSSG	diglutathione
HbA1c	haemoglobin A1c
HBFA	heptafluorobutanoic acid
HCCA	α -cyano-4-hydroxycinnamic acid
HDL	high-density lipoprotein
HG	hydride generation
HPLC	high-Performance Liquid Chromatography
HR	high resolution
HSA	human serum albumin
IAA	iodoacetamide
ICP-MS	inductively coupled plasma-mass spectrometry
IDA	isotope dilution analysis
iNOS	inducible nitric oxide synthase
IP	ion-pairing
KED	kinetic energy discrimination
K-S test	Kolmogorov-Smirnov test
LA	laser ablation
LDL	low-density lipoprotein
LOH	loss of heterozygosity
LR	logistic regression
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
MCA	multiple correspondence analysis
MCMS	multiple collector mass spectrometer
MD%	mass bias per mass unit
MeOH	methanol

MIP-AES	microwave induced plasma atomic emission spectrometry
MR	medium resolution
MSe	methylselenol
Mw	molecular weight
NCC	nested case-control
NHRMC	National Health and Medical Research Council
NOAEL	No Observed Adverse Effect Level
NR	Normative Requirement Estimate
OLS	ordinary least squares
OES	optical emission spectrophotometry
ON-IDA	on-line isotope dilution analysis
OR	odds ratio
ORS	octopole reaction system
PBIT	S,S'-(1,4-phenylenebis[1,2-ethanediyl])bisisothiourea
PCA	principal component analysis
PD	Parkinson's disease
PhB	phosphate buffer
PLE	pressurized liquid extraction
PMSF	phenylmethanesulfonylfluoride
PPAR γ	peroxisome proliferator-activated receptor gamma
PRI	Population Reference Intake
PTPs	tyrosine phosphatases
PVD	vascular perfusion disease
QMS	quadrupole mass spectrometer
RBCs	red blood cells
RCHT	radiochemotherapy
RDA	Recommended Dietary Allowance
RF	radiofrequency
RNI	Reference Nutrient Intake
ROS	reactive oxygen species
RP	reversed phase
RR	relative risk
RSD	relative standard deviation
RTILs	room-temperature ionic-liquids
SA	survival analysis
SCCH	stratified case-cohort
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SeAlb	seleno-albumin
SE	standard error
SEC	size-exclusion chromatography
SeCys	seleno-cysteine
SeIP	seleno-protein P
Se-MCys	SeMethylSeCysteine
SeMet	seleno-methionine
SeOMet	seleno-methionine Se-oxide
Se-PAPS	Se-isologue of 3-phosphoadenosine 5-phosphosulfate
SFMS	sector field mass spectrometer
SPME	solid phase microextraction

SSIDA	species-specific isotope dilution analysis
S-W test	Shapiro-Wilk test
TB	Tris-HCl extraction buffer
TBD	Tris-HCl extraction buffer with detergent
TFA	trifluoroacetic acid
TIMS	thermal ionization mass spectrometry
TMAH	tetramethylammonium hydroxide
TMSe	trimethylselenide
TNM	tumour node metastasis
Tris	Tris(hydroxymethyl)aminomethane
TrxR	thioredoxin reductase
Trxs	oxidized thioredoxins
UL	upper level
UPLC	ultra performance liquid chromatography
UV	ultraviolet
WHO	World Health Organization

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I. BIOCHEMISTRY OF SELENIUM

I.1. Introduction

It has long been known that the proper functioning of life is critically dependent on trace elements in a number of different ways. Some metals (e.g. Hg, Pb) and metalloids (As) are highly toxic, whereas others (e.g., Mo, Mn, Fe, Co, Cu, Zn, Se), are considered essential for life processes [1]. A number of other elements (e.g., V, Cr, Ni) are recognized as carrying beneficial effects in some specific chemical forms and at low concentration.

Biological systems are very complex environments, rich of a large variety of ligands, and free metal ions are of low probability occurrence. The essential or toxic action of metals and metalloids is highly controlled by complexing the metal ion by electron-pair donating biological ligands [2]. The chemical form in which the metal is complexed determines its uptake, transport, accumulation and depletion.

The biomolecular ligands range within a large interval of molecular weight. They include citrate, tartarate and oxalate [3,4]; amino acids, oligo- and polypeptides (proteins) among which ferritin, β -amylase, alcohol dehydrogenase, carbonic anhydrase, metallothioneins and phytochelatin [5-7]; heterocyclic nucleobases, oligo- and polynucleotides, and -nucleosides [8,9]; macrocycling chelating molecules (i.e. chlorophyll and products of its degradation [10,11], cobalamins, and porphyrins [12] including the heme group of haemoglobin, myoglobin, cytochromes and peroxidases);

polysaccharides and glycoproteins. A further class of complexes includes exogenous metallodrugs recently introduced for diagnostic and therapeutic proposals, and their metabolites [13].

A distinct category includes biosynthesized molecules with the metal(metalloid)-carbon bond. Some of these molecules are known and widely investigated from several decades, as is the case of coenzyme B₁₂ (Co-corrin unit), but only recently an increasing number metal-carbon bonds in biomolecules have been proved [14]. Metalloid-carbon bonds are also present in seleno-amino acids and their higher analogues (selenogluthathione and Se-proteins), selenosugars, arsenobetaine and arsenosugars [2,13,15].

Among the essential elements, selenium (Se) has received a special attention in the last years because it is present as an essential component in the active site of mammalian enzymes, named for that seleno-enzymes. Several studies have shown that by means of these proteins, Se is involved in many fundamental biochemical processes, and is associated with incidence and progression of topical human diseases. Thus, the interest in Se-species identification and characterization increased considerably in a wide range of biochemical, nutritional and medical studies.

This chapter introduces the general characteristics of Se chemistry and biochemistry, with particular attention to its metabolic routes in mammals. Selenium-containing proteins and Se-proteins identified in human/rat proteomes are described in details regarding their specific catalytic activity and function. Particular emphasis is given to the glutathione peroxidases 1, 2 and 3, thioredoxin reductase 1, Se-protein P and seleno-albumin, which were studied in this Ph.D. project.

I.2. Biochemistry of selenium

Selenium (Se) is an element of the group 16 and period 4. It has four natural oxidation states: (0) elemental Se, (-2) selenide, (+4) selenite and (+6) selenate. The chemical and physical properties of Se are very similar to those of S as they belong to the same group in the periodic table. These two elements present similar outer valence-shell electronic configuration, atomic size, bond energies, ionization potential and electron affinity.

As Se has many chemical behaviours shared with S, it is easily incorporated in biomolecules instead of it. Nevertheless, the two elements are not fully

interchangeable because some key properties are significantly different. In biological systems, Se can replace S in the amino acid cysteine by means of a selenolic (-SeH) group, forming SeCysteine (SeCys). Selenols have stronger acidity compared with thiols (-SH) by up to three orders of magnitude [16,17] and hence are negatively charged at physiological pH [18], while thiols are mainly neutral. Moreover, the bond stability of organo-Se-compounds is lower than in the organo-S- analogues, resulting in a decreased stability. As a consequence, organo-Se-compounds are more reactive, easily oxidized, more liable to decomposition due to the presence of air and light [19]. SeCysteine is also much more susceptible to degradation under acid hydrolysis conditions than its sulfur analogue.

Several organo-Se-compounds have proved to play an important role in cell biochemistry and in nutrition. The main species of biochemical interest are reported in attachment in Table A - 1 at p. 320.

I.3. Selenium metabolism in mammals

The general metabolism of Se in mammals is schematically represented in Figure I-1. The primary metabolic pathway for the assimilation of Se intake was proposed by Ip [20] and consists of the reduction of the different species to hydrogen selenide (SeH⁻). This specie plays the role of central gateway for both utilization (into Se-proteins) and excretion of Se. Selenium excess detoxification occurs through a mechanism of sequential methylation into dimethylselenide (DMSe), excreted into the breath, and selenosugars and trimethylselenide (TMSe), excreted into the urine.

The absorption of Se occurs mainly in the lower part of the small intestine. All forms of Se, inorganic as well as organic, are readily absorbed with an overall efficiency about 80 %. There are differences, however, between levels of absorption for the different species of the element. Organic compounds are generally absorbed more efficiently than inorganic forms, with uptake from the gastrointestinal tract of more than 90 % for seleno-methionine (SeMet) compared to 60 % for selenite [21,22].

The inorganic forms of assumed Se comprise selenite and selenate. After absorption, selenite is reduced to H₂Se following two possible ways. As first option, it can react non-enzymatically with reduced glutathione (GSH) in the red blood cells (RBCs) to form selenodiglutathione (GS-Se-SG, path a, Figure I-1) [23]:



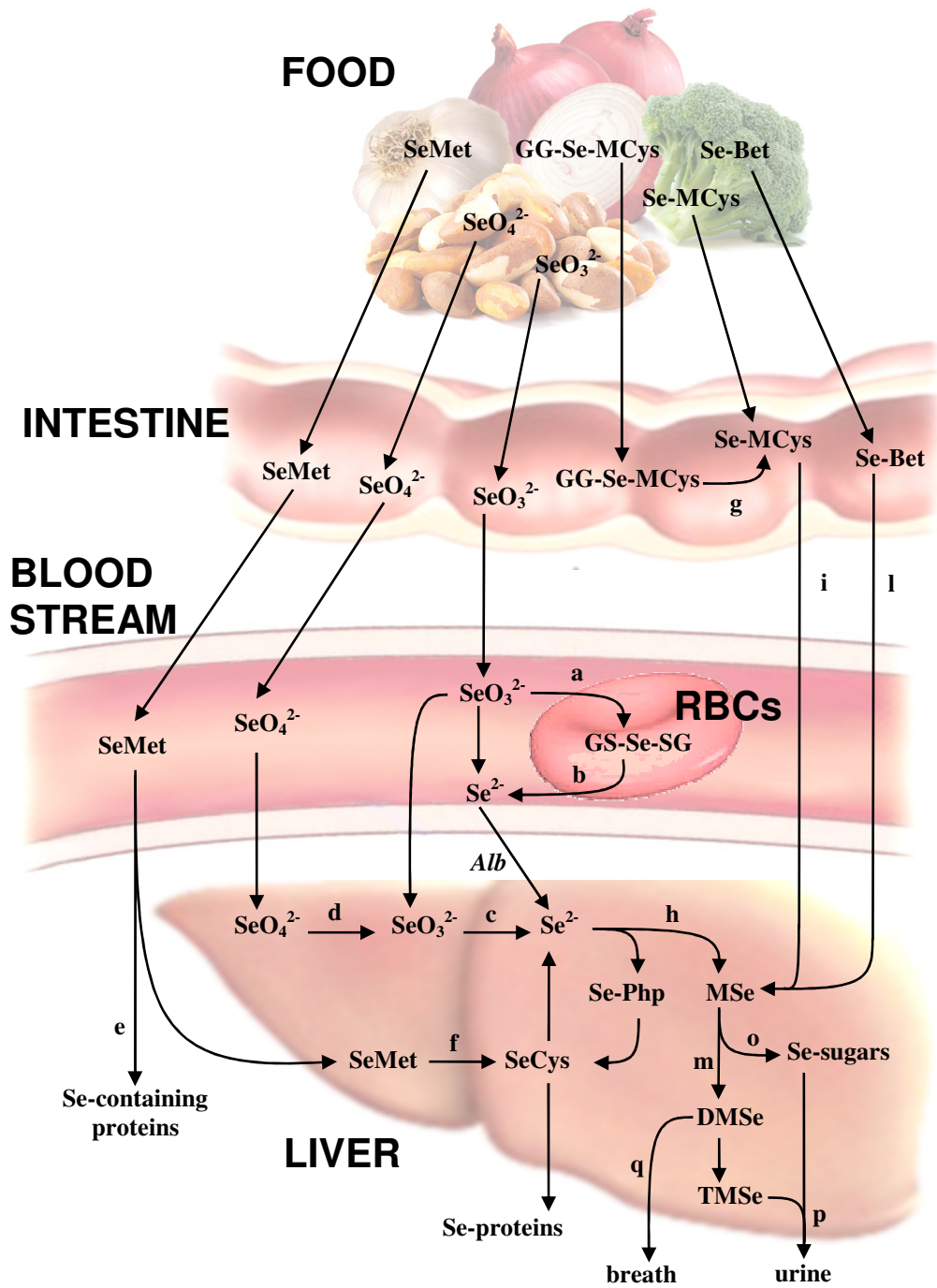
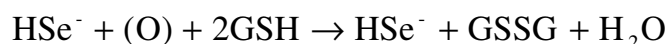
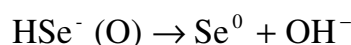
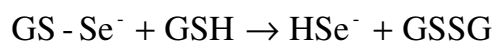
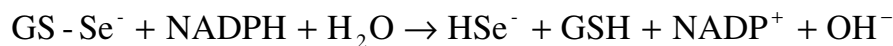
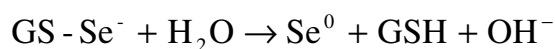
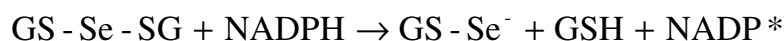


Figure I-1 Schematic representation of Se metabolism in mammals, adapted and integrated from Rayman [1].

GS-Se-SG is subsequently decomposed by glutathione reductase into hydrogen selenide following the steps (path b):



Alternatively, selenite can be a substrate for the thioredoxin system (thioredoxin, NADH and thioredoxin reductase) and directly reduced to hydrogen selenide (path c) following a reactions path similar to that reported above for glutathione reductase [23]. The diglutathione (GSSG) is not a substrate for thioredoxin reductase and is a poor disulfide substrate for reduced thioredoxin. Nevertheless, the insertion of a Se atom renders this compound a highly reactive substrate for the thioredoxin system, capable of redox cycling in the presence of oxygen [23]. Rather, selenate is reduced to selenite (path d), as in sulfate reduction, by ATP sulfurylase *via* the still unidentified Se-isologue of 3-phosphoadenosine 5-phosphosulfate (Se-PAPS) [24], then selenite is transformed into selenide as exposed above (path c).

Among the organic forms of Se assumed with the diet, Se-methionine (SeMet) is the main specie. Ingested SeMet is absorbed into the small intestine *via* the Na⁺-dependent neutral amino acid transport system [25]. Se-methionine can be incorporated non-specifically into proteins such as albumin and haemoglobin by replacing methionine (path e) [21,26], as will be better exposed in the section I.4. Alternatively, it can be transformed into SeCys (path f) and then into hydrogen selenide (path g) *via* the so-called trans-selenation pathway, similar to the trans-sulfuration pathway [27,28], represented in Figure I-2.

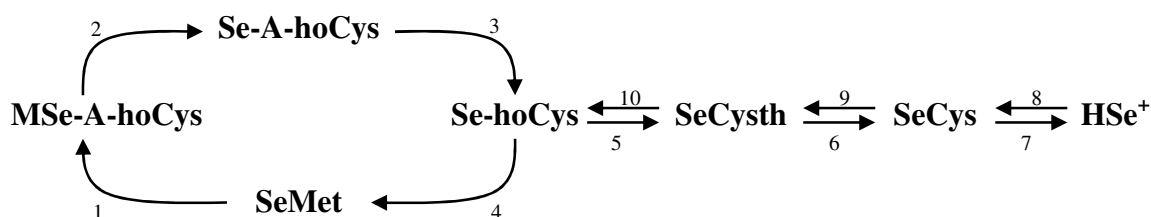


Figure I-2 Se compounds generated by the trans-selenation pathway. Enzymes involved are: 1, SAM synthetase; 2, methyl transferase; 3, SAH hydrolase; 4, methionine synthase; 5, cystathionine β -synthase; 6, cystathionine γ -lyase; 7, cysteine lyase; 8, cysteine synthase; 9, cystathionine γ -synthase; 10, β -cystathionase.

γ -Glutamyl-Se-methylselenocysteine (GGMSC) has been reported to be present in garlic [29,30], *Astragalus bisulcatus* [31] and *Melilotus indica* L [32]. This Se-dipeptide can be assumed to serve mainly as a carrier of SeMethylSeCysteine (Se-MCys). In fact, after ingestion as a dietary constituent, the bulk (not necessarily entirely) of GGMSC is hydrolyzed by γ -glutamyl transpeptidase in the gastrointestinal tract (path g), releasing Se-MCys for absorption and systemic delivery to other tissues [33]. γ -Glutamyl-Se-methylselenocysteine is quantitatively absorbed from the gastrointestinal tract like Se-MCys, while the urinary excretion is apparently the major route for eliminating the excess of Se from GGMSC.

Selenium is mostly excreted into urine (path p) after being methylated stepwise from Se^{2-} (path h), selenobetaine (path i) and Se-MCys (path j) to methylselenol (MSe). Urinary metabolites are known to be monomethylates Se and TMSe. Even if the monomethylated Se specie was initially supposed to be directly MSe, it was finally identified as the selenosugar SeMethyl-*N*-acetylgalactosamine (path p) [34-36]. The ratio of the two Se metabolites in urine changes depending on the Se dose: at lower doses Se is excreted mostly in the form of SeMethyl-*N*-acetylgalactosamine, while at higher doses TMSe increases [37]. In addition, excessive Se can be excreted not only into urine but also into breath in the form of DMSe (path q) [38].

Liver is the foremost organ in Se metabolism, since it synthesizes its intrinsic Se-proteins as well as the secreted Se molecules and excretory metabolites. The Se-protein P (SeIP), produced into the liver, is released into the bloodstream and is responsible for the distribution of Se in the other organs, as is extensively exposed in the paragraph I.5.3. The whole-body Se distribution appears to be regulated in the liver by the allocation of metabolically available Se between the pathways of Se-proteins synthesis and the excretory metabolite synthesis [39]. This process might be passive, with the part of Se that is incorporated into SeIP being destined for retention by the organism, and the part of Se that cannot be accommodated by the Se-proteins synthesis pathway entering the excretory pathway. Active regulation of excretory metabolite synthesis is also possible. Synthesis of intrinsic liver Se-proteins would not be expected to affect the Se pool because their catabolism would return the element to it.

I.4. Selenium-containing proteins

Cereals and forage crops convert Se mainly into SeMet and incorporate it into proteins in place of (sulphur) methionine (Met), because transfer Ribonucleic Acid for methionine (tRNA^{Met}) does not discriminate between Met and SeMet. The replacement of Met by SeMet as a rule does not significantly alter proteins structure but may influence the activity of enzymes if the replacement occurs in the vicinity of the active site. Since the CH₃-Se group of SeMet is more hydrophobic than the CH₃-S-moiety of Met, substrate access may be affected, altering the kinetic parameters. Se-methionine Se-oxide (SeOMet) is easily reduced back to SeMet by glutathione (GSH), therefore the oxidative damage to SeMet is reversible. On the basis of this observation, SeMet and GSH were suggested to act as an antioxidant system, protecting cells against oxidants such as peroxynitrite [40]. However, it is still not certain whether this occurs *in vivo*. Se-methionine showed also to have radioprotective properties [41] and to protect against UV-light-induced skin damage in mice [42].

Higher animals are unable to synthesize SeMet because they do not have an efficient mechanism for Met synthesis. A controversial issue thus arises whether SeMet has specific essential or beneficial functions in the organism. SeMethionine is incorporated into erythrocytes mainly bounded to haemoglobin [43], in plasma in the albumin fraction (SeAlb), and is significantly retained also by brain [44]. The incorporation of SeMet into body proteins allows Se to be stored and reversibly released by normal metabolic processes, thus offering an advantage over other Se compounds. The reported average whole-body half-life of SeMet in humans is 252 days, indicating that SeMet is utilized and reutilized extensively [45,46]. SeMet was also detected in human milk [47]. In nursing mothers, SeMet or Se yeast prevented the decline of plasma Se and glutathione peroxidase activity as well as the decline of Se in milk. Significantly more Se appeared in milk of mothers consuming SeMet than selenate [48,49]. Supplemental SeMet, compared with selenite or selenate, has also differential effects on lymphocyte proliferation and other immunological variables [50,51]. However, despite to the promising evidences, the physiological relevance of SeMet and Se-containing proteins remains still unclear [52].

I.4.1. Seleno-albumin

Human serum albumin (HSA) is a ~66 kDa monomeric protein (see the amino acid sequence in Figure I-3) which constitutes the most abundant protein in human plasma (~640 μM) [53]. Human serum albumin is synthesized in the liver and secreted into the bloodstream, where circulates for 2-3 weeks before being degraded and replaced [54], but is found in some degree in every fluid of the body.

Even if comprises 60 % of the mass of plasma proteins, HSA is responsible for 80 % of the colloid osmotic pressure of the plasma, thus plays a fundamental role in maintaining the osmotic pressure of the circulatory system [55]. It supplies also the most of the acid/base buffering action of the plasma proteins [56].

Human serum albumin is characterized by the propriety to bind a large variety of biologically active molecules. Most strongly bound are hydrophobic organic anions of medium size (100 to 600 Da) such as long-chain fatty acids, haematin, and bilirubin. Smaller and less hydrophobic compounds such as tryptophan and ascorbic acid are held less strongly, but their binding can still be highly specific [57]. Human serum albumin can also bind cations including Cu^{+2} , Ni^{+2} , Ca^{+2} , Mg^{+2} , Zn^{+2} , Cd^{+2} and Hg^{+2} [57]. Furthermore, a wide variety of drugs has two primary sites which overlap with the binding locations of endogenous ligands. Due to these peculiar properties, HSA fundamental function is as carrier for such a variety of endogenous and exogenous compounds in serum [58].

```

MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE
ENFKALVLIA FAQYLQQCPF EDHVKLVNEV TEFKTCVAD
ESAENCCKSL HTLFGDKLCT VATLRETYGE MADCCAKQEP
ERNECFLOHK DDNPNLPRLV RPEVDV MCTA FHDNEETFLK
KYLVEIARRH PYFYAPELLF FAKRYKAAFT ECCQAADKAA
CLLPKLDLDR DEGKASSAKQ RLKCASLQKF GERAFAKAWAV
ARLSQRFPKA EFAEVSKLVT DLTQVHTECC HGDLLCADD
RADLAKYICE NQDSISSKLL ECCEKPLLEK SHCIAEVEND
EMPADLPSLA ADFVESKDVC KNYAEAKDVF LG MFLYEYAR
RHPDYSVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE
FKPLVEEPQN LIKQNCLEFE QLGEYKFQNA LLVRYTKKVP
QVSTPTLVEV SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV
LNQLCVLHEK TPVSDRVTCK CTESLVNRPP CFALEVDET
YVPKEFNAET FTFHADICTL SEKERQIKKQ TALVELVKHK
PKATKEQLKA VMDDFAAFVE KCKKADDKET CFAEEGKKLV
AASQAALGL

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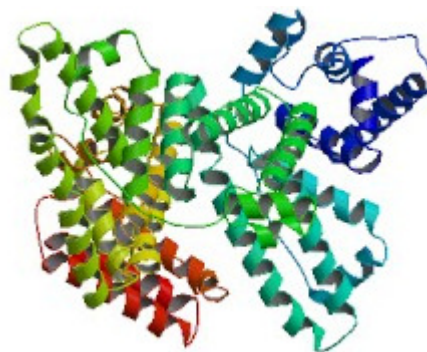


Figure I-3 Amino acid sequence and crystal structure of HSA. The Met residues potentially exchanged to SeMet residues are marked in gray.

In addition to the transport function, HSA acts as a toxic waste handler. It gathers bilirubin from sites of haemoglobin breakdown and delivers it to the liver for excretion, binds and delivers haematin to the liver when its primary vehicle is saturated, and sequesters several exogenous toxins [57].

As mentioned in the paragraph I.3.3., SeMet can replace Met in HSA in six different sites of the amino acids chain, marked in Figure I-3, forming SeAlb.

I.5. Se-proteins

As mentioned before, Se is a component of the active site of several proteins, named for this Se-proteins, which play essential biological functions. In Se-proteins Se is incorporated in the form of the amino acid SeCys. Differently from SeMet, SeCys is recognised as the 21st amino acid because its synthesis is genetically encoded in the ribosome-mediated system.

About 25 Se-proteins have been identified in human proteome [59] and 24 in rat and mouse proteome [60]. The glutathione peroxidases (GPxs) family in mammals comprises at least 6 isoenzymes with general antioxidant function that protect the organism from the oxidative stress by catalyzing the reduction of reactive oxygen species (ROS) at the expenses of GSH [61]. Iodothyronine deiodinases are 3 different enzymes that play an important role in the physiological functions by catalyzing the activation and inactivation of thyroid sulphated hormones [62]. Thioredoxin reductases belong to the pyridine nucleotide-disulphide oxidoreductase family. These enzymes catalyze the reduction of thioredoxins by NADPH and are involved in redox regulation of transcription factors, regulation of apoptosis and immunomodulation [63]. Selenophosphate synthetase is responsible for the regulation of the other Se-proteins production by means of the catalysis of selenophosphate synthesis. Blood serum SelP is mainly responsible for the Se transport through the whole body. Several other Se-proteins have been identified in specific tissues and associated with metabolic function of specific organs, but most of them have a biochemical role which is still unclear [61].

In the next paragraphs are described in details the characteristics, distribution and biochemical/physiological functions of the Se-proteins that have been specifically investigated in this Ph.D. project. All the human Se-proteins are also presented in attachment in Table A - 2 at p. 324.

I.5.1. Glutathione peroxidases family

Glutathione peroxidases (GPxs) are a family of enzymes with antioxidant functions. Eight isoforms are known, but only five of them contain SeCys residues and can catalyze the reduction of hydrogen peroxide and lipid hydroperoxides using glutathione (GSH) as a reducing cofactor [52]. This group comprises the classical cytosolic GPx (GPx1), gastrointestinal GPx (GPx2), plasma GPx (GPx3), phospholipid hydroperoxide GPx (GPx4) and the olfactory epithelium GPx (GPx6).

All the enzymes belonging to this group present a SeCys residue which is oxidized by the peroxide to a selenenic acid, consecutively reduced back to the selenolate by thiols according to the scheme in Figure I-4. The selenolic group of GPx catalytic site is included into a catalytic triad of SeCys-Trp-Gln residues where it is both stabilized and activated by hydrogen bonding as represented in Figure I-4 [63].

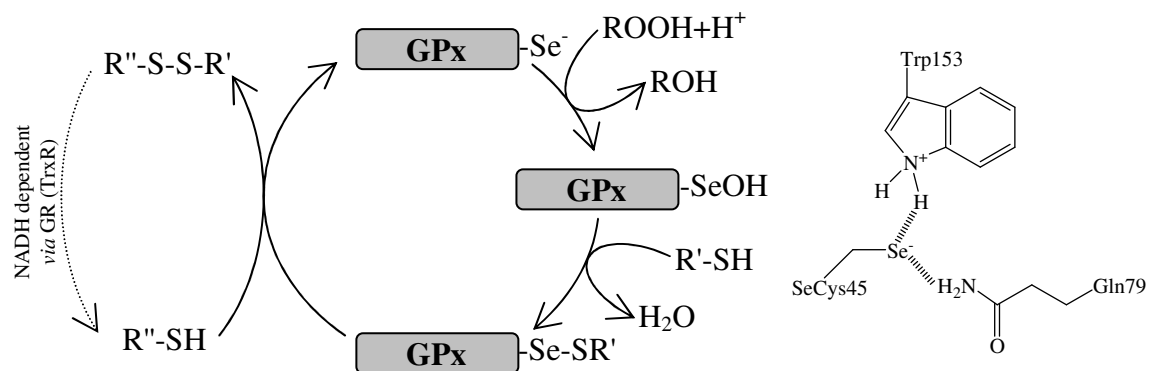


Figure I-4 Reaction scheme of GPx (left) and active site of the protein showing the catalytic triad (right).

GPx1 is a ubiquitous homotetrameric protein located in cytosol and mitochondria, which presents a SeCys residue in position 47 of the amino acid sequence (Figure I-5).

```
MSAARLSAVA QSTVYAFSAR PLAGGEPVSL GSLRGKVLII
ENVASLGGTT TRDYTEMNDL QKRLGPRGLV VLGFPNCQFG
HQENGKNEEI LNSLKYVRPG GGFEPNFTLF EKCEVNGEKA
HPLFTFLRNA LPAPSDDPTA LMTDPKYIIW SPVCRNDISW
NFEKFLVGPD GVPVRRYSRR FRTIDIEPDI EALLSKQPSN
P
```



Figure I-5 Amino acid sequence and crystal structure of human GPx1 monomer. The UGA codon encoding for the SeCys residue is marked in gray.

GPx1 enzyme utilizes exclusively GSH as reducing substrate for the reduction of H₂O₂ and a limited number of organic hydroperoxides including cumene hydroperoxide and *tert*-butyl hydroperoxide [52]. Among its family members, GPx1 is one of the most highly sensitive to changes in both Se status [64] and oxidative stress conditions [65], but evidence suggests that global protein synthesis is reduced under conditions of stress as a means of reserving cellular resources and that GPx1 recovers most rapidly compared to other Se-proteins [59].

GPx2 is a secreted homotetrameric enzyme mainly expressed in the gastrointestinal system mucosa, including the squamous epithelium of the esophagus and, in humans, is also detectable in liver [66]. Its expression in the intestine is not uniform, but it is higher in the crypt grounds and decreases gradually toward the luminal surface, suggesting a role in proliferating cells [67]. The function of GPx2 is mainly to protect intestinal epithelium from oxidative stress. GPx2 exhibits a substrate specificity similar to that of GPx1, that includes hydrogen peroxide, *tert*-butyl hydroperoxide, cumene hydroperoxide, and linoleic acid hydroperoxide, but not phosphatidylcholine hydroperoxide [68]. The expression of GPx2 is much more resistant than GPx1 or GPx3 to dietary Se deficiency [68]. GPx2 location and resistance suggest that this Se-protein may serve as a first line of defence in exposure to the oxidative stress induced by ingested prooxidants or gut microbiota. The SeCys residue of GPx3 is located in position 40 of the amino acid sequence (Figure I-6).

```
MAYIAKSFYD LSAIGLDGK IDFNTRGRA VLIENVASL
GTTTRDYTQL NELQCRFPRR LVVLGFPCNQ FGHOENCQNE
EILNSLKYYR HGGGFQPTFS LTQKCDVNGQ NQHPVFAYLK
DKLPYPYDDP FSLMTDPKLI IWSPVRRSDV SWNFEKFLIG
PEGEPFRYS RTFQTINIEP DIKRLKVAI
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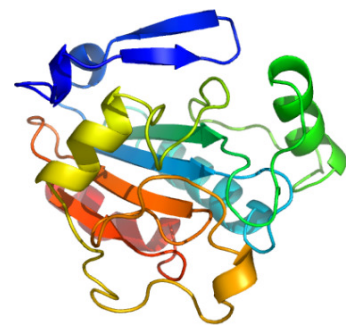


Figure I-6 Amino acid sequence and crystal structure of human GPx2 monomer. The UGA codon encoding for the SeCys residue is marked in gray.

GPx3 is the only extracellular enzyme of the GPxs family. This 90 to 100 kDa glycosylated homotetrameric protein is produced into the cells of the proximal tubular epithelium and in the parietal cells of Bowman's capsule of the kidney, and then secreted into the plasma, where it constitutes approximately 15-20 % of the total Se. Both GPx3 protein and mRNA are also expressed in several tissues, particularly heart and thyroid gland, where this enzyme may play the role of a local source of extracellular antioxidant capacity. The SeCys residue of GPx3 is located in position 73 of the amino acid sequence (Figure I-7).

```
MARLLQASCL  LSLLLAGFVS  QSRGQEKSKM  DCHGGISGTI
YEGALTIDG  EEYIPFKQYA  GKYVLFVNVA  SYUGLTGQYI
ELNALQEELA  PFGLVILGFP  CNQFGKQEPG  ENSEILPTLK
YVRPGGGFVP  NFQLFEKGDV  NGEKEQKFYT  FLKNSCPPTS
ELLGTSDFLF  WPEMKVHDIR  WNF EKFLVGP  DGIPIMRWHH
RTTVSNVKMD  ILSYMRRQAA  LGVKRK
```



Figure I-7 Amino acid sequence and crystal structure of human GPx3. The UGA codon encoding for the SeCys residue is marked in gray.

Differently by GPx1, GPx3 presents a more restricted hydroperoxide substrate specificity. Although it can reduce H_2O_2 and the same organic hydroperoxides, its activity is ~10 fold lower than the activity of GPx1. Considering that GSH is a poor reducing substrate for GPx3 and also the low concentration of reduces thiol groups in human plasma, most likely the extracellular thioredoxin reductases, thioredoxin or glutaredoxin can be reducing substrates better than GSH [69]. As a secreted protein, GPx3 is proposed to be a major scavenger of reactive oxygen species (ROS) in the extracellular space and within the vasculature.

I.5.2. Thioredoxin reductases

The thioredoxin reductases (TrxRs) are homodimeric enzymes belonging to the flavoprotein family of pyridine nucleotide-disulphide oxidoreductases that includes lipamide ehydrogenase, glutathione reductase and mercuric ion reductases [70]. The predicted molecular mass for the human TrxR1 monomer is 54.6 kDa, but mass-spectral analyses of the enzyme have given masses ranging from 54.6 kDa for purified

placental to 56 kDa for TrxR1 isolated from T cells [71]. This suggests that the human enzyme has not undergone post-translational glycosylation. On the contrary, rat liver TrxR1 has an N-linked glycosylation consensus sequence (Figure I-8) that is not present in the human enzyme [72].

```

MNDSSKDAPKS YDFDLIIIGG GSGGLAAAKE AAKFDKKVMV
LDFVTPPLG TNGGLGGTCV NVGCIPKKLM HQAALLGOAL
KDSRNYGWL EDTVKHDWEK MTESVQNHIG SLNWGYRVAL
REKKVYENA YGKFIGPHKI MATNNGKEK VYSAERFLIA
TGERPRYLG PGDKEYCISS DDLFSLPYCP GKTLVVGASY
VALECAGFLA GIGLDVTVMV RSILLRGFDQ DMANKIGEEM
EEHGKIFIRO FVPTKIEQIE AGTPGRLKVT AKSTNSEETI
EDEFNTVLLA VGRDSCRTI GLETVGVKIN EKTGKIPVTD
EEQTNVPYIY AIGDILEGKL ELTPVAIQAG RLLAQRLYGG
STVKCDYDNV PTTVFTPLEY GCCGLSEEKA VEKFGREENIE
VYHSFFWPLE WTVPSRDNNK CYAKVICNLK DNERVVGPHV
LGNAGEVTO GFAAAKCGLT KQQLDSTIGI HPVCAEIFTT
LSVTKRSGGD ILOSGCUG

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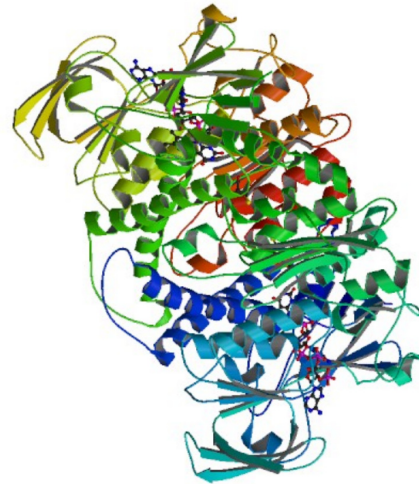


Figure I-8 Amino acid sequence and crystal structure of rat TrxR1. The UGA codon encoding for the SeCys residue is marked in gray, while the N-linked glycosylation consensus sequence is signed in the gray rectangle.

A second human TrxR, TrxR2, has recently been cloned [73,74]. It has a predicted molecular mass of 56.2 kDa and presents a 33-amino-acid N-terminal extension identified as a mitochondrial import sequence.

Like in the other enzymes of the flavoprotein family, each monomer of TrxRs includes a FAD prosthetic group, a NADPH binding site and an active site containing a redox-active disulphide. Electrons are transferred from NADPH *via* FAD to the active site disulphide of TrxR, which then reduces the substrate [70]. TrxRs specifically reduce oxidized thioredoxins (Trxs), a group of small (10 ± 12 kDa) ubiquitous redoxactive peptides that supply reducing equivalents to enzymes such as ribonucleotide reductase, thioredoxin peroxidase [75], and some transcription factors, resulting in their increased binding to DNA and altered gene transcription [76]. Mammalian Trxs have also been shown to act as cell growth factors and to inhibit apoptosis [73,77]. Since TrxRs are the only class of enzymes known to reduce oxidized Trx, it is possible that alterations in TrxR activity may regulate some of the activities of Trxs.

In addition to Trxs, other endogenous substrates have been demonstrated for TrxRs, including lipoic acid [78], lipid hydroperoxides [79], the cytotoxic peptide NK-lysin [80], vitamin K₃ [81], dehydroascorbic acid [82], the ascorbyl free radical [83] and the tumour-suppressor protein p53 [84]. However, the physiological role that TrxRs play in the reduction of most of these substrates is unknown. Some of the most likely functions for TrxRs are summarized in Figure I-9.

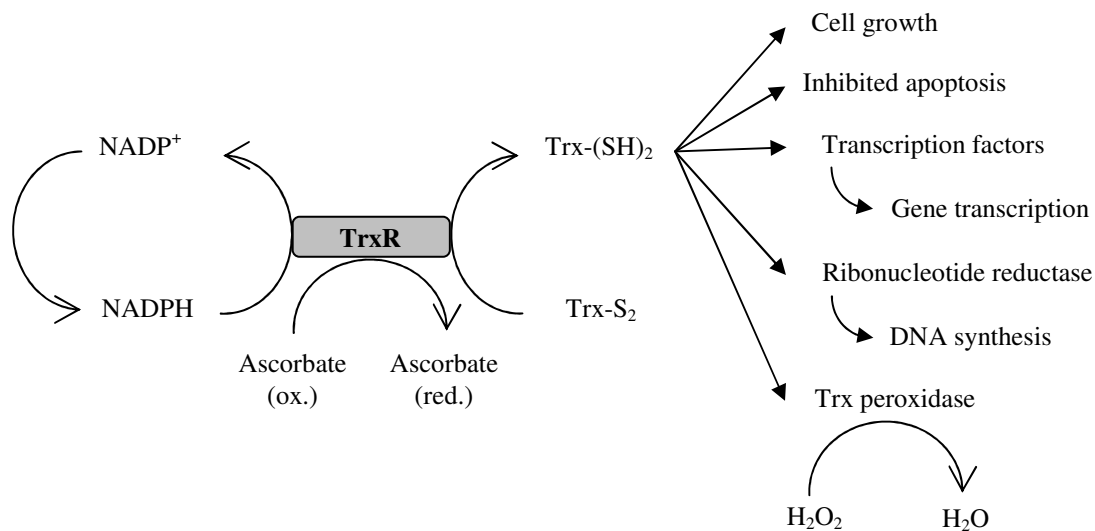


Figure I-9 Reaction scheme and biological functions of TrxR in the cell.

The ability of TrxR to reduce the ascorbyl free radical suggests that TrxR may play an additional action through the recycling of ascorbate. Humans lack the ability to synthesize ascorbic acid, an important antioxidant in the protection of cells from oxidative stress; therefore dietary intake and the recycling of ascorbate from its oxidized forms (dehydroascorbic acid and the ascorbyl free radical) are essential for maintenance of ascorbate levels. The relation between TrxR level and ascorbate cycle has been demonstrated by the observation that maintenance of rats on a Se-deficient diet results in decreased liver ascorbate, glutathione peroxidase and TrxR level [82].

Gene expression for TrxR1 and Trx are showed to increase in the lungs of newborn baboons exposed to air or O₂ breathing [85] and in adult baboon lung explants in response to 95 % O₂. These effects suggest that TrxR may play a protective role against O₂ breathing in the mammalian lung. There have also been reports that TrxR is highly expressed on the surface of human keratinocytes and melanocytes [86],

where it may provide the skin's first line of defence against free radicals generated in response to UV light [87].

The function of TrxR2 is unknown, but it may play a role in protecting the cell against mitochondrial-mediated oxidative stress, an idea that finds credence with the recent identification of a unique mitochondrial form of Trx [88].

Alterations in dietary Se intake affect TrxRs activity due to variation of Se incorporation into the enzyme without a variation in protein synthesis [89]. The consequences of alterations in TrxR Se content have not been thoroughly investigated.

I.5.3. Se-protein P

Se-protein P is a secreted glycoprotein that contains the major part of Se in plasma/serum [90]. The protein has a Mw of about 57 kDa [59] and contains 10 SeCys residues in rats, mice, and humans (Figure I-10) [91].

```

MWRSLGLALA LCLLPSSGTE SQDQSSLCKQ PPAWSIRDQD
PMLNSNGSVT VVALLQASUY LCITQASKLE DLRVKLKKEG
YSNISYIVVN HQGISSRLKY THLKNKVSEH IPVYQQEENQ
TDVWTLNLSG KDDFLIYDRC GRLVYHLGLP FSFLTFPYVE
EAIKIAYCEK KCGNCSLTTL KDEDFCRVS LATVDKTVET
PSPHYHHEHH HNHGHQHLGS SELSENQQPG APNAPTHPAP
PGLHHHHKHK GQHRQGHPEP RDMPASEDLQ DLQKKLCRKR
CINQLLCKLP TDESELAPRSU CCHCRHLIFE KTGSAITUQC
KENLPSLCSU QGLRAEENIT ESCQURLPPA AUQISQQLIP
TEASASURUK NQAKKUEUPS N

```

Figure I-10 Amino acid sequence of human SeIP. The UGA codons encoding for the SeCys residues are marked in gray; the heparine-binding site is the underlined sequence; the N-linked glycosylation sites are signed in the gray rectangles.

A heparin-binding site in the N-terminal domain that contains histidine as 2 of its 5 basic residues give to SeIP the property to bind heparin. That binding is possible only when the pH is below the normal physiologic range, when the histidine becomes protonated. SeIP is a glycosylated protein since it presents 3 occupied N-glycosylation sites and 1 occupied O-glycosylation site [92]. Several disulfide and selenenylsulfide bonds have been also identified in purified rat SeIP [92]. These bonds might have structural functions and might serve for the protection of the selenolic groups reactivity. SeIP purified from rat plasma is present as 4 isoforms containing the lower number of 6 SeCys residues [93,94]. There are evidences for the existence of different

SelP isoforms also in mice [95] and humans [96], but separation and characterization of them in these species has not yet been reported.

As is deeply exposed in the paragraph II.2.2, plasma SelP and GPx3 are used as Se status markers because they are correlated with the dietary Se intake [97] and they can be determined with a scarcely invasive procedure, while tissue Se-proteins cannot be measured without performing a biopsy. The half-life of SelP in plasma is about few hours (3-4 h in rat plasma) [98], therefore the protein concentration reflects the short-term status of Se in the organism. Studies showed that its concentration drops as the severity of Se deficiency increases [99]. Between the two Se-proteins available for measurement, starting from Se deficient intake SelP showed to require a greater Se dose to reach its maximum concentration in respect to GPx3 [100]. This makes it a better marker than GPx3 for the Se nutritional status. However, once reached the normal Se requirement additional increase of the element does not cause an increase in GPx or SelP concentration, therefore these Se biomarkers have only a limited validity [101].

SelP is mainly produced in the liver, but is expressed and probably secreted by other tissues including brain and heart [102,103]. Evidence supports functions of SelP in Se transport and homeostasis through the whole body [98]. SelP knockout mice present very low Se concentration in brain, testis and fetus, with severe pathophysiological consequences in each tissue [98]. In addition, these mice waste moderate amounts of Se through the urine. On the other hand, dietary Se deficiency causes a profound decrease in liver Se concentration, presumably because the liver exports a fraction of its metabolically available Se as SelP, even when the element supply to the organ is drastically decreased [99]. Under dietary Se deficiency, SelP appears to be responsible for maintaining preferentially Se in the brain and testis by a mechanism that is distinct from its effect on the other organs [104,105]. In the kidney and in the muscle, Se concentration falls approximately to the same extent as do the whole-body Se with the deletion of SelP and with dietary deficiency. Since Se is covalently bound, its release requires disruption of SelP to exploit its transport function [106,107].

The specific biochemical activity of SelP remains still unclear. Indications exist about the possible role of the protein in the antioxidant defence. SelP plasma level correlates with prevention of diquat-induced lipid peroxidation and hepatic endothelial cell injury [108], and an association was also reported between SelP and

protection against oxidant injury from GSH depletion in Se-deficient rats [109]. In addition, SelP binds to endothelial cells in the rat, probably through its heparine-binding site [110]. Endothelial cells release primary free radicals NO^\bullet and O_2^\bullet from which peroxynitrite (ONOO^-) and H_2O_2 secondary products are formed. Thus, endothelial cells and their environment have been postulated to be sites of oxidative stress. Localization of SelP in proximity of endothelial cells is consistent with its having an antioxidant defence function, perhaps membranes protection [110].

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II. SELENIUM AND HUMAN HEALTH

II.1. Introduction

As exposed in the Chapter I, Se is a trace element incorporated in many proteins which play fundamental roles for human health. The dietary intake of the element influences all biological functions involved, thus is important in the estimation and regulation of nutritional status to improve the health state. The metabolic pattern and biochemical action of Se are extremely complex, and many aspects need to be taken into account, including all the possible positive and negative associations between Se and human diseases.

Essentiality and toxicity characteristics of Se are discussed in the first part of this chapter. The different criteria that can be adopted to estimate the recommended dietary levels of Se are exposed to introduce the reference values provided in the last years by the major institutions. The current status in dietary Se intake is then presented in relation to the geographical region and to the type of diet and food most commonly consumed. The second part of the chapter comprises an overview of human diseases to which Se has been associated. Particular attention is set on diabetes and cancer, which were object of applicative studies in this Ph.D. project.

II.2. Selenium and nutrition

Se was initially identified during the 1930s as a toxic agent in animal feeds and forages that caused the livestock poisoning named "alkali disease" [1]. The prevailing opinion was that Se was a toxin carrying out negative effects on metabolism, as well as carcinogenic action. The perception of Se began to change in 1957, when Schwarz and Foltz [2] proved that traces of this element in food protect vitamin E deficient rats from developing liver necrosis. Following this observation, many diseases related with Se deficiency were then discovered in a variety of economically important farm animals including turkeys, chickens, sheep, swine, and cattle. In 1979 Se was demonstrated to protect humans against Keshan disease, a cardiomyopathy affecting young children residing in low-Se regions of China [3]. This finding reversed the opinion regarding the role of Se, and catalysed the interest in the element as an essential trace nutrient for human health. Nowadays, Se is considered an essential element that present a very narrow range between deficient, essential and toxic doses [4]. The estimation of minimum element dietary intake is currently a challenging task. Both deficiency and excess of Se cause characteristic diseases that can be used as endpoints for the estimation of lower and upper recommended dietary values. More difficult is the assessment of the relationship between Se dose and its effect on a very wide variety of biological functions or diseases, where the role of the element is extremely complex and in most of the cases still unknown. The Se intake is also strongly dependent on the geological characteristics of each area, ranging in a broad interval. In recent years, many institutions proposed reference values for the Se recommended dietary intake, considering a balance among the most reliable epidemiological studies. A more recent perspective, probably premature for a normative translation, moved from Se intake reference values designed to prevent negative occurrence to doses able to improve the health state. The increased diffusion of Se food supplements consumption needs to be considered mainly in this context.

II.2.1. Essentiality and toxicity of selenium

Selenium deficiency is associated with two endemic diseases diffused in soil Se-poor regions of China and Russia: Keshan disease and Kashin-Beck Disease. Keshan disease is an endemic juvenile cardiomyopathy with myocardial insufficiency, that primarily affects children between 2 and 10 years old [5]. This pathology is

characterized by cardiac enlargement, abnormal electrocardiogram (ECG) patterns, cardiogenic shock, and congestive heart failure, with multifocal necrosis of the myocardium [6]. The Kashin-Beck disease is an osteoarthritis characterized by atrophy, degeneration, and necrosis of cartilage tissue, which occurs primarily in children between the ages of 5 and 13 years. This pathology results in enlarged joints, shortened fingers and toes, and dwarfism in extreme cases [5,7,8].

Acute Se toxicity by inhalatory exposure have been reported to cause stomach pain and headaches, as well as respiratory symptoms such as pulmonary edema, bronchial spasms, symptoms of asphyxiation and persistent bronchitis, elevated pulse rates, lowered blood pressure, vomiting, nausea, and irritability [6]. Acute oral exposure to extremely high levels of Se produces nausea, vomiting, diarrhoea, and occasionally tachycardia [6]. Regarding chronic inhalation exposure, several occupational studies revealed respiratory effects such as irritation of the nose, respiratory tract, and lungs, bronchial spasms, and coughing [6].

Chronic oral intake of very high levels of Se results in selenosis [9]. This pathology is characterized by hair loss, deformation and loss of nails, discoloration and excessive decay of teeth, garlic breath, gastrointestinal disturbances, skin rash, and abnormal functioning of the nervous system (numbness, paralysis and occasional hemiplegia) [10-12]. Other related toxic effects are a disruption of endocrine function, synthesis of thyroid hormones and growth hormones, and an insulin-like growth factor metabolism. Particularly high levels of dietary Se were also significantly associated with diminished T3 levels, impairment of natural killer cells and hepatotoxicity [9].

II.2.2. Assessment of the selenium nutritional status

The assessment of optimum Se dietary requirements is a very complex matter of debate. Until few years ago, most of the studies focusing on Se status assessment investigated only the total level of the element in tissues or body fluids. Plasma or serum Se concentration was generally considered a useful biomarker of both Se status and dietary intake at short-term, while erythrocyte Se reflects better the long-term status. Other tissues were also used to measure long-term Se status, including hair and toenails. Daily urinary excretion was demonstrated to be closely associated with

plasma Se level and dietary intake, accounting for a stable value of 50-60 % of the total amount excreted, and thus was also used as short-term intake measure.

Recently, many authors pointed out that total Se concentration could be not representative of the real functional activity of Se, because it can be incorporated in a large variety of proteins with different biological function (or even without direct biological function, as is the case of SeAlb). The distribution of Se among Se-proteins is strongly dependent by a precise hierarchy in its incorporation, the average dietary intake, the speciation of Se in food, the health state, the age, the lifestyle (smoking and exercise), and also by Se-proteins genetic polymorphism [13]. The measure of individual Se-proteins activity should constitute a more accurate biomarker for the Se functional status. Nowadays, the most used parameter for the assessment of Se status is the maximization of GPx3 activity [1,13]. This criterion has been used in the most recent update of the US and Canadian Dietary Reference Intakes (DRIs). GPx1 activity is also used. Together with GPx3, it reaches its maximum activity at relatively low total Se concentration in blood ($\sim 100 \text{ ng mL}^{-1}$). Because of this reason, they are no longer accurate biomarkers for higher levels. SelP and GPx4 activities have also been proposed as biomarkers alternative to GPx3. In the choice of the Se-protein to be adopted, it is necessary to consider that each of them plays a proper biological function, and thus could provide only limited information. In this context, some authors assumed that the best parameter is expected to be not a single protein, but rather a set of combined biomarkers, to be applied to specific problems associated with suboptimal Se status [13].

An alternative approach for Se status assessment is to compare the dietary intake with specific end-points of disease. The basal Se requirement should be that intake able to prevent pathologically and clinically relevant signs of dietary inadequacy. This approach has been applied to the Keshan disease in Se-deficient areas [14,15]. Recently, evolution towards recognition of an 'optimal nutrition' moved the interest to the possible health effects of Se in larger than minimum intakes, considering the alternative end-points of the promotion of growth, maintenance of good health and the reduction of other diseases not caused by nutritional deficiencies. However, the causal association between Se-proteins and specific diseases is still far to be clarified, and its inclusion in the estimation of the Se RDA appears to be premature.

II.2.3. Recommended selenium intake levels

Depending on the adopted criterion, the reference intake levels can be fixed on different values. An intake of $\sim 20 \mu\text{g day}^{-1}$ for adults is generally accepted as the minimum needed to prevent Keshan disease onset [14,15]. Considering the more general prevention of pathologically and clinically relevant signs of dietary inadequacy, the World Health Organization (WHO) corrected this basal requirement to an average of $19 \mu\text{g day}^{-1}$, corresponding to $21 \mu\text{g day}^{-1}$ for men and $16 \mu\text{g day}^{-1}$ for women, taking into account the body weight [16].

The most important institutions used the GPx3 activity maximization to calculate reference levels named in different way but with a common interpretation. Some of those recommended values are reported in Table II-1.

Table II-1 Recommended dietary standard intake of Se for adults [$\mu\text{g day}^{-1}$].

	Men	Women
Australia RDI Truswell <i>et al</i> , 1990 [17]	85	70
USA and Canada RDA Standing Committee on the Evaluation of Dietary Reference Intakes, 2000 [18]	55	55
UK RNI Department of Health, 1991 [19]	75	60
WHO NR WHO/FAO/IAEA, 1996 [16]	40	30
EU PRI Scientific Committee for Food 1993 [20]	55	55
Germany RNI German Nutrition Society <i>et al</i> , 2000 [21]	30-70	30-70

RDI: recommended dietary intake; RNI: reference nutrient intake; NR: normative requirement estimate; PRI: population reference intake.

The Estimated Average Requirement (EAR) and Recommended Dietary Allowance (RDA) of the US and Canadian RDAs are the most adopted as general reference. They are respectively defined as “*the average daily nutrient intake level estimated to meet the requirement of half the healthy individuals in a particular life stage and gender group*” and “*the average daily dietary nutrient intake level sufficient to meet the nutrient requirement of nearly all (97 to 98 %) healthy individuals in a particular life stage and gender group*” [18]. United Kingdom defined the Reference

Nutrient Intake (RNI) as “*the amount of a nutrient that is adequate to prevent deficiencies in 97.5 % of the population*” [19]. Similar definitions were adopted by the Europe Community referring to the Population Reference Intake (PRI) as “*the level of (nutrient) intake that is adequate for virtually all people in a population group*” [20]; and by the WHO for the Normative Requirement Estimate (NR) as “*the level of intake that serves to maintain a level of tissue storage or other reserve that is judged by the Expert Consultation to be desirable*” [16].

Specific values are then commonly provided for gender, age groups and particular categories. Higher intake levels are recommended under pregnancy ($60 \mu\text{g day}^{-1}$) and lactation ($70 \mu\text{g day}^{-1}$) conditions [18].

The National Health and Medical Research Council (NHMRC) of the Australian Government proposed in 2005 [22] the most updated EAR and upper levels, reported in Table II-2.

Table II-2 Recommended lower and upper levels of dietary standard intake for Se [$\mu\text{g day}^{-1}$] proposed by the NHMRC.

age [years]	Lower limits EAR (RDI)		Upper limits
	Men	Women	
1 - 3	20 (25)	20 (25)	90
4 - 8	25 (30)	25 (30)	150
9 - 13	40 (50)	40 (50)	280
> 14	60 (70)	50 (60)	400
pregnancy		55 (65)	400
lactation		65 (75)	400

The cited institutions proposed also some upper levels (UL) of Se intake, that can be defined as “*the highest average daily nutrient intake level likely to pose no adverse health effects to almost all individuals in the general population*”[22]. The ULs relate to intakes from food and supplements. There are limited data about toxicity in humans. Acute toxic symptoms are associated with extremely high Se intakes of 3200 to $6700 \mu\text{g day}^{-1}$. Milder symptoms such as fingernail changes have been reported for Se intakes of $1260 \mu\text{g day}^{-1}$ [23]. Studies from China and US gave a NOAEL (the level of exposure of an organism at which there is no statistically significant increase in the frequency or severity of any adverse effects in the exposed population) for

adults of $800 \mu\text{g day}^{-1}$, and other researchers reported selenosis with Se intakes of $\geq 850 \mu\text{g day}^{-1}$ [10,24]. The Environmental Protection Agency of USA defined $1262 \mu\text{g day}^{-1}$ as the Se intake at which clinical selenosis appeared. More difficult is to estimate the association between high dietary Se intake and health effects of diseases which are not directly caused by the element, like cancer. The Nutritional Prevention of Cancer Trial [25] showed an increase in the risk of squamous cell carcinoma and total non-melanoma skin cancer in individuals with high basal risk supplemented with $200 \mu\text{g day}^{-1}$ of Se, but there are still doubts on how this would relate to the risk for general population. In a preventive perspective, the NHMRC chose to apply to this latter estimate a factor of 2 to protect sensitive individuals from gaps in the data and incomplete knowledge. The UL was therefore set at $400 \mu\text{g day}^{-1}$ for all adults, as there are no data to suggest increased susceptibility during pregnancy and lactation [22].

II.2.4. Current selenium intake levels

Diet is the major source of Se. Its concentration in food of vegetal origin is highly variable and depends mainly on the soil conditions [26]. Many studies have been performed in different countries and communities throughout the world, some of them reported in Table II-3. The estimated Se intake ranges from $< 10 \mu\text{g day}^{-1}$ in Se-deficient areas, to almost $7000 \mu\text{g day}^{-1}$ in areas characterized by endemic selenosis [10].

Type of food and diet are the most important variables in determining the amount of Se intake [27]. Table II-4 reports the Se content in some food groups of common consumption. Se content in plants is directly affected by levels of the element in the soil where they are grown. The concentration in vegetables is generally low, usually at $< 0.1 \mu\text{g g}^{-1}$ [28], because plants do not require Se for growth and normally do not accumulate it. Some exceptions are the members of the *Allium* family, which includes garlic and onion. These plants are able to accumulate significant amounts of Se, if grown on rich or enriched soil, reaching concentrations of Se up to 68 and $96 \mu\text{g g}^{-1}$, respectively [29]. High amount of Se can be accumulated also by mushrooms and broccoli. The richest natural source of Se is Brazilian nut, with mean concentration reported in the literature up to $83 \mu\text{g g}^{-1}$ [30].

Table II-3 Average total Se intake [$\mu\text{g day}^{-1}$] in several countries.

Country	Se intake	Ref.	Country	Se intake	Ref.
Belgium	52 ± 16	[31]	Mexico	73 - 61	[32]
Bolivia	43 ± 15	[33]	Nepal	23 ± 16	[34]
Burundi (rural pop.)	17	[35]	Netherlands	56 ± 13	[36]
China (Keshan area)	3 - 11	[27]	New Guinea	20	[37]
China	240 - 6990	[10]	Norway	80	[38]
Croatia	27 ± 10	[39]	Poland	30 - 40	[40]
Denmark	56 ± 28	[41]	Portugal (Pinhel)	37	[42]
Finland	28 ± 17	[43]	Slovakia	38 ± 7	[44]
France	48 ± 3	[45]	Slovenia	87 ± 28	[46]
Greece	39	[47]	Sweden	40 ± 4	[48]
Greenland	235	[49]	Switzerland	66	[50]
India	62	[51]	Turkey	52 ± 34	[52]
Italy	51 ± 30	[53]	UK	34	[54]
Japan	129 ± 32	[55]	USA (SD)	174 ± 91	[56]
Lybia	13 - 44	[57]	USA (OH)	85 ± 5	[58]
Lithuania	100	[59]	Venezuela	200 - 350	[60]

Table II-4 Se mean concentration [ng g^{-1} fresh weight] in some food groups, minimum and maximum [29,61].

Food group	Se	Food group	Se
Brazilian nuts	850 - 6860	meat	27 - 450
bread	60 - 740	mushrooms	80 - 130
butter	1 - 14	olive oil	1 - 5
cashew nuts	49 - 80	onion	2 - 10
chocolate	32 - 41	oyster	770
cow's milk	2 - 140	pasta	6 - 50
eggs	56 - 420	pork kidney	849 - 1543
fish	120 - 970	pork liver	256 - 800
fruit	1 - 76	potato	5 - 70
garlic	30 - 250	rice	11 - 182
legumes	18 - 28	yoghurt	22 - 50

The Se content in animal products reflects the level of the element in their diet. As a consequence, Se concentration in meat ranges in a wide range, but it is generally relatively high. Together with fish and eggs, meat contributes to the major part of dietary Se in many countries. Specific organs, such as liver and kidney, can accumulate a particularly high concentration of the element [54].

II.2.5. Selenium supplementation

Due to the low abundance of soil Se in some regions, a wide variety of Se-enriched materials have been commercialized or proposed to supply the population with Se levels adequate to the RDAs. The United States 1994 Dietary Supplement Health and Education Act (DSHEA) defines a dietary supplement as “*a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: (a) a vitamin; (b) a mineral; (c) an herb or other botanical; (d) an amino acid; (e) a dietary substance for use by man to supplement the diet by increasing the total dietary intake; or (f) a concentrate, metabolite, constituent, extract, or combination of any ingredient described in clause (a), (b), (c), (d), or (e)*”[62].

Many strategies have been followed to obtain such products. The use of fertilizers enriched in sodium selenite is one of the most used technique to obtain plant food with high Se concentration. This strategy is used in Finland since 1984 [63]. Supplementing fertilizers with Se is a very effective and readily controlled way to increase the average daily Se intake, because plants growth is generally improved by the element [5], but at the same time it is reduced by feedback mechanisms when its concentration is too high. Plants with the ability of accumulating high concentrations of Se such as broccoli [64,65], garlic [66,67], green onions [68,69], green tea [70,71] as well as mushrooms [72,73], are particularly adapt to obtain natural dietary supplements following fertilization strategy. As a consequence of Se fertilization, increased Se levels in milk, meat, eggs and the whole food chain were also observed [5]. The total Se intake in Japan, Australia, Finland, and USA, as well as Keshan areas in China was significantly increased in the last decades by the diffusion of Se-enriched fertilizers [61]. However, Se speciation in these fertilizing products plays a differential role which has not been clearly elucidated yet [74].

The use of Se-enriched fertilizer resulted effective, but part of the Se in the final products is lost during harvesting and manipulation prior to consumption [4]. An alternative is the supplementation of animals with food enriched in Se content. This strategy includes: a) direct application of Se to pastures to increase Se uptake by plants for animal feed; b) supply of sodium selenite or selenate incorporated into salt blocks or licks; c) direct administration of Se to animals by drenching with Se salt solutions such as sodium selenite; and d) use of Se pellets that slowly release it in the

animal's gut [61]. Recently, a technological process to produce Se-enriched eggs, meat and milk has been developed and successfully introduced in various countries worldwide [75], particularly Korea and Greece [47,76]. Detailed investigations on the possible interactions with other nutrients in Se-enriched food are still missing.

As third strategy, the direct intake of Se supplements by humans received increasing attention in the last years. Two different types of multimicronutrient products can be distinguished: a) multi-vitamin and multi-mineral preparations containing inorganic Se, other trace elements and vitamins, and b) supplements based on *Saccharomyces cerevisiae* yeast (Baker's yeast). Se-enriched yeast is the primary Se dietary supplement, and have been widely studied [4]. Selenized yeast is particularly attractive due to its low cost, facility to grow in different conditions, and ability to assimilate up to 3000 $\mu\text{g g}^{-1}$ of Se from the sodium selenite added to the growth medium. Se speciation in *S. cerevisiae* has been widely investigated, showing that most of the element is accumulated as SeMet. Other organic specie were reported, such as Se-MCys, Se-(Cys)₂, GGMSC, Se-A-hoCys, SeCysth, Se-Lth, SeOMet, DMS₂Se [77,78].

II.3. The role of selenium in human diseases

Se is linked to a large variety of diseases in humans. Most of these associations are due by the role of GPxs and TrxRs enzymes in the reduction of oxidative stress, which has been identified as cause or relevant factor in the development and progression of several pathologies. Some other Se-proteins are involved in specific processes such as calcium signalling, brain functions and spermatogenesis. Alterations in the their genes or underexpression related to Se deficiency were indentified as possible cause of the corresponding pathology. However, definitive studies concerning the biochemical mechanisms of Se-proteins are still rare in the literature, and most of the available data emerge from epidemiological investigations where serum/plasma total Se concentration was compared between patients and control subjects.

II.3.1. Muscle disorders

Keshan and Kashin-Beck diseases, presented in the paragraph II.2.1, are muscle disorders directly caused by severe deficiency of Se. Muscular dystrophy is an other group of pathologies involving slow degeneration of muscle tissue [79]. Some forms of congenital muscular dystrophy, including mutiminicore myopathy, rigid spine muscular dystrophy-1 and desmin-related myopathy with Mallory bodies, have been linked to mutations of the SelN gene [80]. The role of SelN in multiminicore disease has been elusive because its biological function is largely unknown. A recent study showed that the association of SelN with ryanodine receptors is necessary for the proper function of these receptors, which are responsible for calcium signalling [81]. The mutations in SelN gene prevent this association, and thus are responsible for multiminicore disease by inhibition of calcium-stimulated release of calcium from intracellular stores.

II.3.2. Cardiovascular diseases

Oxidative stress damages vascular endothelial cells and exacerbates cardiovascular diseases such as atherosclerosis, hypertension, and congestive heart failure [52]. Se-proteins are involved in the cellular antioxidant defence system, thus the potential prevention effects of Se supplementation on cardiovascular diseases has been widely investigated. Some epidemiological studies revealed lower plasma/serum or urine Se level in patients with various cardiopathies [26,82], but others did not [83]. In most of the cases their experimental design cannot elucidate which is the causal connection under the differences that were observed, therefore the data still do not support a clear interpretation of the role of Se in these pathologies [27]. The precise mechanism by which low plasma Se level influences the diseases is also unknown. Some authors hypothesized that the oxidative stress inhibition carried out by GPx1, GPx4 and TrxR1 in vascular endothelial or smooth muscle cells, protects them by damage and apoptosis from lipid hydroperoxides and oxidized low-density lipoproteins, which exhibits proatherogenic effect [84,85]. Other authors proposed that Se is related to prostaglandin metabolism. By enhancing its synthesis, it could improve thromboxanes in platelets, and diminish prostacyclin concentration in vascular endothelium [86].

II.3.3. Hepatopathies

Some studies revealed significantly lower plasma/serum Se concentration in patients with different grades of hepatocellular injury, than those determined for healthy controls [87,88]. The difference in alcoholic individuals might be due to a low nutritional supply of the element. Most likely, the excessive dietary intake of alcohol simply entails a decrease of the remaining food consumption [89]. The nutritional deficit of Se impairs the GPxs activity, involved in the destruction of ROS which are augmented by the high alcohol intake [90]. Even if the decreased Se levels are not the cause (or chemically related with the causes) of hepatopathies, Se supplementation could be considered potentially beneficial in the care of hepatic diseases [27].

II.3.4. Renal failure

Plasma GPx3 activity and Se level in RBCs and plasma are significantly lower in patients with chronic renal failure (CRF) and in dialyzed uremic patients [91,92]. Decreased dietary intake of Se, increased urinary (or dialytic) loss, impaired intestinal absorption, abnormal binding to Se transport proteins and others mechanisms have been proposed to explain these observations, but clear results are still missing [93]. Plasma Se level decrease may be just a direct or indirect consequence of renal failure, but could also extend the negative effects of the disease by enhancement of oxidative stress and alteration of thyroid hormones metabolism [94]. Oral and intravenous Se supplementation was demonstrated to be effective in improving the immune function of renal patients through reduction of oxidative stress [95]. Therefore, it seems beneficial to monitor Se status in patients with CRF, and to correct the less-than-optimal status with supplementation in such conditions where dietary protein restrictions are necessary [95].

II.3.5. Neurological disorders

Damage from ROS takes place also in neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), ischaemic damage, exposure to environmental toxins and abuse of drugs, brain tumours, multiple sclerosis, Batten's disease and epilepsy [96,97]. All these pathologies result in lower brain expression or activity of Se-enzymes including SeIP, GPxs and TrxRs. Given the importance of Se-

proteins in the control of ROS, the role of Se in brain functions has been widely investigated [96]. Se showed an exceptional tendency to be retained within the brain under conditions of dietary deficiency [98]. The available data support the hypothesis that this preferential supply of the central nervous system with Se may involve primarily a SelP-dependent mechanism [99]. Experiments carried out on transgenic mice with knockout for specific Se-proteins showed that SelP-deficient mice have drastic reduction of brain Se, as well as the activities of GPxs and TrxRs [100]. SelP is also locally expressed in the brain, proving that local functions for SelP have to be considered [101,102].

Oxidative damage to macromolecules is an early indication of AD that can appear before clinical symptoms [103]. Alzheimer's disease patients suffer memory loss, impaired cognitive function and changes in behaviour and personality [168]. The brain of AD patients is characterised by extracellular plaques consisting of the protein amyloid β , as well as by intracellular neurofibrillary tangles. Symptoms similar to those which are typical in AD were observed in mice with the genetic deletion of SelP. The models presented impairment of synaptic function in the hippo-campus, a region involved in memory, and the reduction of spatial learning and long-term strengthening, a cellular model for learning and memory [104]. SelP presents also a characteristic expression pattern within the centre of neuritic (dense-core) plaques [105]. Although a specific action of SelP in AD is still uncertain, its distribution in brain suggests a role in mitigating the oxidation accompanying plaques. The age-related alteration of other Se-proteins activity in brain of AD patients results in increased oxidative stress and reduced protection against neurodegeneration through redox regulation. GPx1, GPx4, TrxR1, SelW, SelH and SelM could be potentially involved in these functions, but their specific mechanism of action is still unknown [97].

The severe loss of dopamine-releasing neurons in the *substantia nigra* is central for PD, an other neurodegenerative disorder [106]. Parkinson's disease is characterized by loss of dopaminergic neurons in *putamen* and *caudate* within the *striatum* from neurons projecting from the *substantia nigra* (the nigrostriatal pathway). The *substantia nigra* and *putamen* have notably high Se concentration in comparison to other brain regions [107]. Evidences revealed that Se deficiency exacerbates the chemical lesions of dopaminergic terminals and neurons in PD mouse models [108], and on the contrary, Se supplementation and overexpression of GPx1 have a

protective action [109]. However, the role of GPxs and other Se-proteins in protecting dopaminergic transmission and preventing PD is still unsupported by the evidence of a direct correlation between proteins expression or function and PD [110].

Epilepsy is a chronic neurological disorder characterized by seizures which cause interruptions in the normal brain function [111]. Epilepsy, ischaemia and brain trauma cause a signal cascade of free radicals and activation of pro-apoptotic transcription factors, resulting in neuronal loss [112]. Thus, this pathologies could be associated with altered Se-proteins activity in ROS reduction. As a support, GPx1 activity appears to be correlated with induced seizure in mice [113,114]. SeLP-knockout mice develop neurological seizures and movement disorders under Se-deficient diet [99], providing further evidence for a possible role of Se-proteins in epilepsy prevention.

II.3.6. Immunity defence and Inflammatory disorders

The immune system relies on many processes including the generation of ROS as a defence against microbial pathogens, coordinated regulation of adhesion molecules and the expression of soluble mediators such as eicosanoids and cytokines and their receptors [1]. Se influences the immunitary response through many mechanisms. As a part of the antioxidant system, it contributes to control the ROS concentration by detoxification of organic hydroperoxides and hydrogen peroxide when they are produced in excessive concentration. Se also regulates the balance of activity in the eicosanoid synthesis pathways, leading to preferential synthesis of leukotrienes and prostacyclins over thromboxanes and prostaglandins, and down-regulates cytokine and adhesion molecule expression. By up-regulation of the interleukin-2 receptor expression, it leads to enhanced activity of both T- and B-lymphocytes, natural killer and lymphokine activated killer [115]. Mice with a T-cell-specific deletion in tRNA^{Sec} result in knockout of all T-cell Se-proteins. This produces many effects, including decrease in functional T-cells, reduced antigen-specific production of immunoglobulins *in vivo*, moderate to severe atrophy of the thymus, spleen and lymph nodes [116]. Se-deficient mice exhibit increased pathology from viral infection, owing to an exaggerate pro-inflammatory immune response [117,118]. Se deficiency or deletion of GPx1 in mice also increases viral mutations and virulence [119].

According to these functions, it is expected that Se carries out beneficial effects on inflammatory conditions such as rheumatoid arthritis. Negative correlations were observed between serum Se level and rheumatoid arthritis, asthma, and immune activation (through soluble interleukin-2 receptor and erythrocyte sedimentation rate) in Crohn's disease [120-122]. Plasma Se and SelP concentration is lower in patients with sepsis at different seriousness level or sepsis-like illness [123,124]. However, the reason for that decrease nor its mechanisms are known with certainty. Intervention studies have been carried out in patients with severe sepsis, but did not confirm that Se supplementation is globally beneficial for patients with sepsis [125] .

A specific Se-protein involved in immune responses is SelS. Its expression in liver cells is regulated by inflammatory cytokines and extracellular glucose concentration [126]. SelS has an antiapoptotic role, and reduces endoplasmatic reticulum stress in peripheral macrophages [127] and brain astrocytes [128]. A particular polymorphism of SelS was proved to be responsible for increased plasma level of the inflammatory cytokines. A possible increased risk of several inflammatory diseases could be the consequence [136,129], but a direct correlation with stroke, autoimmune disorders or inflammatory bowel disease is still missing [110].

II.3.7. AIDS and HIV

The implications of Se in immunity system stimulated the investigation of its role in HIV contrasting. Chronic oxidative stress has been reported during the early and advanced stages of HIV-1 infection, and has been linked to HIV-induced apoptosis of T-cells, development and progression of AIDS, Kaposi sarcoma, and related neural damage [130-132]. Several studies on Se status and HIV-1 progression observed a direct association between low plasma/serum Se concentration or erythrocytes GPx1 activity, and reduced CD4+ counts, progression from AIDS to HIV and risk of death [133-135]. Nevertheless, other studies have not found relatively low serum Se in HIV-1-infected children or adults [136,137], suggesting that its deficiency in HIV-1 infection may be most likely to occur in subjects with poor diets, such as intravenous drug abusers and those who live in poverty. Thus, maintaining an optimal Se status in HIV-1 patients may help to increase the enzymatic defence, improve general health and reduce their risk of hospitalization for opportunistic infections and other problems [138].

II.3.8. Endocrine disorders

Se-proteins play important roles in the production of thyroid hormones and growth factors. GPxs and TrxRs regulate ROS concentration and the redox reactions in the endocrine tissue. SelT may be involved in the control of calcium-dependent release of PACAP hormonal peptide from pituitary [139]. Activation of thyroid hormone is dependent upon the DIOs. These enzymes catalyse deiodination of the pro-hormone thyroxine, or T₄ (tetra-iodo-L-thyronine), to the active hormone T₃ (tri-iodothyronine), and to the inactive metabolites rT₃ (reverse triiodothyronine) and T₂ (di-iodothyronine) [140]. Both Se deficiency and mutations in the SeCys insertion sequence-binding protein 2 can be responsible for deficiencies in thyroid function [110].

II.3.9. Male fertility

Moderate Se deficiency leads to impaired sperm motility and morphological alterations of the midpiece architecture, often resulting in disconnections of heads and tails, while in extreme Se deficiency spermatogenesis is completely abrogated [141]. The Se-protein GPx4 was recognized as one of the possible causes of oligoasthenozoospermia, a form of infertility characterized by reduction of both the number and the motility of spermatozoa [142]. This Se-protein is highly expressed in mitochondria in the midpiece of human spermatozoa. GPx4 plays an important dual role in spermatogenesis: as an active peroxidase in spermatogenic cells and as a structural protein in spermatozoa [143,144]. It is the only known intracellular antioxidant enzyme that can directly reduce lipid hydroperoxides in membranes. Through its action in reducing phospholipid hydroperoxide and hydrogen peroxide, GPx4 protects the rapidly dividing cells against oxidative injury. A decrease in the expression level of GPx4 in the spermatozoa results in a defected incorporation of rhodamine 123, with a loss of mitochondria membrane potential that affects their morphology [142].

II.3.10. Aging

The relationship between Se levels and aging is still controversial. Plasma/serum Se concentration seems to remain stable with the age, but the tissue distribution may

be altered [131]. In general, the association of Se with aging is mainly indirect, due to the fact that most of the biological processes in which Se is involved change with the age. Several studies have shown that aging cells accumulate oxidative damage [145]. Age-related oxidative stress influences many of the processes cited in the previous paragraphs, including damage to both mitochondrial and nuclear DNA, lymphocyte population fall, telomere length decrease in peripheral leukocytes and thyroid hormones alterations.

II.3.11. Diabetes

Diabetes mellitus is a family of metabolic disorders that share hyperglycemia resulting from defects in insulin secretion, insulin action, or both of them [146]. High circulating levels of glucose contribute to increase the risk of a set of associated complications including atherosclerosis, kidney failure, blindness and amputations, coronary heart disease, stroke, peripheral vascular disease and birth defects [146,147]. The two most common forms of diabetes mellitus are type I (insulin-dependent) and type II (noninsulin-dependent). Type I diabetes is caused by cellular-mediated autoimmune destruction of the pancreatic insulin-producing β cells. Type II diabetes is characterized by defects in insulin secretion and action caused by inability of the body cells to respond to the presence of insulin (insulin resistance)[148].

Selenate or Se were demonstrated to have insulin-mimetic properties, being effective in the stimulation of glucose uptake both *in vitro* and *in vivo* [149,150], the regulation of glycolysis, gluconeogenesis, fatty acid synthesis and the pentose phosphate pathway [148,151]. The blood glucose-lowering action of oral selenate is independent from a rise in circulating insulin levels or decrease in food intake and slowing of body weight gain [152]. Se insulin-like action involves partial correction of altered pretranslational regulatory mechanisms in the liver metabolism [153]. Selenate treatment can increase two-to-threefold the hepatic activities and mRNA levels of two key glycolytic enzymes, glucokinase and L-type pyruvate kinase, which are highly reduced in diabetic rats. In contrast, the typically elevated activity and mRNA levels of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase get appreciably reduced [153]. Mueller and Pallauf [154] performed an elegant study in type II diabetic *db/db* mice to show that selenate influences two important mechanisms involved in the insulin resistance state characteristic of type II diabetes

mellitus: (1) it reduces the activity of liver cytosolic protein tyrosine phosphatases (PTPs) as negative regulators of insulin signalling by about 50 %; and (2) increases the expression of the peroxisome proliferator-activated receptor gamma (PPAR γ). These two mechanisms are responsible for the changes in the intermediary metabolism, in particular gluconeogenesis and lipid metabolism.

Se also prevents or alleviates the adverse effects that diabetes has on cardiac [155-157] and renal functions [158,159], micro and macrovascular complications [160], and atherosclerosis progression [161]. The protective function of Se-proteins relies also in this case on their action for ROS reduction. Hyperglycaemia induces oxidative stress through activation of the polyols pathway, which increases the utilization of NADPH and the production of superoxide anions. The toxicity of high glucose levels is also related to free radicals generated by auto-oxidation of sugars, prostanoids metabolism, and proteins glycation. A consistent high oxidative stress level or low antioxidant defence were revealed in patients with diabetes [162], which are responsible for many pathogenic processes of diabetic complications [163,164]. In patients with diabetes an increase of the platelet aggregation has been also observed. Such an effect is potentially associated with the occurrence of microvascular degenerative complications. GPx3 activity decreases the synthesis of thromboxan, a vasoconstrictor product, and decreases platelet aggregation preventing degenerative complications [165,166].

Despite the large amount of indications regarding the biochemical action of Se in diabetes aetiology, no definitive data are reported in the literature about the direct association between levels of Se in plasma/serum and the presence of type II diabetes mellitus. Some studies have shown that mean plasma [147,167-169], serum [170,171] or blood [172] Se concentrations and GPx3 activity [173] are lower in patients with diabetes than in controls. Lower plasma/serum Se levels have also been found in gestational diabetic pregnancies with respect to normal pregnancies [174-177]. However, other works observed no significant difference in plasma Se levels of patients with diabetes [178-181] or even demonstrated higher serum Se levels in diabetic children with respect to healthy subjects [182-185]. Also in a study by Bleys *et al.* [186] Serum Se was not significantly different even if subjects over the highest quintile had an increased prevalence of diabetes compared to those below the first quintile. Whiting *et al.* [173] noticed lower plasma Se levels in patients with diabetes, but only with a disease duration ≤ 2 years. Se urinary excretion has been reported to

be inversely correlated with the degree of diabetes control and insulin administration [187].

II.3.12. Cancer

Mainly due to its antioxidant action, Se has been hypothesized to have chemopreventive properties. The ability of Se in reducing carcinogen induced and spontaneous cancer incidence has been widely investigated in the last 20 years in both animal and human models, in most organs, and against a broad range of cancer forms [188]. Table A - 3 resumes many epidemiological studies carried out to found the possible correlation between Se and cancers. In humans, the results have generally been inconsistent. Meta-analyses were performed as a attempt to extrapolate a conclusive interpretation. Positive effects of high Se levels were revealed for prostate [189,190], bladder [191] and lung [192] cancers. On the contrary, no significant effects were found in Se supplementation randomized control trials for prostate cancer [193] and colorectal cancer [194], as well as in case-control studies regarding primary liver cancer [195]. Gender-dependent effects were found after grouping a set of different cancer forms [196]. Still, such meta-analyses appears to be highly conditioned by the limited design of most of the studies that were examined, in particular regarding sample size, covariates control (age, gender, diet, lifestyle and others) and time dimension.

A particular attention has been given by some authors to investigate the level of total serum or plasma Se in patients with colorectal cancer (CRC). This tumour is the third most commonly diagnosed form of cancer in women and fourth in men worldwide [197]. Many of these studies found a significantly lower Se level in CRC patients with respect to control healthy subjects, while others observed significantly higher risk to develop CRC and lower cumulated cancer-related survival rates for subject with low serum Se levels [198-200]. Similar results were obtained by investigating the total serum Se level in patients affected by colorectal adenoma, the precursor lesion in most CRC cases [201-204]. Se levels have also been associated with the CRC stage (from adenomatous polyps to local and metastatic cancer, respectively) [205]. On the other hand, no such a relationship was found in other studies regarding colorectal adenoma [206-208].

Only a few studies investigated the relationship between cancer and individual serum, plasma or tissue Se-proteins concentration, but again with contradictory conclusions. Inverse correlations were found between plasma GPx3 and CRC [199] or cancer of the uterine cervix [209], plasma SelP and various types of cancer [210], tissue SelP and colorectal adenoma [211], tissue GPx4 and pancreas [212] as well as breast [213] cancers. Some studies suggested that the level of TrxR1 in tumour cells is greater than in normal tissues [214-217]. Other works did not reveal any association between CRC and total Se, GPx3 and SelP levels in plasma or serum [207,218].

More numerous are the studies in genetics and biochemical behaviour of Se-proteins in cancer tissues or cell lines. The biochemical association between Se-species and cancer is mainly mediated by their action in oxidative stress control. Oxidative stress plays an important role in carcinogenesis by means of DNA damage induction and its effects on intracellular signal transduction pathways [219]. Reactive oxygen species can induce almost all forms of DNA damage that were reported in genes dysfunctions which are involved in the genesis of cancer, and play a key role in cancer development by inducing and maintaining the oncogenic phenotypes [220]. As a consequence, the genetic polymorphisms, gain or loss of functions of antioxidant enzymes, such as GPxs, attracted great interest in cancer study and therapy [221]. Loss of heterozygosity of GPx1 gene resulted implicated in lung cancer development [222]. GPx1 polymorphism is associated with increased risk of lung [223], breast [224], bladder [225], hepatocellular [226], prostate [227] carcinomas, and non-Hodgkin's lymphoma [228]. SelP polymorphism is associated to colorectal adenoma [229]. GPx3 hypermethylation was shown to occur frequently in prostate cancer and Barrett's esophagus [188]. Sep15 polymorphism is associated with lung cancer [230]. GPx2 is upregulated in some types of cancer, particularly of gastrointestinal origin [231,232]. A recent study showed that lower expression of GPx2 increased migration and invasion of cancer cell clones, but decreased their growth [233], and thus depends on the stage of tumor development. Finally, TrxR1 is probably the most investigated Se-protein in its relationship with cancer. This Se-protein presents both preventing than promoting properties for cancer. It regulates the redox state in the cell and activates the p53 tumor suppressor [234,235], but on the other hand is targeted by a number of anticancer drugs [236] and its deficiency alters cell morphology [237].

A number of non-proteic Se-species have also been tested in cancer therapy for many clinical aspects. Sodium selenite systemic or topical administration produced

radioprotective effect in normal tissues [238-240]. Such an effect was not observed in the corresponding malignant tissues, where dose-dependent radiosensitizing capacities, including apoptosis induction and cytotoxicity, were on the contrary noticed [241,242]. In general, some authors suggested that substitution of sulfur by Se in cancer chemopreventive agents may result in more effective analogues [243]. This hypothesis was confirmed for the action of a Se-analogue of the chemopreventive agent S,S'-(1,4-phenylenebis[1,2-ethanediyl])bisisothiurea (PBIT), also known to inhibit inducible nitric oxide synthase (iNOS), as an inducer of apoptosis and inhibitor of cell growth in the case of lung cancer [244]. Following this principle, a number of organo-Se compounds have been synthesized and tested as chemopreventive agents [245,246]. The production of monomethylated Se from methylselenocysteine or methylseleninic acid has been postulated as a key step in the mechanism of the anticancer activity of Se-species [247]. In particular, methylseleninic acid synergizes with tamoxifen to induce caspase-mediated apoptosis in breast cancer cells [248].

An additional aspect to be considered is that Se deficiency is nearly the norm in cancer patients treated with radio- and chemotherapy, or even just hospitalized [241]. Supplementation of cancer patients with Se at doses of up to 2000 $\mu\text{g day}^{-1}$, alone or in combination with vitamins, has been suggested as a way to improve their general quality of life [241].

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III. OVERVIEW OF THE ANALYTICAL TECHNIQUES FOR SPECIATION OF SELENIUM IN BIOLOGICAL SAMPLES

III.1. Introduction

The study of the influence of metal ions, ligands, and metal complexes on human health has been subject from the 70ies of the bioinorganic chemistry. This analytical task is extremely complex and currently challenging. Molecular mass spectrometry driven that research, providing instruments able to detect and structurally characterize large biomolecules. However, such techniques are poorly sensitive for analytes present in real complex matrices, and due to the strong dependence of ionization efficiency on the individual molecule, quantitative purposes are basically inaccessible [1]. Elemental detectors exploit the presence of the eteroatom, such as Se, to provide this kind of missing complementary information. If the two approaches are employed in an integrate context, and combined with high resolution separation techniques such as liquid chromatography or capillary electrophoresys, they constitute an extremely powerful tool to elucidate the mechanisms on the bases of the metabolism and the biological role of Se.

This chapter exposes an overview of the main separation, detection and identification techniques applied to speciation of Se in biological samples. Particular

attention is given to their application in integrated approaches. Sample preparation strategies decisively condition the success of analyses, thus are also discussed in details.

III.2. Sample preparation

A sample preparation procedure for speciation of Se requires to be milder than the acid digestion employed for the total element determination, because its distribution among different species must be preserved. The method of choice depends on the matrix, the chemical form of Se expected in the sample and the instrumental set-up selected for further separation and identification of the species. When the sample is liquid, in some cases it can be analysed after a few and simple preparative steps, whereas solid samples requires an extraction procedure to transfer the analytes to a liquid phase. If the studies are aimed to the determination of Se-proteins, particular attention must be paid to the preservation of their structure or activity. For Se-proteins

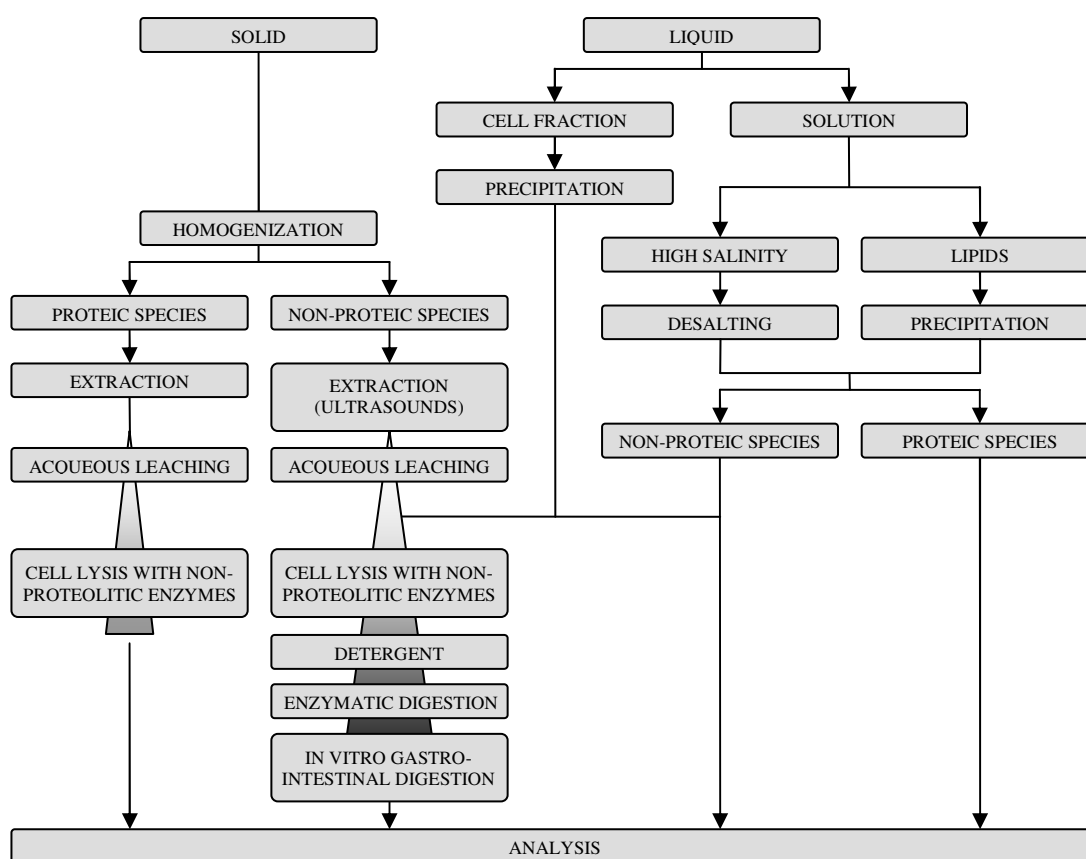


Figure III-1 Flow chart of the sample preparation strategies for speciation analysis of Se in biological samples.

speciation, an additional preparation step comprises also the raw fractionation of proteins on the bases of their molecular weight (Mw), that can be obtained through centrifugal ultrafiltration using different cutoff semi-permeable membranes. Figure III-1 resumes the main conceptual steps to be followed in the design of the sample preparation for speciation analysis of Se in biological matrices.

III.2.1. Body fluids

Blood and blood fractions (plasma, serum and erythrocytes), breast milk and urine are the body fluids most commonly analyzed for Se. Plasma and urine have the advantage of containing soluble compounds, while erythrocytes have to be lysed to release the Se-species. Desalting can be necessary for the analysis of urine, because of the high salt concentration (~1 % NaCl) that could be incompatible with the conditions of chromatographic separation. For this purpose, commercial desalting columns can be used, or the sample can be simply diluted in ultrapure water [2]. Urine has the advantage that all its normal constituents are water-soluble and this permits direct chromatographic separation. To improve the stability of urine samples, the organic matrix can be removed by passing through C18 cartridges [3]. Preconcentration of Se-species in urine can be achieved by crown ether extraction [4]. Plasma/serum samples usually do not require a special treatment, a part of filtration (through 0.45 μm cellulose filters) just before injection into the chromatographic system [5]. For erythrocytes lysis, the most common method is recurring to freeze-thaw cycles, followed by centrifugation and filtering [6,7]. The analysis of milk requires the elimination of lipids. Centrifugation at 3000 rpm for 30 min at 48° C allows to efficiently precipitate lipids, whereas precipitation of casein take place by adding acetate 1 M. The analysis is then performed on the whey. If further separation is needed, dialysis can also be used [8].

III.2.2. Tissues

The main Se-species in biological matrices are Se-amino acids, either or nor incorporated into proteins. Two distinct approaches can be adopted, depending on the focusing on Se-proteins or other organic species. If Se-proteins have to be determined, the extraction must be carried out by particularly soft procedures in order to prevent their degradation.

A wider range of approaches have been used for Se-amino acids and soluble Se-proteins extraction, including aqueous leaching, acid, basic and enzymatic digestion. Some free Se-amino acids or Se-peptides are water soluble and can be extracted with deionized water or various buffers at 25, 37, 60° C with good recoveries. This procedure has been applied for selenized yeast (10-15 % recovery) [9,10], animal tissues (10-76 % recovery) [11-15], plants and seeds (2-73 % recovery) [16-18], and mushrooms (36 % recovery) [19]. The most used media for preparation of cell lysates are Tris-HCl (TB), phosphate (PhB) and Hepes solutions, buffered at physiological pH. The latter is largely used for cell cultures to protect the structure and the activity of the frozen enzymes from cryo-induced variations of pH. The use of buffers at physiological pH is required to avoid dissociation of the complexes. If proteic species need to be preserved in the intact form, additioning reagents to the extraction buffer is also suggested. Ethylenediaminetetraacetic acid (EDTA) inhibits metalloproteases by chelating Mg and other metal ions that are part of their active site. Its use is not recommended for speciation of metalloproteins in general, where EDTA can take away the metal from the species of interest, but this is not a problem for Se-species because Se is covalently bond to them. Proteases inhibitor cocktails are available in commerce and are also widely used in biochemistry. If post-translational modifications of the proteins are under study, sodium orthovanadate and sodium fluoride can be used to inhibit the tyrosine phosphatases and the serin/threonin phosphatases, respectively [20]. Phenylmethanesulfonylfluoride (PMSF) is a serine protease inhibitor also commonly used in the preparation of cell lysates.

To increase the low yield of aqueous leaching procedures, more aggressive leaching media has been applied by several authors. Detergents increase the yield of Se-proteins extracted to the aqueous phase by breaking down the cell membrane and forming ion pairs with the proteins [21]. The anionic detergent sodium dodecyl sulphate (SDS) is the most used, but also Triton-X or Igepal (non-ionic detergents) are employed [22]. Using SDS, recoveries up to 90-100 % for both animal and plant tissues were obtained [12,18]. However, SDS breaks the non-covalent bonds and S-S (or Se-S) bridges in the proteins, denaturing them, and therefore is not recommended where the structure and the activity of the proteins have to be preserved.

Proteins precipitation by organic solvent can also be used as an additional extraction step. The use of ethanol or acetone in aqueous protein-containing extract has a variety of effects, leading to proteins precipitation. High organic solvent

percentage (> 80 %) allows precipitation of both high and low Mw proteins, whereas at 50 % solvent, the proteins of low Mw (< 15 kDa) are likely to remain in solution [17]. Thus, a preliminary fractionation of the analytes can be achieved.

Acid or alkaline hydrolysis has been applied for Se extraction with good recovery values, but in this case degradation or transformation of species can easily occur [23]. Enzymatic hydrolysis is the most used method for Se-species extraction in biological matrices, allowing to operate in temperature (37° C) and pH (7.0) natural conditions of the sample, and thus preventing degradation of the original species. The proteolytic enzymes used in most digestion protocols are proteinase K, [24,25], subtilisine [26,27], pepsine [19,28], protease [29], pronase [19], trypsin [19] or mixture of them [21]. Protease XIV and proteinase K provided extraction yields of ~70 % in mushrooms and plant tissues [19,30,31]. The recovery of Se-amino acids from animal tissues was showed to increase up to 95 % by using a mixture of proteolytic enzymes [32], but for non-proteic Se-amino acids, such as Se-MCys and GGMSC, the efficiency is similar either or nor enzymatic extraction is used [31]. The non-proteolytic enzyme driselase has also been applied for extraction of Se-species which are entrapped physical or chemically within the plant cell wall [21].

A particular application of sequential extraction is the establishment of Se bioaccessibility by using *in vitro* gastrointestinal digestion method. The procedure was firstly proposed by Luten *et al.* [33], and comprises two steps. In the first step, gastric digestion, the sample is submitted for 4 h, 37° C at the action of gastric juice consisting of 1-6 % w/v pepsine. In the following step, intestinal digestion, the solid residue from step one is treated for 4 h, 37° C with intestinal juice containing 1.5-3 % w/v pancreatine, 0.5-1.5 % w/v amylase and 0.15-1 % w/v bile salts. This procedure has been successfully applied to selenized yeast and fish samples [34,35].

Ultrasound energy can be employed for different purposes: dispersion of agglomerates, homogenization, cell disruption, emulsification. These effects are based on the cavitation process where high temperatures and pressures are generated. Due to the favoured contact between the enzymes and cellular components, the enzymatic incubation using ultrasonic probe allows quantitative extraction of SeMet from selenized yeast [36] and chicken liver [11] in time ranged within 30 s to 3 min. This method is extremely rapid in comparison with the conventional enzymatic hydrolysis, that needs at least 48 h to be performed with the same results. However, species transformation could take place in some cases, and temperature increasing has to be

controlled by performing the procedure in ice and by quick cycles and interruptions instead of a unique step. Another approach for decreasing sample treatment is the use of pressurized liquid extraction (PLE). This technique has been applied for the extraction of Se-species from yeast samples in a water:methanol mixture in 10 min [37].

Se-amino acids such as SeCys and SeMet are susceptible to oxidative degradation. To prevent oxidation, a feasible approach is the addition of iodoacetic acid (SeCys is converted into its carboxymethyl derivative), or reducing agents such as dithiothreitol (DTT) and β -mercaptoethanol (BME). These agents protect the selenolic group from oxidation, for instance reducing the SeOMet generation from SeMet to ~3 % [38], but at the same time could produce Se-species transformations and/or hamper the later separation and identification of the species. Notably, it was also observed that the degree of SeMet transformation into SeOMet is more remarkable when ultrasonic probe is applied for the extraction [39].

III.3. Separation and detection techniques

Two major techniques are employed for Se-species separation in biological samples: high performance liquid chromatography (HPLC) and electrophoretic techniques. Each method guarantees different separation power (i.e. the number of compounds that can be separated in one single run) depending primarily on the Mw range of the analytes. In general, HPLC is more effective for small molecules such as inorganic species, small metabolites, individual Se-amino acids and peptides. For studies where only few expected Se-species have to be monitored, and eventually quantified, affinity -HPLC or multi-dimensional HPLC methods offer higher specificity, and technical flexibility to be adapted for a large variety of proteins. Big proteins are better separated by electrophoretic techniques, among which capillary zone electrophoresis (CZE) is a powerful and versatile tool for the separation proteins at extremely low amount. Gel electrophoresis (GE) has the ability to isolate more than thousand proteins in a single run, and is still the most powerful separation technique for screening of Se-proteins in complex samples with unknown composition. Gas chromatography (GC) is an other separation technique that can be employed, which is specifically dedicated to the separation of volatile Se-compounds.

For Se-species detection, the most sensitive, accurate and precise techniques are elemental-specific detectors for the heteroatom (Se) present in them. Some elemental detectors can be used in combination with the separation techniques introduced above, recurring to direct on-line coupling. The so-called “hyphenated methods” allow to detect in a single analytical run individual Se-species, eliminating all the possible steps of fractions handling before detection, and hence reducing at minimum the analyte loss/degradation. Inductively coupled plasma-mass spectrometry (ICP-MS) detector is nowadays retained as the most powerful in terms of resolution and sensitivity for Se determination. This technique is multielemental, guarantees an extremely wide linearity interval for quantification, and it allows also easy coupling with HPLC, GC and CZE separation techniques. Other detection methods such as atomic absorption and fluorescence spectrometry (AAS or AFS), or inductively coupled plasma-atomic/optical emission spectrophotometry (ICP-AES/OES), can be used for Se speciation purposes, likewise hyphenated with separation techniques. Even if these methods offer lower sensitivity than ICP-MS, their reduced costs render them competitive for routine analyses and small laboratories. Finally, non-hyphenated approaches are helpful for specific requirements. Gel electrophoresis does not allow direct on-line coupling with highly sensitive detectors, but its use in off-line combination with ICP-MS or laser ablation (LA) -ICP-MS offers several advantages for Se speciation in biological samples.

III.3.1. HPLC hyphenated to ICP-MS

HPLC can be directly applied to non-volatile compounds of high and low Mw, providing a great versatility derived from several separation mechanisms. Se in biological and food samples mainly occurs in non-volatile compounds, such as inorganic species, small metabolites, Se-amino acids and Se-proteins; thus HPLC has rapidly become the most used separation method in proteomics. Its coupling to ICP-MS is mainly influenced by the composition of the mobile phase which is used to perform the chromatographic separation, and hence by the mechanism to be adopted [40].

Size exclusion chromatography (SEC) is often applied to separate Se-biomolecules with Mw in the range 10-1,000 kDa among them or from inorganic Se-species and small metabolites. SEC does not guarantee sufficient resolution for quantitative

purposes, and thus is mainly used in preliminary studies of complex samples with unknown composition. The first investigations aimed to elucidate the Se metabolism through the whole body were carried out using this technique, coupled to ICP-MS [41,42]. SEC columns with different Mw range can be tested in order to explore an interval as larger as possible, like Kannamkumarath *et al.* [18] performed for the preliminary analysis of brasil nut extracts. Several studies used SEC for the screening of Se-amino acids and other soluble Se-compounds in a variety of samples including yeast [43], plants [17,44,45], fish [46], cow serum [47] and human breast milk [48]. Most of these investigations were based only on SEC-HPLC methods hyphenated to ICP-MS, and did not provide an identification of the Se-species corresponding to the peaks that were observed. Nevertheless, useful additional information can be also achieved. Daun *et al.* [14] compared the SEC profiles of muscle extracts from seven animal species, discussing their differences in the Mw distribution of Se-species and in relation to meat quality and nutritional value. Palacios and Lobinski [49] exploited the properties of SEC to investigate the degradation of human serum Se-proteins into lighter species (protein monomers and inorganic Se), as function of storage time [44]. By a similar SEC profiles comparison, Pedrero *et al.* [45] evaluated the accumulation and distribution of Se in different parts of plants, and Kapolna *et al.* [50] studied the Hg-Se interactions in chicken liver. An additional advantage of SEC is that its separation mechanism is not based on chemical interaction between analytes and the stationary phase. Samples can be eluted by buffers with the same composition of their original conditions, and therefore the best preservation of the species is achieved. This property allows to easily employ SEC as a preliminary purification step, followed by fractions collection and their subsequent separation by other types of chromatography in a multidimensional scheme.

Even if reverse phase (RP) is probably the most diffuse separation mode used in HPLC, its coupling with ICP-MS is in principle problematic because it requires the use of eluents with high percentage of organic solvents, that negatively affects plasma stability and sensitivity. The diffusion of micro- and nano-flow HPLC systems and nebulizers allowed to overcome this problem, maintaining the absolute amount of organic solvent carried to the ICP source at a low level [1]. A μ HPLC system was used with C18 stationary phase for Se-metabolites speciation in human urine [51]. An other strategy adopted to make more feasible the RP-HPLC separation coupled to ICP-MS, consists in the addition of ion-pairing (IP) agents to the mobile phase. These

reagents are large ionic molecules that form ion pairs with the polar analytes to increase their retention in the stationary phase. Their addition allows to separate both charged and uncharged compounds in the same analytical run by recurring to mobile phases with lower organic content [40]. In this way, hydrophilic species like Se-amino acids and a wide class of Se-compounds became separable also by RP. The most used ion-pairing agents for Se speciation are trifluoroacetic acid (TFA) and heptafluorobutanoic acid (HFBA)[44,52,53]. Other compounds have been recently proposed, among which the room-temperature ionic-liquids (RTILs), that showed to efficiently separate six Se-species (selenite, selenate, Se-(Cys)₂ SeMet SeMCys and Se-Ethionine) in yeast extract by isocratic elution with 99.2 % of water [54]. Other authors proposed to improve plasma stability by decreasing the RF power to 1300 W, in combination with IPRP, and applied this strategy to isolate the water-soluble Se-species from yeast and plant extracts [52,55,56]. Working on Se-metabolites speciation in human urine sample, RP has been also used coupled to ICP-MS recurring to on-line microwave digestion-hydride generation interface [2,57]. Recently, Bendahl *et al.* [58] proposed a new system based on IPRP ultra performance liquid chromatography (UPLC), that allows separations on columns packed with particles of 1.7 µm of diameter, reaching pressures up to 15,000 psi. By coupling with ICP-MS detector, separation of Se-compounds standards and Se-metabolites in urine was achieved reducing the analysis times tenfold without losses in resolution and detection limits.

Ionic exchange chromatography (IE) is an other separation mechanism widely used not only for the separation of inorganic ions of Se, but also for other easily ionizable Se-compounds. The anion exchange (AE) mode is the most employed. It was used to separate standards of selenite, selenate, Se-(Cys)₂, SeMet, SeMCys, GGMSC; and to investigate species transformation during plant growth [45,59]. Similar methods were used to speciate Se in fish and oysters [60], cow milk [53], human urine [61] and human serum [62]. Cation exchange (CE) mode requires low pH loading conditions, which could result in low resolution of the weakly charged compounds. Anion and cation chromatographies were compared to speciate Se-compounds in white clover and yeast extracts, confirming higher resolution for AE [63,64]. However, other authors showed that by optimizing the elution conditions, CE can be successfully applied to speciate soluble Se-compounds in a wide variety of biological tissues [26,56,63-65].

In recent years, affinity (AF) chromatography emerged as a new powerful separation mechanism, designed to retain individual Se-proteins with high specificity. Stationary phases with multiaffinity properties have been explored to selectively remove the major (interferents) proteins from serum [66]. Double AF HPLC systems were proposed by Hinojosa-Reyes *et al.* [62] and subsequently refined obtaining efficient and robust methods for Se speciation in human serum or plasma [5,67]. Despite its advantages, AF-HPLC application in Se-proteomics are still very rare, because of the high costs and the scarce knowledge of such complex interaction mechanisms.

Finally, hydrophilic interactions liquid chromatography (HILIC) is a relatively new separation method, particularly adapt to highly polar compounds, which has been recently proposed for speciation of Se in enriched yeast samples [43,68].

Since each separation mode offers different advantages and is effective in the retention of specific classes of molecules, the modern trend is directed to the adoption of multidimensional approaches, where one or more additional columns are exploited to further separate the fractions isolated by first one. Two-dimensional chromatography using SEC for the first separation was applied in Se speciation studies carried out on cow milk [53], mushrooms [69] and plant samples [17,59]. Two-dimensional AF-SEC HPLC coupled to ICP-MS were used for the separation of human and mouse serum Se-proteins [70]. SEC followed by IPRP were adopted for the study of Se distribution among amino acids in plasma. The first dimension allowed to isolate the amino acids fraction, and the second dimension to separate SeMet and derivatized SeCys [71]. Moreno *et al.* [26] extracted the water soluble fractions from various marine animal tissues, and performed a first purification step by SEC-HPLC. The fractions were collected, enzymatically digested and further separated by CE-HPLC, coupled to ICP-MS detection. SeMet was detected in all tissues, TMSe was detected in oyster, mussels and trout, while inorganic Se was found in krill. A similar approach was also applied by Ruiz Encinar *et al.* [71] to speciate Se in human blood. In this case, enzymatic digestion was carried out directly on the sample, and SEC separation was performed of the digests after reduction and derivatization steps. After collection of the Se-amino acids fraction, SeMet and SeCys were separated by capillary RP-HPLC. A particular type of SEC columns, endowed with double separation mechanism (SEC and AE) in a unique stationary phase, has been recently manufactured following the principles of multi-dimensional separation,

and has been successfully applied for the speciation of Se in plants [45,59,72], mushrooms [73] and yeast [34].

III.3.2. GC hyphenated to ICP-MS

As previously mentioned, GC can be used to separate those Se-compounds which are volatile or can be readily derivatized. An elegant approach is the use of solid phase microextraction (SPME) for preconcentration. SPME was combined to GC-ICP-MS to determine several volatile alkylselenides and their sulfur analogues in *Brassica juncea* seedlings [74]. The same analysis was performed also on lupine, yeast and indian mustard grown in Se-enriched media [75,76]. Among the identified species there were DMSe, allyl methyl selenide, methanesulfenoselenoic acid methyl ester and 2-propenesulfenoselenoic acid methyl ester. GC-MS has also been applied to non-volatile Se-amino acids after derivatization with methyl chloroformiate [77] or cyanogen bromide (CNBr) [78], but these methods resulted time-consuming and poorly effective for oligopeptides.

III.3.3. CZE hyphenated to ICP-MS

Capillary zone electrophoresis is a separation technique based on differences in the electrophoretic mobility of target analytes and on electroosmotic properties. Very recently, it has been successfully proposed for coupling to ICP-MS detection system. CZE offers attractive features for Se-species separation, including high resolution, amount of sample below 1 ng, absolute detection limits at pg levels and the possibility of analysing relatively labile species. Since CZE has been coupled to ICP-MS for the first time, the main critical aspect of this set-up was adapting the flow rates of CZE (nL min^{-1}) to those required by the ICP source (mL min^{-1}) [79]. Several studies were focused on the design of efficient interfaces, some of them nowadays commercially available [80-83]. CZE-ICP-MS has been applied for chiral separation of L- and D-SeMet previously derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in enzymatically digested yeast samples, being only L-SeMet detected in the samples [84]. Bendhal and Gammelgaard [85] proposed the use of a capillary coated with polyvinylsulphonate for the separation of Se-species in aqueous extracts of selenized yeast, reaching to isolate more than 20 Se-compounds in 13 min and detection limits in the $\mu\text{g L}^{-1}$ range. CZE-ICP-MS analysis was carried out on yeast extracts by

Monicou *et al.* [86], but in this case 30 % of the Se-compounds did not elute from the column before 30 min of analysis, whereas effective was the separation of inorganic Se, SeMet and SeCys in nuts proteolytic extracts. The only application regarding human samples was proposed for urine, where 10 metabolites were effectively separated by an interesting combination of HPLC and CZE-ICP-MS [87,88]. SEC-HPLC-ICP-MS, followed by fractions digestion and further separation of the peptides by CZE-HPLC allowed to detect SeMet, TMS₂Se and inorganic Se in tissues of a variety of marine organisms [26]. In general, few applications of hyphenated techniques based on CZE for Se-speciation have been proposed until now, however this strategy appears to be one of the most promising trends in the field [89].

III.3.4. Other hyphenated techniques

Although coupled systems based on ICP-MS detectors offer the best performances in terms of sensitivity and specificity for determination of Se, many analytical laboratories cannot support such equipment because of their high price and expensive maintenance. In this context, AAS and AFS attracted some interest as an alternative to MS techniques for Se speciation in routine laboratories [90]. Graphite furnace and electrothermal AAS (GF-AAS/ET-AAS) are rapid and efficient methods that allow determination of Se in biological samples at levels below the $\mu\text{g g}^{-1}$ [91]. These techniques are affected by problems related to matrix interferences, particularly from the presence of phosphates, as well as excessive volatilization. Procedures for overcoming these drawbacks have been proposed, among which the most used is hydride generation (HG) [92-95]. ICP-AES/OES are other available methods that allow simultaneous multielemental analysis with accuracy similar to that of ET-AAS.

Some studies proposed hyphenated techniques based on HPLC separation and these detectors. Se-amino acids, selenite and selenate were determined in yeast extracts, breast and formula milk and shellfish by two dimensional on-line HPLC (RP and IE) systems coupled with microwave digestion (MD) -HG-AFS [37,96,97], which allows to reach lower detection limits [98]. The on-line transformation of Se-species into Se(IV) and reduction to H₂Se, can be carried out using K₂S₂O₈-NaOH [99], KBrO₃-HBr [100] or KBr-HCl [101]. Speciation of TMS₂Se, DMSe, Se-amino acids, selenite and selenate was carried out on plant tissues by AE-HPLC also coupled to

HG-AAS. AFS coupled to HPLC was applied for the speciation of Se in edible mushrooms [102].

Se-MCys and SeMet from enzymatic extracts of yeast were derivatized by ethylchloroformate to produce volatile ethylated species, determined then by GC-AED and GC-MS [103-105]. SPME was combined to multicapillary GC-microwave induced plasma (MIP)-OES allowing to identify at least seven Se-species, among which DMDS₂Se was predominant [106]. As garlic contains many volatile Se-species, GC-AED and GC-MS has been extensively used for speciation of Se in this sample [75,76,107]. GC-AED was also used to confirm the identification of SeMet and Se-MCys in *Brassica juncea* plants, previously revealed by IPRP-HPLC-ICP-MS [108]. Dietz *et al.* [106] performed an interesting comparison between hyphenation of (SPME) multicapillary GC to different detectors, including ICP-MS, MIP-AES and AFS, for speciation of volatile organo-Se compounds in yeast.

Only few publications reported the hyphenation of CZE with atomic detectors others than ICP-MS. Hydride-generation interfaces have been adopted for coupling CZE with ICP-AES/AFS in speciation of inorganic Se standards [109,110].

III.3.5. Non-hyphenated techniques

When the on-line separation-detection instrumental set-up is unavailable, it is possible to carry out Se speciation analysis by using the same separation techniques described above, followed by fractions collection and subsequent off-line analysis by a large variety of detectors for total Se determination. By adopting this approach, multidimensional RP/SEC-HPLC was combined to ICP-MS and atmospheric pressure chemical ionization (APCI) -MS detection, allowing separation of the main metabolites of Se in human urine, and identification of selenomethyl-N-acetylhexosamine as the main specie [57]. The same strategy was adopted also by Cao *et al.* [111]. The off-line approach allows also to employ GE as separation method. Sodium dodecyl sulphate polyacrylamide GE (SDS-PAGE) is the most common mode. It is particularly adapt to Se-proteins because these species does not loss the eteroatom (Se) despite the denaturation of the secondary protein structure that the method requires. Once the Se-proteins have been separated by the electrophoretic run, Se-containing bands (in one-dimension GE) or spots (in two-dimensions GE) can be localized by autoradiography or laser ablation (LA) -ICP-MS. Autoradiography was

used after two-dimensional GE to screen the radiolabelled Se-proteins in yeast grown in presence of ^{75}Se [112,113], but the use of this technique is restricted because sample handling is hazardous. Laser Ablation is a feasible alternative that allows also to exploit the high sensitivity of ICP-MS for detection of Se. Its combination with GE was used for first time in 2002 to identify the Se-proteins in extracts from an avocet embryo and a bass ovary collected from Se-rich waters [114]. More studies were done afterwards, applying LA-ICP-MS to one-dimensional SDS-PAGE to detect GPx1 from RBCs [115], and other Se-proteins in plants extracts [116] as well as in *Escherichia coli* culture cells [117]. The method was then extended to two-dimensional SDS-PAGE to detect Se-proteins in yeast extracts [115,118]. The cheapest solution comprises GE separation applied in combination with AAS, as was done for the first determination of Se distribution among human plasma proteins [119,120].

III.4. Identification techniques and integrate approaches

Element-specific detectors provide information concerning the presence of Se in fractions, bands or spots that were isolated. Chromatographic identification of the Se-species can be based on retention time matching with pure standards. However, this approach is often unfeasible due to the lack of appropriate standards, particularly if the species of interest are Se-proteins or unknown molecules. Molecular MS techniques such as electrospray ionization (ESI) -MS and ESI-MS/MS, and matrix assisted laser desorption ionization-time of flight (MALDI-TOF) -MS are a useful tool by which structural information can be achieved to univocally reach the identification of Se-species.

MALDI-TOF-MS is a technique by which the exact Mw of both intact proteins and peptides can be determined. Theoretically, it allows to observe the isotopic pattern of Se, but in practice this is relatively difficult for peptides, and impossible for intact proteins larger than 7-8 kDa due to inadequate resolution [118]. However, proteins can be identified from their digests by peptide mass fingerprint, as was done by Roveri *et al.* [121] to identify the proteins which resulted differentially expressed in prostate cancer cells grown in Se-enriched media. MALDI-TOF can be alternatively applied to extract other information than the exact mass of proteins or peptides. The comparison of mass spectra for haemoglobin bounded and unbounded to Se revealed

the site, functional groups and mechanisms of Se-haemoglobin binding into RBCs [122]. A similar analysis was carried out by Ma *et al.* to locate the *N*- and *O*-glycosylation sites of rat SeIP [123], and to demonstrate that together with GPx3, this protein loses Se through conversion of SeCys residues to dehydroalanine during purification and characterization [124].

For Se-species identification, ESI-MS and ESI-MS/MS are more used, since they allow high specificity and relative sensitivity for the unambiguous identification of low Mw species by the exact mass and fragmentation pattern, as well as easy on-line coupling with HPLC and CZE [1]. ESI MS/MS was used to identify SeMet as the major Se-specie in *Lentinula edodes* mushrooms [73], GGMSC in selenized odorless garlic, shallot [72] and yeast [55], Se-sugars in urine [57] and nine to twelve different Se-metabolites in selenized yeast [43,68]. Intact Se-proteins identification by ESI-MS is difficult, mainly due to relative concentration in the sample [118], thus is better to perform the analysis at level of peptides, *previa* enzymatic digestion carried out either before or after separation. Carboxymethylation is essential in this case to protect the selenolic groups in SeCys, even if the derivatization agent may react with SeMet producing artefact peaks [71]. The efficiency of species separation is also crucial, because a minor Se-peptide must arrive virtually pure at the ESI source to be detected. Knowledge of its retention time in hyphenated HPLC and CZE set-up is extremely important unless the protein is known and a particular ion can be searched for. Parallel ICP-MS detection is an extremely powerful tool to provide information about the number of compounds that should be identified, their concentration, the digestion efficiency and the columns recovery [125,126]. A similar approach can be adopted in the case of intact proteins separated by two-dimensional GE, where LA-ICP-MS allows to locate the Se-containing spots, to be digested and analyzed by ESI-MS. Such integrated ICP-MS-assisted approaches, also defined as “synergic” or “multimode”, and schematically resumed in Figure III-2, are the most actual trend in proteomics [1,127]. The use of HPLC-ICP-MS and narrowbore HPLC-ESI-MS/MS allowed also the determination of low Mw compounds in yeast, such as SeMet, Se-MCys, GGMSC, Se-(Cys)₂, Se-Lth, SeCysth, Se-A-hoCys and selenodiglutathione [128-130]. Combining HPLC-ICP-MS and HPLC-ESI-MS/MS, SeMet and Se-(Cys)₂ were determined as the major species in brazil nut extracts [25,131]. By the same strategy, SeMet, Se-MCys and GGMSC were determined in garlic and onion [132,133]; Se-A-hoCys and SeCysth in the Se-accumulating plant *Brassica genus*

[31,134], SeMet and Se-(Cys)₂ in sunflower and lupine grown in Se-enriched soil [135]. CZE was also coupled to nanoHPLC-ESI-MS/MS for speciation of Se in urine [88]. One- and two-dimensional GE, LA-ICP-MS followed by nanoHPLC with parallel ICP-MS and ESI-MS/MS detection were used for identification of rat TrxR1, bovine GPx1 and Se-methionyl calmoduline [117,127,136].

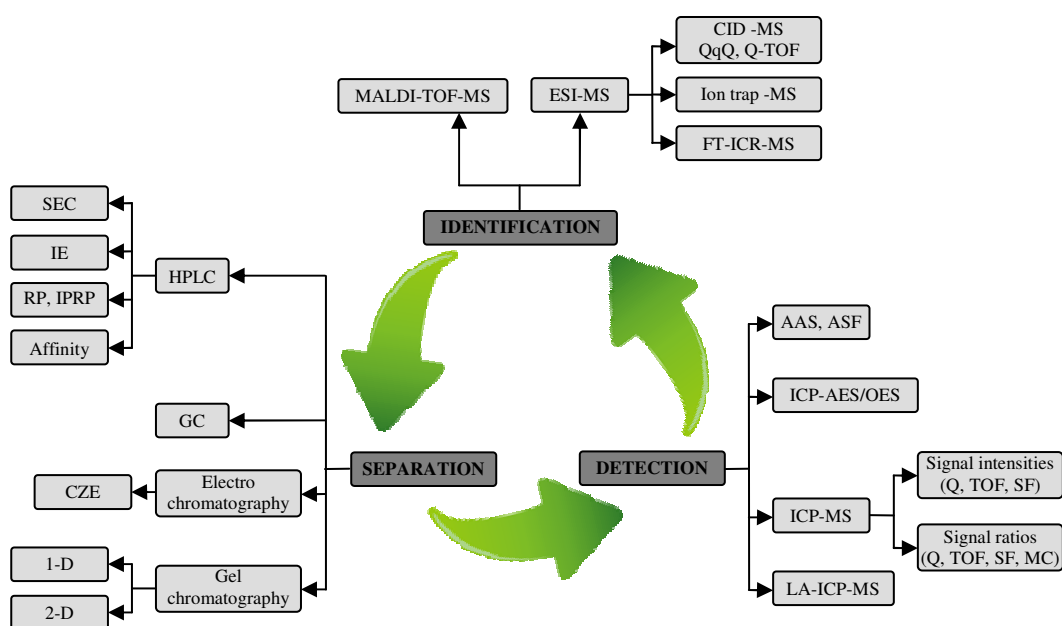


Figure III-2 Flow chart of the methodologies for speciation analysis of Se in biological samples, adapted from Szpunar [1].

Further integrated strategies in proteomics combine MALDI-MS and ESI-MS/MS analysis to obtain complementary and complete structural information. A representative example of this integrated strategy for proteomics of Se is the work carried out by Ruiz Encinar *et al.* [10]. The Se compounds in yeast were extracted in different fractions by successive steps. All fractions were analyzed by SEC-ICP-MS. The Se fractions were collected, pooled and submitted to tryptic digestion. The peptides were determined by RP-HPLC-ICP-MS. The Se-peptides were then analyzed by MALDI-TOF-MS in order to select target ions for collision-induced dissociation (CID) -MS, carried out by ESI- quadrupole (Q) -TOF-MS, enabling their *de novo* sequencing. A new family of Se-containing proteins was identified, and confirmed by MALDI-TOF-MS analysis of the original (non-digested) protein fraction. In an other similar research by Dernovics *et al.* [137], Se-peptides from yeast digests were isolated by two-dimensional SEC-RP-HPLC and detected by on-line coupling with

ICP-MS. Their identity was studied by a set of techniques including MALDI-TOF-MS and nanoHPLC-ESI-Q-TOF-MS/MS. The investigation proved the possibility of loss of Se from SeMet by degradation to vinylglycine, and the formation of new Se-peptides by methylation on carboxylic residues.

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IV. INSTRUMENTATION

IV.1. Introduction

In speciation analysis, a separation technique is coupled on-line with a detector capable to convert the “presence” of the analyte in an electric signal, then processed by the computer. As mentioned in the Chapter III, for the separation of complex molecular species, such as proteins or peptides, HPLC is one of the best choices, since it allows to operate with liquid samples in conditions close to their natural state. On the other side, ICP-MS is an ideal technique for the absolute quantification of the species of interest as it presents high specificity and sensitivity for the detection of elements like Se. This chapter presents the characteristics and the operating principles of these instruments, employed in the Ph.D. project for Se speciation in biological matrices. The technical aspects of HPLC systems and mass spectrometers with ICP and MALDI sources are briefly presented.

IV.2. High Performance Liquid Chromatography

High Performance Liquid Chromatography is a chromatographic method that allows to separate two or more analytes on the bases of their different interaction equilibrium between a stationary phase, packed into a chromatographic column, and a liquid mobile phase (eluent), flowing through the column itself [1]. The analytes are desolved into an appropriate solution, and the sample is injected into a mobile phase

stream passing through the column. A solvent delivery system (HPLC pump) provides the necessary highly controlled flow speed and chemical composition, by mixing up to four different mobile phases, under high pressure conditions.

An Agilent 1100 series HPLC system (Agilent Technologies, Yokogawa Analytical Systems, Tokyo, Japan) was employed for the chromatographic separation of Se species in biological matrices, and coupling with the ICP-MS detectors. The instrument, shown in Figure IV-1, has a modular structure.

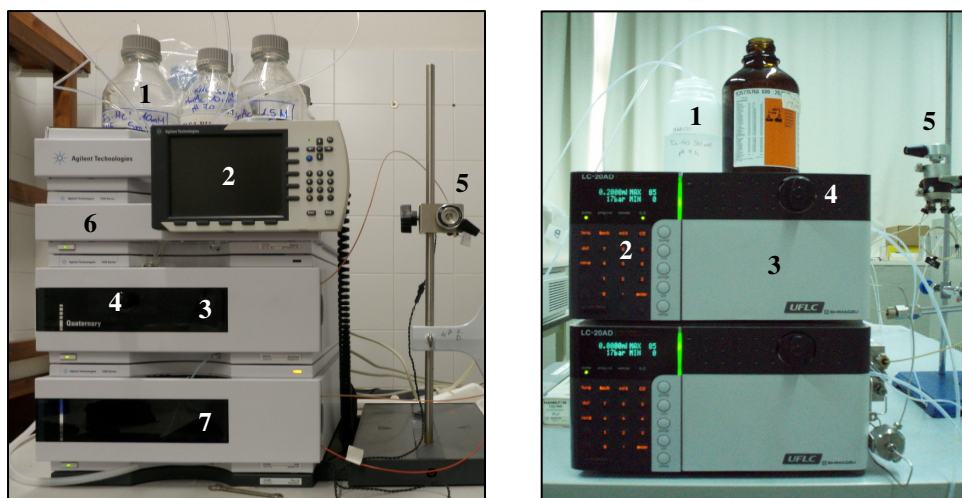


Figure IV-1 Picture of a HPLC Agilent 1100 equipped with DAD (left) and of a HPLC Shimadzu LC-30AD (right): 1) eluent bottles; 2) control panel; 3) HPLC pump module; 4) purge valve; 5) injection valve; 6) degasser module; 7) DAD module.

The solvent delivery module is a quaternary pump with a multi-channel gradient valve, designed to mix at all proportions and in gradient mode up to four eluents, and is equipped with an on-line vacuum degasser. The detector module consists in a diode-array detector (DAD) with deuterium and tungsten lamps that ensure to acquire signals from 190 to 950 nm. The detector comprises 1024 diodes and a programmable slit to optimize wavelength resolution [2]. A Shimadzu binary HPLC pump (Shimadzu LC-30AD, Shimadzu Corporation, Kyoto, Japan, Figure IV-1), equipped with Micro-volume double plunger pump [3], was also used for ICP-MS coupling. Both HPLCs were equipped with a six-way Rheodyne injection valve model 9125 (IDEX Health & Science, Wertheim-Mondfeld, Germany) [4].

Several separation mechanisms, both physical and chemicals, can be employed for the HPLC separation of the analytes, including affinity interactions, size-exclusion, ion exchange, reverse and normal phase, and hydrophobic interactions.

Affinity chromatography (AF) was used to separate serum/plasma Se-proteins (Chapters VII-IX). Affinity chromatography separates biomolecules on the basis of a reversible interaction between the protein (or group of proteins) and a specific ligand coupled to a chromatographic matrix [5,6]. The interactions between ligand and target molecule can be electrostatic or hydrophobic, van der Waals' forces and/or hydrogen bonding. To elute the target molecule from the affinity medium, the interaction can be reversed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity of the mobile phase. This technique is highly selective, widely adaptable by changing the ligand of choice, and offers high recoveries without proteins degradation.

Size-exclusion chromatography (SEC) was used in this studies as first dimension for the separation of Se-species in rat and human colon extracts (Chapters X, XI). The SEC stationary phase is a inert porous media with controlled pore size [7]. The biomolecules in the sample do not interact chemically with the stationary phase, but are separated on the basis of their differential permeability into the stationary phase depending on the molecular size (or, more correctly, their hydrodynamic diameter) [8]. Small biomolecules are able to penetrate into the pores as they pass through the column, while the large molecules remain in the interstitial space. By this mechanism, the larger the molecules are, the rapidly they are eluted from the column. The advantage of SEC is that a large variety of mobile phases can be employed to preserve the biological activity of the particles to be separated, without interferences with the filtration process. On the other hand, SEC offers usually poor resolution, and is most commonly used for preparative purposes or as preliminary separation step.

Ion (anion/cation) exchange chromatography (AE/CE) was used to separate Se-proteins in tissue extracts (Chapters X, XI). This technique allows to separate biomolecules as function of their net surface charge [9,10]. Charged target molecules interact reversibly with an oppositely charged stationary phase on the bases of their titration curve characteristics, mobile phase pH and ionic strength. The target molecules are then eluted by increasing the ionic strength, as the salt ions (typically Na^+ or Cl^-) compete with the bound components for charges on the surface of the

medium. The higher is the protein net surface charge, at the selected pH, the higher will be its retention time.

IV.3. Inductively coupled plasma-mass spectrometry

ICP-MS elemental detection is a technique that offers low detection limits (sub ng L⁻¹), a wide linear dynamic range, multi-elemental capability and easy coupling with HPLC and CZE. The ICP mass spectrometers consist in compartments including sample introduction system, ionization compartment, mass analyzer and detector. In this Ph.D. project the Agilent 7500 series and the Thermo Element2 ICP-MS instruments, showed in Figure IV-2, were used.



Figure IV-2 Pictures of the ICP-MS Agilent 7500cx (left) and Thermo Element2 (right): 1) spray chamber assembly; 2) peristaltic pump; 3) ionization compartment; 4) mass analyzer compartment.

The two mass spectrometers share a similar sample introduction system and ionization source, but differ in the type of mass filter they are based on: Agilent models are equipped with quadrupole mass analyzer, while the Element2 has a sector-field mass analyzer. The main technical characteristics of these instruments are exposed in the next paragraphs of this chapter.

IV.3.1. Principles of the method

In ICP-MS the analyte is atomized and ionized in a plasma source, the different ions are separated on the bases of their mass to charge ratio (m/z) in a mass analyzer, and finally converted into electrical pulses by a detector (a discrete dinode electron multiplier) [11,12]. The output of the instrument is therefore a mass (over charge)

spectrum. The magnitude of the electrical pulses corresponds to the number of analyte ions present in the sample, which is then used for trace element quantification by comparing the ion signal with known calibration or reference standards.

Se has six stable isotopes with a large variety of abundances, offering several options for the optimization of the measurement by ICP-MS in terms of signal precision for both individual isotope and isotopic ratios determinations [13,14]. However, its detection by ICP-MS is seriously hampered by spectral interferences, particularly in biological matrices. As reported in Table IV-1, the major sources of interference are argon-based molecular ions matching the m/z of Se isotopes, but also Cl and Br, which are typically abundant in biological samples, generate interferences on the isotopes 77 and 82 of Se.

Table IV-1 Main spectral interferences in the determination of Se by ICP-MS.

isotope	abundance [%]	main interferences	required resolution [M/ Δ M]
$^{74}\text{Se}^+$	0.89	$^{36}\text{Ar}^{38}\text{Ar}^+$	9,476
$^{76}\text{Se}^+$	9.37	$^{38}\text{Ar}^{38}\text{Ar}^+$	12,145
		$^{40}\text{Ar}^{36}\text{Ar}^+$	7,083
		$^{40}\text{Ar}^{35}\text{Cl}^1\text{H}^+$	3,825
$^{77}\text{Se}^+$	7.63	$^{38}\text{Ar}^2\text{H}^+$	3,167
		$^{40}\text{Ar}^{37}\text{Cl}^+$	9,190
		$^{76}\text{Se}^1\text{H}^+$	10,797
$^{78}\text{Se}^+$	23.77	$^{40}\text{Ar}^{38}\text{Ar}^+$	9,975
		$^{40}\text{Ar}^{36}\text{ArH}^{2+}$	2,755
		$^{40}\text{Ar}^{37}\text{Cl}^1\text{H}^+$	4,142
		$^{77}\text{Se}^1\text{H}^+$	7,471
$^{80}\text{Se}^+$	49.61	$^{40}\text{Ar}^{40}\text{Ar}^+$	9,699
		$^{40}\text{Ar}^{38}\text{Ar}^1\text{H}^{2+}$	3,297
		$^{40}\text{Ar}^2\text{H}^{2+}$	3,456
$^{82}\text{Se}^+$	8.73	$^{81}\text{Br}^1\text{H}^+$	11,055

The spectral interferences, the relatively low ionization efficiency of Se in the argon plasma (31 %), and the typically low concentration of Se in many biological matrices, render the determination of this element by ICP-MS a task requiring fine optimization procedures. Several technical strategies have been developed to resolve the spectral interferences on Se. Among them, high resolution mass analyzers and collision/reaction cell technology offer the best advantages. Their principal features are presented in the paragraphs IV.3.3 and IV.3.4, respectively, while their

performances are discussed in details in the Chapter VII. An alternative method that has been investigated for reducing the Ar-based interferences is the use of a cold plasma. Operating the plasma at low power (i.e. as low as 600 W) has the ability to reduce, but not completely eliminate, argon ion interferences [15]. However, under low power plasma conditions, the sensitivity and the mass bias are completely dependent on the sample matrix and the ability to ionize most elements is limited. These factors, combined with the low concentration of Se in real samples have made cold plasma techniques unsuitable for Se measurement [16].

IV.3.2. Sample introduction system, ionization source and interface

A view of these compartments is shown in Figure IV-3. Sample introduction devices are available in ICP-MS analysis for both solution-phase samples and solid samples. For both total Se determination and coupling to the HPLC, the liquid sample is introduced into a pneumatic nebulizer concentric tube type (Micromist), where a high-velocity argon gas flow dissociates the sample into fine droplets. The nebulizer is connected to a quartz spray chamber, thermostated at 2° C, which selects for gravity the light drops, and carries them to the plasma through a flow of Ar [17]. The plasma is an electrically neutral mixture of molecules, ions, electrons and atoms at high temperature (6,000-10,000 K). In ICP-MS the plasma is generated at the end of a

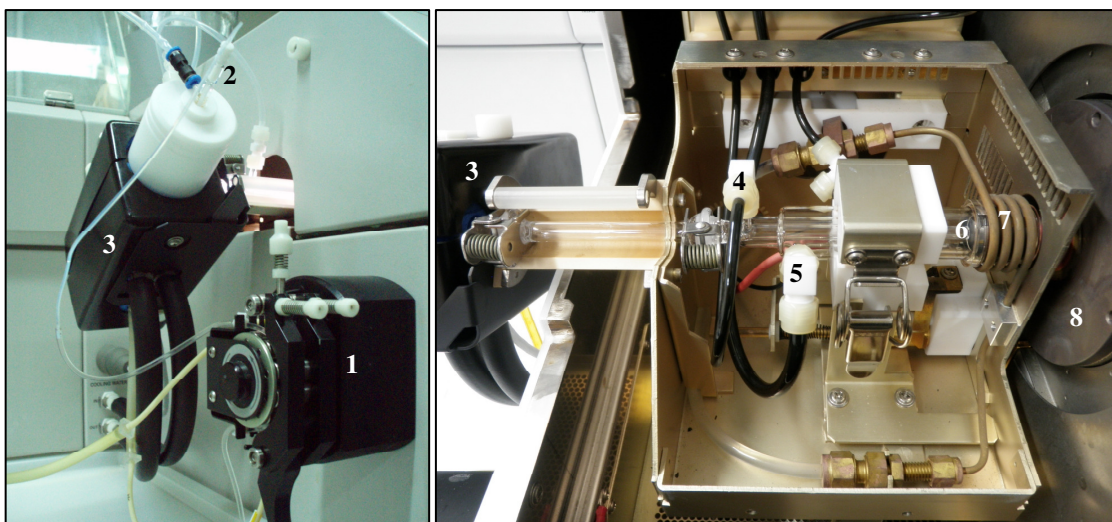


Figure IV-3 Left: sample introduction system set-up for total Se analysis by ICP-MS Agilent 7500cx: 1) peristaltic pump; 2) concentric nebulizer; 3) spray chamber compartment. Right: picture of the ionization compartment with the plasma torch of an ICP-MS Agilent 7500cx: 3) spray chamber compartment; 4) auxiliary gas inlet; 5) plasma gas inlet; 6) torch; 7) induction coil; 8) sampler cone.

torch, which operates at atmospheric pressure [18]. The plasma torch consists of three concentric tubes made from quartz, named outer tube, middle tube, and sample injector [17].

The Ar gas flow that is used to form the plasma is passed tangentially between the outer and middle tubes at a flow rate of $\sim 12\text{-}17\text{ L min}^{-1}$. A second Ar gas flow (auxiliary gas) passes tangentially between the middle tube and the sample injector at $\sim 1\text{ L min}^{-1}$ and is used to sustain the plasma and change the position of its base relative to the tube and the injector. A third gas flow (nebulizer gas), also at $\sim 1\text{ L min}^{-1}$, carries the aerosol from the sample introduction system. A three-turn water-cooled load copper coil surrounds the top end of the torch and is connected to an RF generator. The RF power applied to the coil (between 1.2 and 1.6 kW) generates an oscillating alternate current that produces an intense magnetic field at the top of the torch. With Ar gas flowing through the torch, a high-voltage spark is applied to the gas, causing some electrons to be stripped from their Ar atoms. These electrons, which are caught up and accelerated in the magnetic field, then collide with other Ar atoms, stripping off still more electrons. This collision-induced ionization of the Ar continues in a chain reaction, breaking down the gas into Ar atoms, Ar ions, and electrons, forming what is known as an inductively coupled plasma discharge. The sample aerosol is then introduced into the plasma through the inner tube of the torch. As first step the droplets evaporate and the sample becomes a solid particle, than it changes into gaseous form and consequently into ground-state atoms. As final process the atoms are converted to ions by collision with the energetic electrons of the plasma gas. The ions then emerge from the plasma and are direct into the interface of the mass spectrometer.

Since the plasma source operates at atmospheric pressure (760 torr), while the mass analyzer requires vacuum conditions (10^{-6} torr), an interface region is needed to transport the ions efficiently, consistently, and with electrical integrity from the plasma to the mass spectrometer analyzer. The interface consists of two metallic (nickel or platinum, more tolerant to corrosive liquids) cones, named sampler and skimmer, with orifices between 0.8 and 1.2 mm id., maintained at ground potential and pressure of $\sim 1\text{-}2$ torr by a mechanical pump [11]. The ions emerge from the skimmer cone and are directed through the ion optics.

Three major ion lenses blocks are used in a Agilent 7500 ICP-MS: extraction lenses, Einzel lenses and Omega lenses [17]. The extraction lenses block extracts the

ions passed through the skimmer cone, accelerate them and repels electrons. Einzel lenses block focuses and accelerates the ion beam to the omega lenses block, which separates photons and ions, and conducts the ions onto the mass analyzer. In the ICP-SFMS Thermo Element2 instrument a high voltage for acceleration is required. A special lenses system is used both to focus the ions into the mass analyser and to shape the circular peak profile of the ion beam behind the skimmer to the rectangular profile of the entrance slit [19,20]. This system consists of a quadrupole lens with an electrode configuration similar to a quadrupole mass analyser, but operates with direct current voltages only.

IV.3.3. Octopole reaction system

The Agilent 7500cx ICP-QMS is equipped with an octopole reaction system (ORS) for polyatomic interferences removal. A collision/reaction cell is positioned between the ion lenses assembly and the quadrupole mass filter. The cell contains an octopole guide for ions focusing, as represented in Figure IV-4 [17].

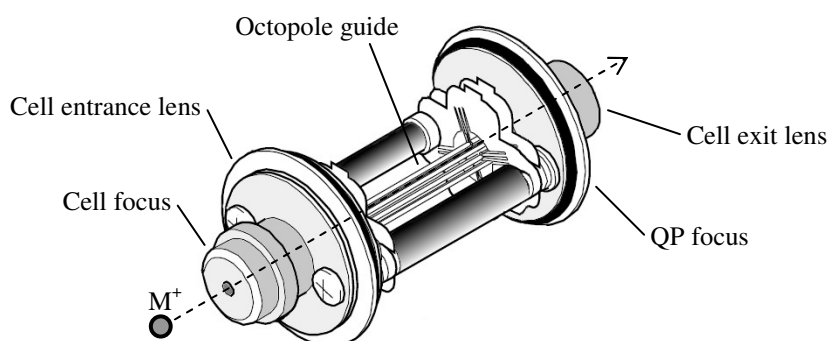


Figure IV-4 Schematic representation of the octopole collision/reaction cell of an Agilent 7500cx.

The cell can operate in collision mode by pressurizing with an inert gas like He (the most commonly used), Ar or Xe, or can operate in reaction mode if gasses like H_2 , NH_3 or CH_4 are used. The reduction of spectral interferences can take place by several processes.

In collision mode, the larger polyatomic species (greater ionic radii) collide more frequently with the cell gas, lose more energy than the smaller analyte species, and are prevented from entering the mass filter by a positive potential step at the cell exit. This filter effect is called kinetic energy discrimination (KED). In addition, in some of

the polyatomic interferences, such as ArNa^+ and ArO^+ , the bond energies are lower than their collision energy with the He atoms. In this case the polyatomic interferences are removed by collisional induced dissociation (CID).

In reaction mode the interferences reduction can take place by two processes. In charge transfer an electron is transferred from neutral molecules of the reaction gas to the interfering ion, resulting in a neutral interfering specie, which is not focused by the analyzer. Alternatively, a proton transfer can take place to or from the interfering species. Typically, the new species have low energy and get lost through ED processes. Hydrogen reaction processes are quite specific and mostly effective on argon-based polyatomic species, such as Ar^+ , ArAr^+ , and ArO^+ [21].

Inside the cell, an octopole guide guarantees ions focusing and transmission to the mass analyzer. The use of 8 rods in the octopole allows greater ion transmission efficiency compared to 6 rods (hexapole) or 4 rods (quadrupole) systems.

IV.3.4. Mass analyzer

Quadrupole

Quadrupole design mass analyzers were the first to be commercialized and nowadays comprise approximately 85 % of all ICP-MS worldwide [11]. Quadrupole ICP-MS (QMS) technology is very robust and particularly adapt for routine trace element monitoring. A QMS consists of four cylindrical or hyperbolic metallic rods of the same length and diameter, as represented in Figure IV-5. The rods are

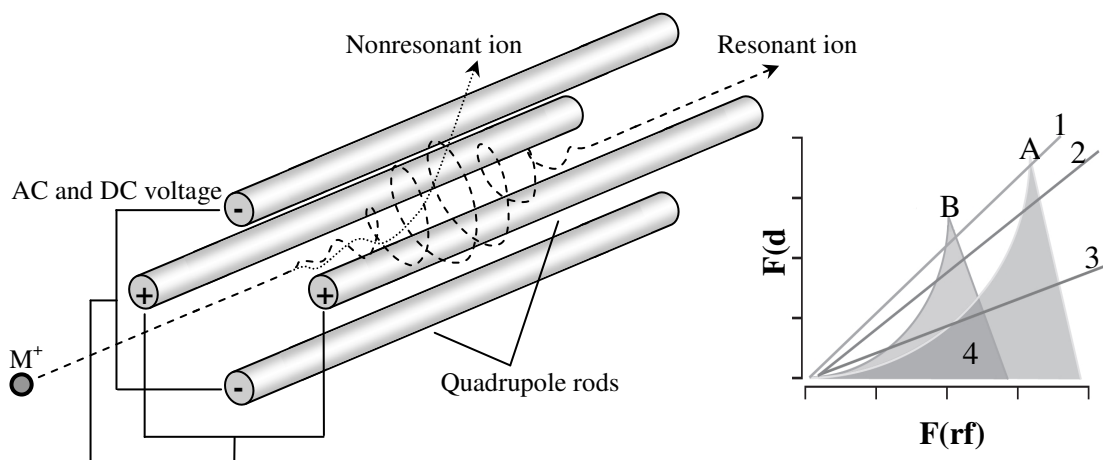


Figure IV-5 Left: schematic representation of the quadrupole mass analyzer and the behaviour of resonant and nonresonant ions. Right: simplified Mathieu stability diagram of the QMS, showing separation of two different masses A and B; (1) high resolution; (2) low resolution; (3) inadequate resolution; (4) overlapping region.

made of stainless steel or molybdenum, and can be treated with a ceramic coating for corrosion resistance. A quadrupole mass filter operates by placing both a direct current (DC) field and a time-dependent alternating current (AC) of radio frequency (typically 2-3 MHz) on opposite pairs of the four rods. For each analyte (m/z) of interest, a specific AC/DC potential ratio is optimized to allow that positive or negative bias on the rods electrostatically steer the ion down the middle of the four rods to the end, where it emerges and is converted to an electrical pulse by the detector. The other ions, with different m/z values, become unstable during the quadrupole crossing, pass through the spaces between the rods and are ejected (see Figure IV-5). Multielemental analysis is achieved by scanning the AC/DC voltage. Theoretically, a quadrupole scan rate is in the range of 2500 amu per second and can cover the entire mass range (0-300 amu) in about 100 ms. In practice, 25 elements can be determined in duplicate with good precision in 1-2 min, depending on the analytical requirements. The ICP-QMS Agilent 7500 used in this Ph.D. project operates in low resolution (300, corresponding to 0.3 % valley definition).

Double focusing sector field

In double focusing sector field mass analyzers (SFMS) a magnetic field, which is dispersive with respect to ion energy and mass, focuses the ions with diverging angles of motion from the entrance slit. An electrostatic analyzer, which is only dispersive with respect to ion energy, then focuses the ions onto the exit slit, where the detector is positioned (see Figure IV-6).

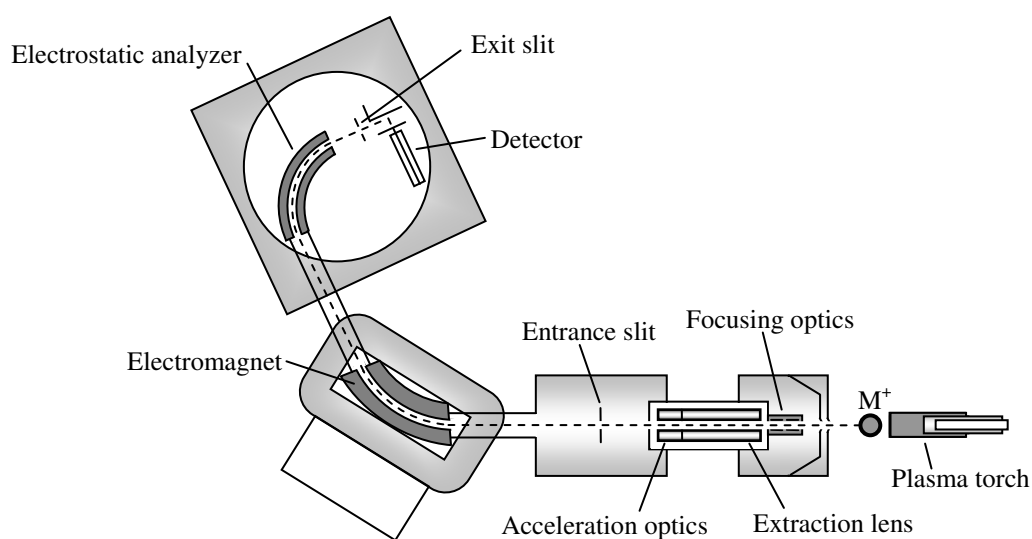


Figure IV-6 Schematic representation of the Element2 ICP-SFMS.

If the energy dispersion of the magnet and electrostatic analyzers is equal in magnitude but opposite in direction, they focus both ion angles (first focusing) and ion energies (second or double focusing) when combined together. Changing the electric field in the opposite direction during the time cycle of the magnet (in terms of the mass passing the exit slit) has the effect of “freezing” the mass for detection. Then, as soon as a certain magnetic field strength is passed, the electric field is set to its original value and the next mass is “frozen.” The voltage is varied on a per-mass bases, allowing the operator to scan only the mass peaks of interest rather than the full mass range.

Typical speeds for a full mass scan (0-250 amu) of a magnet are in the order of 200 ms. In addition, it takes much longer for a magnet to slow down, settle, and stop to take measurements, typically 20 ms compared to 1-2 ms for a quadrupole [18].

A double-focusing magnetic sector instrument involves focusing ion angles and ion energies, therefore operating mass resolution is changed by varying the conditions of two mechanical slits, at the entrance and at the exit of the mass analyzer. Most commercial magnetic sector ICP-MS systems, including the Thermo Element2 used in this Ph.D. project, can operate at high resolving power (10,000, corresponding to 10 % valley definition), medium resolving power (4,000, corresponding to 4 % valley definition) or low resolving power (300). However, similarly to optical spectrometry, as the resolution is increased the transmission decreases, therefore operating at higher resolution entails a decreasing of sensitivity.

IV.4. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry

IV.4.1. Matrix-assisted laser desorption/ionization

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique where ions are produced in two steps [12]. In the first step, the compound to be analysed is dissolved in a solvent containing small organic molecules, called matrix, that presents a strong absorption at the laser wavelength. A small amount (~ 0.5 μL) of the mixture is deposited over a support (sample plate) and slowly dried at room air and temperature before analysis. The result is a deposit of analyte-doped matrix crystals where each molecule of the analyte is embedded throughout the matrix, and

isolated from the others. The second step occurs when the sample plate is placed under vacuum conditions inside the source of the mass spectrometer. Intense laser pulses over a short duration are then directed to the matrix. Irradiation by the laser induces rapid heating of the crystals by the accumulation of a large amount of energy in the condensed phase through excitation of the matrix molecules. The rapid heating causes localized sublimation of the matrix crystals, ablation of a portion of the crystal surface, and expansion of the matrix into the gas phase, entraining intact analyte in the expanding matrix plume. Complex ionization reactions, still partially unknown, can occur under these vacuum conditions, including gas-phase photoionization, excited state proton transfer, ion-molecule reactions and desorption of preformed ions. The ions in the gas phase are then accelerated by an electrostatic field towards the analyser. In the crystal deposition, the number of matrix molecules exceeds widely those of the analyte, thus separating the analyte molecules and thereby preventing the formation of sample clusters that inhibit the appearance of molecular ions. The matrix also minimizes sample damage from the laser pulse by absorbing most of the incident energy and increases the efficiency of energy transfer from the laser to the analyte. The Voyager-DE mass spectrometer used in this Ph.D. project includes a nitrogen laser that operates at 337 nm and produces 3 ns-duration pulses [22]. An attenuator varies the intensity of the laser beam reaching the sample by deflecting the laser beam into the ion source.

Since the process is independent of the absorption properties and size of the compound to be analysed, MALDI allows the desorption and ionization of analytes with very high molecular mass, in excess of 100,000 Da. Typical MALDI spectra include mainly the monocharged molecular species by protonation in positive ion mode. More easily deprotonated compounds are usually detected in negative ion mode. Some multiply charged ions, some multimers and very few fragments can also be observed.

IV.4.2. Time-of-flight mass analyzer

Contrary to most other ionization sources that yield a continuous ion beam, MALDI is a pulsed ionization technique that produces ions in bundles by an intermittent process. The pulsed nature of the MALDI source is well suited for the time-of-flight (TOF) mass analyser. In addition, the TOF analyser has the ability to

scan ions over a wide mass range and thus is adapt to analyse the high-mass ions generated by MALDI. In this Ph.D. project, the Voyager-DE STR Biospectrometric Workstation MALDI-TOF-MS in Figure IV-7 was used for characterization of the rat colon extract AE fractions.

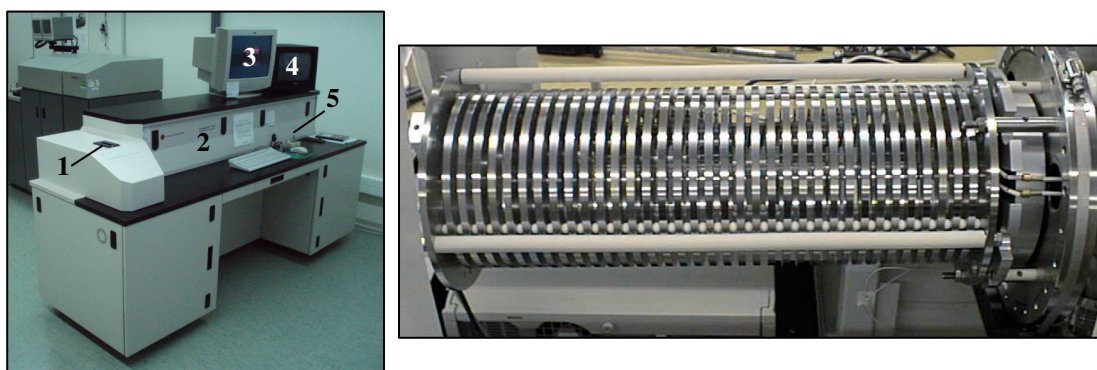


Figure IV-7 Left: picture of the Voyager-DE STR Biospectrometry Workstation MALDI-TOF-MS; 1) sample loading chamber; 2) mass spectrometer components; 3) computer monitor; 4) video monitor; 5) control stick. Right: reflectron.

After ions acceleration by an electric field, the TOF analyser separates them according to their velocities when they drift in a free-field region that is called flight tube. As represented in Figure IV-8, ions are expelled from the source in bundles

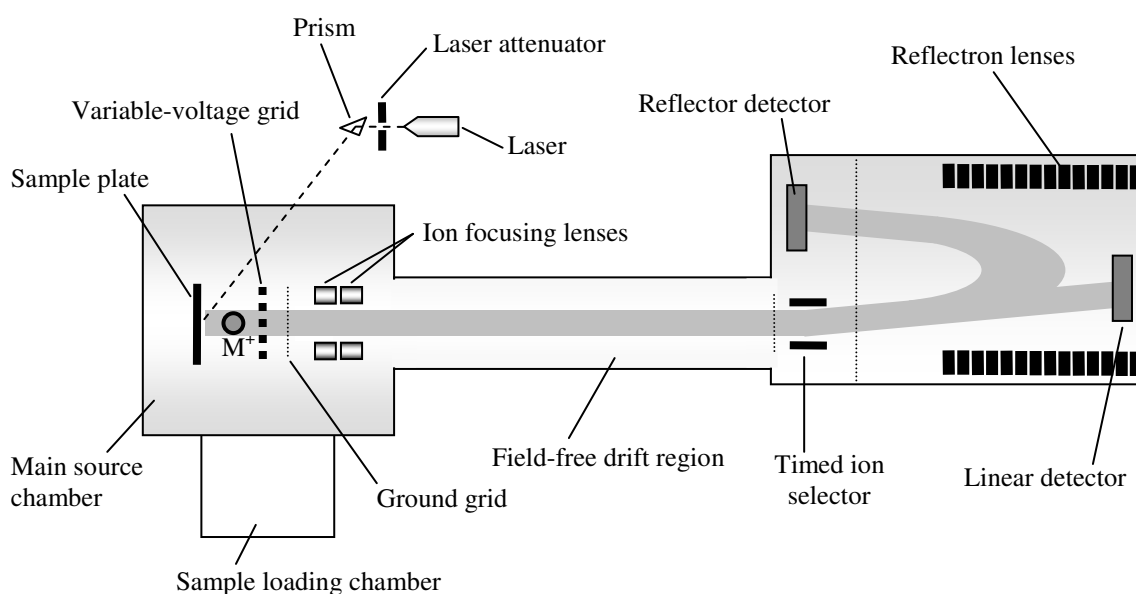


Figure IV-8 Schematic representation of the Voyager DE-STR Biospectrometry Workstation MALDI-TOF-MS.

which are produced by the intermittent process of laser desorption. These ions are then accelerated towards the flight tube by a difference of potential applied between an electrode and the extraction grid. As all the ions acquire the same kinetic energy, ions characterized by a distribution of their masses present a distribution of their velocities. When leaving the acceleration region, they enter into the flight tube where they are separated according to their velocities, inversely proportional to the square root of their masses, before reaching the detector. Mass-to-charge ratios are determined by measuring the time that ions take to move through the flight tube.

A linear detector is positioned at the end of the flight tube. It is a hybrid high-current detector consisting of a single microchannel plate, a fast scintillator, and a photomultiplier. The linear detector can be excluded if the reflector, also positioned at the terminal of the flight tube, is activated. The reflectron (Figure IV-7) is a single-stage, gridded mirror that focuses energy by a uniform electric field. Ions reflection filters out neutral molecules, corrects time dispersion due to initial kinetic energy distribution and provides greater mass accuracy and resolution. The reflectron deviates ions to two multichannel plate reflectron detectors that send a signal to the digitizer for conversion. The Voyager-DE instrument is also equipped with a timed ion selector (TIS) that allows only ions of a selected mass of interest to reach the detector. The TIS device is a Bradbury-Nielson gate positioned approximately 676 mm from the ion source. This prevents ions deflected by the gate from entering the reflector and reduces background noise in the detector. When the TIS is turned on, voltage is applied to it for ions deflection. At the time that corresponds to the ion of interest, voltage is turned off to allow it entering the detector, then voltage is turned on again.

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V. STATISTICS FOR DATA ANALYSIS IN EPIDEMIOLOGICAL STUDIES

V.1. Introduction

As part of this Ph.D. project, some of the analytical methods previously developed by our research group for Se-proteins speciation analysis in human serum/plasma have been applied to carry out studies of epidemiological interest. Serum or plasma Se-proteins pattern was determined in patients affected by type II diabetes (Chapter VIII) and colorectal cancer (Chapter IX), and compared with the pattern of healthy control subjects. Following the goal of detecting possible associations among individual Se-proteins concentration, presence/progression of the disease and clinical parameters, several statistical methods proper of epidemiology have been applied. The learning of their main theoretical fundamentals, synthetically exposed in this chapter, has been essential to appropriately use these tools, and correctly interpret the results in such a delicate issues.

V.2. Normality: S-W test

In order to compare two independent groups on the basis of the measure of a continuous variable, a necessary preliminary step consists in testing the normal distribution of the data in the groups being compared. The assumption of normality

can be verified by the Shapiro-Wilk (S-W) test [1]. This test is based on the null hypothesis that the values of the variable for a given group are normally distributed vs. the alternative hypothesis that they are not. The test statistic W is calculated as:

$$W = \frac{\left(\sum_{i=1}^n a_i x_{(i)}\right)^2}{\left(\sum_{i=1}^n (x_i - \bar{x})\right)^2} \quad (\text{eq. V-1})$$

where $x_{(i)}$ is the i^{th} order statistic (i.e., the i^{th} -smallest value in the group), \bar{x} is average value of the variable and the constant values a_i are give by:

$$(a_1, \dots, a_n) = \frac{m^T V^{-1}}{\sqrt{m^T V^{-1} V^{-1} m}} \quad (\text{eq. V-2})$$

with

$$m = (m_1, \dots, m_n)^T \quad (\text{eq. V-3})$$

m_1, \dots, m_n are the expected values of the order statistics of independent and identically-distributed random variables sampled from the standard normal distribution, and V is the covariance matrix of those order statistics. The null hypothesis is rejected if the value of W is lower than the critical value corresponding to the same degrees of freedom and to the chosen confidence level (typically $\alpha = 0.05$).

The assumption of normality needs to be checked before the application of the t -test.

V.3. Paired groups comparison: t -test and K-S test

Once the normal distribution has been verified, the t -test can be appropriately applied for the comparison of the two groups. The test is built on the null hypothesis that the mean values of the variable in the two groups are not statistically different, with an alternative hypothesis that the two means are statistically different. The assumptions underlying a t -test are that:

- the data used to carry out the test should be sampled independently from the two groups being compared;
- each of the two groups being compared should follow a normal distribution.

In the most general case of unequal variances and samples number for the two groups, the statistic t is calculated as follows:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}} \quad (\text{eq. V-4})$$

where \bar{x}_1 and \bar{x}_2 are the average values of the variable for the two groups, s_1 and s_2 the standard deviations, and n_1 and n_2 the corresponding number of samples. The null hypothesis is rejected if the value of t is higher than the critical value calculated for the corresponding degrees of freedom with the appropriate confidence level (typically $\alpha = 0.05$).

On the contrary, if the data resulted not normally distributed by the S-W test, the t -test cannot be appropriately applied. In this case, a possible way to carry out the group comparison is transforming the data by an appropriate formula in order to convert them in a normally distributed variable. Several alternative transformations can be applied for this purpose, including logarithm, square root and Box-Cox (a family of power transformations). In general there are no general rules for the choice of the best transformation formula, and neither the guarantee that normal distribution can be obtained. An alternative option is the use of non parametric tests, which are not affected by basics assumptions on the data distribution. These tests are more widely applicable, but if the same conditions are respected (i.e. normality) they are less robust in comparison with parametric tests like the t -test. A frequently used non parametric test for groups comparison is the Kolmogorov-Smirnov (K-S) test. The Kolmogorov-Smirnov statistic D for two-sample test is definite as:

$$D_{n_1, n_2} = \sup_x |F_{1, n_1}(x) - F_{2, n_2}(x)| \quad (\text{eq. V-5})$$

where F_{1, n_1} and F_{2, n_2} are the empirical distribution functions of the first and the second group, respectively. The K-S test checks whether the two data samples come from the same distribution, without specifying what distribution is (e.g. normal or not normal). The null hypothesis that the two distributions are not statistically different is rejected at the confidence level α if:

$$\sqrt{\frac{n_1 \cdot n_2}{n_1 + n_2}} D_{n_1, n_2} > K_\alpha \quad (\text{eq. V-6})$$

V.4. Multiple groups comparison

V.4.1. Analysis of variance

The purpose of one-way analysis of variance (ANOVA) is to test differences in means (for groups or variables) for statistical significance by partitioning the total variance of the data into two sources. The within-group variance is computed by adding together the sums of squared deviations within each group, while the between-group variance is given by subtracting it from the sum of squared deviations based on the overall data. The first component is due by the difference between mean values among the groups. Under the null hypothesis that there are no mean differences between groups in the population, the variance estimated based on within-group variability should be about the same as the variance due to between-groups variability. Those two estimates of variance are compared *via* the *F*-test, which tests whether their ratio is significantly greater than 1.

V.4.2. Kruskal-Wallis analysis of variance

The Kruskal-Wallis ANOVA by Ranks test is a nonparametric alternative to the one-way ANOVA [2,3]. It assumes that the variable under consideration is continuous and that it was measured on at least an ordinal (rank order) scale. The test assesses the hypothesis that the different samples in the comparison were drawn from the same distribution or from distributions with the same median. Thus, the interpretation of the Kruskal-Wallis test is basically identical to that of the parametric one-way ANOVA, except that it is based on ranks rather than means. The statistic is give by:

$$K = (N - 1) \frac{\sum_{i=1}^g n_i (\bar{r}_i - \bar{r})^2}{\sum_{i=1}^g \sum_{j=1}^{n_i} n_i (r_{ij} - \bar{r})^2} \quad (\text{eq. V-7})$$

where: n_g is the number of observations in the group g , r_{ij} is the rank of the observation j within the group i , N is the whole sample size. r_{ij} is computed as:

$$\bar{r}_i = \frac{\sum_{j=1}^{n_i} r_{ij}}{n_i} \quad (\text{eq. V-8})$$

The associated probability is given by: $P(\chi_{g-1}^2 \geq K)$

V.5. Correlation

V.5.1. Linear correlation

Pearson (linear) correlation between two variables assumes that they were measured on at least interval scales, and it determines the extent to which values of the two variables are linearly related to each other. The value of correlation (correlation coefficient) does not depend on the specific measurement units.

V.5.2. Spearman's rank correlation

Spearman's rank correlation coefficient ρ is a nonparametric measure of statistical dependence between two variables [4]. It assesses how well the relationship between two variables can be described using a monotonic function. To calculate ρ the n raw scores X_i, Y_i are converted into ranks x_i, y_i , and the differences $d_i = x_i - y_i$ between the ranks of each observation on the two variables are calculated. If there are no tied ranks, then ρ is given by:

$$\rho = 1 - \frac{6 \sum d_i^2}{n(n^2 - 1)} \quad (\text{eq. V-9})$$

If tied ranks exist, Pearson's correlation coefficient between ranks should be used for the calculation:

$$\rho = \frac{\sum_i (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_i (x_i - \bar{x})^2 \sum_i (y_i - \bar{y})^2}} \quad (\text{eq. V-10})$$

The same rank has to be assigned to each of the equal values, that is an average of their positions in the ascending order of the values. The sign of the Spearman's correlation indicates the direction of association between X (the independent variable) and Y (the dependent variable). The Spearman's correlation increases in magnitude as X and Y become closer to being perfect monotone functions of each other. When X and Y are perfectly monotonically related, the Spearman correlation coefficient becomes 1. Such a perfect monotone increasing relationship implies that for any two pairs of data values X_i, Y_i and X_j, Y_j , $X_i - X_j$ and $Y_i - Y_j$ have always the same sign. A perfect monotone decreasing relationship implies that these differences always have opposite signs.

V.6. Test for confounding factors: analysis of covariance

In epidemiological studies, the result of a test for groups comparison can be affected by the influence of variables that lead the action of confounding factors due to their nature of covariates in respect to the grouping parameter. The analysis of covariance (ANCOVA) tests whether certain factors (both categorical and continuous) have an effect on the outcome variable after removing the variance for which covariates account [1,5,6]. ANCOVA is a general linear model with one continuous outcome variable (quantitative) and one or more factor variables (qualitative). Like ANOVA, this method is based on the assumption that the errors are normally distributed and homoscedastic. In addition, ANCOVA assumes also that: (1) covariates and grouping variables are independent; (2) the relationship of the dependent variable to the independent variable(s) is linear. The first additional assumption is particularly important since it implies that the overall regression model is true for all groups of measurements. ANCOVA is conducted by fitting a linear regression model to the entire data set, ignoring to which group a measurement belongs.

V.7. Multivariate analysis

V.7.1. Factor analysis

Factor analysis (FA) refers to a set of latent variable models and methods for fitting them to data [7,8]. Factors are latent variables: unobservable constructs presumed to underlie the measured variables. The objective of FA is to identify the number and the nature of factors that produce covariances or correlations among variables. The variance of each measured variable is partitioned into common variance, shared with the other variables, and specific variance, which is both random error and systematic unshared variance. In this picture, common factors represent common variance and specific factors represent specific variance.

The FA model is:

$$\sum_{xx} = \Lambda\Phi\Lambda^T + \mathbf{D}_\psi \quad (\text{eq. V-11})$$

where \sum_{xx} is the $p \times p$ covariance matrix among the measured variables x_1, x_2, \dots, x_n ; Λ is a $p \times m$ matrix of regression coefficients, called factor loadings, that relate

each factor to each measured variable; Φ is an $m \times m$ matrix of correlations among m factors; and \mathbf{D}_ψ is a $p \times p$ diagonal matrix of specific variances (one for each measured variable).

Classic FA is applicable to continuous measured variables, and is analogous to multivariate linear regression with the exception of the predictors are unobservable. Assumptions comparable to those made in linear regression are made in FA: (1) common and unique factors are presumed to be uncorrelated; (2) specific factors are presumed to be uncorrelated with each other; (3) the measured variables are assumed to be linearly related to the factors. Additional assumptions are needed to identify the model because latent variables have no inherent scale. The scale of the common factors is often identified by fixing the mean and variance to 0 and 1, respectively. This implies the necessity to standardize the data before apply the FA.

Exploratory FA (EFA) is performed when investigators are unable or unwilling to specify the number and the nature of the common factors, as is most commonly the case. A key task is to select the number of common factors (m) that best accounts for the covariance among measured variables. Several models with differing m are fitted to the same data and both statistical information and substantive interpretability are used to select a model. The goal is to identify the number of major common factors such that the solution is not only parsimonious, but also plausible and well matched to the data.

Once the parameters of a model with a particular m were estimated, the solution is rotated to improve substantive interpretability. Rotated, but not unrotated, factor loadings aid in the selection of m . Factorial analysis models can be represented graphically in a m -dimensional space with an axis for each factor and a point for each measured variable. In this space, axes rotation is aimed to obtain that: (1) each factor is represented by a distinct subset of measured variables with large factor loadings; (2) subsets of measured variables defining different factors overlap minimally; (3) each measured variable is influenced by only a subset of common factors. Numerous rotation methods have been developed [9]. One major distinction among them is whether factors are permitted to correlate. Orthogonal rotations force factors to be uncorrelated, whereas oblique rotations permit nonzero correlations among factors. One of the most used orthogonal rotations is called Varimax, and consists in

maximizing the sum of the variances of the squared factor loadings on each factor [10].

Because the EFA model is rotationally indeterminate, an additional criterion is imposed when the parameters are estimated, so that initial factor loadings are specific. By this criterion, each common factor should account for the maximum possible amount of variance in the measured variables. Only one Λ satisfies the principle factors criterion, and it is estimated using eigenvalues and eigenvectors. An eigenvalue is equal to the sum of squared loadings down each column of Λ , therefore it is interpreted as the proportion of variance accounted for by each factor [11].

V.8. Multiple correspondence analysis

Multiple correspondence analysis (MCA) is used to analyze a set of observations described by a set of nominal variables. Each nominal variable comprises several levels, and each of these levels is coded as a binary variable. For example, gender is a nominal variable with two levels. The complete data table is composed of binary columns with one and only one column taking the value “1” per nominal variable. Multiple correspondence analysis can also accommodate quantitative variables by recoding them as “bins.” The coding scheme of MCA implies that each row has the same total, which for correspondence analysis (CA) implies that each row has the same *mass*. As general application, MCA allows to evaluate whether in a sample categorical measured variables (i.e. presence *vs.* absence of a given set of disease symptoms) tend to be distinct into groups in which they behaves in a correlate way. In practice, MCA is an extension of CA which can be seen as an adaptation of principal component analysis (PCA) when the variables to be analyzed are categorical instead of quantitative. Both MCA and PCA methods are based on decompositions of centred and normalized matrices, using either the eigenvalue-eigenvector decomposition of a square symmetric matrix. Similarly to PCA, MCA provides eigenvalues that are squared singular values (called “principal inertias” in MCA), percentages of explained variance (percentages of inertia), factor loadings (correlations with principal axes), and communalities (percentages of explained inertia for individual rows or columns) [12,13].

V.9. Logistic Regression Analysis

Logistic regression (LR) is a modelling tool frequently used in case-control studies for the assessment of risk factors in rare disorders. This method is basically adapted to ordinary least squares (OLS) regression when the dependent variable is categorical instead of quantitative. In both OLS and LR, the fitted models consist of linear combinations of the independent (multiple) variables. Since in LR the dependent variable is categorical, the principle of least squares cannot be used as fitting criterion like in OLS, but the principle of maximum likelihood is rather applied. On its bases, the intercept and slope coefficients are estimated by searching iteratively for the values that maximize the probability of obtaining the observed data [11,14].

The LR model output is an estimate of the probability for a set of observations to belong to one of the levels (denoted as “=1”) defined for the dependent variable D . This probability is denoted by the conditional probability statement:

$$P(D = 1 | X_1, X_2, \dots, X_k) = P(\mathbf{X}) \quad (\text{eq. V-12})$$

and is expressed as:

$$P(\mathbf{X}) = \frac{1}{1 + e^{-(\alpha + \sum \beta_i X_i)}} \quad (\text{eq. V-13})$$

Where α and β_i are the parameters estimated by the maximum likelihood principle. Once the model has been built, the probabilities p_1 , p_0 for a set of observations to belong to one of the two levels of the dependent variable are estimated. Then, for each independent variable can be calculated an odds ratio OR :

$$OR = \frac{p_1 / (1 - p_1)}{p_0 / (1 - p_0)} \quad (\text{eq. V-14})$$

V.10. Survival Analysis

Survival analysis (SA) is a statistical procedure for data analysis where the outcome variable of interest is follow-up time until an event occurs [15-17]. Such an event is called survival, but can be in general a binary variable like death or failure. Most survival analyses must consider a key analytical problem called censoring. In essence, censoring occurs when some information regarding the individual survival time is only partial. Censoring can occur because: (1) a person does not experience the

event before the study ends; (2) a person is lost to follow-up during the study period. In these cases the person's exact survival time becomes incomplete at the right side of the follow-up period, therefore this kind of data are considered right-censored. Left-censoring is less common in epidemiological studies, and can occur when a person's true survival time is less than or equal to that person's observed survival time. This is the case, for example, of diseases which are diagnosed with an unknown delay.

Survival analysis is based on the survivor function $S(t)$, which gives the probability that a person does not experience the event before than a specified time t : that is, $S(t)$ gives the probability that the random variable T exceeds the specified time t :

$$S(t) = P(T > t) \quad (\text{eq. V-15})$$

The survival function is estimated by the observed relative frequencies using the Kaplan-Meier method, and has the form of a step function. Giving the opposite side of the information of the survivor function, the hazard function $h(t)$ expresses the instantaneous potential per unit time for the event to occur, given that the individual survived until the time t . The hazard function is defined as:

$$h(t) = \lim_{\Delta t \rightarrow 0} \frac{P(t \leq T < t + \Delta t | T \geq t)}{\Delta t} = - \left[\frac{dS(t)/dt}{S(t)} \right] \quad (\text{eq. V-16})$$

Once the survivor curves have been obtained for two groups of subjects (defined by a different level of an independent variable), they can be compared by the log-rank test of statistical significance, in order to establish if they significantly differ, that is if the independent variable significantly influences the survival time. The log-rank test is a large-sample chi-square test that makes use of observed *vs.* expected cell counts over categories of outcomes. The categories for the log-rank statistic are defined by each of the ordered failure times for the entire set of data being analyzed. The log-rank test statistic is made by computing the observed and expected number of events in one of the groups at each observed event time, and then adding them to obtain an overall summary across all time points, where there is an event. Given $j = 1, \dots, J$ as the time of observed events in either group; N_{1j} and N_{2j} as the number of subjects "at risk" (that have not yet experienced an event or been censored) at the start of period j in the two groups being compared; and O_{1j} and O_{2j} as the observed number of events in the two groups at time j . If O_j events happened across both groups at time j , under

the null hypothesis that the two groups have identical survival and hazard functions, O_{1j} has a hypergeometric distribution with expected value:

$$E_{1j} = O_j N_{1j} / N_j \quad (\text{eq. V-17})$$

and variance:

$$V_j = \frac{O_j (N_{1j} / N_j) (1 - N_{1j} / N_j) (N_j - O_j)}{N_j - 1} \quad (\text{eq. V-18})$$

The log-rank statistic is then defined as:

$$Z = \frac{\sum_{j=1}^J (O_{1j} - E_{1j})}{\sqrt{\sum_{j=1}^J V_j}} \quad (\text{eq. V-19})$$

If the two groups have the same survival function, the log-rank statistic is approximately standard normal. A one-sided level α test will reject the null hypothesis if $Z > z_\alpha$, where z_α is the upper α quantile of the standard normal distribution.

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VI. METHODS FOR THE QUANTIFICATION OF SELENIUM BY ICP-MS

VI.1. Introduction

Many methods for the quantification of elements by ICP-MS are usually available, including external and internal calibration, matrix-matching and isotope dilution analysis. The analysis of biological samples, particularly by HPLC-ICP-MS coupling, entails the necessity to take into account possible matrix and species-specific sensitivity effects that can dramatically affect the accuracy of determinations. For Se-proteins speciation, the study of different quantification strategies becomes particularly important due to the almost total lack of commercially available pure standards. In the Chapter VII, external calibration and isotope dilution analysis are extensively investigated and compared for the determination of serum/plasma Se-proteins. External calibration was adopted in the epidemiological studies concerning patients with diabetes and colorectal cancer (see Chapters VIII and IX, respectively), while isotope dilution analysis was chosen for method development regarding Se speciation in rat and human colon tissue (see Chapters X and XI, respectively).

In this chapter, the main theoretical features of each quantification strategy are briefly exposed. Particular emphasis is given to isotope dilution analysis, which constitutes the most innovative method, and also the more extensively used in this Ph.D. project. The estimation and optimization of instrumental parameters for isotope

dilution analysis, exposed in this chapter, are an important part of the experimental work carried out prior to the methods development and application. Instrumental optimizations for Agilent 7500cx ICP-MS were carried out by using two identical devices, located at the University of Oviedo and at the University of Venice. For individual instrument-dependent parameters, in the next paragraphs the location of the device is specified.

VI.2. External calibration

In external calibration a linear calibration graphs is obtained by independently measuring a set of calibration standard solutions. External calibration can be species-specific if the chemical form of the calibrant is the same of the analyte to be determined, whereas for species-unspecific calibration the calibrant is different in its chemical form.

In elemental analysis by ICP-MS, it is well known that the matrix can strongly influence the ionization process. Such influence can be complex and non-linear, moreover it can be highly dependent on the chemical form of the element. Therefore, external calibration should be ideally carried out by using the same species of the analyte, prepared in the same matrix of the sample. Unfortunately, in proteomics it is very common to investigate analytes that are not commercially available, as is the case of most of the Se-proteins. In this cases, a careful study of the behaviour of different candidate calibrants should be carried out in order to choose the one that shows the most similar behaviour in comparison to the analyte.

In total Se determination (direct analysis by flow injection), the problem of matrix effects can be partially reduced by diluting the sample in water or 1 % HNO₃ water solution, but in chromatographic mode (HPLC-ICP-MS coupling) the use of specific eluents-buffers often imposes strong matrix effects that cannot be avoided. Moreover, if chemical forms of the element others than the analyte are used for external calibration, in chromatography it is highly probable that they are eluted at a different retention time, and if gradient elution is used this means different matrix conditions. These aspects have been deeply investigated during this Ph.D. project, particularly by comparing the use of Se-(Cys)₂, SeMet and inorganic Se as external calibrants for the quantification of human serum Se-proteins. As is exposed in the paragraph VII.5.3, effects of sensitivity variation were evidenced to be function of the chemical form of

the species for calibration, HPLC eluents and flow, and also the detector (Agilent 7500cx or Element2) to be used.

One more drawback associated with the use of external calibration consists in the absence of control under the possible variation of instrumental conditions (i.e. nebulisation conditions, plasma temperature, cones and lenses cleaning) during routine analyses, which can further influence sensitivity and matrix effects. The only way to relatively compensate this common variations, is the analysis of a standard sample (like the certified reference material BCR-637 for human serum samples) or a standard calibration solution every 5-10 real samples, in order to qualitatively detect significant drifts.

VI.3. Internal standardization

In this approach, a known amount of a internal standard is added to all samples, calibration standards and blanks. The internal standard is a compound closely related to the analyte, but different enough to be quantified separately. The analyte content is then calculated from the relative response of the analyte and the internal standard. Given that ratio of signals is measured, the fluctuation of the signal between the calibration standards and the real samples, caused by matrix effects or instrumental drift, is theoretically compensated by the same fluctuation for the internal standard signal. Internal standards can be added to the sample also before the preparation steps. In this way possible losses of the analyte can be compensated, and recoveries can be also estimated.

In case of ICP-MS analysis, the internal standard should be as close in atomic mass (m/z) and/or first ionization potential to the analyte, if correction for matrix effects is to be successful. For general applications, the internal standards mostly used in ICP-MS are Li, In, Rh, Sc, Y, and Bi. However, for specific application, the list of the internal standard used might be extended or reduced.

VI.4. Standard addition

In this internal calibration method, a standard of the analyte is added in increasing known amounts to at least three to five aliquots of the same sample. The amount of analyte contained in the original sample is then calculated from the standard-added

linear calibration function by extrapolation of the value of signal intensity at concentration zero.

Standard addition method is carried out for a specific sample and cannot be extended to the analysis of another one, since each of them requires its own calibration. This is a crucial drawback for the application in routine analysis of samples available only in low amount and/or in which the analytes are present at concentration close to the detection limit. The problem is particularly important in the case of analysis of biological samples by ICP-MS for determination of Se, since this element is typically present at extremely low levels, and the instrumental sensitivity is low itself. Moreover, biological samples can be available only in a very low amount. Blood/plasma/serum are usually easy to collect in relatively relevant volume from humans, but only ~0.5 mL is the total volume of serum that can be obtained from a rat (killing him). For tissues, the problem of sample amount is even more crucial. The colon of rats can weight about 3-5 g in total, but only part of the tissue can be used for the analyses, particularly for homogeneity criterion satisfaction. Tissues from humans are even more difficult to obtain. If collected from living individuals by biopsy, only ~ 0.5/1 g of tissue is typically removed; while surgical interventions (i.e. cancerous tissue removing) can supply larger amounts. An alternative can be recurring to sampling from dead subjects. For human samples, additional aspects needs to be also considered, including legislation, ethical implications and collaboration with medical doctors.

The standard addition method can only be applied if the following conditions are respected:

- the analytical method is selective enough; that is, the response is entirely due to analyte;
- a linear relationship exists between signal intensity and concentration of the analyte;
- method sensitivity unchanges with the additions;
- blank value can be corrected for.

VI.5. Isotope dilution analysis

Isotope dilution analysis (IDA), can be used in ICP-MS for the determination of elements with at least two isotopes free from spectral interferences. This technique is

based on the principle that an isotopically enriched analogue (for inorganic MS) or an isotopically labelled analogue (for organic MS) of the analyte is used as an internal standard for its quantification. After addition of an accurately known amount of the isotopic analogue to the sample, the mixture is analyzed, and the analyte concentration can be calculated from the ratio between signal intensities for the two isotopes.

Isotope dilution analysis presents considerable advantages for ICP-MS applications:

- is considered an absolute method due to its precision, accuracy and provision of definable uncertainty values.
- Once equilibration of the spike and analyte isotopes has been achieved, the total recovery of the analyte is not required, because the determined value is based on measuring the ratio between the analyte and the isotopic analogue (spike), that theoretically behaves in the same way as the analyte. This feature allows to compensate for bias sources originated by loss of analyte during the preparation or pre-treatment of the sample.
- The analyte and its isotopic analogue behave in the same way also in the ICP source. This allows to compensate also plasma fluctuations, instrument instabilities and matrix effects.
- Once acquired the necessary mathematical tools, the methodology is less time consuming and can provide greater accuracy than other methods like external/internal calibration and standard addition.

On the other hand, drawbacks of the method can be found in:

- cost and availability of suitable isotopic materials.
- Possible differences in the physical (e.g. solvation) and chemical properties (e.g. pK value) between the analyte and the isotopic analogue, that can affect the ions generated in the ICP source (most applicable to organic analysis).
- Utmost importance of the analyst training.

VI.5.1. Mathematical elaborations for IDA

The analyte concentration can be calculated recurring to the following formula:

$$C_S = C_{SP} \cdot \frac{m_{SP}}{m_S} \cdot \frac{M_S}{M_{SP}} \cdot \frac{{}^a A_{SP} \cdot (R_M - R_{SP})}{{}^b A_S \cdot (1 - R_M \cdot R_S)} \quad (\text{eq. VI-1})$$

where:

C_S : concentration of analyte in the sample (ng mL^{-1})

C_{SP} : concentration of analyte in the spike (ng mL^{-1})

m_{SP} : mass of spike added to the sample (g)

m_S : mass of the sample (g)

M_S : molar mass of the sample

M_{SP} : molar mass of the spike

${}^a A_{SP}$: abundance of the enriched isotope (a) in the spike

${}^b A_S$: abundance of the reference isotope (b) in the sample

R_M : isotopic ratio in the mixture (isotope b/ isotope a)

R_{SP} : isotopic ratio in the spike (isotope b/ isotope a)

R_S : isotopic ratio in the sample (isotope a/ isotope b)

If ${}^{76}\text{Se}$ is used as enriched isotope and ${}^{78}\text{Se}$ as natural isotope Eq. VII-1 becomes:

$$C_S = C_{SP} \cdot \frac{m_{SP}}{m_S} \cdot \frac{M_S}{M_{SP}} \cdot \frac{{}^{76}A_{SP} \cdot (R_M - R_{SP})}{{}^{78}A_S \cdot (1 - R_M \cdot R_S)} \quad (\text{eq. VI-2})$$

where

${}^{76}A_{SP}$: abundance of ${}^{76}\text{Se}$ in the spike

${}^{78}A_S$: abundance of ${}^{78}\text{Se}$ in the sample

R_M : isotopic ratio ${}^{78}\text{Se}/{}^{76}\text{Se}$ in the mixture

R_{SP} : isotopic ratio ${}^{78}\text{Se}/{}^{76}\text{Se}$ in the spike

R_S : isotopic ratio ${}^{76}\text{Se}/{}^{78}\text{Se}$ in the sample

In ICP-MS various phenomena affect the variation of relative ion intensities as a function of the ion mass. Such mass discrimination effects can occur during extraction, transmission, or detection of the ions.

Detector dead time. Detector dead time is defined as the interval during which the detector and its associated counting electronics are unable to resolve successive pulses [1]. If another ion strikes the detector surface within the time required for handling the first ion pulse, the second ion will not be detected and hence the observed count rate will be lower than the actual count rate.

It is known that, if the detector dead time can be determined accurately, these signal losses can be appropriately corrected for by using the equation:

$$\frac{1}{\text{observed count rate}} = \frac{1}{\text{actual count rate}} + \text{dead time (s)} \quad (\text{eq. VI-3})$$

If two measured isotopes have different abundance, the inaccurate estimation of detector dead time determines that the losses of signal during electronic handling are not proportional, and therefore the calculated isotope ratio will be affected by a bias. Up-to-date instruments like the Agilent 7500cx or the Thermo Element2 are equipped for the automatic internal correction for dead time, nevertheless this parameter needs to be periodically determined. Its estimation can be achieved by measuring an isotope ratio as a function of the analyte concentration and is then considered as a characteristic of the individual instrument.

For the instruments used in this Ph.D. project, the detector dead time was determined as proposed by Vanhaecke *et al.* [2]. The dead time correction was forced to values between 0 and 100 ns with step of 20 ns, and the main isotopes of Se (^{76}Se , ^{77}Se , ^{78}Se , ^{80}Se and ^{82}Se) were measured for standard solutions containing 100, 200, 400, 800 and 1200 ng g^{-1} of the element, obtaining the curves represented in Figure VI-1.

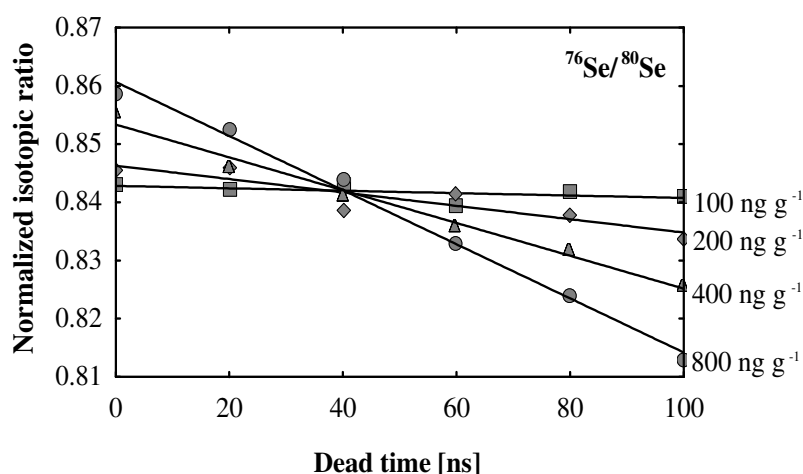


Figure VI-1 Normalized (in respect to the theoretical) isotope ratio as function of the dead time correction for 100, 200, 400 and 800 ng g^{-1} Se standard solutions. The intensities were measured by the ICP-QMS Agilent 7500cx instrument in Venice.

Dead time values of 39.4 ns and 47.2 ns were obtained for the Agilent 7500cx instruments used at the University of Venice and at the University of Oviedo,

respectively. The obtained value was used to correct the measured intensities in further experiments.

Blank subtraction. A preliminary step required for data treatment is blank subtraction: the average intensity of the signal detected for all the masses in a blank solution have to be subtracted to the signals obtained from the sample-spike mixture.

Correction for BrH^+ and SeH^+ spectral interferences. Even if the major spectral interferences generated in the ICP source are then removed by H_2 in the reaction cell, some of them cannot be avoided. The formation of SeH^+ and BrH^+ ions was observed by Boulyga *et al.* [3] when using a hexapole cell with H_2 , by Hinojosa Reyes *et al.* [4] using an octopole cell with H_2 , and also by Sloth *et al.* [5] when using a dynamic reaction cell pressurized with methane. These authors suggested that SeH^+ and BrH^+ formation could be derived from the sample matrix (i.e. water, methanol, etc.) or from impurities of water in the cell gases. As is shown in Figure VI-2, in our studies we observed interferences of BrH^+ on Se also in the ICP-MS Thermo Element2, which is not equipped with a collision/reaction cell. Thus, this effect confirms that such interferences are at least directly generated into the plasma by interaction with the matrix.

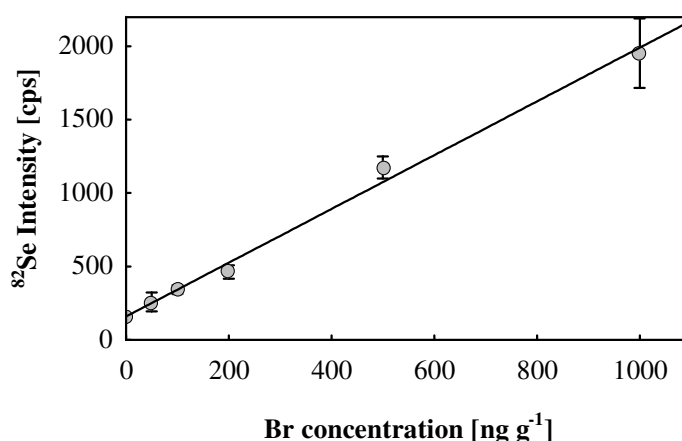


Figure VI-2 Linear relation between ^{82}Se intensity signal (generated by $^{81}\text{BrH}^+$) and concentration of Br in standards solution. The analyses were carried out by ICP-SFMS Element2 in medium resolution mode. $R^2 = 0.994$.

Obviously, the use of a reaction cell flowed with H_2 does not remove efficiently interference by hydrides, but in reverse promotes them by further hydration.

Mathematical correction of the spectral interferences is therefore necessary to obtain accurate isotope ratios. In this studies, the interferences were corrected using the method proposed by Hinojosa Reyes *et al.* [4]. Other than all the masses of Se, $^{79}\text{Br}^+$, $^{81}\text{Br}^+$ and $^{82}\text{SeH}^+$ were monitored, then the following system of equations for intensity correction was applied:

$$\begin{cases} {}^{77}\text{Se} = {}^{76}\text{I} - f_{\text{Se}} \cdot ({}^{76}\text{Se}) \\ {}^{78}\text{Br} = {}^{78}\text{I} - f_{\text{Se}} \cdot ({}^{77}\text{Se}) \\ {}^{79}\text{Br} = {}^{79}\text{I} - f_{\text{Se}} \cdot ({}^{78}\text{Se}) \\ {}^{80}\text{Se} = {}^{80}\text{I} - f_{\text{Br}} \cdot ({}^{79}\text{Br}) \\ {}^{81}\text{Br} = {}^{80}\text{I} - f_{\text{Se}} \cdot ({}^{80}\text{Se}) \\ {}^{82}\text{Se} = {}^{81}\text{I} - f_{\text{Br}} \cdot ({}^{81}\text{Br}) \end{cases} \quad (\text{eq. VI-4})$$

where f_{Se} is the ratio SeH^+/Se^+ based on the measured $^{83}\text{I}/^{82}\text{I}$ ratio in a 100 ng g^{-1} Se standard solution, and f_{Br} is the ratio BrH^+/Br^+ based on the measured $^{81}\text{I}/^{82}\text{I}$ ratio in a 200 ng g^{-1} Br standard solution. No correction for krypton was necessary as $^{83}\text{Kr}^+$ ions are neutralized in the cell. We observed that, at H_2 flow rate of 4 mL min^{-1} , the measured f_{Se} was always $< 2 \%$, a slightly lower level in respect to the 3% observed by Hinojosa Reyes *et al.* [4]. Nevertheless, a high within-day variation in the f_{Se} factor was observed in this study considering 10 consecutive measurements, as showed in Figure VI-3. As a consequence, monitoring of the m/z 83 and f_{Se} calculation is strongly recommended to be carried out for each sample.

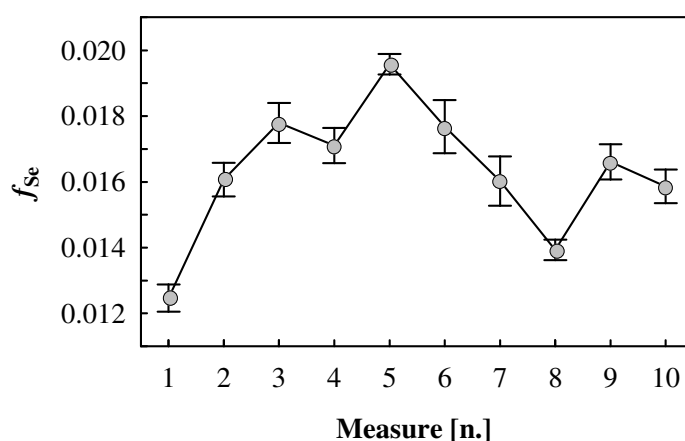


Figure VI-3 Within-day variation of the f_{Se} parameter for the Agilent 7500cx instrument at the University of Oviedo.

Correction for mass bias. Further bias in the Se isotopic ratio is also produced by isotope selective phenomena occurring in the mass spectrometer, after the plasma generation. When positively charged ions of an element formed in the plasma leave the skimmer cone, the lighter isotopes are deflected from the central ion beam more than the heavier isotopes, due to mutual repulsion of ions. This is called the “space-charge” effect [6,7]. As a result, the heavier isotopes transmit more efficiently than the lighter ones to the detector. The differential transmission of ions of different masses leads to mass discrimination and causes a mass bias in the measured isotope ratios. Moreover, even if the use of a collision/reaction gas can reduce ion kinetic energies and improve the sensitivity for Se ions, it is also known that the gas pressure can alter the mass discrimination in the collision/reaction cell [3,7-10]. Depending on the mass of the analyte ion, the gas flow rate used and the sample matrix, light isotopes are preferentially scattered compared to heavier isotopes, resulting in an appreciable trajectory change. This effect leads to further inaccurate and fluctuating isotope ratio estimation [11].

The mass bias effect can be corrected introducing a factor determined by the following exponential equation:

$$\left(\frac{I}{\Delta M}\right) \cdot \ln\left(\frac{R_{\text{exp}}}{R_{\text{theo}}}\right) = K \quad (\text{eq. VI-5})$$

where I is the signal intensity for the isotope ^xSe , ΔM is the mass difference between the isotope ^xSe and reference isotope (in our case ^{78}Se), R_{exp} is the experimental ratio of $^x\text{Se}/^{78}\text{Se}$ isotopes intensities, R_{theo} is the theoretical ratio of $^x\text{Se}/^{78}\text{Se}$ isotopes intensities and K is the mass bias correction factor. Once K has been determined, the corrected isotope ratio R_{corr} can be calculated using the formula:

$$R_{\text{corr}} = R_{\text{exp}} \cdot e^{-(K \cdot \Delta M)} \quad (\text{eq. VI-6})$$

The factor K can therefore be estimated by linear regression between the logarithm of the corrected measured isotopic ratios and the mass difference between the measured isotopes from the ^{78}Se reference isotope. The instrument mass bias per mass unit (MD %) for the measured isotope ratio is then calculated according to the following equation:

$$\text{MD \%} = (e^{-K} - 1) \cdot 100 \quad (\text{eq. VI-7})$$

Figure VI-4 shows the linear relationship obtained in single days for the instruments Agilent 7500cx at the Universities of Oviedo and Venice. The mass bias factors, derived from the slope of the regression lines, were 0.03630 and 0.04192, respectively, corresponding to an MD % of 3.7 % and 4.3 %.

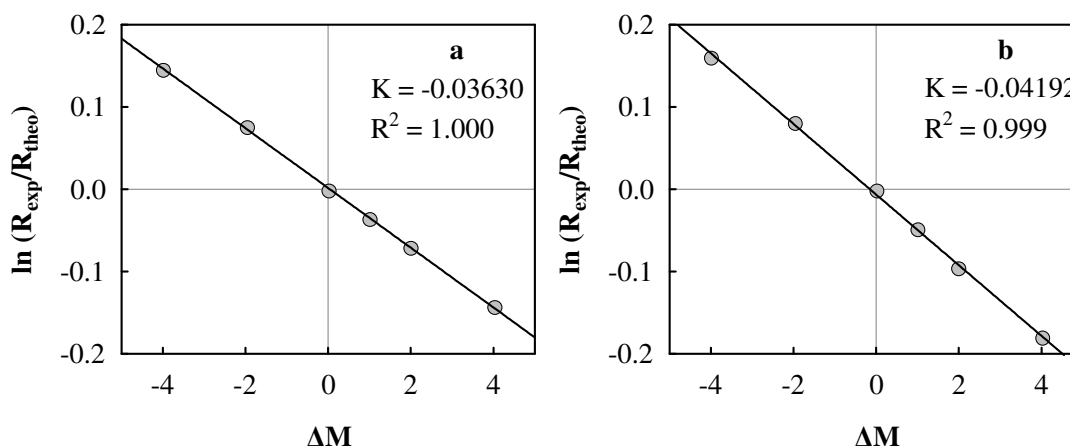


Figure VI-4 Linear relationship between corrected measured isotopic ratios and the mass difference between the measured isotopes from the ^{78}Se reference isotope, calculated for the instruments Agilent 7500cx at the Universities of Oviedo (a) and Venice (b).

Similar values have been obtained in other studies regarding Se determination by ICP-MS, as reported in Table VI-1. As expected, in all cases the MD % values are >1 , indicating that the lighter isotopes are more discriminated than heavier isotopes.

Table VI-1 Mass bias per mass unit (MD %) for the Se isotope ratio calculated by different ICP-MS instruments.

MD %	Instrument	Cell gas	Reference
2.7	Perkin-Elmer SCIEX ELAN 6100	CH_4	[5]
3.4	Agilent 7500cx	H_2	[12]
3.3	Agilent 7500ce	$\text{H}_2 + \text{He}$	[13]
2.7 to 3.3*	Thermo-Finnigan MAT Neptune	-	[14]
3.6	Micromass Platform ICP	$\text{H}_2 + \text{He}$	[7]
2.6	Thermo-Finnigan MAT Element2	-	[7]

* depending on the sample introduction system

The values for different ICP-MS instruments were also found to vary within-day and between-days [7]. Concerning the Agilent 7500cx instrument, Hinojosa Reyes *et al.* [4] reported that a variation in the mass discrimination factor was not significant

within a period of time of 2 h, but recommended to carry out the mass discrimination correction every 2 h when analysing real samples. In our study we investigated also the stability of between-days variation of the factor. The data, showed in Figure VI-5, demonstrated that even if in a 2-3 days of instrument use the mass bias can be considered stable, with a 4-6 days of interval this effect can change significantly, in particular if those days are dedicated to other analysis, carried out with different experimental conditions and instrumental set-ups.

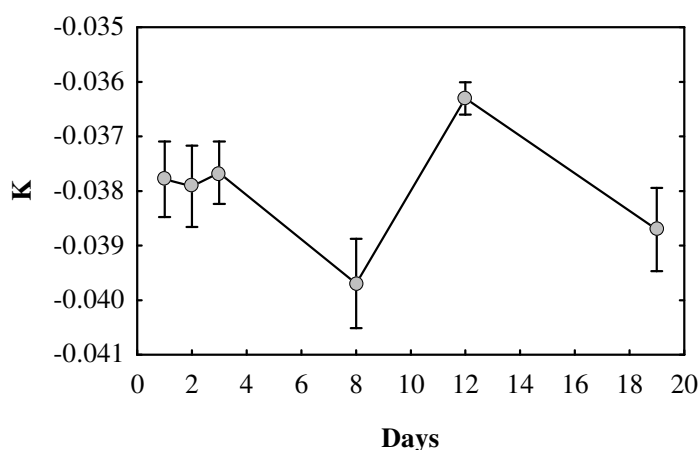


Figure VI-5 Mass bias factor estimated in different days for the instrument Agilent 7500cx at the University of Oviedo.

VI.5.2. Isotopically enriched spike characterization

The accurate knowledge of isotopic abundances distribution in both sample and enriched spike solution is the first preliminary step that needs to be carried out for the application of IDA. Since the sample has not been manipulated in its isotopic abundances distribution, it is assumed that its distribution coincides with that considered “natural”, as reported by Rosman and Taylor [15] and showed in Table VI-2. Rather, the Se isotopically enriched spikes are supplied from Cambridge Isotope Laboratories in metallic form, matching with an indicative enrichment (percentage) factor, as that reported in Table VI-3.

Table VI-2 Isotopic abundances [atom, %] of natural Se as reported by Rosman and Taylor [15].

Mass	Abundance
74	0.89 ± 0.04
76	9.37 ± 0.29
77	7.63 ± 0.16
78	23.77 ± 0.28
80	49.61 ± 0.41
82	8.73 ± 0.22

Table VI-3 Se isotopically enriched (metal) standards available from Cambridge Isotope Laboratories and corresponding enrichment factor [%].

Mass	Enrichment factor
74	99 +
76	97
77	96
78	97
82	92

The isotopic abundance of the spike solution requires to be accurately and precisely known, therefore an experimental characterization is necessary to estimate them in the individual standards to be used. In this studies, the purchased elemental powders were dissolved in a minimum volume of sub-boiled nitric acid and diluted with ultra-pure water to the appropriate volume to obtain a $\sim 100 \text{ ng g}^{-1}$ concentration. Then, such solutions were analyzed by ICP-MS using the operating conditions reported in Table X-1 at p. 232.

Table VI-4 Isotopic abundances calculated from 100 ng g^{-1} standards of isotopically enriched Se.

Mass	^{76}Se enriched	^{77}Se enriched	^{82}Se enriched
74	0.170 ± 0.001	0.007 ± 0.001	0.149 ± 0.002
76	99.599 ± 0.005	0.098 ± 0.003	0.202 ± 0.004
77	0.177 ± 0.008	98.702 ± 0.006	0.168 ± 0.005
78	0.026 ± 0.005	1.103 ± 0.005	0.563 ± 0.003
80	0.022 ± 0.006	0.064 ± 0.001	1.839 ± 0.006
82	0.006 ± 0.001	0.026 ± 0.001	97.079 ± 0.007

As reported in Table VI-4, the experimental abundances are significantly different from the enrichment factors provided by the producer. Moreover, the high sensitivity of IDA method requires to determine the exact values with an high precision that is not provided with the product information.

Other than the isotopic abundances, the concentration of spike standard solutions needs to be exactly known in order to accurately apply the IDA method. Since the enriched Se is provided in metallic powder and all the subsequent dilutions are home-

made, even if by weighing in analytical balance, such concentration are not certificated. On the contrary, standards of natural Se solutions can be found in commerce, provided with a certificated concentration value. Therefore, it is possible to internally certify the concentration of an isotopically enriched Se solution by spiking it with an accurately known amount of natural Se standard, and by applying the reverse IDA formula:

$$C_{SP} = C_S \cdot \frac{m_S}{m_{SP}} \cdot \frac{M_{SP}}{M_S} \cdot \frac{{}^b A_S \cdot (1 - R_M \cdot R_S)}{{}^a A_{SP} \cdot (R_M - R_{SP})} \quad (\text{eq. VI-8})$$

If ${}^{76}\text{Se}$ is used as enriched isotope and ${}^{78}\text{Se}$ as natural isotope eq. VII-8 becomes:

$$C_{SP} = C_S \cdot \frac{m_S}{m_{SP}} \cdot \frac{M_{SP}}{M_S} \cdot \frac{{}^{78} A_S \cdot (1 - R_M \cdot R_S)}{{}^{76} A_{SP} \cdot (R_M - R_{SP})} \quad (\text{eq. VI-9})$$

VI.5.3. Estimation of the optimum spike-to-sample ratio and choice of the enriched isotope

The appropriate amount of enriched Se spike to be added to the sample can be estimated by applying the error propagation theory [16] in order to minimize the uncertainty on the final measured isotopic ratio. According with García Alonso [17], the relative error of element concentration in the sample can be approximated (in the case of samples containing natural elements and when a certified enriched isotope is used as the spike) as:

$$\left[\frac{s(C_S)}{C_S} \right]^2 = f(R_M) \cdot \left[\frac{s(R_M)}{R_M} \right]^2 \quad (\text{eq. VI-10})$$

Thus, it depends on the relative error of the isotopic ratio (R_M) measure and on the factor $f(R_M)$, called error magnification factor, expressed as:

$$f(R_M) = \left[\frac{R_M \cdot (1 - R_{SP} \cdot R_S)}{(R_M - R_{SP}) \cdot (1 - R_M \cdot R_S)} \right]^2 \quad (\text{eq. VI-11})$$

In order to minimize the error magnification factor, since R_{SP} (for a given enriched spike) and R_S are imposed, only R_M can be optimized. Considering the isotopic abundances of natural Se and the three isotopically enriched spikes available, reported

in Table VI-4, the dependence of $f(R_M)$ from R_M is represented in Figure VI-6 for different couples of reference/enriched isotopes.

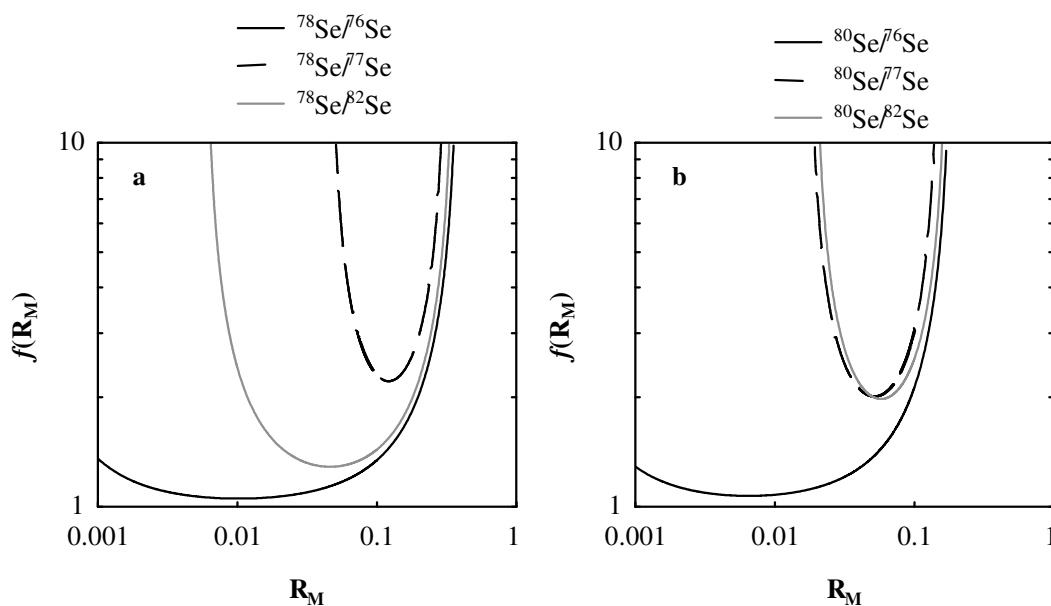


Figure VI-6 Variation of the error magnification factor f as function of the measured isotopic ratio R_M , for the determination of natural Se using the isotopically enriched spikes of ^{76}Se , ^{77}Se , ^{82}Se ; and ^{78}Se (a) or ^{80}Se (b) as reference isotope.

The optimum R_M can be easily calculated from eq. VII-11 as follows:

$$R_M^{\text{opt}} = \sqrt{\frac{R_{SP}}{R_S}} \quad (\text{eq. VI-12})$$

In Table VI-5 are reported the parameters R_M^{opt} calculated for all the couples of reference/enriched isotope of Se considered in this study.

Table VI-5 Optimum measured isotopic ratio (R_M^{opt}) for determination of Se by IDA, calculated by minimization of the magnification error function.

Spike	Reference	
	^{78}Se	^{80}Se
^{76}Se	0.010	0.006
^{77}Se	0.121	0.052
^{82}Se	0.046	0.058

From the R_M^{opt} is consequently possible to estimate the optimal amount of spike to be added to the sample, using the equation:

$$R_{\text{mass}}^{\text{opt}} = \frac{R_M^{\text{opt} \cdot a} A_S^{-b} A_S}{A_{SP}^{-b} A_{SP}^{-a} R_M^{\text{opt}}} \quad (\text{eq. VI-13})$$

where $R_{\text{mass}}^{\text{opt}}$ is the ratio: amount of Se in the added spike/amount of Se in the sample (ng/ng). The error magnification factor can be also directly expressed as function of R_{mass} , and therefore the optimum ratio $R_{\text{mass}}^{\text{opt}}$ directly calculated by minimizing this function $f(R_{\text{mass}})$, obtaining that:

$$R_{\text{mass}}^{\text{opt}} = \frac{A_S}{A_{SP}} \cdot \frac{\left[\sqrt{\frac{R_{SP}}{R_S}} - R_S \right]}{\left[R_{SP} - \sqrt{\frac{R_{SP}}{R_S}} \right]} \quad (\text{eq. VI-14})$$

The dependence of $f(R_{\text{mass}})$ from R_{mass} is represented in Figure VI-7 for all the couples of reference/enriched isotopes, while in Table VI-6 are reported the corresponding $R_{\text{mass}}^{\text{opt}}$ values obtained by the eq. VI-14.

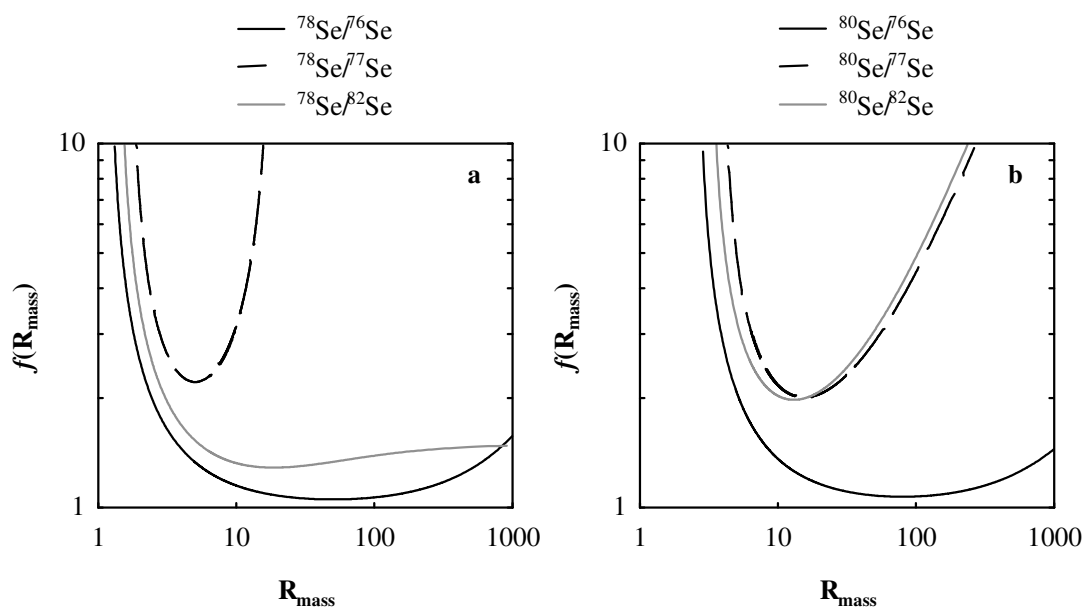


Figure VI-7 Variation of the error magnification factor f as function of the mass ratio R_{mass} , for the determination of natural Se using the isotopically enriched spikes of ^{76}Se , ^{77}Se , ^{82}Se ; and ^{78}Se (a) or ^{80}Se (b) as reference isotope.

Table VI-6 Optimum mass ratio (R_{mass}^{opt}) for determination of Se by IDA, calculated by minimization of the magnification error function.

Spike	Reference	
	^{78}Se	^{80}Se
^{76}Se	24.05	79.75
^{77}Se	3.25	15.42
^{82}Se	5.97	13.04

VI.5.4. Detection limits comparison for external calibration and isotope dilution

According with Yu *et al.* [18] and with the error propagation theory, the detection limit (LOD) for isotope dilution analysis can be expressed as:

$$LOD = \frac{\sqrt{LOD_a^2 + LOD_b^2 / R_{SP}^2}}{|^a A_S - ^b A_S / R_{SP}|} \quad (\text{eq. VI-15})$$

where LOD_a and LOD_b are the linear calibration detection limits for isotopes a and b , respectively, calculated as:

$$LOD_a, LOD_b = \frac{3\sigma}{\rho} \quad (\text{eq. VI-16})$$

where σ is the standard deviation of six blank measurements, and ρ is the slope of the calibration curve calculated from 12 standards from 0.01 to 50 ng g⁻¹. Table VI-7 reports the $LODs$ calculated for both EC and IDA by the analysis of 12 Se standard solutions prepared in an acid digestion reagent blank (see paragraph X.3.4). Even if IDA presents crucial advantages in terms of accuracy, as is discussed in the Chapter VII, due to the effects of error propagation theory its LOD is in general higher than for EC.

Table VI-7 Limit of detection for Se calculated for EC and IDA.

Isotope	EC	IDA	
	LOD [ng g ⁻¹]	Reference isotope	LOD [ng g ⁻¹]
74	0.143	78	0.077
76	0.017	80	0.032
77	0.026		
78	0.018		
80	0.016		
82	0.044		

VI.5.5. Optimization of integration time for Se isotopic ratios measurement

The influence of integration time was evaluated in order to optimise this acquisition parameter for the best precision of isotopic ratios, by analysing a standard solution of 100 ng g^{-1} of natural Se. The RSD % ($n = 6$) of $^{76}\text{Se}/^{78}\text{Se}$ and $^{76}\text{Se}/^{80}\text{Se}$ ratios as function of the integration time per mass is shown in Figure VI-8.

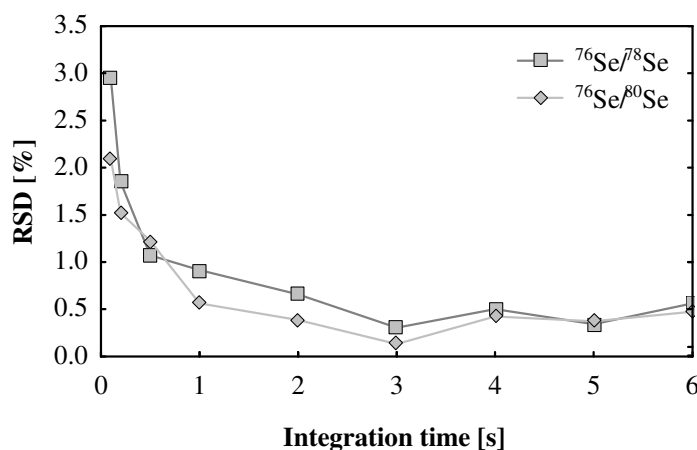


Figure VI-8 Relative standard deviation (RDS) of the ratios $^{76}\text{Se}/^{78}\text{Se}$ and $^{76}\text{Se}/^{80}\text{Se}$ intensities as function of the integration time.

The measured RSD decreased from 2-3 % at 0.1 s integration time to < 0.5 % at integration time > 3 s. These results are in agreement with those found by Hinojosa Reyes *et al.* [19]. According with their study, we finally selected an optimum integration time of 4 s per mass.

VI.6. References

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VII. DEVELOPMENT OF ANALYTICAL METHODOLOGIES FOR SE SPECIATION IN HUMAN PLASMA/SERUM¹

VII.1. Introduction

The determination of Se-proteins in human plasma or serum is currently an analytical challenge. Due to their low concentration and the matrix complexity, only the recent development of selective separation systems based on HPLC, coupled to very sensitive detection systems such as ICP-MS, resulted in accurate, precise and robust analytical approaches for Se speciation in plasma/serum.

One of the most used among these methods is based on the enzymatic hydrolysis of the whole serum, followed by separation and quantification of the two Se-amino acids SeCys and SeMet by RP-HPLC coupled on-line to ICP-MS [1,2]. As already exposed in the Chapter III, this approach presents some drawbacks affecting the accuracy of determination, including incomplete hydrolysis process, unavailability of SeCys standards and reduction of the original Se-proteins pattern to only two amino acidic species. An alternative approach has been recently proposed, aimed to the HPLC separation of intact plasma/serum Se-proteins using a double affinity (2AF) system

¹ This work is published in: Jitaru P., Roman M. et al. *Microchim Acta* 2009;166:319-27; and Jitaru P., Roman M. et al. *Accredit Qual Assur* 2010;15(6):343-50.

[3]. In this case, GPx3, SelP and SeAlb can be individually determined, allowing to study the complete and original Se-proteins pattern.

The most critical issue for intact Se-proteins determination in human serum is method validation. None of the proteins of interest are commercially available as pure standards for calibration and quality control, therefore the use of surrogate standards or species-unspecific IDA is necessary. Certified reference materials for individual Se-species concentration or at least indicative levels are also unavailable. In this context, all the published methods dealing with the determination of Se-proteins have been applied to the analysis of control (volunteers) serum samples, rather than commercial materials. This limits drastically their usefulness from a metrological point of view because such serums are rarely available to other laboratories, and hence methods inter-comparison is impossible. Other factors limit the application of these methods for epidemiological investigations, comprising the lack of a set of alternative versions, accurately compared in terms of performances, which hamper the application in laboratories where different instruments (particularly ICP-MS) are available. In a routine analysis perspective, time of analysis (currently at least 20 min) and sample amount (currently at least 100 μ L)[4] need also to be reduced, particularly for studies carried out on mouse or rat, where only an extremely low sample volume is available.

VII.2. Goals of the study

The aim of this study was the development of new analytical methods for the simultaneous speciation analysis of GPx3, SelP and SeAlb in human serum. Instrumental set-ups based on 2AF-HPLC were explored with the goal of separation system miniaturization, in order to reduce the sample consumption and the time for analysis. Coupling with ICP-MS was realized in order to test for the first time the SFMS and ORS-QMS detectors potentiality for Se-proteins determination, in comparison with the traditional QMS. All the methods were fully optimized and characterized, paying particular attention to the comparison of EC and IDA quantification approaches. A human serum reference material (BCR-637), certified for total Se content, was finally analyzed by the newly developed methods, and by the methods previously investigated by our group, in order to assess accuracy and precision. Through an interlaboratory comparison, all the results were cross-checked

following a metrological approach in order to estimate for the first time the indicative concentration of GPx3, SelP, and SeAlb (in terms of Se) in the human serum BCR-637.

VII.3. Plasma/serum Se-proteins separation by 2AF-HPLC

The separation of Se-proteins was carried out by a 2AF-HPLC system, exploiting the proteins' selectivity towards heparin (HEP) and Cibacron™ Blue F3G-A (BLUE) stationary phases.

Heparin is a highly sulphated glycosaminoglycan with a structure as shown in Figure VII-1, presenting the ability to bind a very wide range of proteins. When bound to agarose, HEP constitutes a stationary phase able to retain biomolecules including:

- DNA binding proteins such as initiation factors, elongation factors, restriction endonucleases, DNA ligase, DNA and RNA polymerases;
- serine protease inhibitors such as antithrombin III, protease nexins;
- enzymes such as mast cell proteases, lipoprotein lipase, coagulation enzymes, superoxide dismutase;
- growth factors such as fibroblast growth factor, Schwann cell growth factor, endothelial cell growth factor;
- extracellular matrix proteins such as fibronectin, vitronectin, laminin, thrombospondin, collagens;
- hormone receptors such as oestrogen and androgen receptors;
- lipoproteins.

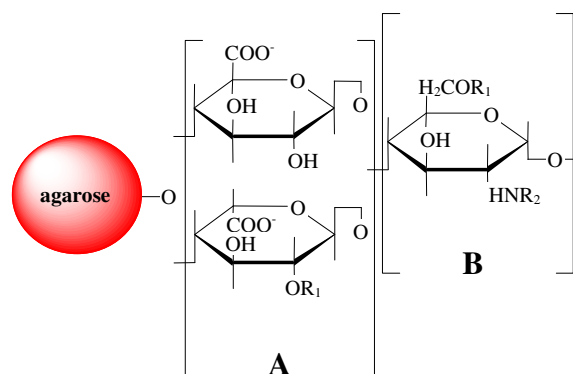


Figure VII-1 Structure of the HEP stationary phase. The heparin polysaccharide consisting of alternating hexuronic acid (A) and D-glucosamine residues (B). The hexuronic acid can either be D-glucuronic acid (top) or its C-5 epimer, L-iduronic acid (bottom). R₁ = -H or -SO₃⁻, R₂ = -SO₃⁻ or -COCH₃.

Heparin has two modes of interaction with proteins: a) with DNA-binding proteins, it mimics the polyanionic structure of the nucleic acid; b) with coagulation factors, such as antithrombin III, heparin acts as an affinity ligand. In both cases, the interaction can be weakened by increases in ionic strength.

Cibacron™ Blue F3G-A, is a synthetic polycyclic dye that acts as an aromatic anionic ligand, binding specifically the albumin *via* electrostatic and/or hydrophobic interactions. Cibacron™ Blue F3G-A is linked to Sepharose to create Blue Sepharose affinity stationary phase, as in Figure VII-2.

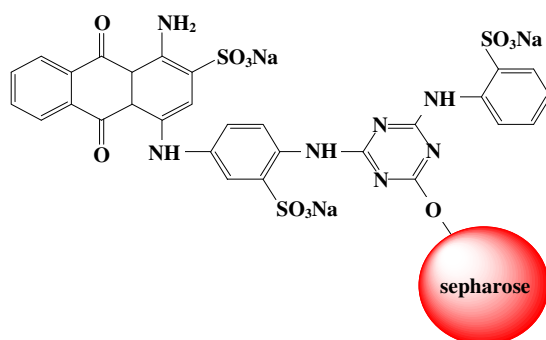


Figure VII-2 Partial structure of BLUE Sepharose stationary phase.

VII.3.1. Columns preparation

The columns with HEP and BLUE stationary phase were purchased from GE Healthcare (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and are named HiTrap™ Heparin HP and HiTrap™ Blue HP, respectively. The columns, shown in Figure VII-3, are low-pressure plastic, with a maximum allowed back pressure of ~3 bar.



Figure VII-3 HiTrap™ Heparin HP (left) and HiTrap™ Blue HP (right) columns.

Since high-pressure columns based on those stationary phases are not commercially available, in this study they were home-made by filling empty stainless steel columns with the stationary phase removed from the corresponding plastic products. In order to reduce both sample volume and time of analysis in respect to the state-of-the-art of the method [4,5], in this study microbore (1 mm i.d. × 5 cm length) and narrowbore (2 mm i.d. × 5 cm length) columns were used. The self-made AF columns were prepared by the procedure represented in Figure VII-4 and hereinafter explained.

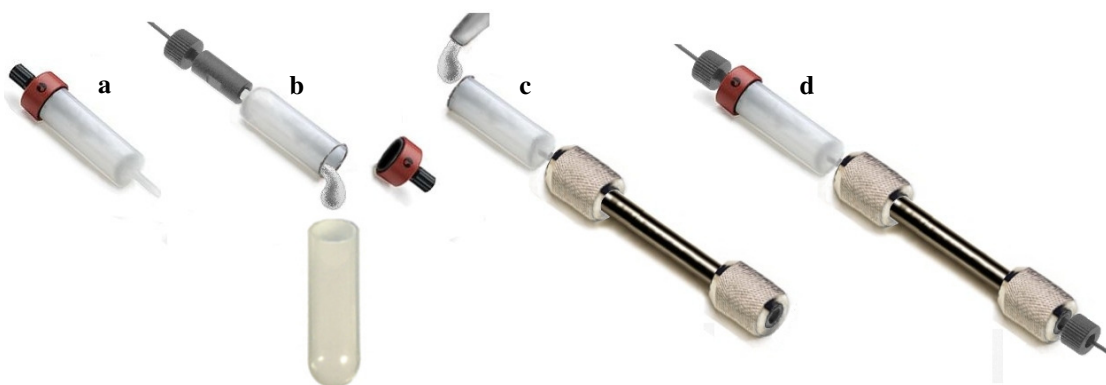


Figure VII-4 Procedure for the preparation of the self-made affinity HEP and BLUE columns.

The stationary phases from the commercial columns (a) were obtained by removing the plastic cap, reverse-connecting the column to the HPLC system, flushing a 5 % MeOH in H₂O solution at low flow (0.1 mL min⁻¹) and collecting the slurry in a plastic tube (b). Then, the original plastic column and the new empty column were connected on-line, by previously removing the head-frit from the steel column. The plastic column was filled with the slurry of the stationary phase by using a pipette (c). The system was then connected again to the HPLC and a low flow of the same 5 % MeOH in H₂O solution was flushed until the whole stationary phase was transferred the steel column, or until no additional transfer was visible (d). The system was disconnected from the HPLC, the steel column removed, the missing frit added and the new column finally assembled.

VII.3.2. HPLC instrumental set-up

As reported elsewhere [4], the serum/plasma Se-proteins separation can be achieved by using both HEP and BLUE stationary phases with a binding mobile phase of ammonium acetate (AmAc) 50 mM buffered at pH 7.0 (buffer A). In such conditions, the HEP column retains selectively SeIP, the BLUE column retains both SeIP and SeAlb, whereas GPx3 is not retained. Subsequent releasing of the proteins can be achieved by increasing the ionic strength of the eluent. In this study, AmAc 1.5 M buffer solution at pH 7.0 (buffer B) was used as elution buffer. However, truly on-line use of the two columns for the separation of GPx3, SeIP and SeAlb is impossible. If HEP and BLUE columns are directly connected on-line (in this order), the elution of SeIP from the HEP column loads the BLUE column with elution buffer, and hence SeIP is not retained anymore onto this column (it co-elutes with SeAlb). The only feasible approach to separate the three Se-proteins in the same analytical run, relies on the exclusion of the BLUE column from the chromatographic system during the elution of SeIP from the HEP column, followed by its reintegration into the system for the consequent elution of SeAlb. The corresponding instrumental set-up is represented in the schemes in Figure VII-5.

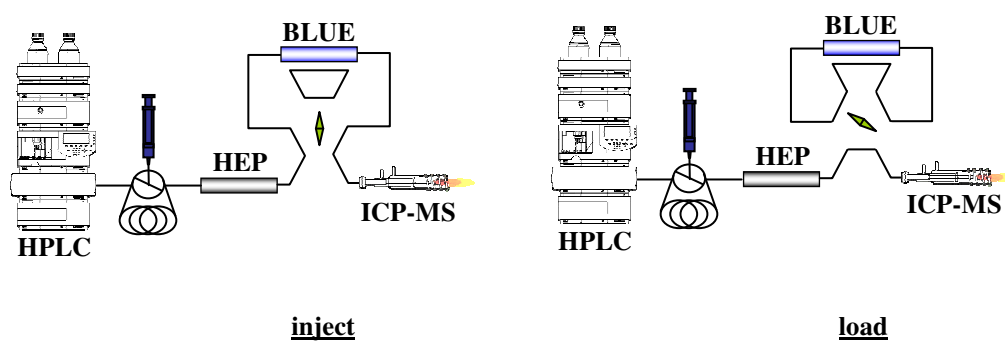


Figure VII-5 Instrumental set-up for speciation of plasma/serum Se-Proteins by 2AF-HPLC (coupled with ICP-MS). In switching valve position "inject" the BLUE column is connected on-line to the HPLC line after the HEP column; in position "load" the BLUE column is excluded from the HPLC line.

A switching valve module Microneb 2000 Direct Injection Nebulizer (Cetac Technologies Inc, Omaha, Nebraska, USA) was used for the (automated) exclusion of the BLUE column from the HPLC line.

VII.4. Speciation of plasma/serum Se-proteins by 2AF-HPLC-SFMS

VII.4.1. Instrumentation and set-up

Speciation of plasma/serum Se-proteins was achieved in this study by coupling the microbore 2AF-HPLC system with the ICP-SFMS Element2. A scheme of the instrumental set-up is represented in Figure VII-6.

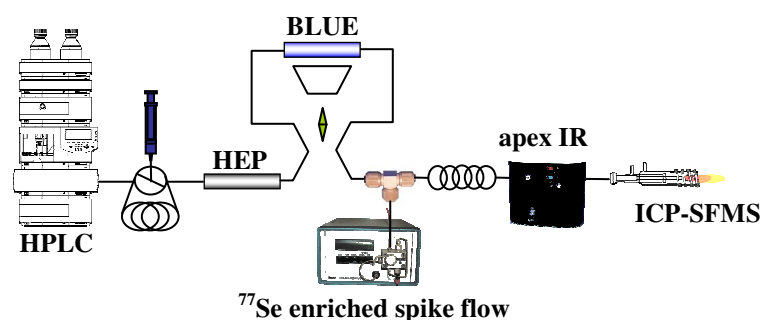


Figure VII-6 Instrumental setup for Se-proteins determination by microbore 2AF-HPLC-apex IR-ICP-SFMS and IDA.

The 2AF-HPLC system consisted of the microbore HEP and BLUE affinity columns, with a sample loop of 5 μL . The elution sequence was as follows: 0-1 min 100 % buffer A (BLUE column connected on-line); 1-5 min 100 % buffer B (BLUE column excluded); 5-6 min 100 % buffer B (BLUE column connected on-line); 6-7 min 100 % buffer A (BLUE column connected on-line) for re-conditioning of the columns. The flow rate was 0.3 mL min^{-1} .

A Thermo Element2 ICP-SFMS was used as on-line coupled detector. Apex IR sample introduction system was used as interface for the on-line coupling of HPLC and ICP-SFMS instruments. A PEEK T was used to mix the 2AF-HPLC output flow with a ^{77}Se enriched spike solution (2 ng g^{-1}) pumped by a solvent delivery system (S1100 type, Sykam GmbH, Eresing, Germany). In order to ensure the equilibration of the 2AF-HPLC eluent with the spike solution, a custom-made knotted reactor of 100 cm linear length of Teflon tubing (0.25 mm id) was used as a transfer line between 2AF-HPLC and the apex IR.

VII.4.2. Optimization of ICP-SFMS sample introduction system

As introduced in the Chapter IV, the use of HR mode in ICP-SFMS allows to resolve most of the spectral interferences on Se, but leads to a significant loss in sensitivity, which is a critical point in trace analysis. This effect is particularly relevant for low-ionizing elements such as Se, and when the available sample amounts are very low. In order to compensate the sensitivity decrease, the high-efficiency sample introduction system apex IR was used in this study to replace the common double-pass Scott-type spray chamber. The apex IR device is more typically used for isotope ratio analysis, since it can guarantee higher signal stability, as well as signal increasing. As shown in Figure VII-7, it consists in a quartz flow path that includes an additional mixing chamber, that homogenizes and stabilizes the sample aerosol stream, resulting in a stabilization of the signal from the ICP-MS. Sample transport efficiency is enhanced by nebulizing liquid samples into a heated cyclonic spray chamber using a PFA MicroFlow nebulizer. A low-volume three-stage Peltier-cooled desolvation system is then incorporated for on-line removal of solvent vapour.

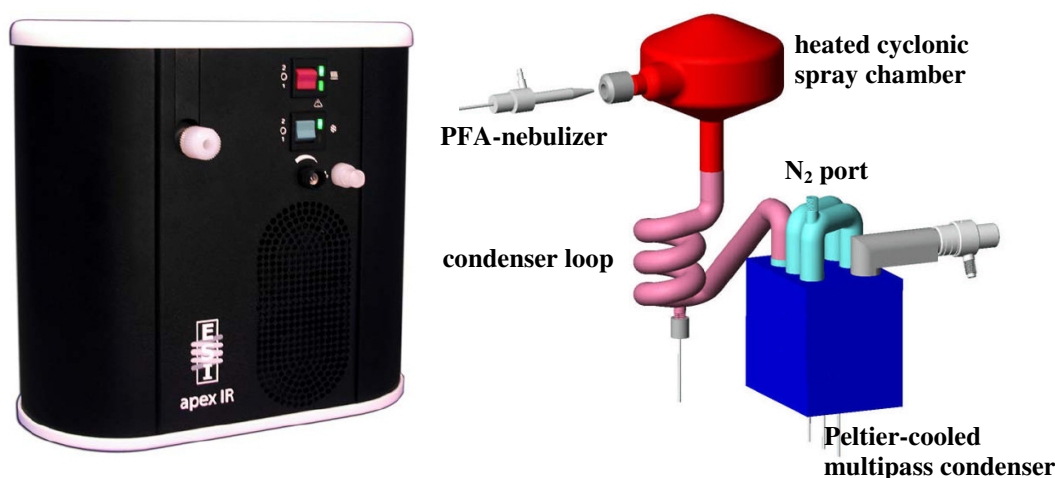


Figure VII-7 Picture (left) and scheme (right) of the apex IR device.

Optimization of the apex IR-ICP-SFMS system was carried out daily using a solution of 1 ng g^{-1} In and 5 ng g^{-1} Se. Preliminary experiments were performed using N_2 as a make-up gas for the apex IR device, as recommended by the manufacturer. By this set-up, intensities up to $8\text{-}10 \times 10^6$ cps and 8×10^5 cps were usually obtained in low resolution for ^{115}In and ^{78}Se , respectively, whereas in high resolution mode the sensitivity dropped to ~ 105 cps for ^{115}In and $\sim 5,000$ cps for ^{78}Se . A human serum

sample (the certified reference material BCR-637) was then injected into the HPLC-apex IR-ICP-SFMS coupled system to assess the performances in analysis mode. Despite the use of apex IR, the obtained chromatograms showed a very low sensitivity and bad peaks shape for quantification. Also, mass discrimination between isotopes was noticed, with a ratio $^{82}\text{Se}/^{77}\text{Se}$ varying from ~ 1.1 (the theoretical value is 1.1435) up to ~ 5 . In order to investigate these unexpected effects, Ar was also used instead of N_2 as make-up for apex IR and an increasing in signal intensity about fivefold has been observed, as shown in Figure VII-8. Most likely, the addition of N_2 in the plasma gas inhibits the efficient ionization of Se.

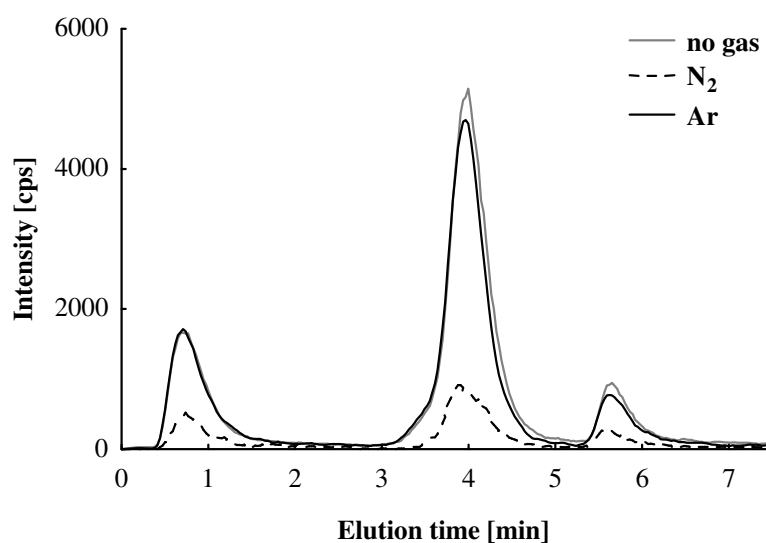


Figure VII-8 Chromatograms of human serum BCR-637 obtained for ^{77}Se by microbore 2AF-HPLC hyphenated to ICP-SFMS (HR) using the apex IR system without make-up gas, and with N_2 or Ar as make-up gases.

The same sensitivity increase was also achieved by just removing the make-up gas addition to the apex IR. Since by introduction of Ar a slight worsening in repeatability was noticed, further experiments were carried out without any make-up gas addition into the apex IR device.

A chromatogram obtained by microbore 2AF-HPLC hyphenated to ICP-SFMS (HR) by means of the optimized apex IR system is shown in Figure VII-9a, whereas a chromatogram obtained by using a Scott-type spray chamber cooled at 2°C is shown in Figure VII-9b. As can be seen, an increase in sensitivity of more than one order of magnitude can be achieved using the apex IR as interface between the microbore 2AF-HPLC and the ICP-SFMS.

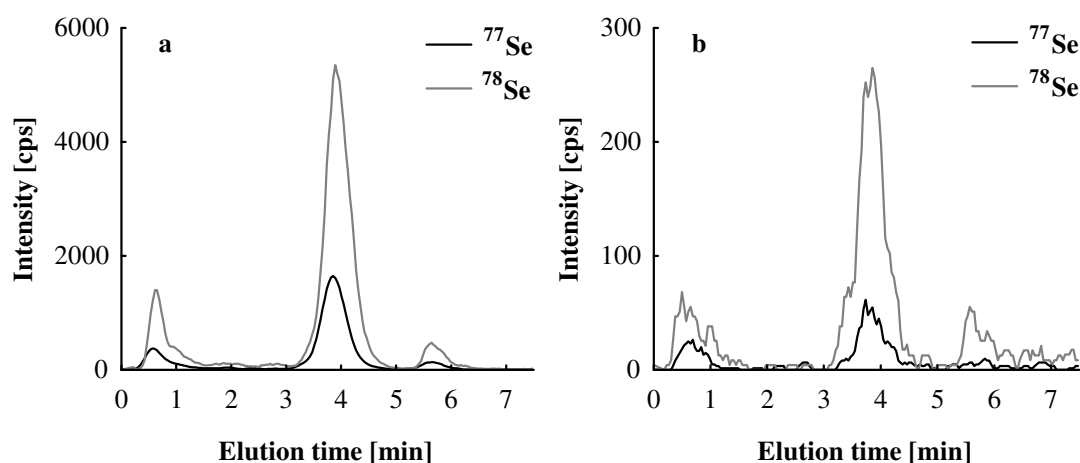


Figure VII-9 Chromatograms of human serum BCR-637 obtained for ^{77}Se and ^{78}Se by microbore 2AF-HPLC hyphenated to ICP-SFMS (HR) using the apex IR system without make-up gas (a), and a Scott-type 2°C cooled spray chamber (b).

VII.4.3. Final operating conditions

The instrumental parameters for HPLC and ICP-SFMS coupling are summarized in Table VII-1. Both HR and MR modes were used in this study, as is exposed afterwards.

Table VII-1. Optimum operating conditions for the microbore 2AF-HPLC-apex IR-ICP-SFMS instrumental set-up.

apex IR-ICP-SFMS		
RF power	1550 W	
Nebulizer flow rate	1.0 ± 0.2 L min ⁻¹ (daily optimization)	
Additional gas (Ar)	0.15 ± 0.05 L min ⁻¹ (daily optimization)	
Auxiliary gas flow rate	1.0 L min ⁻¹	
Plasma gas flow	16 L min ⁻¹	
apex IR spray chamber temperature	140 °C	
apex IR cooler temperature	2 °C	
Detection mode	counting	
Integration type	average	
Isotopes monitored	^{77}Se , ^{78}Se , ^{82}Se	
	HR	MR
Run/passes	200/1	250/1
Mass window	125	125
Samples per peak	20	20
Search window	60	50
Integration window	60	60

Table VIII-1. (continuation)

2AF-HPLC	
Stationary phases	Hitrap TM Heparin HP (HEP) Hitrap TM Blue HP (BLUE)
Columns	5 cm × 1.0 mm id
Sample loop	5 µL
Flow rate	0.3 mL min ⁻¹
Elution mode	Step gradient
Mobile phases	Binding buffer (A): AmAc 0.05 M (pH 7.0) Elution buffer (B): AmAc 1.5 M (pH 7.0)

VII.4.4. Interferences elimination by SFMS in high resolution mode

In the application of the 2AF-HPLC system for Se speciation in human plasma/serum, GPx3 is not retained by the columns, and hence it co-elutes in the void volume with other non retained species, such as Cl⁻ and Br⁻. As was introduced in the paragraph IV.3.1, these species seriously interfere the measurement of the masses 77, 80 and 82 of Se. This aspect is particularly relevant for the plasma/serum matrix, where Cl⁻ is present at level from 3.4 to 3.8 mg g⁻¹ [6] and Br⁻ at level from 3.2 to 5.6 µg g⁻¹ [7]. Interferences alleviation can be performed by off-line sample clean-up using AE solid phase extraction (SPE) [5] and multi-affinity media [8], or by on-line chromatographic separation of Se-proteins from Br/Cl by AE-2AF-HPLC before ICP-MS detection [4]. However, these approaches introduce additional steps in the analytical process, increasing the time for analysis and the number of uncertainty sources. The unique approach theoretically allowing interference-free direct quantification of Se-proteins, without additional sample preparation and/or mathematical corrections, relies on the spectral resolving of interferences by ICP-SFMS operating in HR or MR modes, whereas classical detectors such as ICP-QMS operate in LR mode.

Referring to the main interferences on Se isotopes reported in Table IV-1, none of them can be theoretically resolved in MR. In HR the interference of ⁴⁰Ar³⁷Cl⁺ on ⁷⁷Se⁺ can be resolved ($M/\Delta M = 9,190$), thus this isotope of Se is accurately measurable. On the other hand, even if the interferences of ⁴⁰Ar³⁸Ar⁺ on ⁷⁸Se⁺ and ⁴⁰Ar⁴⁰Ar⁺ on ⁸⁰Se⁺ could be theoretically resolved ($M/\Delta M = 9,975$ and $9,699$, respectively), in fact the necessity of perfect instrument conditions and tuning,

difficult to obtain, and the very high abundance of interferents preclude this possibility.

Chromatograms shown in Figure VII-10 were realized by injecting BCR-637 human serum and pure standards of Cl and Br at normal serum level in order to verify if the interferents generated some signal corresponding to the masses 77 and 82, respectively, in HR.

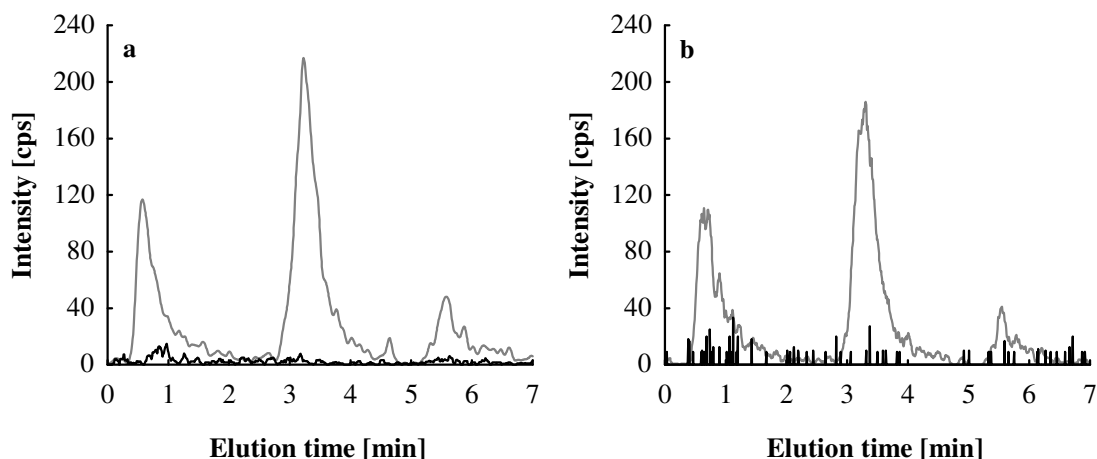


Figure VII-10 Chromatograms for (a) ^{77}Se of human serum BCR-637 (gray line) and a Cl standard (black line); and (b) ^{82}Se of human serum BCR-637 (gray line) and a Br standard (black line); obtained by microbore 2AF-HPLC-apex IR-ICP-SFMS (HR).

No significant signals from the interferents were obtained. Hence, HR allows to completely resolve the interferences on $^{77}\text{Se}^+$ due by $^{40}\text{Ar}^{37}\text{Cl}^+$, but surprisingly also on $^{82}\text{Se}^+$ due by $^{81}\text{Br}^1\text{H}^+$. The resolving power required for this latter interference (11,055) is higher than that can be reached by the instrument, even if in HR, however the Br concentration in the sample appears to be enough low to allow a partial resolution of the false signal, rendering it negligible. A similar effect was observed for $^{78}\text{Se}^+$, which is interfered by $^{40}\text{Ar}^{38}\text{Ar}^+$: the low abundance of the isotope ^{38}Ar (0.063 %) makes this interference quantitatively negligible even if incompletely resolved. On the contrary, $^{40}\text{Ar}^{40}\text{Ar}^+$ resulted too abundant as well as impossible to resolve from $^{80}\text{Se}^+$, thus this isotope is actually the only that cannot be monitored. In order to fully investigate the effects of interferents concentration and use of the apex IR interface, further analysis were carried out in HR, but also in MR modes monitoring the masses 77, 78 and 82.

VII.4.5. Accuracy assesment

Accuracy assessment was carried out in both MR and HR by analysing the human serum BCR-637. For quantification, EC with Se-(Cys)₂ and species-unspecific IDA were applied to several isotopes/isotopic ratios. The results are summarized in Table VII-2.

Table VII-2 Concentration (average \pm SD, n = 3) of GPx3, SelP, SeAlb and their sum (total Se) in BCR-637 obtained by microbore 2AF-HPLC coupled to apex IR-ICP-SFMS in HR and MR. Certified total Se level: 81 ± 7 ng mL⁻¹.

		mass/ratio	Se [ng mL ⁻¹]			
			GPx3	SelP	SeAlb	Total Se
HR	IDA	78/77	18 \pm 1	59 \pm 2	15 \pm 1	92 \pm 2
		82/77	16 \pm 1	57 \pm 3	16 \pm 1	89 \pm 3
	EC	77	28 \pm 3	71 \pm 8	16 \pm 2	115 \pm 9
		78	26 \pm 3	68 \pm 1	15 \pm 1	109 \pm 3
		82	26 \pm 2	69 \pm 3	14 \pm 1	109 \pm 4
MR	IDA	82/77	18 \pm 1	61 \pm 3	13 \pm 1	92 \pm 3
		77	18 \pm 1	62 \pm 9	9 \pm 1	89 \pm 9
	EC	78	19 \pm 3	62 \pm 8	10 \pm 1	91 \pm 9
		82	18 \pm 1	61 \pm 7	8.7 \pm 0.3	88 \pm 7

The human serum BCR-637 is certified for total Se level (81 ± 7 ng mL⁻¹) but not for individual Se-proteins concentration. The only approach for accuracy assessment can be to consider the sum of the three Se-proteins as a valid estimate for total Se level in the sample, and to compare it with the certified value. In this sense, a relatively good agreement was obtained between the measured total Se and the certified total Se content in BCR-637 by IDA either in HR and MR, and using both ⁷⁸Se/⁷⁷Se and ⁸²Se/⁷⁷Se ratios. The results obtained in this study for total Se, SelP and SeAlb are also comparable with those obtained in a previous work by IDA using AE-2AF-HPLC (normalbore columns) and detection by ICP-QMS [4]. Among the results, the use of ⁷⁷Se and ⁸²Se for quantitative purposes appeared feasible either in HR and MR. In the latter case, the apex IR interface most likely contributes to alleviate the Cl⁻ and Br⁻ interferences prior to plasma generation. This assumption is confirmed by the highly comparable results for GPx3 (interfered by Br⁻ and Cl⁻) either by on-line IDA and EC in MR for all the isotopes. Consistently higher results were obtained for GPx3 compared to that reported in an other work [4]. Nevertheless, given the lack of

biological material certified for individual Se-proteins level, such accuracy assessments can be considered only indicative. External calibration led also to a significant positive bias for total Se, GPx3 and SeAlb, when the detection was carried out in HR. The latter results are not completely understood yet, but most likely EC fails in the accurate assessment of the response factor of Se in HR (with considerably lower sensitivity) because of the strong influence of high carbon content of proteins in the ionization efficiency, whereas this effect is compensated by IDA. This effect was observed also in the study of plasma conditions influence on Se sensitivity and accuracy that is presented in the paragraph VII.5.3.

VII.4.6. Determination of columns capacity

The capacity of the microbore 2AF-HPLC columns used in this work was assessed by analyzing volumes of BCR-637 serum in the range 1-15 μL . A minimum sample loop of 5 μL was employed for this study, and the volumes of 1 and 2 μL investigated here correspond to 5 and 2.5 times, respectively, dilution of the serum just before analysis (loops with volumes of 1 and 2 μL introduce a high uncertainty when a conventional manual injection valve is used). As can be seen in Figure VII-11, the response was linear up to 10 μL . A 5 μL sample loop was chosen for further experiments, as a compromise between the minimum sample consumption and ensuring sufficient sensitivity for accurate speciation analysis of GPx3, SeIP and SeAlb.

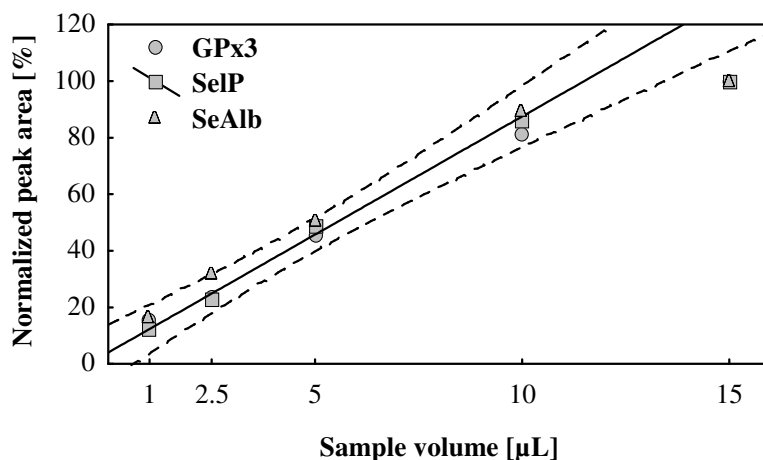


Figure VII-11 Normalized peak area of GPx3, SeIP and SeAlb in BCR-637 as function of the injected sample (loop) volume. The chromatograms were obtained by microbore 2AF-HPLC-ICP-apex IR-ICP-SFMS.

VII.4.7. Determination of columns recovery

The recovery factor for serum Se-proteins was determined by injecting the human serum BCR-637 into the HPLC system with the two columns assembled alone. The chromatograms obtained by microbore HEP and BLUE columns individually are shown in Figure VII-12 a and b, respectively.

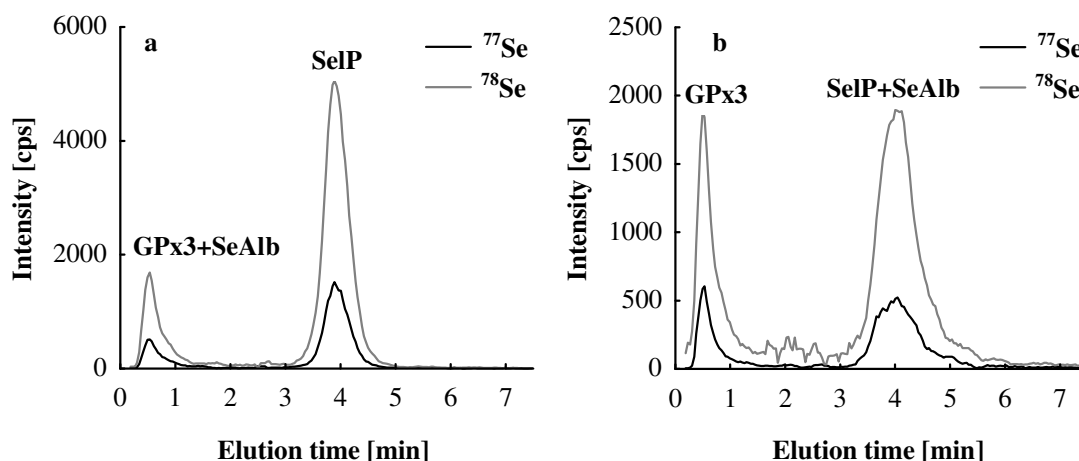


Figure VII-12 Chromatograms obtained for the analysis of 5 μ L of human serum by microbore HEP (a) and BLUE (b) AF-HPLC-apex IR-ICP-SFMS (HR).

When using the HEP column alone, two peaks were obtained, the first attributed to GPx3+SeAlb (not retained, eluted with the void volume by means of the binding buffer A) and the second for SeIP (eluted with buffer B). Two peaks were also obtained by using the BLUE column, the first corresponding to GPx3 and the second for SeIP+SeAlb. Due to the lack of Se-proteins standards, it was impossible to estimate the recovery of each individual specie. The overall column recovery of all Se-proteins was determined by comparison of total peak area (corresponding to total Se) with that of the peak obtained by analysis of the same amount of serum in flow injection (FI) mode (no column used). It is worth noting that enhanced sensitivity is commonly obtained when introducing elevated carbon concentration into the plasma and hence, in order to avoid discrimination between signals obtained with different elution (buffers A and B), the recovery factor was calculated using only buffer B, both in chromatographic and FI modes. A recovery of $\sim 100\%$ was estimated for total Se from HEP, whereas only $\sim 80\%$ of Se was recovered from the BLUE column. The latter result is most likely the consequence of the low recovery of SeIP from the

BLUE column, as explained further. As can be seen in Figure VII-12b, the intensity of the second peak (SeIP+SeAlb) is lower compared to the second peak in Figure VII-12a (SeIP only). That is an indication of low recovery for SeIP from the BLUE column. In order to prove this assumption, the recovery factor of SeIP was calculated by rationing the intensity of SeIP obtained from the BLUE column to that obtained from the HEP column (assuming a quantitative recovery of SeIP from the HEP column), as following:

$$R_{\text{SeIP, BLUE}} (\%) = \frac{\text{SeIP}_{\text{BLUE}}}{\text{SeIP}_{\text{HEP}}} \times 100 \quad (\text{eq. VII-1})$$

where SeIP_{HEP} is the 2nd peak in Figure VII-12a, and $\text{SeIP}_{\text{BLUE}}$ is calculated using the following equation:

$$\text{SeIP}_{\text{BLUE}} = [\text{GPx3}]_{\text{BLUE}} + [\text{SeIP} + \text{SeAlb}]_{\text{BLUE}} - [\text{GPx3} + \text{SeAlb}]_{\text{HEP}} \quad (\text{eq. VII-2})$$

Assuming that $[\text{GPx3}]_{\text{HEP}} = [\text{GPx3}]_{\text{BLUE}}$ and $[\text{SeAlb}]_{\text{BLUE}} = [\text{SeAlb}]_{\text{HEP}}$. Please note that the symbols of Se-proteins (GPx3, SeIP and SeAlb) in eq.VIII-1,2 represent peaks area. A recovery of ~65 % of SeIP from the BLUE column was calculated, according to the ~80 % average recovery of SeIP + SeAlb. Due to the low recovery provided for SeIP by the BLUE column, this stationary phase cannot be used for the separation of SeIP. Nevertheless, in the particular case of the 2AF-HPLC system, it is excluded from the HPLC line before the elution of SeIP from the HEP column (SeIP does not pass through the BLUE column), hence its use for the simultaneous determination of GPx3, SeIP and SeAlb is appropriate.

VII.4.8. Analytical performance characteristics

Repeatability (RSD) for 7 consecutive injections of BCR-637 serum was calculated either from the mass flow ($^{78}\text{Se}/^{77}\text{Se}$) and intensity (^{78}Se) chromatograms. Values of 5 % for GPx3, 6 % for SeIP and 7 % for SeAlb were calculated from the mass flow chromatogram measurements, comparable with those from the peak area of the intensity chromatograms (5 % for SeIP and 7 % for GPx3 and SeAlb).

Linearity was assessed by measuring Se-(Cys)₂ standards with concentration up to 250 ng mL⁻¹ (at 10 different levels); the calibration graph was linear with $R^2 > 0.999$.

A method detection limit of 2.5 ng mL⁻¹ was calculated as threefold the SD of the y-intercept (divided for the slope) of a calibration curve obtained by analyzing Se-

(Cys)₂ in the range 10-100 ng mL⁻¹, using the same experimental set-up as for the determination of Se-proteins.

VII.5. Speciation of plasma/serum Se-proteins by 2AF-HPLC-ORS-QMS

VII.5.1. Instrumentation and set-up

The method based on microbore 2AF-HPLC coupled to ICP-SFMS allowed to significantly reduce both sample volume and time for analysis. However, it requires complex instrumental set-up, which needs time expensive tuning procedures, and get easily dirty due to the high complexity of the matrix. The apex IR device is particularly affected by deposition of material in the internal surface of the cyclonic spray chamber, a process that influences the efficiency of the desolving system. The cleaning procedure for apex IR is also time expensive and difficult to handle.

In order to simplify the HPLC-ICP-MS interface and to improve the detection system, but preserving at the same time the advantages of 2AF-HPLC miniaturization, a different coupling system was tested, based on narrowbore HPLC columns and ICP-ORS-QMS detector. As presented in the paragraph IV.3.3, the ORS technology allows to remove the interferences due by Cl⁻, Br⁻ as well as the Ar dimmers, without require a high resolution mass analyzer. The new instrumental set-up, represented in Figure VII-13, consisted in the narrowbore columns 2AF-HPLC, coupled on-line with an ICP-ORS-QMS and with IDA strategy adopted.

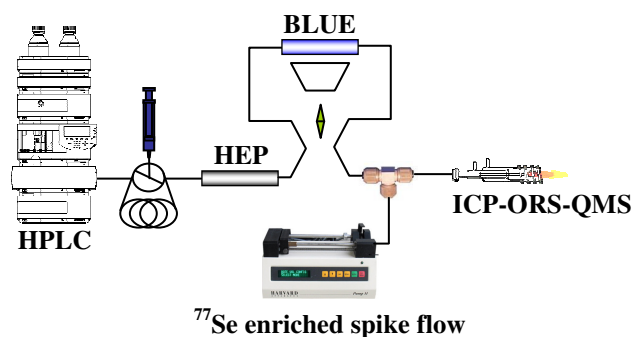


Figure VII-13 Instrumental set-up for Se-proteins determination by narrowbore 2AF-HPLC-ORS-QMS and IDA.

The HPLC flow was 0.75 mL min^{-1} , and the elution sequence was: 0-3 min 100 % buffer A (BLUE column connected on-line); 3-6 min 100 % buffer B (BLUE column excluded); 6-9 min 100 % buffer B (BLUE column connected on-line); 9-12 min 100 % buffer A (BLUE column connected on-line).

VII.5.2. Interferences elimination by ORS technology

The collision gases He and Ar, as well as the reaction gas H_2 , were tested for removing effect on the Se spectral interferences. The optimum cell gas flow rate was estimated by minimizing the background equivalent (BEC) for all the masses of Se in standard solutions 5 ng g^{-1} , and the corresponding interferences solution (Cl and Br) or a blank (for argides). The graphs in Figures VIII-14-16 represent the trend for signals and corresponding BEC as function of the gas flow rate. The optimum flow for He was estimated as 5 mL min^{-1} . This collision gas allowed to efficiently reduce the interference of $^{40}\text{Ar}^{37}\text{Cl}^+$ on $^{77}\text{Se}^+$, but was ineffective for the interferences on the masses 82, 78 and 80. Argon is a collision gas heavier than He, and consequently its optimum flow for the same BEC reduction is consistently lower. Since the instrument was equipped with a manometer unable to manage accurately cell gas flows lower than 1 mL min^{-1} , this minimum was chosen as starting condition. The use of Ar allowed to efficiently reduce the interference of $^{40}\text{Ar}^{37}\text{Cl}^+$ on $^{77}\text{Se}^+$ and of $^{40}\text{Ar}^{38}\text{Ar}^+$

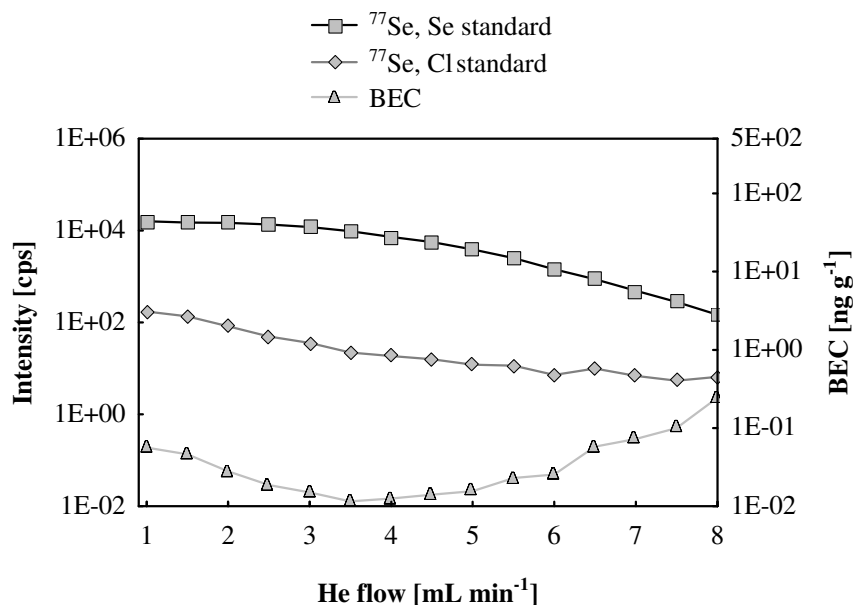


Figure VII-14 Signal and background equivalent (BEC) for ^{77}Se in standards of Se(VI) 10 ng g^{-1} and Cl $3.5 \text{ } \mu\text{g g}^{-1}$ as function of collision gas (He) flow.

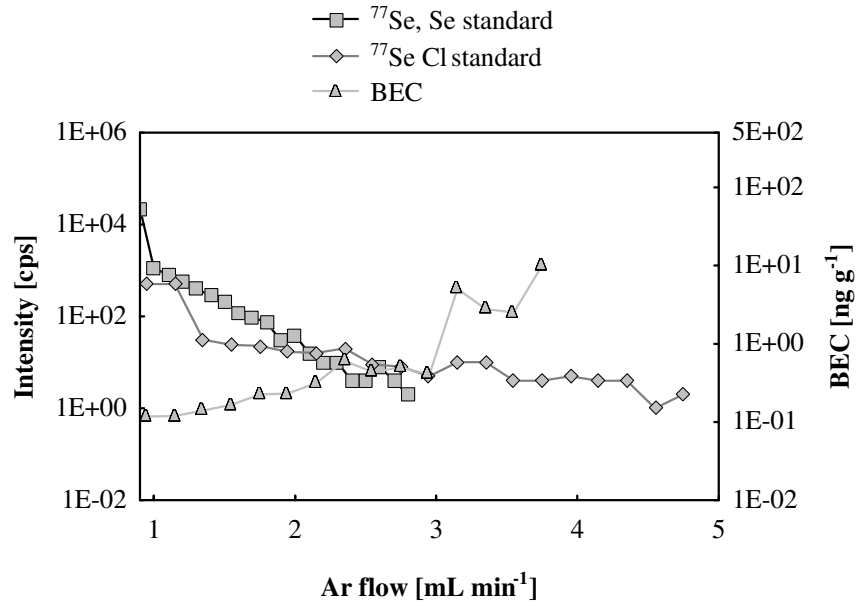


Figure VII-15 Signal and background equivalent (BEC) for ^{77}Se in standards of Se(VI) 10 ng g^{-1} and $\text{Cl}^- 3.5 \mu\text{g g}^{-1}$ as function of collision gas (Ar) flow.

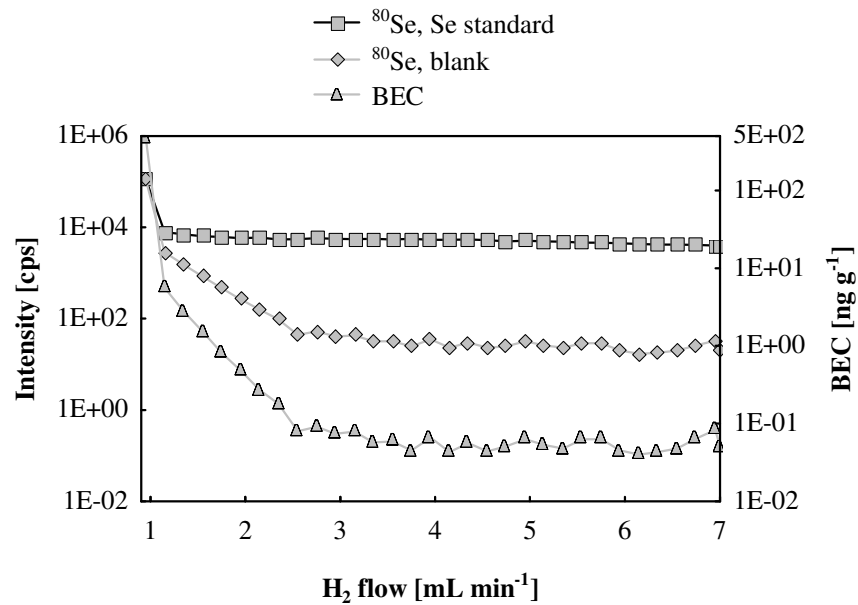


Figure VII-16 Signal and background equivalent (BEC) for ^{80}Se in standards of Se(VI) 10 ng g^{-1} and blank solution (milli-Q water + HNO_3 1% v/v) as function of reaction gas (H_2) flow.

on $^{78}\text{Se}^+$, but still was ineffective for $^{40}\text{Ar}^{40}\text{Ar}^+$ on ^{80}Se . On the contrary, H_2 removed very efficiently all the spectral interferences on Se, particularly for $^{40}\text{Ar}^{40}\text{Ar}^+$ on $^{80}\text{Se}^+$, with a low loss in sensitivity at an optimum flow estimated as $\sim 4\text{ mL min}^{-1}$.

An important variable to take into account for operative parameters optimization, is the effect of collision/reaction cell stabilization time on the instrumental sensitivity. This effect was assessed by firstly optimizing the instrument in no-gas conditions. Then, a standard solution of $5\text{ ng g}^{-1}\text{ Se}$ and $1\text{ ng g}^{-1}\text{ Y}$ as internal standard was pumped into the instrument. After ^{77}Se , ^{82}Se and ^{89}Y signals stabilized in no-gas conditions, the collision/reaction gas was flowed into the cell at the previously optimized rate, and the three masses immediately monitored for 1 h. Signal intensities for Se were normalized for Y intensity, and the graphs in Figure VII-17 were obtained for the gasses He, Ar and H_2 , independently.

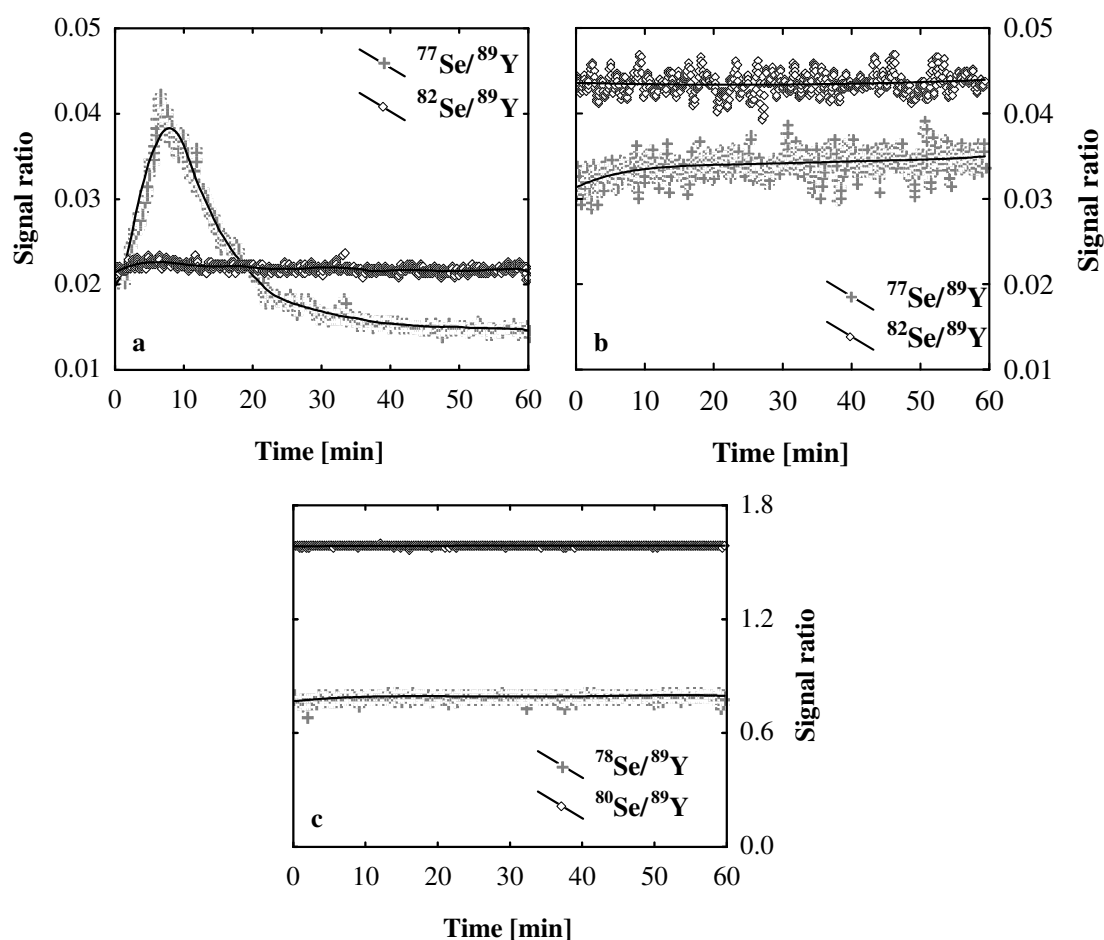


Figure VII-17 Signal ratio Se/Y as function of collision cell running time using the gases: a) He, flow 5 mL min^{-1} ; b) Ar, flow 1 mL min^{-1} ; c) H_2 , flow 4 mL min^{-1} .

Helium showed to need flowing at working rate at least for 30 min to reach stable signal ratios, particularly for the mass 77. Argon required a shorter time to stabilize in the cell, and in general the stabilization time effect is less relevant. However, also for Ar it is suggested to wait at least for 30 min before starting the analysis. Also for H₂, 30 min of stabilization time are suggested, but the possibility to acquire the masses 78 and 80 (more abundant than the masses 77 and 82), as well as the notably bigger instrumental sensitivity, guarantee higher stability of the signal ratios.

VII.5.3. Study of plasma conditions

Optimization of plasma RF power and methanol percentage in the eluent

Apart from the plasma parameters that were daily optimized (carrier and auxiliary gas flows), the radiofrequency power (RF) was the main factor influencing the signal intensity for Se-proteins in the ICP-ORS-QMS. As Se presents high ionization potential, the signal intensity is significantly influenced by the plasma temperature,

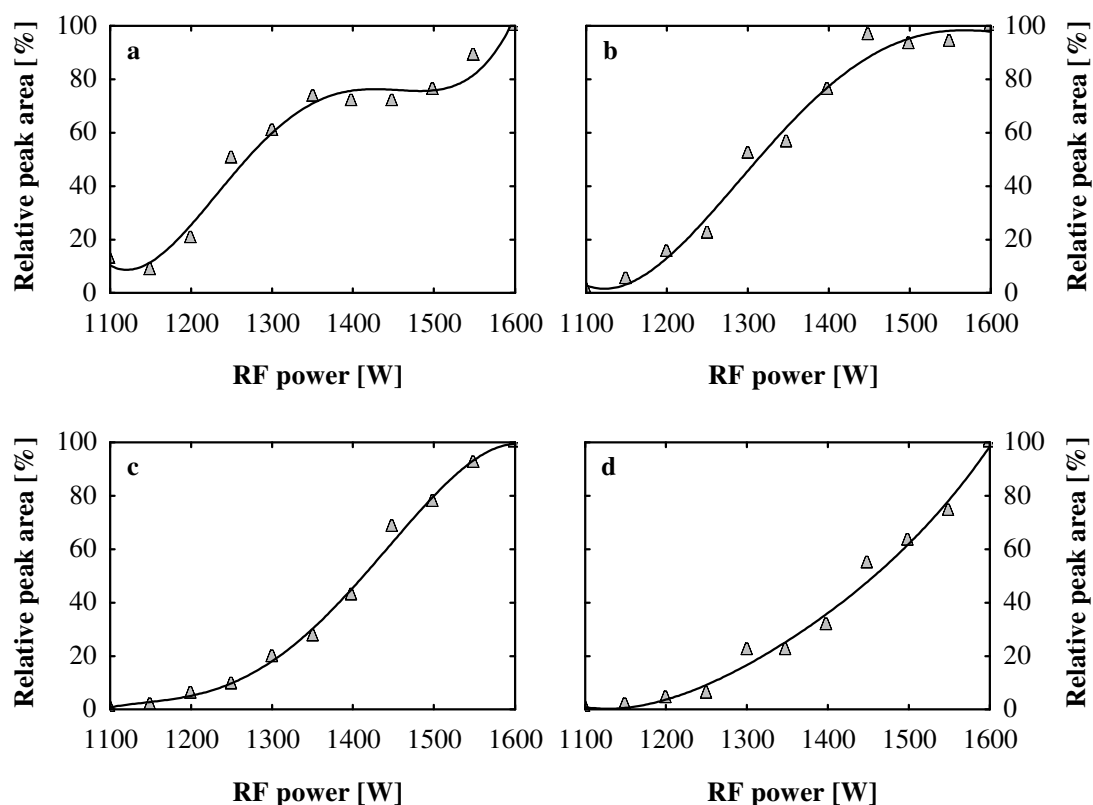


Figure VII-18 Relative peak area as function of the plasma RF power for: (a) Se-(Cys)₂ 10 ng g⁻¹ standard; (b) GPx3; (c) SeIP; (d) SeAlb in BCR-637. The area was calculated from chromatograms obtained by narrowbore 2AF-HPLC-ICP-ORS(Ar)-QMS. The Se-(Cys)₂ standard was eluted with buffer A.

which is in turned conditioned by both RF power and organic carbon content in the gas-nebulised drops mixture. The influence of RF power was studied in the range between 1100 and 1600 W (the upper limit for the instrument). Standard solutions of 10 ng g^{-1} Se-(Cys)₂, and BCR-637 sample were injected into the narrowbore 2AF-HPLC system coupled with the ICP-ORS-QMS using Ar as collision gas (flow rate 1 mL min^{-1}). Peak areas were calculated for the Se-(Cys)₂ standard and the individual Se-proteins in BCR-637 as function of plasma RF power. As shown in Figure VII-18, the ionization efficiency for all the four species increased with RF power within the entire range investigated, but with notably different trends.

Plasma temperature is also increased by the organic carbon content in the mobile phases/sample solution. The addition of organic phases affects also the surface tension of the solution, causing an improvement of nebulisation efficiency, which contributes to a further enhancement of plasma conditions. To evaluate this effect, buffers A and B were prepared in methanol with proportions ranging from 1 to 20 % (v/v)

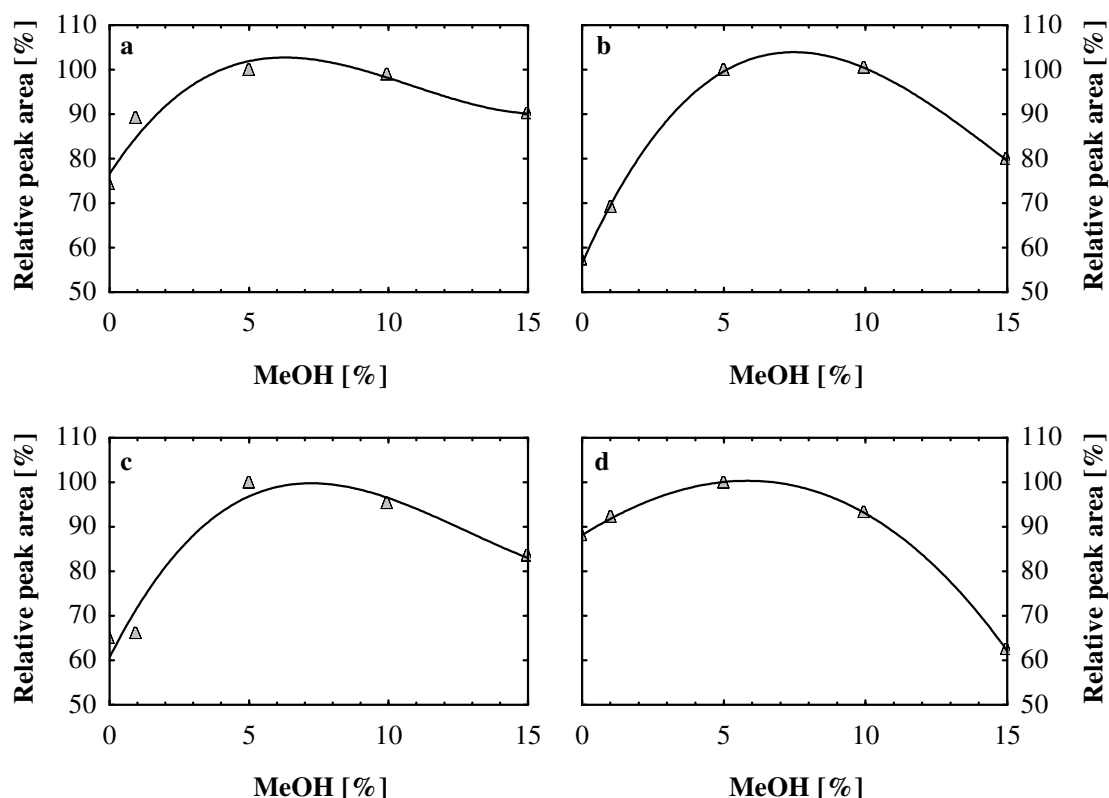


Figure VII-19 Relative peak area as function of the MeOH percentage in the eluent for (a) Se-(Cys)₂ 10 ng g^{-1} standard; (b) GPx3; (c) SeIP; (d) SeAlb in BCR-637. The area was calculated from chromatograms obtained by narrowbore 2AF-HPLC-ICP-ORS(Ar)-QMS. The SeCys standard was eluted with buffer A.

(introduction of methanol over 15 % extinguished the plasma due to increasing of the reflected power), and again standards of Se-(Cys)₂ and BCR-637 serum samples were analyzed by 2AF-HPLC coupled with the ICP-ORS-QMS(Ar). Relative peak area was represented as function of methanol percentage in the graphs in Figure VII-19.

Maximum sensitivity enhancement of ~10 % was obtained for all the species by using binding/elution buffers prepared in 5 % methanol, whereas higher percentage caused a drop in all signal intensities. Nevertheless, poor precision between consecutive replicates was noticed when using methanol.

Considering that investigation of plasma RF power and methanol content in the mobile phases was carried out by analysing BCR-637 samples, the effect of such parameters was evaluated not only in term of sensitivity, but also in terms of analytical accuracy for applicative purposes. This is particularly important if EC is used, because Se species such as Se-(Cys)₂ (the potential calibrant) and individual Se-proteins showed to behave in different way as function of the plasma conditions.

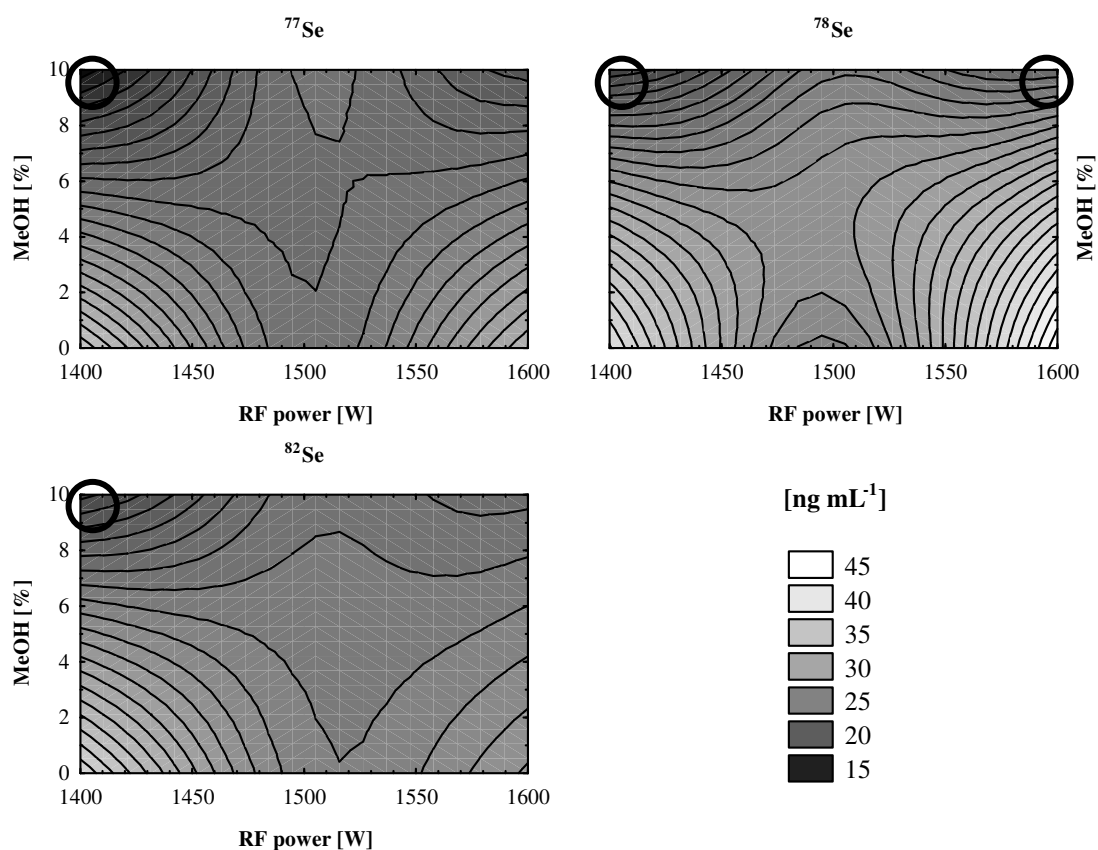


Figure VII-20 Concentration of GPx3 in BCR-637 obtained by EC with Se-(Cys)₂ standards, as combined function of plasma RF power and methanol percentage in the mobile phases. The areas where the response surface is closer to the indicative reference level (15 ± 4 ng mL⁻¹) are marked by the circles. The analysis was performed by narrowbore 2AF-HPLC-ICP-ORS(Ar)-QMS.

External calibration based on Se-(Cys)₂ was used to quantify the Se-proteins concentration in BCR-637, as combined function of plasma RF power and methanol percentage in the mobile phases. Dolehert experimental design was employed to optimize the study. Bivariate graphs for each Se isotope like those shown in Figures VIII-20,22 were obtained.

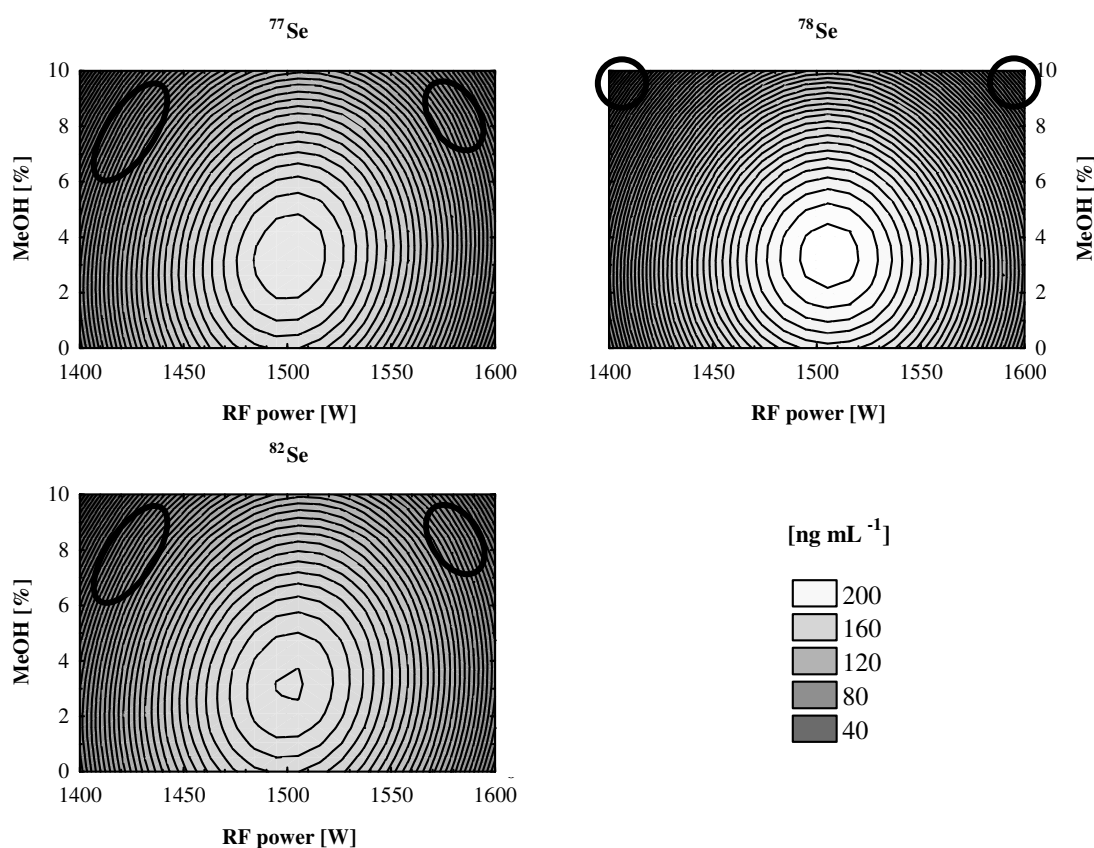


Figure VII-21 Concentration of SeIP in BCR-637 obtained by EC with Se-(Cys)₂ standards, as combined function of plasma RF power and methanol percentage in the mobile phases. The areas where the response surface is closer to the indicative reference level ($60 \pm 7 \text{ ng mL}^{-1}$) are marked by the circles. The indicative reference level is The analysis was performed by narrowbore 2AF-HPLC-ICP-ORS(Ar)-QMS.

All the serum Se-proteins were accurately determined in correspondence of minimum (1400 W) or maximum (1600 W) RF power, and maximum methanol percentage (10 %). The surfaces of each protein are characterized by different shapes, even if the maxima tend to locate in correspondent zones. For some proteins a strong difference among isotopes was also observed, particularly for SeAlb (see Figure VII-22), most probably due to the influence of plasma conditions on independent spectral interferences incompletely resolved.

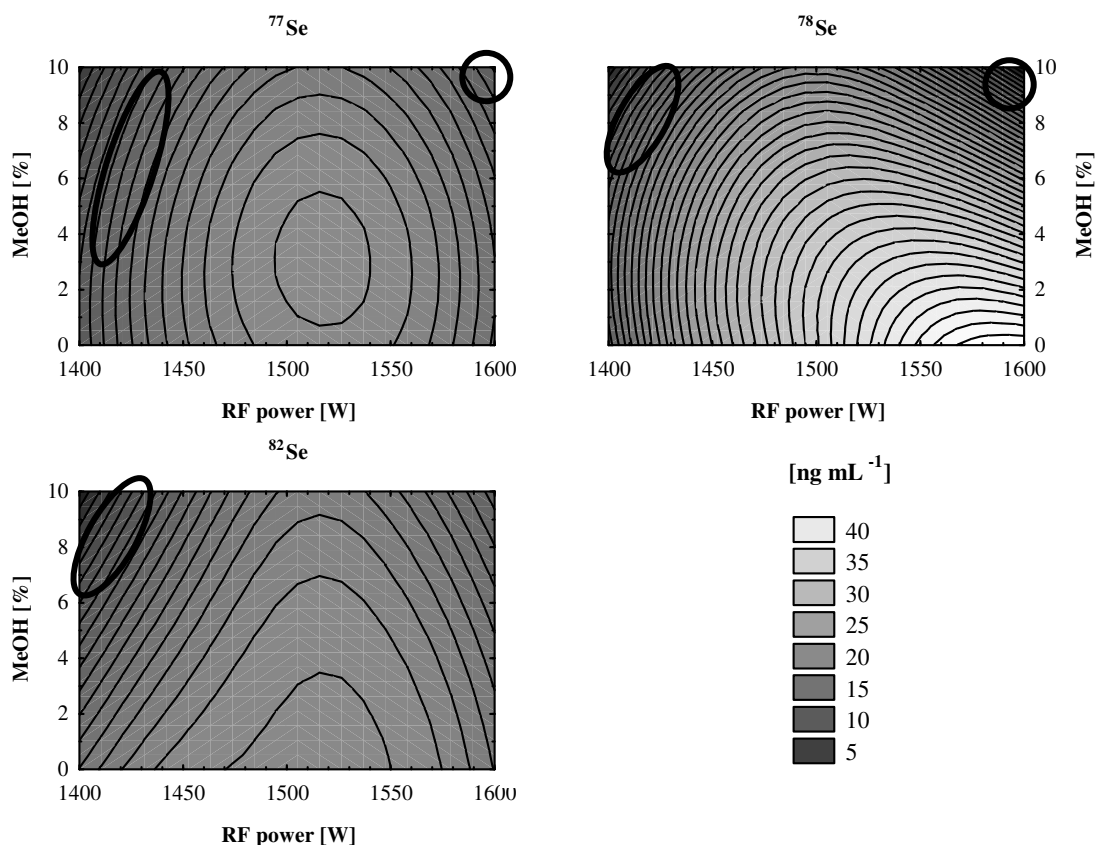


Figure VII-22 Concentration of SeAlb in BCR-637 obtained by EC with $\text{Se}(\text{Cys})_2$ standards as combined function of plasma RF power and methanol percentage in the mobile phases. The areas where the response surface is closer to the indicative reference level ($13 \pm 4 \text{ ng mL}^{-1}$) are marked by the circles. The analysis was performed by narrowbore 2AF-HPLC-ICP-ORS(Ar)-QMS.

Optimization of methane percentage in the optional plasma gas

Organic carbon content into the plasma can also be increased by using an optional gas flow containing methane. Variable percentages of methane were tested in the range 0-20 % monitoring the sensitivity for $\text{Se}(\text{Cys})_2$ standards and Se-proteins in BRC-637. A relative increase in sensitivity was observed using 5 % methane for GPx3 and SelP (see Figure VII-23), but an inverse association was observed between Se sensitivity and methane percentage for $\text{Se}(\text{Cys})_2$ and SeAlb. Therefore, addition of methane in the optional plasma gas was in general unsuitable for improving Se ionization efficiency.

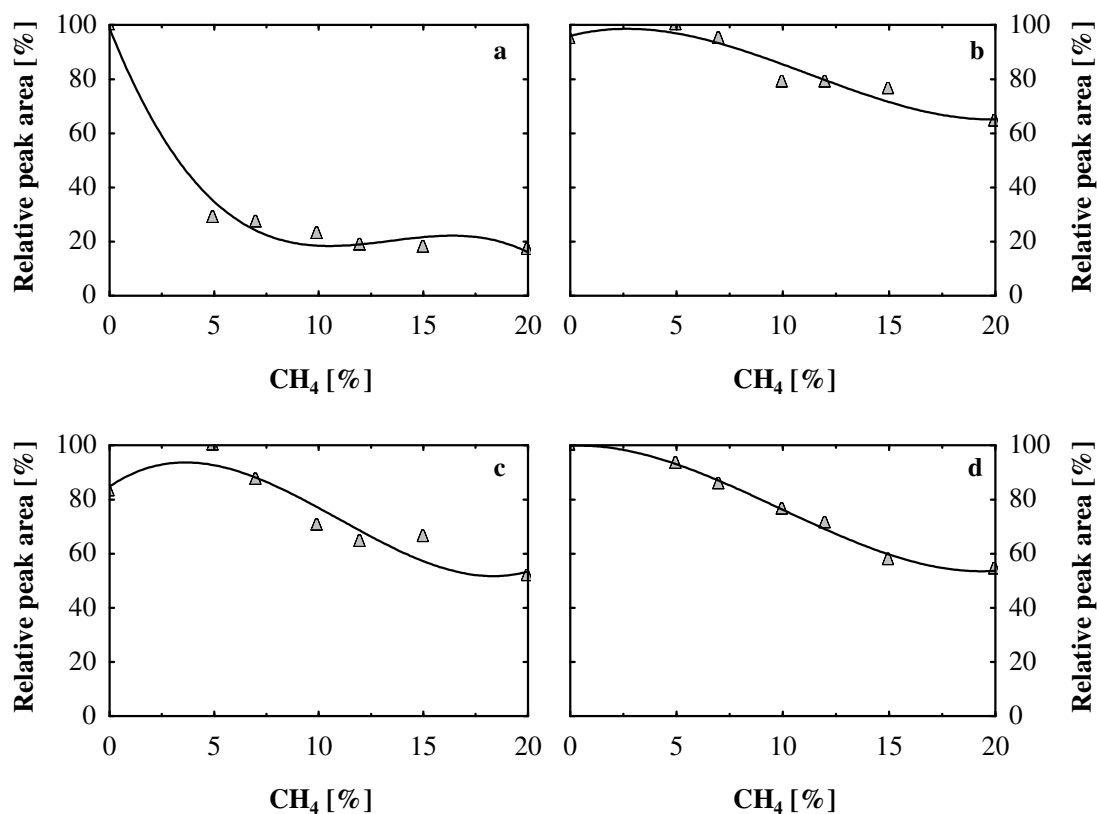


Figure VII-23 Relative peak area % as function CH₄ percentage in the plasma optional gas flow for (a) SeCys 10 ng g⁻¹ standard; (b) GPx3; (c) SeIP; (d) SeAlb in human control serum. The area was calculated in chromatograms obtained by narrowbore 2AF-HPLC-ICP-ORS(Ar)-QMS. The SeCys standard was eluted with buffer A.

Study of matrix effects produced by mobile phase composition

As pointed out in the previous paragraphs, plasma conditions may highly affect the instrumental sensitivity for Se, also with different behaviour among species. Methanol was added to the mobile phases to evaluate the changes in Se sensitivity as function of matrix organic content. The direct influence of the mobile phases A and B on sensitivity for different inorganic species of Se was then investigated. Calibration curves were realized by injecting of Se-(Cys)₂ standards in the 2AF-HPLC system with normalbore and microbore columns. Changing the columns diameter allowed to assess the matrix effects also as function of the flow rate (1.0 mL min⁻¹ for the normalbore columns, and 0.3 mL min⁻¹ for the microbore), which entails variations in nebulisation efficiency and total solvent/sample amount carried to the plasma. As can be seen in Figure VII-24, buffer B entailed an increase of sensitivity due by enhancement of plasma temperature, caused in turn by the higher carbon content.

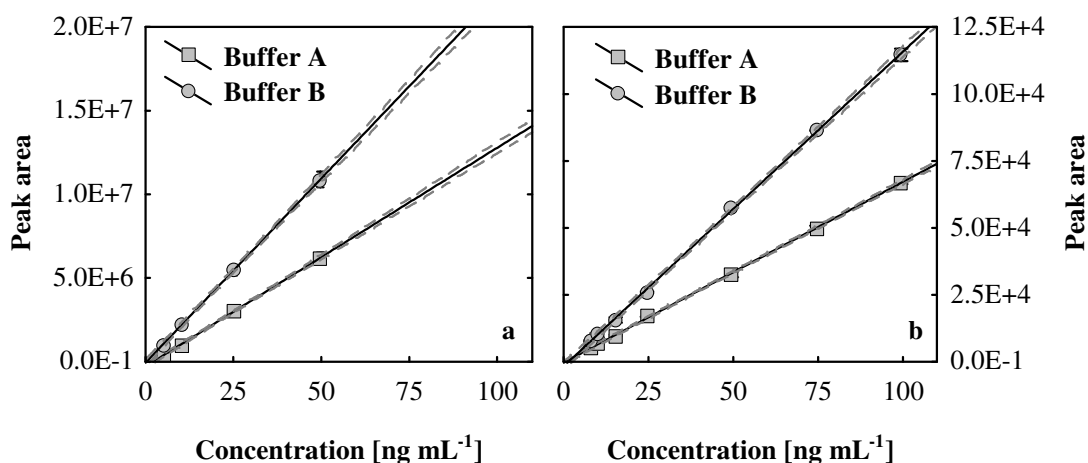


Figure VII-24 ^{82}Se calibration curves for Se-(Cys)₂ standards eluted in buffers A and B, obtained by 2AF-HPLC-ORS(H₂)-QMS with: (a) normalbore columns, flow 1.0 mL min⁻¹; (b) microbore columns, flow 0.3 mL min⁻¹.

This effect is comparable to that shown for addition of methanol in the mobile phase. A lower flow entails decrease of analyte amount introduced into the nebulizer, but on the other side is characterized by increasing of nebulisation efficiency, and hence by higher ratio Se-to-the-plasma/Se-to-the-nebulizer. As a consequence, the signal intensity is lower, but not as a linear function of flow rate. In this study, the ratio of the slopes buffer B/buffer A at 1.0 mL min⁻¹ was notably higher (1.69) in respect to the same ratio at 0.3 mL min⁻¹ (1.28). This result proved that decreasing the flow rate reduces the gap of Se sensitivity in mobile phases with different carbon content.

The effect of mobile phase carbon content was also tested on different Se species by eluting Se-(Cys)₂ and Se(IV) standards in the same buffers A and B with normalbore and microbore columns. As shown in Figure VII-25, Se-(Cys)₂ and Se(IV) curves presented significantly different slope, with higher sensitivity for Se-(Cys)₂ in both buffers. By increasing of mobile phase carbon (buffer B), the discrimination effects between species was notably reduced.

Considering the effects on Se sensitivity related to both mobile phase composition and Se specie, decreasing the HPLC flow rate and increasing the mobile phase carbon content reduced, even if did not eliminate, matrix effects and species discrimination.

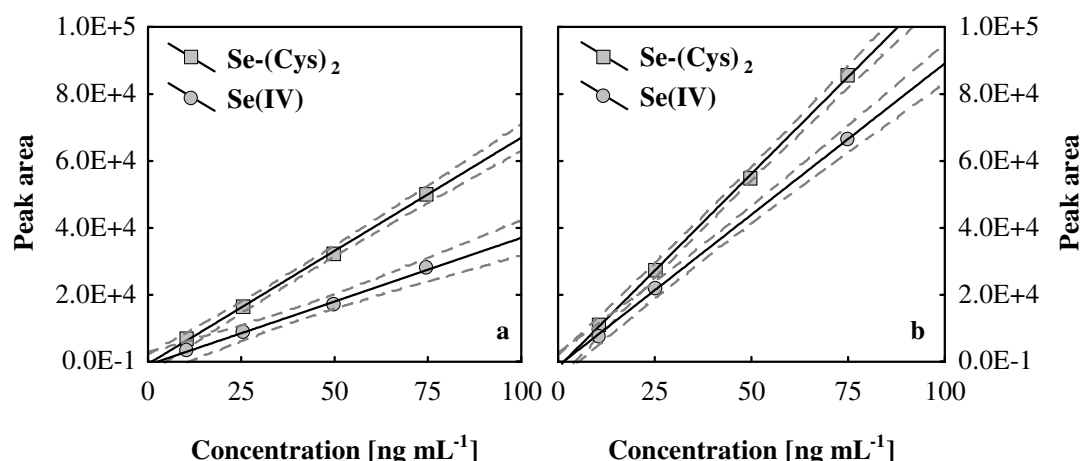


Figure VII-25 Calibration curves for Se-(Cys)₂ and Se(IV) standards eluted in buffers A (a) and B (b) obtained by microbore 2AF-HPLC-ORS(H₂)-QMS, flow 0.3 mL min⁻¹.

VII.5.4. Final operating conditions

The finally selected instrumental parameters for HPLC and ICP-ORS-QMS coupling are summarized in Table VII-3.

Table VII-3. Optimum operating conditions for the narrowbore 2AF-HPLC-ICP-ORS-QMS instrumental set-up.

ICP-ORS-QMS	
RF power	1550 W
Nebulizer flow rate	1.0 ± 0.2 L min ⁻¹ (daily optimization)
Auxiliary gas flow rate	1.2 L min ⁻¹
Plasma gas flow rate	16 L min ⁻¹
Detection mode	counting
Integration type	average
Isotopes monitored	⁷⁷ Se, ⁷⁸ Se, ⁸⁰ Se, ⁸² Se
Acquisition time per point	1 s
Samples per peak	3
Cell gas flow (H ₂)	4 mL min ⁻¹
2AF-HPLC	
Stationary phases	Hitrap™ Heparin HP (HEP) Hitrap™ Blue HP (BLUE)
Columns	5 cm × 2.0 mm id
Sample loop	10 μL
Flow rate	0.75 mL min ⁻¹
Elution mode	Step gradient
Mobile phases	Binding buffer (A): AmAc 0.05 M (pH 7.0) Elution buffer (B): AmAc 1.5 M (pH 7.0)

VII.6. Methods interlaboratory comparison for assessment of Se-proteins indicative concentration in BCR-637 human serum CRM

VII.6.1. Comparison of the analytical methods

As mentioned in the introduction of this chapter, 'speciated' serums certified for individual Se-protein concentration have not yet been produced, thus making the method validation virtually impossible. As we carried out in this study, an alternative is the analysis of a serum certified for total Se: with the assumption that Se is almost entirely bound to proteins, the agreement between the level of total protein bound Se and the certified value can be considered a valuable tool for method validation.

In the past years, our research group developed some methods for the determination of Se-proteins in human serum. Each of them was applied to the analysis of the commercially available BCR-637. All the data obtained by using 13 different methods were finally grouped for an interlaboratory comparison, in order to derive reliable indicative levels of the Se-proteins in BCR-637. The Laboratoire National de Métrologie et d'Essais (LNE, Paris, France) was involved in this work, providing part of the data. A brief description of each method is provided below:

- M1. serum off-line clean-up by AE SPE, followed by normalbore 2AF-HPLC coupled to ICP-QMS [5].
- M2-M3. tandem normalbore AE-2AF-HPLC coupled to ICP-QMS [4].
- M4. tandem normalbore AE-2AF-HPLC followed by off-line SFMS (HR) analysis of the GPx3, SeIP, and SeAlb fractions [9].
- M5-M10. microbore 2AF-HPLC coupled to apex IR-ICP-SFMS [9].
- M11. normalbore 2AF-HPLC coupled to ICP-ORS-QMS. A mixture of He-H₂ (4.5-2.5 mL min⁻¹ flow rate) was used as collision/ reaction gases [10].
- M12. the same as M11 with the difference that microbore 2AF columns were used.
- M13. normalbore 2AF-HPLC coupled to ICP-ORS-QMS. Argon was used as collision gas (1 mL min⁻¹ flow rate).

Further differences among methods consisted in the choice of EC or IDA for quantification, and monitoring different masses or calculating different isotopic ratios. Table VII-4 reports the adopted quantification strategy for each method, and the corresponding results for individual Se-proteins concentration and total Se.

Table VII-4 Concentrations (average \pm SD, $n = 3$) of GPx3, SeIP, SeAlb and total Se in BCR-637 obtained by 13 different methods.

Method	Quantification	mass/ratio	Se [ng mL ⁻¹]			
			GPx3	SeIP	SeAlb	Total Se
M1	EC	77	9.9 \pm 0.1	51 \pm 1	18 \pm 3	79 \pm 3
M2	IDA	82/77	13 \pm 1	65 \pm 5	14 \pm 1	92 \pm 5
M3	EC	82	13 \pm 1	59 \pm 1	16 \pm 1	88 \pm 2
M4	EC	82	10 \pm 1	57 \pm 2	7 \pm 1	74 \pm 2
M5	IDA	78/77	18 \pm 1	59 \pm 2	15 \pm 1	92 \pm 2
M6		82/77	16 \pm 1	57 \pm 3	16 \pm 1	89 \pm 3
M7	IDA	82/77	18 \pm 1	61 \pm 3	13 \pm 1	92 \pm 3
M8		77	18 \pm 1	62 \pm 9	9 \pm 1	89 \pm 9
M9	EC	78	19 \pm 3	62 \pm 8	10 \pm 1	91 \pm 9
M10		82	18 \pm 1	61 \pm 7	8.7 \pm 0.3	88 \pm 7
M11	EC	80	11 \pm 1	54 \pm 2	12 \pm 1	77 \pm 2
M12	EC	78	15 \pm 1	62 \pm 1	13.1 \pm 0.3	90 \pm 2
M13	EC	78	14 \pm 1	66 \pm 2	11 \pm 1	91 \pm 4
Certified level						81 \pm 7

The Se-proteins concentration obtained by using methods M1-M13 for the analysis of the BCR-637 serum were normally distributed, according to the K-S test. The average concentration and the combined standard uncertainty (u_c) of GPx3, SeIP, and SeAlb (as well as their sum) in the BCR-637, within all the methods are summarized in Table VII-5.

Table VII-5. Mean concentration \pm combined standard uncertainty ($n = 13$) of Se-proteins in the BCR-637 CRM human serum determined by using M1-M13.

Se [ng mL ⁻¹]	
GPx3	15 \pm 4
SeIP	60 \pm 7
SeAlb	13 \pm 4
Total Se	87 \pm 9 (90 \pm 7)*

* recalculated after outliers' elimination ($n = 10$)

It is worth noting that the level of total protein bound Se is not significantly different than the certified value (81 ± 7) ng mL⁻¹. This is an indication of the methods' accuracy, and hence the indicative concentration values for Se-proteins calculated in this work could be tentatively used for validation in studies where 'speciated' CRMs are not available. The distribution of Se-proteins concentration determined by using the methods M1-M13 is shown in Figure VII-26. A relatively high spread of the results was obtained: the RSD was 27 % for GPx3 and SelP, and 35 % for SeAlb, respectively. This confirmed that the determination of Se-proteins in a complex matrix such as the human serum is still a difficult analytical task.

Among all the methods, the following outliers were detected using the Hempel test: SelP in M1 and total Se in M1, M4, and M11. Once removed the outlier methods, the average data remained unchanged for SelP, whereas for total Se the mean value and u_c changed slightly (see Table VII-5).

In order to establish which method is the best in terms of accuracy and repeatability, the Mandel's h and k tests were applied by considering the whole pooled values of GPx3, SelP, SeAlb and total Se. h and k plots, represented in Figure VII-27, indicated that the best overall accuracy and repeatability were both gained by M12. A repeatability exceeding the critical value for 5 % level of significance was revealed by M8, M9, and M10 for total Se, and by M9 for SelP; 1 % level of significance was exceeded by M1 for SeAlb, M8 for SelP, and M9 for GPx3. Mandel's h and k tests showed that all the methods provided satisfactory accuracy with respect to the overall mean, whereas the best method for Se-proteins determination in human serum among those considered in this study was M12, which is based on microbore 2AF-HPLC coupled to ICP-ORS-QMS (see description above).

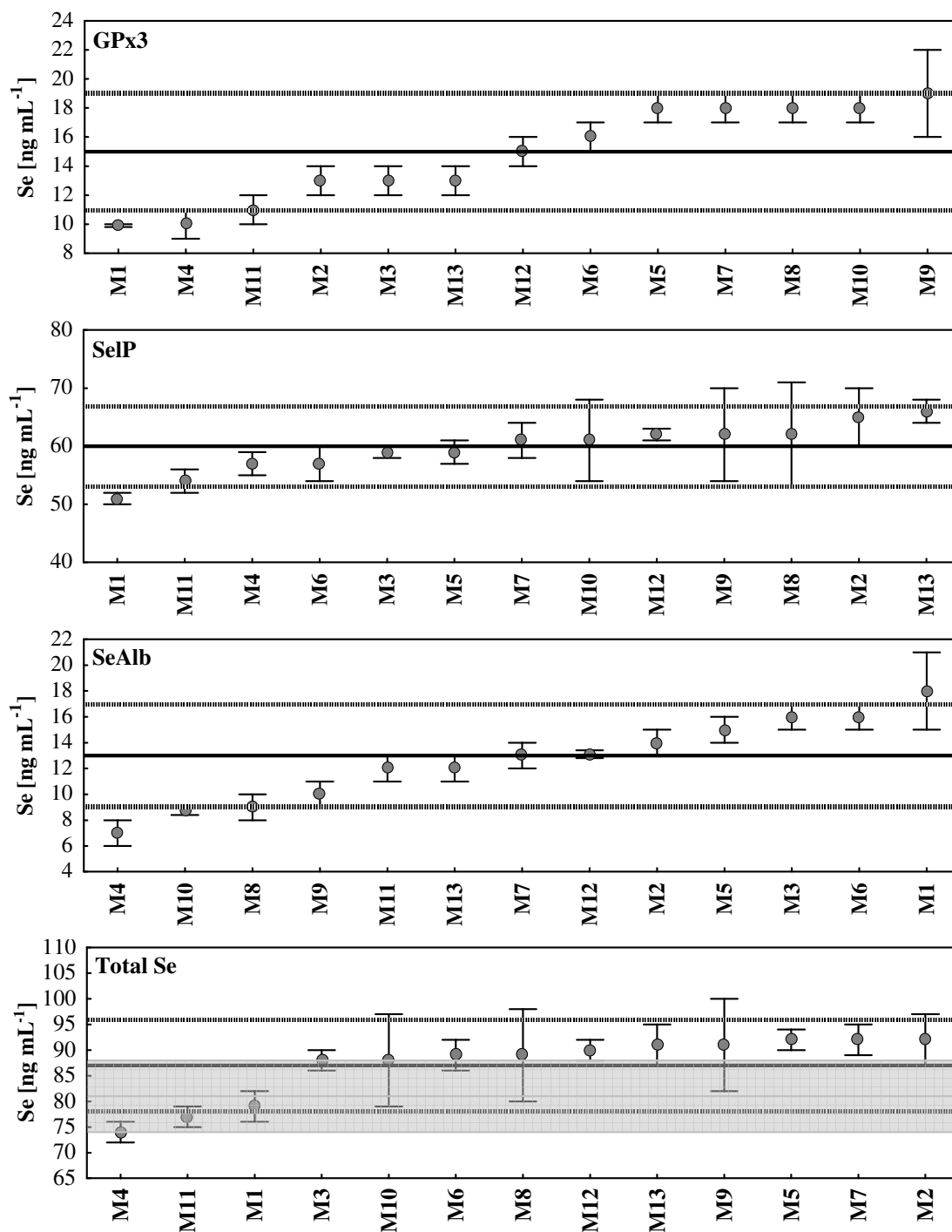


Figure VII-26 Se-proteins concentration in BCR-637 human serum CRM obtained using 13 different methods based on 2AF-HPLC coupled to ICP-MS. The central line represents the average of all 13 results (M1-M13), whereas the dotted lines correspond to the combined standard uncertainty (u_c). The bars shown for each result correspond to the standard deviation calculated for three independent serum replicates. In (Total Se), the central line in the gray area represents the certified value of total Se whereas the gray area represents the confidence interval (95 %).

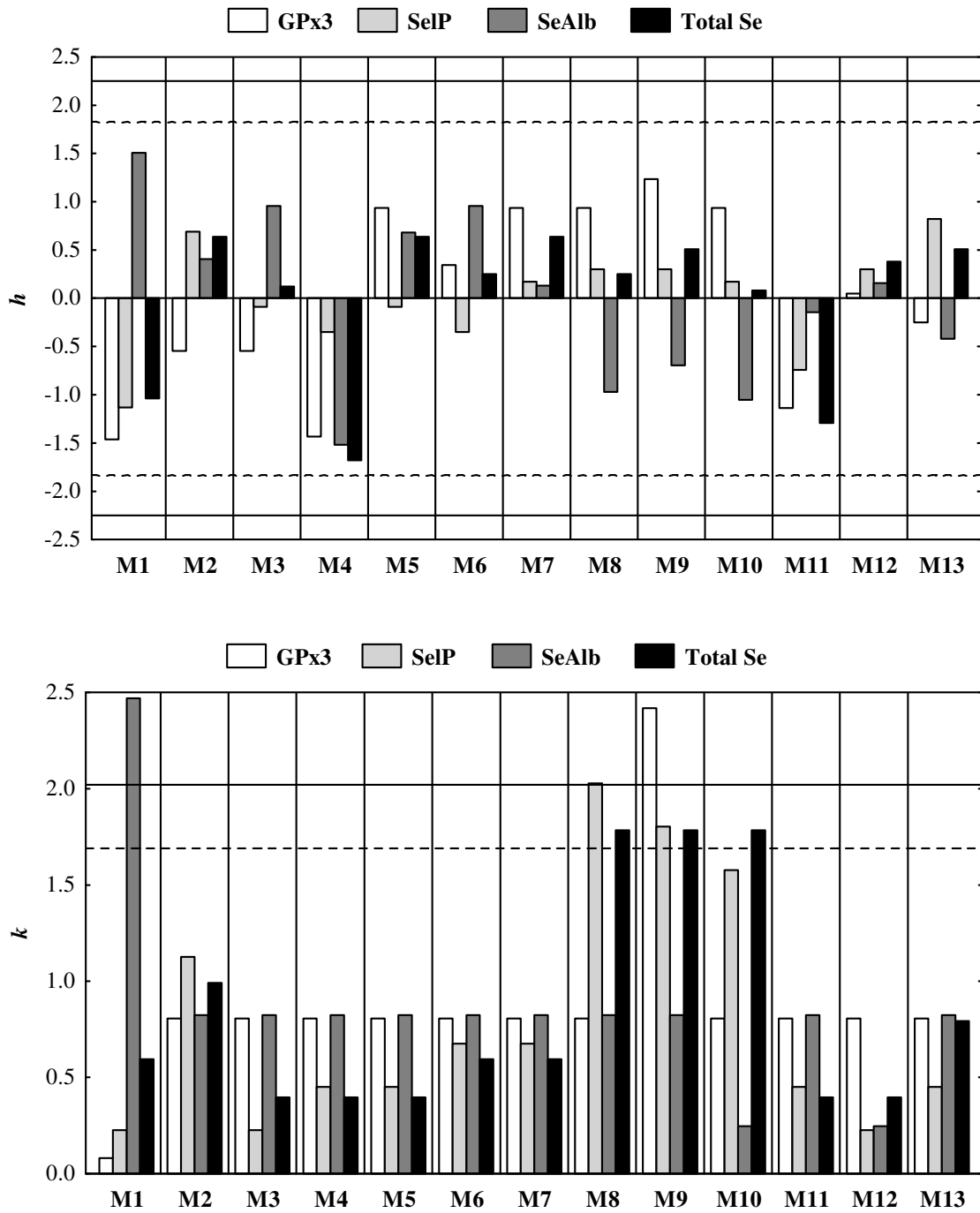


Figure VII-27 Values of Mandel's h (above) and k (below) statistics applied to the Se-proteins concentration in BCR-637 obtained by the analytical methods M1-M13. The bold horizontal lines mark the critical value at $\alpha = 0.01$, whereas the dotted horizontal lines mark the critical value at $\alpha = 0.05$.

VII.7. Conclusions

A deep investigation and optimization of instrumental parameters for Se determination by HPLC-ICP-MS, using SFMS and ORS-QMS detectors, was carried out. Useful information were obtained about the behaviour of Se-containing analytes in the plasma source. The results can be used to maximize the analytical performances in future determinations, and overcome classical problems in Se quantification by ICP-MS, such as low sensitivity and matrix effects.

A complete new analytical method was developed and characterized for speciation of Se-proteins in human serum, based on microbore 2AF-HPLC hyphenated to ICP-SFMS in HR mode using on-line IDA. The method allowed to perform interference-free simultaneous determination of Se-proteins in a sample volume (5 μ L) twentyfold lower and a time for analysis (7 min) twofold lower than those required by the previous state of the art. No correction equations or additional sample preparation steps were necessary, but a high efficiency sample desolvation/introduction system was necessary to compensate for the loss in sensitivity when HR was employed. This method could be particularly useful for applications in which the amount of serum available is limited, such as in bio-medical studies based on mouse/rat models, where the amount of blood that can be sampled daily is commonly of a few microliters (≤ 20).

A full interlaboratory comparison, in terms of analytical performances, was also carried out considering 13 different methods for Se-proteins determination in human serum by 2AF-HPLC-ICP-MS. The data elaborations allowed to select the best analytical method available nowadays for this specific task, and provided the first indicative concentration (in terms of Se) of individual Se-proteins in a commercially available human serum. These data can be useful for future methods validation and/or intercomparison.

Two journal articles, in attachment, were published regarding the studies presented in this chapter.

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VIII. STUDY OF HUMAN PLASMA SE-PROTEINS CONCENTRATION IN TYPE II DIABETES MELLITUS²

VIII.1. Introduction

The importance of collecting information not only on the presence/absence of proteins as specific biomarkers of disease, but also on their concentration/activity in tissues and body fluids has emerged in recent years. The determination of proteins present at very low level in such complex matrices is a very complex task. A large amount of work has been done in the last few years for the development of new accurate, precise and fast methods for proteins analysis by HPLC and ICP-MS coupling. However, at present very few of these methods have been applied to real biomedical studies.

An example of potentially interesting application is the investigation of plasma Se-proteins in patients with type II diabetes. Other detection systems such as atomic fluorescence spectrometry and atomic absorption spectrometry was used in this field, but due to the complicated on-line coupling with a separation technique, such as HPLC, they have been employed only for the total Se determination [1-9].

² This work is published in: Roman M. et al. Transl Res 2010;156(4):242:50

Alternatively, the activity of plasma GPx3 in patients with diabetes has been determined by enzyme-linked immunosorbent assay (ELISA) [3] or other enzymatic assays [9]. However, these techniques are difficult to handle, time consuming, imprecise, and SeAlb cannot be assayed because it has no enzymatic activity (from the point of view of Se). On the contrary, ICP-MS allows easy coupling with HPLC, resulting in high specificity and sensitivity. A wide range of methods based on these techniques have been successfully developed and validated by our research group, also in the context of this Ph.D. project (see paragraph VII.6), for individual Se-proteins determination in human plasma/serum.

VIII.2. Goals of the study

In this study, one of the methods previously developed for the determination of plasma Se-proteins GPx3, SeLP and SeAlb, was applied for the cross-sectional investigation of the possible relationship among the complete plasma Se-proteins pattern, presence of type II diabetes mellitus and clinical parameters in 40 patients with diabetes and 15 healthy control subjects. A relatively low number of samples was analyzed in order to carry out a pilot study, which can be used as a suitable design of broader investigations.

VIII.3. Patients and Methods

VIII.3.1. Patients and study protocol

The study involved 40 patients with type II diabetes regularly attending the Diabetes Clinic of the Department of Medical and Surgical Sciences at the University of Padova (Italy). The control group included 15 healthy subjects with normal glucose tolerance as assessed by an oral glucose tolerance test according to ADA recommendations [10]. All the enrolled patients with type II diabetes and normal control subjects followed a dietary regimen of 50 % carbohydrates, 30 % fats and 20 % proteins. This dietary program was not modified for at least 6 months preceding the analysis. All patients with type II diabetes were treated with oral hypoglycaemic drugs. Informed written consent was obtained from all subjects participating in the study according to the Helsinki Declaration.

Blood samples were collected in the morning, after a 12 h overnight fast, and centrifuged at $3,000 \text{ rev min}^{-1}$ for 10 min. Fasting plasma glucose, haemoglobin A1c and the albumin excretion rate were immediately measured, whereas the remaining samples were divided into aliquots of 1.5 mL and stored at $-20 \text{ }^{\circ}\text{C}$ until analysis for determination of total cholesterol, triglycerides, azotemia, creatinine and Se-proteins content. Albumin-to-creatinine ratio was also assessed in the spot urine collection in the morning.

VIII.3.2. Determination of clinical parameters

The clinical parameters reported below were determined by the research group of Prof. Lapolla at the Diabetes Clinic of the Department of Medical and Surgical Sciences at the University of Padova.

Fasting plasma glucose (FPG) was determined by a glucose-oxidase method [11]. A mixed enzyme-acceptor reagent containing glucose oxidase, peroxidase, 1 % o-dianisidine (in 95 % ethanol), NaH_2PO_4 and heparin was added to the whole blood diluted 100:1. After 60 minutes incubation in thermostatically controlled bath at $35\text{-}37^{\circ}\text{C}$, the sample was analyzed spectrophotometrically.

Haemoglobin A1c (HbA1c) was measured by a liquid chromatography method (Bio-Rad Laboratories, Milan, Italy) [12]. The procedure included an erythrocyte lysis reagent which eliminates the labile aldimine component (pre-A1c) and a two-stage ion-exchange chromatographic step which separates HbA1a+b from HbA1c. This procedure also includes calibrant materials which aid in correcting for temperature fluctuations during the analysis.

Total cholesterol, low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol were measured by a colorimetric enzyme method (CHOD-PAP method, Roche, Milan, Italy) [13]. Cholesterol esters were hydrolyzed to free cholesterol by cholesterol ester hydrolase. The free cholesterol was then oxidized by cholesterol oxidase to cholest-4-en-3-one with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500 nm.

Plasma triglycerides were measured by GPO-PAP colorimetric enzyme test (Roche Diagnostic System) [14]. Serum triglycerides were hydrolyzed by lipase, and

the released glycerol was assayed in a reaction catalyzed by glycerol kinase and L- α -glycerol-phosphate oxidase in a system that generates hydrogen peroxide. The hydrogen peroxide was monitored in presence of horseradish peroxidase with 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone as the chromogenic system, with maximum absorbance at 510 nm.

Urinary albumin and creatinine [15] were determined on morning spot urine samples by a DCA 2000 plus system (Bayer, Milan, Italy), in which albumin is detected immunoturbidimetrically [16]. Creatinine was measured colorimetrically by the Benedict-Behre reaction turbidimetric immunoassay [16]. The urinary albumin ($\mu\text{g mL}^{-1}$)-to-creatinine (mg mL^{-1}) ratio (ACR) was calculated, and microalbuminuria was defined as ACR between 30 and 300 $\mu\text{g mg}^{-1}$.

VIII.3.3. Determination of plasma Se-proteins

Determination of GPx3, SelP and SeAlb in human plasma was achieved simultaneously by HPLC coupled on-line with ICP-MS. The method details including the standards/reagents and the optimum parameters were described in Jitaru *et al.* [17]. In this version of the method, the interferences Cl^- and Br^- were removed from the sample prior to the analysis by AE-SPE. Se-proteins separation was then achieved by normalbore 2AF-HPLC, whereas for detection a coupled ICP-QMS was used.

VIII.3.4. Complications

Patients with diabetes are often affected by complications, that means additional diseases for which diabetes increases the onset. In this study some complications were also taken into account when investigating the association between plasma Se-proteins and diabetes. They included: coronary artery disease (CAD), hypertension, retinopathy, proteinuria, coronary heart disease (CHD), vascular perfusion disease (PVD) cerebral perfusion disease (CVD), neuropathy and carotid arteriopathy (CA). Therapy was also considered as potential associated factor.

VIII.4. Results

The patients' clinical characteristics, Se-proteins concentration and percentage are reported in Table VIII-1. Patients affected by diabetes showed a poor metabolic

control, as evidenced by the mean values of FPG and HbA1c. All patients had normal renal function as shown by the mean values of creatinine, azotemia and ACR. The corresponding clinical parameters, Se-proteins concentration and percentage for the healthy control individuals are reported Table VIII-2.

Table VIII-1 Clinical parameters, plasma Se-proteins concentration and percentage in patients with type II diabetes mellitus (n = 40).

gender							
female		16					
male		24					
		Mean	SD	Min	Max	Skewness	Kurtosis
age [years]		69	4	24	89	-1.27	3.49
BMI [kg m ⁻²]		29	5.3	17	44	0.44	0.46
FPG [mg dL ⁻¹]		179	59.2	108	351	1.66	2.69
HbA1c [%]		8.0	1.7	5.5	13.5	0.78	0.84
triglycerides [mg dL ⁻¹]		130	70	54	415	2.28	6.70
total cholesterol [mg dL ⁻¹]		191	38	102	273	0.23	0.25
HDL [mg dL ⁻¹]		56	14	38	102	1.54	3.26
azotemia [mmol L ⁻¹]		71	35	5	102	-0.34	-1.68
creatinine [mg dL ⁻¹]		0.95	0.23	0.51	1.71	0.92	1.94
ACR [mg g ⁻¹]		62.9	123.5	1.2	607.6	3.40	12.4
mean disease duration [years]		10	7	1	27	1.08	0.71
GPx3 [ng mL ⁻¹]		9	2	5	12	-0.04	0.11
SeIP [ng mL ⁻¹]		58	9	42	75	0.12	-0.94
SeAlb [ng mL ⁻¹]		11	2	6	16	-0.03	0.17
Total Se [ng mL ⁻¹]		78	12	54	99	0.03	-0.82
GPx3 [%]		11	1	8	14	-0.08	-0.84
SeIP [%]		74	2	69	79	-0.22	-0.15
SeAlb [%]		14	2	11	19	-0.01	-0.93

Table VIII-2 Clinical parameters, plasma Se-proteins concentration and percentage in healthy control subjects (n = 15).

gender							
female		12					
male		3					
		Mean	SD	Min	Max	Skewness	Kurtosis
age [years]		46	3	41	52	0.39	-0.37
BMI [kg m ⁻²]		26	2	22	29	-0.54	-0.28
FPG [mg dl ⁻¹]		81	6	70	90	-0.10	-1.37
HbA1c [%]		5.4	0.3	4.9	5.9	-0.04	-0.38
triglycerides [mg dL ⁻¹]		96	48	45	196	0.79	-0.41
total cholesterol [mg dL ⁻¹]		189	16	147	200	-1.52	1.87
HDL [mg dL ⁻¹]		58	11	42	76	0.07	-1.31
azotemia [mmol L ⁻¹]		0.74	0.14	0.53	0.94	0.09	-1.43
creatinine [mg dL ⁻¹]		5.4	2.0	2.1	8.9	-0.13	-0.22
ACR [mg g ⁻¹]		26	2.0	22	29	-0.54	-0.28
GPx3 [ng mL ⁻¹]		11	3	7	15	0.06	-0.82
SelP [ng mL ⁻¹]		56	8	47	74	0.81	0.00
SeAlb [ng mL ⁻¹]		18	8	8	38	1.21	2.03
Total Se [ng mL ⁻¹]		85	16	62	125	0.87	1.35
GPx3 [%]		13	2	10	17	0.51	-1.05
SelP [%]		67	5	59	77	0.16	-0.85
SeAlb [%]		21	5	12	30	0.44	-0.78

VIII.4.1. Comparison between patients with type II diabetes and healthy subjects based on their Se-proteins status

Se-proteins level and percentage. The S-W test was applied to evaluate the normality of both Se-proteins concentration and percentage distribution in diabetes and control group. As reported in Table VIII-3, all variables were normally distributed, therefore the *t*-test was applied for groups comparison. Lower level and percentage of GPx3 and SeAlb ($p < 0.01$) were revealed in patients with diabetes, whereas in the same patients the SelP percentage was significantly higher ($p < 0.01$, see Table VIII-3). These differences can be also graphically confirmed by observing the box-plots represented in Figure VIII-1.

Table VIII-3 Results of the S-W test for normality and *t*-test for paired group comparison concerning the plasma Se-proteins level in patients with type II diabetes (*n* = 40) and healthy control subjects (*n* = 15). The values marked in gray are significant.

	S-W test				<i>t</i> -test	
	diabetes		healthy		<i>p</i>	age-corrected <i>p</i>
	W	<i>p</i>	W	<i>p</i>		
concentration						
GPx3	0.99	0.97	0.94	0.40	< 0.01	0.01
SeIP	0.96	0.20	0.96	0.63	0.65	0.86
SeAlb	0.99	0.91	0.89	0.06	< 0.01	< 0.01
Total Se	0.97	0.47	0.82	0.01	0.06	0.06
percentage						
GPx3	0.97	0.36	0.94	0.31	0.04	0.19
SeIP	0.98	0.85	0.94	0.34	< 0.01	< 0.01
SeAlb	0.97	0.26	0.97	0.76	< 0.01	< 0.01

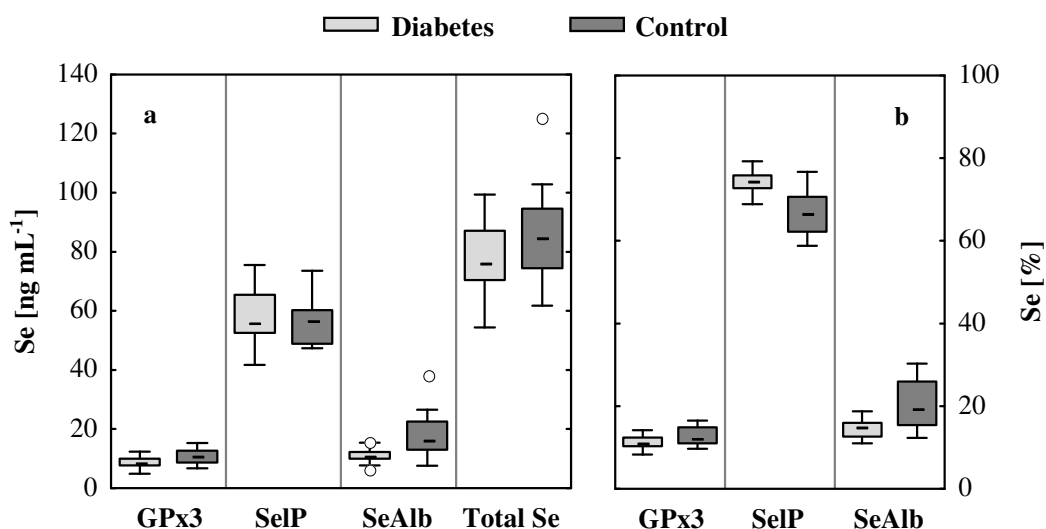


Figure VIII-1 Box plots made by percentiles for Se-proteins concentration (a) and percentage (with respect to their sum) (b) in healthy subjects and patients with type II diabetes.

As reported in Tables IX-1,2 the healthy control subjects presented a significantly ($p < 0.01$) lower mean age (46 ± 3 years) in comparison with the group with diabetes (69 ± 12 years). In order to test the possible confounding effect of that factor on the Se-proteins level and percentage distributions, the ANCOVA method was adopted to express the age as function of the observed variables. The coefficients of GPx3 ($\beta = -0.04 \pm 0.02$, $p = 0.02$) and SeAlb ($\beta = -0.13 \pm 0.05$, $p < 0.01$) concentration were

significant. The p adjusted for age (see Table VIII-3) were generally higher with respect to the uncorrected values, but the observed difference between GPx3 and SeAlb level remained significant. All Se-proteins percentage was significantly correlated with age (GPx3 %: $\beta = -0.04 \pm 0.02$, $p = 0.02$; SeLP %: $\beta = 0.16 \pm 0.04$, $p < 0.01$; SeAlb %: $\beta = -0.12 \pm 0.04$, $p < 0.01$). The age-adjusted p are also reported in Table VIII-3. While SeLP and SeAlb percentages were still significantly different between patients with diabetes and healthy group, GPx3 was no longer statistically different ($p = 0.19$). The age-adjusted data have been adopted for all the statistical elaborations subsequently applied in order to increase their validity.

The healthy subjects group had a relevant asymmetry in distribution between genders (12 females and 3 males). Therefore, gender was also tested as a confounding factor by ANCOVA for both Se-proteins concentration and percentage. Non-significant difference for the β coefficients was observed in the data between females and males, signifying that adjustments for gender are unnecessary.

In general, percentages can be a useful tool for raw data interpretation, but are calculated by introducing an artificial correlation between variables. For this reason they do not satisfy the fundamental assumptions required by the more advanced statistical methods. Therefore, all the subsequent data analyses were only applied to the concentrations.

Logistic regression analysis. Logistic regression models based on Se-proteins concentration were developed in order to deepen the comparison between diabetes and healthy groups. Different input sets were tested by combining GPx3, SeLP, SeAlb concentration and their products in order to capture possible further effects. Models structure and performances are summarized in Table VIII-4.

In the comparison of patients with diabetes and healthy group, GPx3, SeLP and SeAlb levels were initially individually tested, and then combined. Only GPx3 (OR 3.61; CL 1.50-8.71) and SeAlb (OR 14.33; CL 2.78-73.92) were significant (t -test, $p < 0.01$). The model based only on SeAlb level gave a sensitivity of 100 % and a specificity of 60 %, whereas for the GPx3 model the maximum specificity reached 23 %. When combined, the three variables allowed a sensitivity of 100% and specificity increased to 67 % or 73 % (see Table VIII-4). The introduction of the products GPx3·SeAlb and SeLP·SeAlb generated a slight inhibition effect on model performances. Such an effect on the probability of a subject classification in one of

the patients with diabetes or healthy groups, suggested that the SeAlb level acts in a distinct way on the level of the other Se-proteins.

Table VIII-4 Classification performances of the logistic regression models.

model	variable	estimate	Wald test	OR (CL 95%)	sensitivity [%]	specificity [%]
A	GPx3	1.28	8.18	3.61 (1.50-8.71)	100	23
B	SeIP	-0.14	0.21	0.87 (0.48-1.57)		
C	SeAlb	2.66	10.11	14.83 (2.78-73.62)	100	60
D	GPx3	-0.96	2.21	0.38 (0.11-1.36)	100	67
	SeIP	1.66	5.63	5.25 (1.36-20.66)		
	SeAlb	-3.22	6.60	0.04 (0.01-0.47)		
E	SeIP	1.68	5.80	5.34 (1.37-20.90)	100	73
	GPx3+SeAlb	-3.56	11.33	0.03 (0.01-0.23)		
F	GPx3	2.88	0.76	17.84	100	67
	SeIP	3.49	4.26	32.68		
	SeAlb	-2.92	0.16	0.05		
	SeIP·SeAlb	-9.28	1.44	0.00		
	GPx3·SeAlb	-2.84	1.38	0.06		

VIII.4.2. Association between level of plasma Se-proteins and clinical parameters

Clinical parameters (see Tables IX-1,2) in the diabetes and healthy groups were analysed by S-W test in order to appropriately apply the *t*- or K-S tests. The results are reported in Table VIII-5. Body mass index and creatinine were normally distributed in both groups and consequently the *t*-test was applied. Body mass index was not statistically different, whereas creatinine was significantly higher in patients with diabetes (0.95 ± 0.23 vs. 0.74 ± 0.14 mg dL⁻¹ in healthy subjects). The K-S test, applied to the other non-normal parameters, revealed significantly higher values in patients with diabetes for FPG (179 ± 59 vs. 81 ± 6 mg dL⁻¹), HbA1c (8.0 ± 1.7 vs. 5.4 ± 0.3 %) and ACR (46.9 ± 80.4 vs. 5.5 ± 1.9 mg g⁻¹).

The possible association between plasma Se-proteins level and clinical parameters was investigated by the calculation of correlation coefficients within all subjects. The S-W test showed that the parameters were not normally distributed with the exception of total cholesterol. The linear correlation coefficient was calculated for this

parameter, whereas Spearman rank order correlation was calculated for the others. The coefficients are reported in Table VIII-6.

Table VIII-5 Results of the S-W test for normality, *t*-test and K-S test for paired group comparison concerning the clinical parameters in patients with type II diabetes (n = 40) and healthy control subjects (n = 15). The values marked in gray are significant.

	S-W test				t-test		K-S test
	diabetes		healthy		t	p	p
	W	p	W	p			
BMI	0.98	0.69	0.94	0.39	1.55	0.13	
FPG	0.82	< 0.01	0.94	0.34			< 0.01
HbA1c	0.88	< 0.01	0.96	0.63			< 0.01
triglycerides	0.78	< 0.01	0.89	0.07			> 0.10
total cholesterol	0.98	0.69	0.75	< 0.01			< 0.10
HDL	0.87	< 0.01	0.93	0.27			> 0.10
azotemia	0.95	0.08	0.93	0.25	3.25	< 0.01	
creatinine	0.51	< 0.01	0.96	0.78			< 0.01
ACR	0.98	0.69	0.94	0.39	1.55	0.13	

Table VIII-6 Correlation coefficients for plasma Se-proteins level and clinical parameters in the whole sample. The values marked in gray are significant.

	BMI	FPG	HbA1c	triglycerides	total cholesterol	HDL	creatinine	ACR
GPx3	-0.42	-0.34	-0.41	-0.11	0.14	0.14	-0.27	-0.50
SeIP	-0.06	0.02	-0.08	0.10	0.07	0.07	-0.08	-0.05
SeAlb	-0.23	-0.38	-0.45	-0.07	0.09	0.06	-0.30	-0.32
Total Se	-0.23	-0.18	-0.28	0.01	0.09	0.09	-0.24	-0.23
BMI		0.17	0.28	0.34	-0.14	-0.34	0.02	0.23
FPG			0.77	0.18	0.00	-0.09	0.27	0.58
HbA1c				0.32	0.02	-0.12	0.29	0.49
triglycerides					0.23	-0.35	0.21	0.14
total cholesterol						0.09	-0.07	-0.05
HDL							-0.14	-0.17
creatinine								0.30

GPx3 was negatively correlated with BMI, FPG, HbA1c and ACR. A negative correlation was also observed between SeAlb and FPG, HbA1c, creatinine and ACR.

Total Se was correlated with HbA1c and ACR. The correlation coefficients were also independently calculated within the diabetes and healthy groups. The S-W test showed normal distributions of BMI and total cholesterol for patients with diabetes, while for healthy subjects all the parameters were normally distributed except the total cholesterol. GPx3 and ACR were significantly correlated for patients with diabetes ($r = -0.38$), while in healthy subjects GPx3 was positively correlated with HbA1c ($r = 0.54$), and SelP as well as the total Se were positively correlated with triglycerides ($r = 0.59$, and $r = 0.52$, respectively).

Further couples of groups were selected for each clinical parameter considering the subjects who presented values in the 1st and 4th quarters of its distribution in the whole sample. The S-W test was applied to the Se-proteins level for patients in each group, obtaining normal distributions in all cases. The *t*-test was then applied for the comparison of Se-proteins concentration between the two groups for each clinical parameter. The results, reported in Table VIII-7, confirmed all the previously observed correlations by a significant difference in Se-proteins level for subjects in the 1st and 4th quarter.

Table VIII-7 *p*-values of the *t*-test for plasma Se-proteins level between subjects in the 1st and 4th quarter of the group for each clinical parameter. The values marked in gray are significant.

	BMI	FPG	HbA1c	triglycerides	total cholesterol	HDL	creatinine	ACR
Whole sample								
GPx3	0.01	0.02	0.01	0.50	0.82	0.27	0.01	0.06
SelP	0.85	0.92	0.90	0.64	0.99	0.48	0.98	0.24
SeAlb	0.03	< 0.01	0.01	0.76	0.48	0.96	0.02	0.02
Total Se	0.18	0.13	0.08	0.96	0.89	0.54	0.16	0.04
Patients with type II diabetes								
GPx3	0.11	0.80	0.14	0.37	0.31	0.14	0.22	0.05
SelP	0.82	0.77	0.55	0.54	0.75	0.03	0.22	0.44
SeAlb	0.67	0.52	0.18	0.44	0.28	0.17	0.94	0.86
Total Se	0.65	0.93	0.36	0.46	0.56	0.03	0.27	0.39

The same analysis was then applied to only consider the patients with diabetes. In this case, significantly lower concentrations of SelP and total Se (53 ± 8 and 72 ± 12

ng mL⁻¹, respectively) were found in patients with HDL < 47 with respect to patients with HDL > 61 (62 ± 7 and 84 ± 8 ng mL⁻¹, respectively). Due to the low sample size, it was inappropriate to carry out the same comparison between quarters within the healthy subjects group. Notably, most of the significant correlations or differences revealed by considering the sample as a whole disappeared when healthy subjects and patients with diabetes were investigated separately. This effect supports the hypothesis that many of the observed correlations for the whole sample are mostly due to a bimodal distribution, rather than by a continuous relationship among variables

Multivariate analysis. FA was performed to check the possible qualitative classification of healthy and diabetic groups by means of Se-proteins level, coupled with their correlated clinical parameters. Two factors were selected on the basis of the cumulative explained variance (83 %). Fasting plasma glucose, HbA1c and ACR were the significant variables in the first factor (loading > 0.70), whereas creatinine, GPx3 and SeAlb were significant in the second factor. Figure VIII-2 shows the scores and loadings plots, where healthy subjects and patients with type II diabetes are separated in two distinct areas. This analysis provides only indicative results because

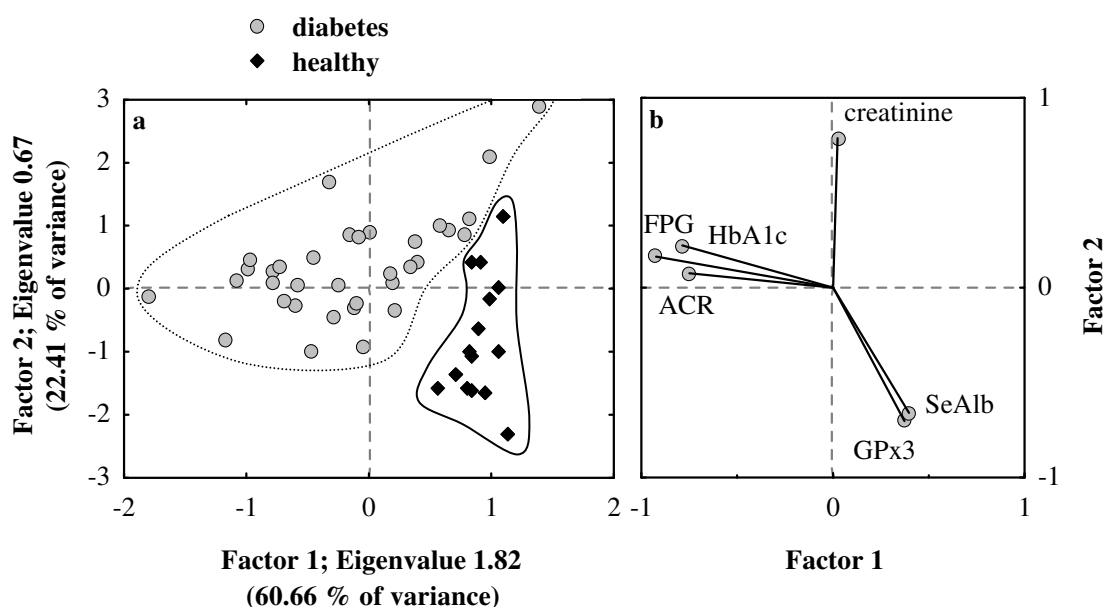


Figure VIII-2 Scores plot (a) and loadings plot (b) for the first two components of the FA obtained for patients with diabetes and healthy subjects. Ovals indicate the boundary of the areas where patients with type II diabetes (broken line) and healthy subjects (unbroken line) are distributed.

of the low number of subjects, particularly for the healthy group ($n = 15$). Nevertheless, it depicts the association among GPx3, SeAlb and some of the most important clinical parameters used in the diagnosis of type II diabetes.

VIII.4.3. Association between level of plasma Se-proteins and complications

The distribution of patients with diabetes into the different categories for each complication is reported in Table VIII-8. Even if patients are prevalently grouped in one of the 2/3 possible categories for most of the complications, an exploratory analysis was carried out in order to compare by *t*-test (following up the S-W test) both the clinical parameters and the Se-proteins level for subjects who presented or did not each complication.

Table VIII-8 Complications in patients with diabetes ($n = 40$).

CAD		proteinuria			CVD		
yes	no	yes	no	ua	TIA	no	ua
15	25	2	34	4	3	36	1
hypertension		CHD			neuropathy		
yes	no	yes	no		yes	no	
31	9	9	31		3	37	
retinopathy		PVD					
yes	no	BK	yes	no			
1	35	4	6	34			
CA							
yes	no	BS	patch				
9	25	3	3				
therapy							
OAD	OAD+insulin	insulin	diet	none			
22	5	5	5	3			

BK: background; ua: unavailable; TIA: transient ischemic attack; BS: bilateral stenosis; OAD: oral antidiabetics.

The *p*-values of the test, reported in Table VIII-9, showed that BMI was significantly higher in subjects with hypertension (30 ± 5 vs. 25 ± 5 kg m⁻²), while HDL and SeAlb were significantly lower in patients with neuropathy (42 ± 1 vs. 57 ± 14 mg dL⁻¹ and 11 ± 2 vs. 8 ± 2 ng g⁻¹, respectively). GPx3 and SelP, as well as total Se and HbA1c were also significantly different as function of the therapy. Both GPx3

and SeIP were higher (10 ± 1 and 68 ± 3 ng g⁻¹, respectively) in patients who were untreated, in respect to all the other therapies, particularly insulin (8 ± 1 and 48 ± 5 ng g⁻¹, respectively). HbA1c was higher in patients treated with OAD+insulin (9.4 ± 0.4 %) in respect with those treated with diet (6.3 ± 0.6 %) or without treatment (6.5 ± 0.4 %).

Table VIII-9 *p*-values for comparison of Se-proteins level and quantitative clinical parameters among the groups defined for each categorical clinical parameter; correlations among categorical clinical parameters themselves. The values marked in gray are significant.

	CAD	hypertension	retinopathy	CHD	PVD	CVD	neuropathy	therapy
GPx3	0.57	0.40	0.98	0.19	0.39	0.07	0.67	0.03
SeIP	0.82	0.81	0.81	0.57	0.44	0.15	0.29	0.03
SeAlb	0.53	0.33	0.92	0.65	0.25	0.89	< 0.01	0.12
Total Se	0.89	0.79	0.83	0.73	0.35	0.17	0.16	0.02
BMI	> 0.10	< 0.03	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	0.89
FPG	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	0.20
HbA1c	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	0.01
triglycerides	> 0.10	< 0.10	> 0.10	< 0.10	> 0.10	> 0.10	> 0.10	0.37
total cholesterol	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	0.12
HDL	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	< 0.03	0.38
azotemia	> 0.10	> 0.10	> 0.10	> 0.10	-	> 0.10	-	0.39
creatinine	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	0.17
MCR	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	< 0.10	0.23

Se-proteins and CAD, Hypertension, Retinopathy, CHD, PVD, CVD, Neuropathy: *t*-test. Se-proteins and Therapy: one-way ANOVA. Quantitative clinical parameters and CAD, Hypertension, Retinopathy, CHD, PVD, CVD, Neuropathy: K-S test. Quantitative clinical parameters and Therapy: Kruskal-Wallis ANOVA.

Multiple correspondence analysis. MCA was applied to investigate the possible underlying structure in the categorical data set of complications. Two dimensions were selected, corresponding to a cumulative inertia of 29 %. Figure VIII-3 shows the two dimensional scores plot. A dense group of variables, mainly corresponding to the absence of complications, tends to concentrate around the origin of the axes, indicating a strong association. Positive complications distribute around a wider area, with negative score in the first dimension. No specific association groups were

detectable in this case, a part of that constituted by presence of bilateral stenosis, proteinuria and neuropathy.

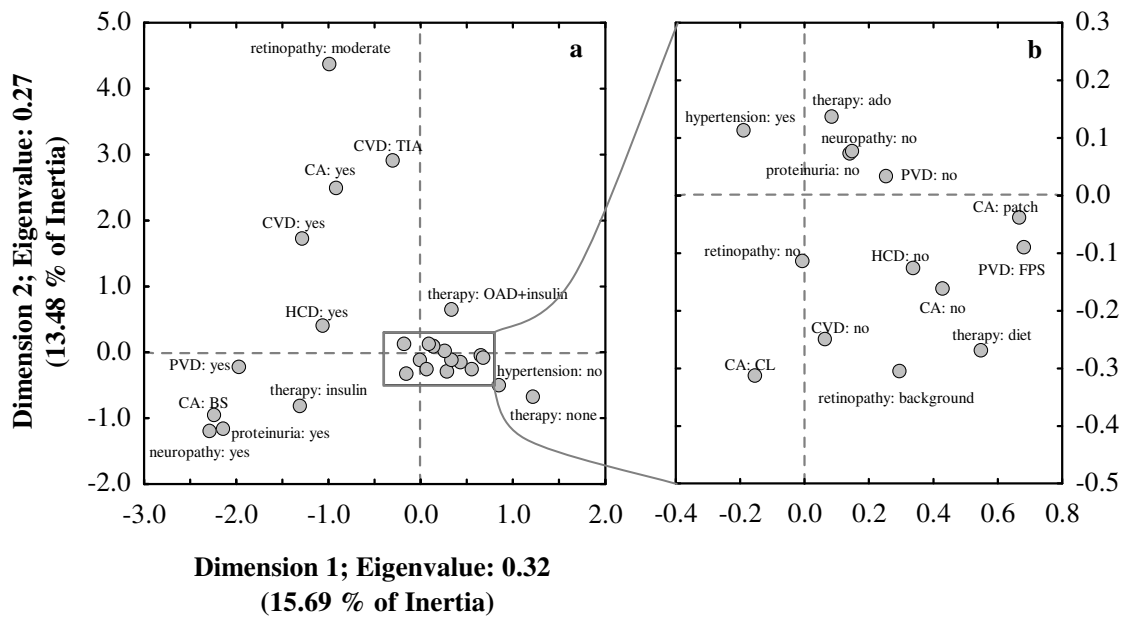


Figure VIII-3 Scores plot (a) and zoom (b) for the first two dimensions of the MCA obtained for complications in patients with diabetes.

VIII.5. Discussion

In this study, GPx3 and SeAlb levels were found to be significantly different ($p < 0.05$) in patients with type II diabetes compared to healthy subjects. Since SelP plays the fundamental role of a Se carrier through the body [18], whereas SeAlb is not an active protein, the total available Se from dietary intake should be preferentially incorporated into SelP, subsequently into GPx3 and only as a last option into SeAlb. As a consequence, under low Se intake or higher oxidative stress conditions, the three Se-proteins in theory are hierarchically ordered in Se incorporation. SeAlb and GPx3 should be the most sensitive proteins to decrease in such altered conditions. The observed significantly ($p < 0.05$) lower level and percentage of SeAlb and GPx3 in patients with type II diabetes, as well as the correlations between SeAlb and FPG and HbA1c, seem to indicate that altered plasma Se-proteins status could be a mirror of bad metabolic control. A negative correlation between the plasma contents of Se and HbA1c was also observed by Ruiz *et al.* [19]. Since plasma Se-proteins level could be a potential biomarker for oxidative stress, this hypothesis is in agreement with the association between oxidative stress and glycaemic control reported in some other

studies. Such a relationship is still controversial in the literature, probably due to the fact that not all the adopted oxidative stress biomarkers biosynthesises *via* pathways which are directly related to glycaemic control [20]. Many of our patients with type II diabetes are overweight or obese, and therefore may be affected by insulin resistance. Lower concentration of SeIP and total Se were found in patients with diabetes having HDL < 47 mg dL⁻¹ compared to patients with HDL > 61 mg dL⁻¹. Studies carried out on rats have shown a beneficial effect of Se supplementation on lipid abnormalities in plasma, aorta and adipose tissue suggesting that Se potentially can also reduce the risk of cardiovascular disease in patients with diabetes [21]. Natella *et al.* [22] have shown that Se supplementation can cause a reduction of atherogenic negative LDL in the postprandial phase in healthy subjects. The positive correlation found in this study between Se and HDL, a well known marker of high risk atherosclerosis development, supports the previous observations.

A recent study by Kornhauser *et al.* [3] revealed an inverse association between ACR and serum GPx3 as well as total Se level in patients with type II diabetes, suggesting that lower serum Se concentration in these patients may be implicated in diabetic nephropathy. Our study confirmed this negative relationship for GPx3 but not for total Se. However, the K-S test applied to compare the plasma GPx3 levels in patients with microalbuminuria (ACR > 30) showed no significant difference. Most likely, the possible biochemical relationship is relatively weak and it cannot be revealed by a *t*-test due to its low power, which is related to the relatively small number of cases we evaluated. In fact, for the *t*-test we obtained a *p* close to 0.05, even if higher (0.08), as well as Kornhauser *et al.* [3], considering 114 individuals, had a *p* close to 0.05 even if lower (0.04).

GPx and SeAlb levels were significantly correlated with the age of the subjects. Other studies observed the same association, which may be due to the less efficient absorption or increased elimination of Se in older persons [23]. However, in this study the differences in plasma Se-proteins concentration between patients with diabetes and healthy subjects were unaffected by the potential confounding effect of age and gender. It is important to state that a further potential confounding factor for the Se-proteins distribution, and particularly for SeAlb, is the Se dietary intake. Nevertheless, the relationship between individual plasma Se-proteins and the dietary intake is very difficult to assess and still controversial in the literature [24,25]. Even if in this study the dietary Se intake was not monitored, all the recruited subjects were treated with

the same controlled diet for at least 6 months prior to the analysis, and moreover were resident in the same (non deficient) Se region [26]. Therefore, we can reasonably assume that the significant observed differences are only due to the health of the subjects.

Few data have been available up to now on the effect of Se on insulin resistance in patients with type II diabetes. Experimental evidences from *in-vitro* and *in-vivo* studies in animals suggested that Se may mediate many insulin-like actions and thus enhance insulin sensitivity [27,28] (see paragraph II.3.11), but this is still unconfirmed for patients with type II diabetes. The biochemical mechanism through which Se carries out its action is still unknown. The logistic model performances in our study have shown the potential of plasma Se-proteins status as a combined biomarker for type II diabetes, in contrast with the misleading picture given by the total Se concentration. This study provides the first evidence for the possible role of Se in diabetes under unsupplemented conditions, which is a requisite that makes the results closer to the real behaviour of Se in diabetes biochemistry.

VIII.6. Conclusions

The results showed that patients with diabetes present significantly lower levels of GPx3 and SeAlb with respect to healthy subjects. Significant negative correlations were also revealed between GPx3 and SeAlb, and clinical parameters including FPG, HbA1c and ACR. Our findings suggested an association between individual Se-proteins concentration, presence of type II diabetes, and several clinical parameters. At this stage, it cannot currently be ascertained whether the altered Se-proteins status is a consequence or a causative factor for diabetes. However, this study demonstrated the potential applicability of a method, developed by our research group, for the individual plasma Se-proteins determination in the investigation of the biochemical relationship between Se and type II diabetes.

A journal article, in attachment, was published regarding the study presented in this chapter.

VIII.7. References

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IX. STUDY OF HUMAN SERUM SE-PROTEINS CONCENTRATION IN COLORECTAL CANCER³

IX.1. Introduction

As previously mentioned in the paragraph II.3.12, colorectal cancer (CRC) is one of the forms of tumour that showed to be associated with the total concentration of Se in plasma/serum or tissue and with expression and activity levels of several Se-enzymes [1]. This pathology is the third most commonly diagnosed form of cancer in women and fourth in men worldwide [2]. Similarly to type II diabetes (see Chapter VIII), this disease is an example of the potentially promising applications for HPLC-ICP-MS based methods, that however has still not been investigated.

Trials regarding the cancer preventive action of Se by supplementation revealed beneficial effects on the incidence of several forms of cancer, including CRC [3-5]. However, most of the results obtained so far seem to be inconsistent or pathology-specific [6]. Most of the studies involved in this topic investigated the level of total serum or plasma Se in patients with CRC. Some of them revealed a significantly lower Se level in CRC patients with respect to healthy subjects, or a significantly higher risk to develop CRC and lower cumulated cancer-related survival rates for subject with low serum Se level [1,7,8]. Similar results were obtained by investigating

³ This work is on writing for publication in: Roman et al. J Pharm Biomed Anal 2011.

the total serum Se level in patients affected by colorectal adenoma, the precursor lesion in most CRC cases [9-12]. Selenium levels have also been associated with the CRC progression (from adenomatous polyps to local and metastatic cancer, respectively)[13]. On the other hand, no such a relationship was found in other studies regarding colorectal adenoma [14-16]. The reasons for these discrepancies in the Se status related to CRC remain still unclear.

Few other investigations took into account the enzymatic activity, expression level or concentration of specific plasma/serum Se-proteins in CRC. Also in this case, in some studies an inverse correlation was revealed between CRC (and other types of cancer) and GPx3 or SelP [1,17,18], but other works did not reveal any association [15,19]. To our knowledge only a single study took into account the complete plasma Se-proteins status in relation with CRC. In this study by Early *et al.*[15], GPx3, SelP and total Se levels were determined in distinct steps recurring to analytical methods based on enzymatic reactions, radioimmunoassay and fluorimetry, respectively, while the SeAlb concentration was calculated by difference. Given the complex matrix of serum, the low levels of Se-proteins and also the sequential determination of various Se-species correlated with the use of laborious procedures, such study should be validated by using modern methodologies, which rely on fast and simultaneous determination of Se-proteins in a single analytical run.

IX.2. Goals of the study

The aim of this study was to apply a methodology for the quantification of GPx3, SelP and SeAlb in human serum [20] to the investigation of Se-proteins pattern in CRC patients. Samples from 42 patients affected by CRC and 20 healthy control subjects were analyzed in this preliminary work, in order to assess the possible association between Se-proteins status and the presence of cancer, progression stage, prognostic criteria and survival rate. A comprehensive statistical approach was addressed for the data analysis in order to extract the maximum information provided by the Se-proteins status to explain the development and progression of CRC.

IX.3. Patients and methods

IX.3.1. Patients and study protocol

The study group consisted of 42 patients affected by CRC. All patients underwent colonoscopy control for colic disorder and had a histologically proven colorectal adenocarcinoma. The diagnosis was performed at the University Hospital of Padova (Italy), and the surgery was carried out at Clinica Chirurgica II (in the same hospital). In order to avoid overlapping the pathologic stages, for this study we selected patients at stage I (infiltration of bowel wall without lymph nodes or distant metastases) and at stage IV/metastatic cancer (presence of distant metastases), according to the tumour node metastasis (TNM) classification of the American Joint Committee on Cancer (AJCC) [21]. The control group included 20 healthy subjects unrepresenting genetic syndrome, and who underwent colonoscopy control in the same hospital for colic disorder (haemorrhoids, diverticulosis and functional disorders), but resulted negative for CRC and inflammatory pathologies. Colorectal cancer patients and healthy subjects were residents in the region of Veneto (80 %) or adjacent areas (20 %), in Italy. The characteristics of the study groups are presented in Table IX-1. Informed consent was obtained and the study was performed in conformance with the Declaration of Helsinki ethical guidelines.

IX.3.2. Serum samples collection

Blood was collected just before surgery (CRC patients) or after the colonoscopy (healthy control subjects). The serum was obtained by centrifugation of the blood at 3,000 rev min⁻¹ for 10 min, divided into aliquots of 1.5 mL and stored at -20 °C until analysis. The human serum certified reference material BCR-637 was used throughout for the quality control of Se-proteins determination.

Table IX-1 Subjects, tumour and treatment characteristics of the study population.

Healthy control subjects (n = 20)			
age (mean \pm SD; range)	58 \pm 14; 31 to 78		
gender			
female	13		
male	7		
family history			
sporadic ^a	20		
CRC patients (n = 42)			
age (mean \pm SD; range)	63 \pm 12; 32 to 82	grading ^h	
gender		I	11
female	19	II	25
male	23	III	4
family history		ua	2
sporadic	31	TNM	
s-HNPCC ^b	4	I	22
FAP ^c	6	IV	20
ua ^d	1	MSI ⁱ	
preoperative treatment ^e		S	37
no	19	L	2
yes	9	healthy	1
ua	14	ua	2
tumour site		LOH ^j	
rectum	14	no	28
colon	28	yes	12
histotype ^f		ua	2
mucinous	4	status	
NOS	38	dead	22
infiltrated ^g		alive	16
no	14	ua	4
yes	27	recurrent ^k	
ua	1	no	33
vascular invasion		yes	5
no	26	ua	4
yes	15		
ua	1		
max. diameter (mean \pm SD)	3.82 \pm 2.08 cm		

^ano family members of first or second degree affected by colorectal cancer; ^bsuspected hereditary non-polyposis colorectal cancer; ^cfamilial adenomatous polyposis; ^dunavailable; ^epatients who underwent chemoradiotherapy (RCHT) before the surgery; ^fhistotype NOS (not otherwise specified); ^gperitumoural lymphocytic infiltration; ^hdegree of tumour differentiation; ⁱmicrosatellite instability test; ^jloss of heterozygosity: allelic deletions of the chromosomes 17 and 18; ^kcancer recurrence after radical surgery.

IX.3.3. Determination of serum Se-proteins and quality control

Determination of GPx3, SelP and SeAlb in human serum was achieved by using tandem AE-2AF-HPLC separation coupled on-line with ICP-QMS. Details regarding this method, the standards/reagents and the optimum parameters were described in a previous study [20].

The quality control for Se-proteins quantification was ensured by the analysis of the serum BCR-637 (certified total Se level: $81 \pm 7 \text{ ng mL}^{-1}$). The concentration of GPx3, SelP and SeAlb in BCR-637 was determined in a previous work [20] by using the same method as employed in this study and hence its use for quality control can be considered satisfactory. The Se levels associated with the Se-proteins in BCR-637 obtained as described above were: $13 \pm 1 \text{ ng mL}^{-1}$ for GPx3, $59 \pm 3 \text{ ng mL}^{-1}$ for SelP and $16 \pm 2 \text{ ng mL}^{-1}$ for SeAlb. The BCR-637 serum was analyzed every 10 samples and the average value ($n = 5$) obtained for Se-proteins was $12 \pm 3 \text{ ng mL}^{-1}$ for GPx3, $55 \pm 4 \text{ ng mL}^{-1}$ for SelP and $16 \pm 2 \text{ ng mL}^{-1}$ for SeAlb. These values and the reference levels were compared by *t*-test and by calculation of difference between averages in percentage, confirming the accuracy of the analysis in this study (p for *t*-test < 0.05 , difference between averages $< 10 \%$). The total Se in BCR-637 ($83 \pm 2 \text{ ng mL}^{-1}$) was also in good agreement with the certified level Se ($81 \pm 7 \text{ ng mL}^{-1}$).

IX.4. Results

IX.4.1. Comparison between CRC patients and healthy subjects based on their Se-proteins status

Se-proteins level and percentage. GPx3, SelP, SeAlb concentration and their sum (considered a reliable estimate of total Se) in the study group are reported in Table IX-2. Figure IX-1 shows the box-plots made by the percentiles of Se-proteins level and percentage. The concentrations were compatible with other results in the literature [15,22]. The CRC samples were also divided into two categories based on disease progression stage (TNM I and IV).

Table IX-2 Mean concentration \pm SD of GPx3, SelP, SeAlb and total Se [ng mL^{-1}] in serum from healthy subjects and CRC patients.

Protein	healthy (n = 20)	CRC patients		
		All (n = 42)	TNM I (n = 22)	TNM IV (n = 20)
GPx3	18 \pm 4	17 \pm 3	17 \pm 3	17 \pm 4
SelP	59 \pm 10	62 \pm 13	65 \pm 13	59 \pm 12
SeAlb	16 \pm 3	18 \pm 4	19 \pm 3	16 \pm 4
Total Se	93 \pm 16	97 \pm 16	101 \pm 17	92 \pm 15

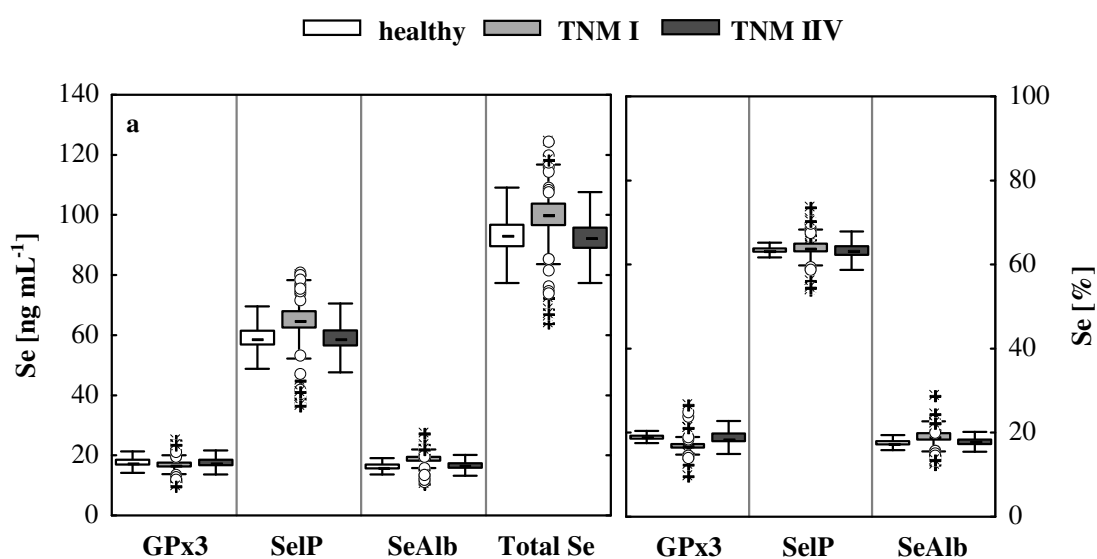


Figure IX-1 Box plots made by percentiles for Se-proteins concentration (a) and percentage (with respect to their sum) (b) in healthy subjects and CRC patients divided into TNM I and IV stages.

All the variables were tested for normality in each group by S-W test, with positive result ($p > 0.05$). The t -test was then applied for groups comparison on the bases of Se-proteins concentration. The results of the test are reported in Table IX-3. No significant differences were revealed in the level of GPx3, SelP, SeAlb, nor in total Se, between CRC and healthy groups. We noticed just a slightly lower GPx3 mean level and higher SelP, SeAlb and total Se mean level in CRC samples, although these differences did not reach the statistical significance. Rather, significantly higher SeAlb concentration was observed for TNM stage I patients in comparison with healthy ($p < 0.01$) and TNM IV ($p < 0.05$) subjects.

Table IX-3 p -values for the t -test between groups according to the Se-proteins concentration, in brackets the results for the test carried out on levels adjusted for RCHT. The values in gray are significant. The values marked in gray are significant.

Protein	healthy	healthy	healthy	TNM I
	vs. CRC	vs. TNM I	vs. TNM IV	vs. TNM I
GPx3	0.46	0.40 (0.66)	0.67 (0.62)	0.72 (0.96)
SeIP	0.42	0.15 (0.09)	0.91 (0.12)	0.14 (< 0.01)
SeAlb	0.13	< 0.01 (0.02)	0.90 (0.42)	0.03 (< 0.01)
Total Se	0.46	0.17 (0.23)	0.87 (0.27)	0.12 (0.02)

As reported by Chan *et al.* [23], the long-course preoperative treatment by radiochemotherapy (RCHT) may alter the TNM classification by downstaging of T and N parameters. Nine patients in our study undergone preoperative treatment, therefore the possible confounding effect of this prognostic criterion was also evaluated by calculating the adjusted p , which are reported in brackets in Table IX-3. When adjusted for RCHT, TNM I and IV stages were significantly different not only in terms of SeAlb level ($p < 0.01$) but also for SeIP ($p < 0.05$) and total Se ($p < 0.05$), while GPx3 remained not significant ($p > 0.05$). Thus, individual Se-proteins level and total Se were not significantly different between RCHT treated and untreated patients ($p > 0.05$ in all cases), and RCHT treated patients were not significantly concentrated in one of the two TNM groups (4 RCHT treated patients were in TNM I stage and 5 in TNM IV stage), RCHT interacts with the TNM stage enough to increase the SeIP, SeAlb and total Se level significance by TNM stage. In order to correct the comparison between healthy subjects and TNM I/IV groups, patients with no information regarding their RCHT treatment (10 from TNM I group and 4 from TNM IV group) were removed from the data set. For the remaining patients, GPx3, SeIP and SeAlb levels were adjusted for RCHT before the application of the t -test. The adjusted p (see Table IX-3) were in some cases highly different from the unadjusted data set, but again only SeAlb remained significantly different between healthy subjects and TNM I patients. Of course, the further reduction of sample size may affect this results.

Age (classified in thirds) and gender were also tested as confounding factors by ANCOVA, but no significant effects were observed. However, SeAlb slightly decreased with the age in healthy subjects, but did not in CRC patients. The same

behaviour can be seen considering the skewness of the ratio Age/SeAlb in different groups: 0.57 for CRC, 0.23 for TNM I, 0.66 for TNM IV and 0.86 for healthy subjects. All groups were right-skewed (skewness > 0), but the healthy subjects had higher skewness respect to CRC patients, in particular the TNM I group, therefore they tended to be less dispersed in higher Age/SeAlb ratios. The kurtosis of the ratio Age/SeAlb was 0.70 for CRC, 0.61 for TNM I, 0.21 for TNM IV and 1.82 for H. The higher value of kurtosis in healthy subjects confirms the tendency of the ratio to be more concentrated around the median.

Logistic regression analysis. Logistic regression models based on RCHT adjusted Se-proteins concentration were developed in order to extend the comparison between CRC, healthy and TNM I/IV groups. Different input sets were tested by combining GPx3, SelP, SeAlb concentration, the ratio of GPx3/SeAlb and the product SelP·SeAlb to assess their possible confounding and interacting effects. The results are summarized in Table IX-4.

Table IX-4 Classification performances of logistic regression models.

groups	input variables	sensitivity	specificity
		[%]	[%]
healthy vs. CRC	GPx3, SeAlb, GPx3/SeAlb	93	50
	GPx3, SelP, SeAlb, GPx3/SeAlb, SelP·SeAlb	100	50
healthy vs. TNM I	GPx3, SelP, SeAlb, GPx3/SeAlb, Age	92	90
healthy vs. TNM IV	GPx3, SeAlb, GPx3/SeAlb	88	50
	GPx3, SelP, SeAlb, GPx3/SeAlb, SelP·SeAlb	88	50

In the models construction to compare CRC patients and healthy subjects, GPx3, SelP and SeAlb levels were tested individually and subsequently combined. SelP, as individual input variable and also combined with the other proteins, was not significant ($p > 0.05$). This implies that it could be uncorrelated with the presence of cancer, and does not capture the effect of the other variables. Therefore, the model was built starting from GPx3 and SeAlb level. Both of them were confounding (if one was missing, the other was not significant) as well as interacting (the introduction of

their product entailed a synergic effect, in this case dominant with respect to the confounding effect). The introduction of SelP and the product SelP·SeAlb, even if they were not significant, entailed a synergic effect on the other variables, and allowed a rise in sensitivity up to 100 %. Still, the introduction of age produced only a slight inhibitory effect. A logistic regression model was also investigated as screening tool to discriminate the TNM I, TNM IV and healthy groups. In this case, SelP level was significant ($p < 0.05$), but the introduction of the product SelP·SeAlb showed an inhibitory effect. Age became also significant ($p < 0.05$), but without interacting effects with SeAlb. In terms of models performances, CRC and TNM I groups were identical, but the model made to discriminate TNM I patients and healthy persons reached notably higher specificity (90 %), even if not optimal sensitivity (92 % only).

IX.4.2. Association between level of serum Se-proteins and prognostic criteria in CRC patients

Correlations. GPx3 was not significantly correlated with any of the criteria considered in this study. SelP showed a significant positive correlation with infiltration ($\rho = 0.57$, $p < 0.01$), considered a positive prognostic factor, and interestingly, although not statistically significant, it was correlated with LOH ($\rho = 0.33$, $p = 0.05$). The presence of LOH is associated with a higher recurrence probability and a lower survival rate. SeAlb was significantly correlated with infiltration ($\rho = 0.37$, $p < 0.05$) and degree of cellular differentiation (grading: $\rho = -0.37$, $p < 0.05$).

Groups comparison. On the basis of correlations, tumour infiltration, tumour grading and LOH were selected as grouping variables for comparison of Se-proteins by S-W and subsequent *t*-test. All data were normally distributed (S-W test: $p > 0.05$). Significantly ($p < 0.05$) higher level of SelP was found in patients without infiltration ($69 \pm 9 \text{ ng mL}^{-1}$) in comparison with those with infiltration ($57 \pm 12 \text{ ng mL}^{-1}$). The same result was obtained for patients without LOH ($64 \pm 12 \text{ ng mL}^{-1}$) in comparison with patients who presented it ($55 \pm 12 \text{ ng mL}^{-1}$). SeAlb had significantly ($p < 0.05$) higher concentration in patients without infiltration ($19 \pm 3 \text{ ng mL}^{-1}$) in comparison with patients who presented infiltration ($17 \pm 3 \text{ ng mL}^{-1}$), and in patients with grading I ($19 \pm 2 \text{ ng mL}^{-1}$) compared with patients presenting grading $> \text{I}$ ($17 \pm 3 \text{ ng mL}^{-1}$).

IX.4.3. Survival analysis

Survival probability curves, represented in Figure IX-2, were estimated for CRC patients in the 1st and in the 4th quarters of the each Se-protein distribution, respectively 15 ng mL⁻¹ and 20 ng mL⁻¹ for GPx3, 54 ng mL⁻¹ and 72 ng mL⁻¹ for SeIP, 15 ng mL⁻¹ and 20 ng mL⁻¹ for SeAlb, 86 ng mL⁻¹ and 109 ng mL⁻¹ for total Se.

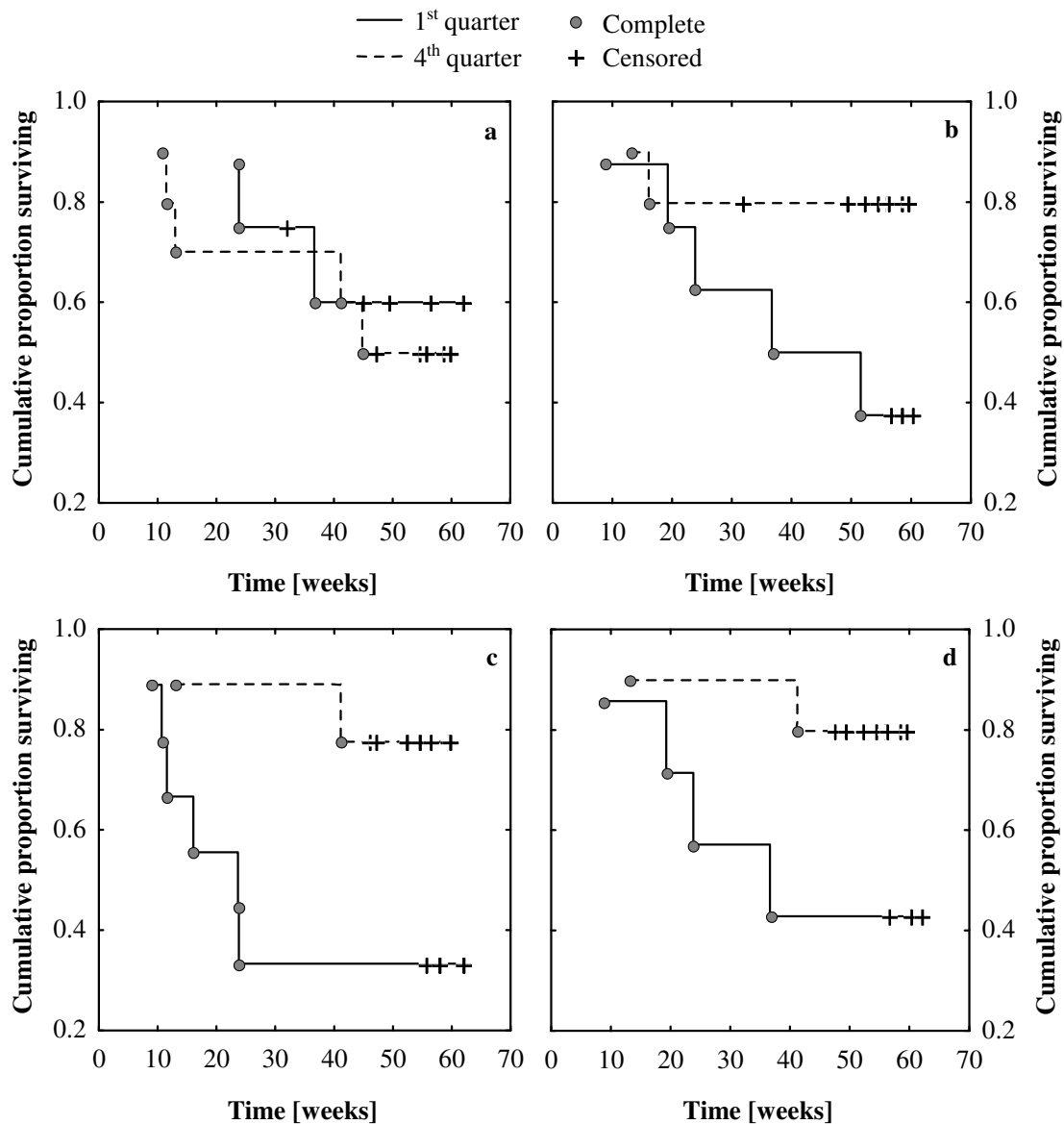


Figure IX-2 Cancer-related survival curves for CRC patients with GPx3 (a), SeIP (b), SeAlb (c) and total Se (d) level in the 1st quarter (unbroken line) and in the 4th quarter (broken line) of the distribution. The follow-up time was 62 weeks. Censored data correspond to the (n = 20) CRC patients who were still alive at the end of the study (since there are no information about the subsequent mortality, the value of the observation is only partially known), while the complete data correspond to the 22 patients died during the time of the study.

For SeAlb, the curves were different by means of log-rank statistic ($Z = -2.02$, $p < 0.05$), confirming the association conjectured from the correlation analysis. No significant nor indicative differences were found among survival curves for GPx3 ($Z = 0.45$, $p = 0.65$), SelP ($Z = -1.53$, $p = 0.13$) and total Se ($Z = -1.64$, $p = 0.10$), although also for SelP and total Se a higher concentration was qualitatively associated with higher survival cumulative probability. Survival analysis was also performed to compare TNM I and TNM IV patients (not shown). As expected, the cumulative survival proportion was significantly higher for patients in stage I than for patients in stage IV ($Z = 3.74$, $p < 0.01$).

IX.5. Discussion

A wide range of screening tests is currently available for CRC diagnosis, but there are still doubts regarding their confidence, large-scale availability, cost-effectiveness balance and patients compliance. In order to overcome these drawbacks, less invasive screening tests for the early detection of CRC have been studied on the bases of identification and validation of new tumour markers [24-26]. Amongst these markers, individual Se species could potentially reflect the complex relationship between Se status and functional alterations occurring in cancer cells.

No significant differences between Se-proteins level in CRC patients and the control group were found in our study. It is worth noting that the CRC patients constitute a heterogenic category, characterized by a high variability of Se status during the disease progression. When CRC patients were divided in two groups, according the disease progression stage, some interesting differences were revealed. The SeAlb level was significantly higher in TNM stage I patients than in the TNM stage IV or healthy subjects groups, and SelP showed a tendency to increase in the same way. On the other hand, the increase in SeAlb level is compensated by a decrease of GPx3 level. This behaviour could be the consequence of a shift of Se-proteins status balance caused by the increase of oxidative stress correlated with the illness stage. As already observed for Se-proteins status in type II diabetic patients (see paragraph VIII.5), the response of the Se system under stress conditions is protein-specific. Theoretically a decrease of the SelP residence time in serum should occur. However, since SelP plays also the role of a Se carrier through the whole body [27], this effect might produce both an increase or a decrease of the protein level

in serum as a function of the specific balance between depletion rate, production rate and the distribution velocity. Therefore, SeLP level solely is not significantly correlated with the general presence of cancer. As an effect of oxidative stress, SeAlb and consequentially GPx3 levels were expected to decrease. The relatively higher concentration of SeLP and lower concentration of GPx3 in TNM stage I patients confirmed the hierarchy between these two proteins, but, still, it remains difficult to explain the enhanced SeAlb level in serum of TNM stage I patients. It is not clear yet if this behaviour is a cause or a consequence of CRC, nor what are its biochemical bases, but it might be interpreted as an anomalous Se-proteins system regulation. In this context, it is possible to explain the subsequent re-normalization of all Se-proteins level in the more advanced cancer stage (TNM IV) as the effect of a further increase of stress toward the system, which produces the annulment of the response to cancer. Such explanation is compatible with the general model of the anticarcinogenic action of Se proposed by Combs *et al.* [28].

Also in this study the potential confounding role of Se dietary intake was not assessed. However, similarly to the study regarding type II diabetes (see paragraph VIII.3), all the recruited subjects were residents in the same (non deficient) Se region [29], and in this case underwent the same intestinal preparation during 2-3 days before blood collection. Again, we assumed that diet confounding effect is negligible with respect to the observed correlations.

SeLP and SeAlb showed interesting associations with prognostic criteria, which were investigated here for the first time. Peritumoural lymphocytic infiltration is considered a significant positive factor in prognosis effectiveness and survival of CRC patients, due to its important role in the immune response to cancer cells [30]. The decrease of SeLP and SeAlb level in patients with infiltration is compatible with the effect of Se in improving the activation and proliferation of B-lymphocytes and enhancing the T-cells function [31]. On the other hand, high tumour grading is an index of cancerous cells aggressiveness, thus is negatively correlated with good prognosis [32]. This study can be improved by taking into account the specific connection between serum Se-proteins level and the immune response mechanisms to colorectal cancer.

Accordingly with the study of Se-proteins pattern in type II diabetic patients (see paragraph VIII.5), SeAlb level showed a tendency to reduce with the age in healthy persons. This effect was not observed for GPx3 and SeLP, confirming that SeAlb is

probably the first depleted Se-protein in case of Se drop, but still, it is difficult to establish if aging is the cause or a covariate of this trend. For CRC patients, such a correlation was not seen, and SeAlb level had higher variability than in healthy persons. Other studies regarding Se status in CRC patients investigated age as a possible confounding factor [12,15]. One of them [33] noticed for SeAlb the same relationship that we observed, and other works showed a similar correlation between total serum Se level, CRC and the age [11,33]. This association may be due to a less efficient absorption or increased elimination of Se with aging [34]. If the decrease of SeAlb level with aging is assumed to be a natural process, the lack of correlation observed in this study for CRC patient supports the hypothesis that the presence of cancer reduces the homeostatic capability of the Se-proteins system.

Logistic model performances showed the potentiality of Se-proteins status assessment in the early cancer stage detection, even if these models needs to be validated over a larger sample size. On the other hand, considering correlation analysis with prognostic criteria and survival analysis results, we assume that the correlation between SeAlb concentration and survival rate has to be seen as a covariance relationship. This implies that the high SeAlb level and the high survival probability are likely a consequence of the early cancer stage at the moment of surgery and hence at this stage it is difficult to propose a direct causal correlation between these two variables.

IX.6. Conclusions

The results of this study mark the importance to move from total Se to individual Se species determination for the identification and the functional characterization of new biomarkers of CRC. More studies are needed to investigate the complex association among Se-proteins status in serum and tissues, presence of cancer and prognostic criteria as well as determination of proteins containing essential trace metals other than Se. The best biomarker for CRC is expected to be not a single (Se-protein) variable but rather a selected group of proteins. The present study can be useful for selection of the most interesting parameters and the design of the optimal sample size in further comparisons between CRC patients and healthy persons. Other investigations should be carried out in order to deep the causal relationship between

CRC and serum Se-species, which could provide more accurate epidemiological and nutritional data in order to enhance the quality of prevention and treatment for CRC.

A journal article regarding the study presented in this chapter is on writing for publication.

IX.7. References

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X. DEVELOPMENT OF METHODOLOGIES FOR SE-PROTEINS SPECIATION IN RAT COLON TISSUE⁴

X.1. Introduction

As mentioned in the paragraph I.5, about 25 Se-proteins have been identified in human proteome [1] and 24 in rat and mouse proteome [2]. According as their specific function/location in the organism, those carry out antioxidant action or cellular redox signalling can constitute promising biomarkers for global oxidative stress status, as is the case of serum/plasma Se-proteins, but also may be important for organ-specific diseases. As previously shown (see Chapter IX.5), colorectal cancer presence and progression revealed associations with some serum Se-proteins, confirming that Se can play a role in the incidence and development of the disease. However, the precise biochemical role of Se in tumour cells remains still unclear. Serum Se-proteins could be appropriate biomarkers for short-term global Se status, but are probably far to describe appropriately what happens at colon tissue level, under oxidative stress conditions.

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Most of the published studies regarding tissues are focused on Se speciation in foodstuff and supplements, among which yeast and wheat flour [3], rice [4] and fish [5-7]. Therefore, accurate and precise analytical methodologies for the quantitative speciation analysis of Se in animal tissues are strongly required nowadays.

As discussed in the paragraph III.2., in order to carry out chemical speciation studies in solid samples, the first required step is the extraction of Se-species to a liquid phase, without any alteration of its chemical form. Many approaches have been proposed in the literature to extract Se compounds from biological samples. Leaching by aqueous solutions and aqueo-organic mixtures allows to extract free Se amino acids as well as soluble and weakly bound Se-species from several biological matrices, but with low recoveries [8-15]. The addition of sodium dodecyl sulfate (SDS) showed to increase the extraction efficiency for the recovery of Se-containing protein fraction [8,16,17]. This reagent denatures the proteins and, by forming ion pairs, renders them water soluble. Proteolytic enzymes mixtures have been also widely used for the extraction of Se in a variety of solid biological matrices [8,9,13,15,17-21]. In this case, quantitative recovery of Se can be obtained, but since proteases break the peptidic bonds, all the information concerning the original Se-containing proteins get lost.

For the separation and detection of Se-species in the extracts, a system based on HPLC coupled on-line with a Se-specific and sensitive detector as ICP-MS is, constitutes the best option [22]. As extensively exposed in this thesis (see Chapter VI), the quantification of Se-proteins in chromatographic mode might be a critical issue because often there are no commercially available standards for the analytes, and matrix effects can invalidate the use of other external calibrants. These drawbacks are extremely relevant also for tissue extracts. In this context, the most powerful approach to get reliable determinations consists of the application of species-unspecific IDA carried out on-line with HPLC-ICP-MS [23]. Such a methodology has been recently applied for Se speciation in yeast and wheat flour enzymatic extracts [3], and cod muscle certified reference material [24].

X.2. Goals of the study

This study was aimed to the development of an analytical methodology for speciation of Se in colon tissue. Healthy rat colon samples were used as model for

humans in the first stage of the investigation, presented here. We studied different extraction procedures in order to optimize the balance between efficiency maximization and species preservation. A method for the separation of Se-species was then developed, based on two-dimensional (SEC and AE) HPLC coupled on-line with ICP-ORS-QMS detector. We then carried out the quantification of both total Se and individual Se-species by IDA, and identified the isolated species by MALDI-TOF analysis of the collected HPLC fractions.

X.3. Experimental

X.3.1. Instrumentation

Solid samples digestions were carried out by a Milestone microwave oven model Ethos-1 (Microwave Laboratory Systems, Socisole, Italy) with an EM-457(A) extractor module and an AC-100 open/close module with medium pressure PTFE vessels. Tissue extractions were carried out by using Reax2 Stirrer (Heidolph, Kelheim, Germany), ultrasonic bath Ultrasons (JP Selecta, Barcelona, Spain), Sonicator up200s (Hielscher, Teltow, Germany) and Ultra-Turrax T-8 homogenizer (Ika, Staufen, Germany). Alkaline digestion of the extraction residues was carried out in a controlled thermostatic bath Digiterm 100 (JP Selecta, Barcelona, Spain).

A HPLC system Agilent 1100 series equipped with autosampler and DAD was used as solvent delivery system for method development, while a Shimadzu LC-10AD HPLC pump was used for coupling with the ICP-MS. Injections were made using a model 7725 Rheodyne valve fitted with a 100 μ L loop. The SEC columns were a Superdex 75 10/300 GL with Mw range of 3,000-70,000 Da (Amersham Biosciences, Uppsala, Sweden); a Shodex Asahipak GS-520 HQ SEC with Mw range of 10,000-300,000 Da (Showa Deko, Tokyo, Japan); and a Bio-Rad Bio-Sil SEC 125-5 (Bio-Rad, Richmond, California, USA) with a Mw range of 5,000-100,000 Da. The AE column was a Mono QTM 5/50 GL (Amersham Biosciences, Uppsala, Sweden). The CE column was a Zorbax 300-SCX (Agilent Technologies, Wilmington, DE). All the HPLC columns were connected to the ICP-MS nebulizer with PEEK[®] tubing (30 cm \times 0.25 mm i.d.). A syringe pump model Pump 11 (Harvard Apparatus, Edenbridge, UK) was used for spike addition in on-line post-column IDA. HPLC operating conditions are summarized in Table X-1.

The ICP-ORS-QMS Agilent 7500cx was used for Se determinations and HPLC coupling. Plasma operating conditions and acquisition parameters are summarized in Table X-1. The Se-species identification was carried out by MALDI-TOF mass spectrometer model Voyager-DE STR Workstation.

Table X-1 HPLC and ICP-ORS-QMS operating conditions.

SEC-HPLC	
Mobile phase	Tris-HCl 50 mM, pH 7.4
Gradient	Isocratic
Flow rate	1 mL min ⁻¹
Injection volume	100 µL
AE-HPLC	
Mobile phases	A - Tris-HCl 25 mM, pH 8.8 B - Tris-HCl 25 mM, pH 7.4 C - Tris-HCl 25 mM + NH ₄ Cl 1 M, pH 7.4
Flow rate	1 mL min ⁻¹
Injection volume	100 µL
ICP-ORS-QMS	
RF power	1500 W
Plasma gas flow rate	15 L min ⁻¹
Auxiliary gas flow rate	1.1 mL min ⁻¹
Ions lens setting	Optimized daily for best sensitivity of 10 ng mL ⁻¹ Li, Co, Y and Tl, 1 % (w/w) HNO ₃ solution
Reaction cell H ₂ gas flow	4 mL min ⁻¹
<i>Total Se determination</i>	
Monitored masses	76, 77, 78, 79, 80, 81, 82 and 83
Points per peak	3
Acquisition time per point	4 s
Replicates	5
<i>HPLC coupling</i>	
Monitored isotopes	76, 77, 78
Points per peak	1
Acquisition time per point	0.5 s

X.3.2. Reagents and materials

Ultra-pure water was obtained from a Milli-Q System (Millipore Co., Bedford, MA, USA). All reagents of analytical grade were used. Acetonitrile (AcN) HPLC/MS grade was purchased from Romil Ltd. (Cambridge, U.K.). HNO₃ (additionally purified by sub-boiling distillation), HCl and H₂O₂ were purchased from Merk (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (Tris) base, NH₄Cl, 25 %

(w/v) tetramethylammonium hydroxide (TMAH) solution in water, urea, CaCl_2 , NH_4HCO_3 , DTT, iodoacetamide (IAA) and acetic acid were purchased from Sigma-Aldrich (Milan, Italy).

All the Tris-buffered solutions were prepared in ultra-pure water, and the pH was adjusted dropwise by HCl. TritonX-100 and proteases inhibitor cocktail containing (2-aminoethyl)benzenesulfonylfluoride (AEBSF), pepstatin, E-64, bestatin, leupeptin, and aprotinin (both from Sigma-Aldrich) were used for extraction buffers preparation. In the extraction buffer (TB) Tris-HCl 50 mM, pH 7.4, proteases inhibition cocktail 1 % in weight was added just before the extractions. For the preparation of extraction buffer with detergent (TBD) an aliquot of the TB solution was transferred in a plastic tube and Triton X-100 was added in ~1 % (v/v). All the buffers were store at 4° C and de-gassed immediately prior to use.

Amicon Ultra centrifuge filter devices (cutoff: 30,000 Da) were purchased from Millipore (Billerica, MA, U.S.A.).

Gel Filtration Standard mix for SEC column calibration containing the proteins: Thyroglobulin (670,000 Da), Bovine γ -globulin (158,000 Da), Chicken ovalbumin (44,000 Da), Equine myoglobin (17,000 Da), and Vitamin B₁₂ (1,350 Da), was purchased from Bio-Rad (Richmond, California, USA). Standard pure solutions of Thioredoxin Reductase from rat liver and Glutathione Peroxidase from bovine liver were purchased from Sigma-Aldrich (Milan, Italy). Peptide Calibration Mixture 2 containing angiotensin I, ACTH 1-17, 18-39, 7-38 clips and bovine insulin was purchased from Applied Biosystems. Sinapinic acid, α -cyano-4-hydroxycinnamic acid (HCCA) and TPCCK-treated trypsin from bovin pancreas was also purchased from Sigma. The trypsin used is a sequencing-grade enzyme that has been modified to inhibit autolysis and to minimize non-tryptic protease activities. A standard solution of 1000 mg L⁻¹ of SeO_3^{2-} stabilized in 2 % (v/v) HNO_3 Suprapur was purchased from Merck. Enriched ⁷⁶Se was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder, was dissolved in a minimum volume of sub-boiled nitric acid, and diluted to volume with ultra-pure water. The concentration of this solution was determined by reverse IDA using a natural abundance standard. The isotopic composition of the Se standard solution was considered to be of natural isotopic abundance, as reported by Rosman and Taylor [25].

X.3.3. Samples collection

Rat colon samples (from healthy individuals) were purchased from the Animal Unit Laboratory of the University of Oviedo (Spain). The biological reference materials Bovine Liver NIST SRM 1577a, from the National Institute of Standards and Technology (Gaithersburg, MD, USA) was used as quality control for microwave digestion procedure.

X.3.4. Procedures

Samples collection. Animals were killed by cervical dislocation, colons were dissected into portions, opened longitudinally and the faeces were removed. The tissue was washed with cold (4° C) TB solution, dried gently with paper and immediately stored at -20° C until extraction.

Microwave digestion. Approximately 0.1 g of sample were digested with 9 mL of HNO₃ diluted 1:3 in water and 1.5 mL of H₂O₂ in a microwave oven. An appropriate amount of ⁷⁶Se spike (calculated by the error magnification theory [26]) was added directly into the vessels before the digestion. A previously developed program for digestion of biological samples was used [23].

Extraction procedure study. A pooled sample (obtained from 3 individuals) was frozen in liquid N₂ and homogenized by grinding in mortar. Three aliquots of 0.1 g of the homogenized sample were used for total Se determination by microwave digestion. Aliquots of 0.1 g of the homogenized sample were placed into a 1.5 mL Eppendorf tube, 0.4 g of extraction buffer (TB or TBD, at 4° C) were added and kept in ice in order to prevent thermal degradation of the species. Extraction was carried out on different aliquots of the sample by stirring (in ice), ultrasonic bath (in 4° C water), ultrasonic probe (in ice) and Ultra-Turrax (in ice). Extractions by stirring and in ultrasonic bath were performed sequentially for 30 min, 1 h, 2 h, 3 h. The ultrasonic probe was used in continuous pulse mode (power 100 %) for 10 s of extraction followed by 50 s of pause, where the cycle was repeated 3 and 6 times. The Ultra-Turrax homogenizer was used at speed 6 for 10 s of extraction followed by 50 s of pause. The cycle was repeated 4 times. After the extraction, samples were centrifuged at 10,000 g, 4° C for 30 min and the supernatant was transferred to a clean Eppendorf

tube for immediate storage at -20°C until analysis. For of all the sequential extractions, each step was performed by adding new 0.4 g of buffer to the residue of the previous extraction. The residues of the last sequential extraction were digested by direct addition of 5 mL of 25 % TMAH solution, and incubation at 60°C for 4 h.

Separation of Se-species in the extracts. Two-dimensional chromatographic separation of Se-species in the extracts was performed by SEC followed by AE - HPLC, both of them coupled on-line to the ICP-MS. The chromatographic conditions are reported in Table X-1.

Three different SEC columns were calibrated by injecting 100 μL of the calibration mixture diluted in the mobile phase. The chromatographic profile was monitored by UV detector at 280 nm. The behaviour of calibration mixture separation by the Superdex column was tested with different eluents. The variation of the calibration curves with eluent changing was evaluated. After selection of the best column, the main SEC fraction was collected in ice, centrifuged in Amicone filters (cutoff: 30,000 Da) at 10,000 g, 4°C and washed with ~ 8 mL of 25 mM Tris-HCl buffer at pH 8.8 (previously stored at 4°C and saturated with N_2 for 10 min), until reaching a concentration twofold higher than the originally injected.

The AE chromatographic separation was achieved by injecting 100 μL of the SEC fraction and using the operative conditions also reported in Table X-1. The elution sequence consists of the following steps: 0-5 min 100 % buffer A; 5-13 min 35 % buffer A, 65 % buffer B; 13-21 min 90 % buffer B, 10 % buffer C; 21-25 min 80 % buffer B, 20 % buffer C; 25-30 min 100 % buffer C; 30-35 min 100 % buffer A.

Determination of total Se in digested samples and extracts. Microwave digested samples were diluted by weight to ~ 20 g with ultra-pure water, while the alkaline digested residues and the extracts were spiked with the appropriate amount of ^{76}Se enriched solution, and diluted 1:2 with ultra-pure water. All the samples were directly analyzed by ICP-MS and total Se was determined by IDA. The $^{78}\text{Se}/^{76}\text{Se}$ isotopic ratio was preferred to $^{80}\text{Se}/^{76}\text{Se}$ to avoid further corrections due to the BrH^+ interference on the ^{80}Se signal [23]. Signal intensities were corrected for SeH^+ formation and detector dead time, and the ratio was corrected for mass bias.

Quantification of Se-species. Se-species concentration in the fraction collected from SEC, and chromatographically separated by AE, was determined by ON-IDA. For this purpose, a ^{76}Se -enriched solution of the appropriate concentration was continuously added to the eluate (flow 0.01 mL min^{-1}) through a T-connection. The $^{78}\text{Se}/^{76}\text{Se}$ isotopic ratio was calculated and corrected as previously mentioned for total Se determination.

In-solution proteins digestion protocols.

Reagents. All of the reagents were prepared immediately prior to use. The water used in all components of the procedure was MilliQ-type. The reagents were prepared in 20 mL glass vials with Teflon-lined caps of 1.5 mL plastic microfuge tubes. The prepared solutions were:

- 50 mL of Tris stock solution 0.2 M, CaCl_2 40 mM at pH 7.6 adjusted with HCl 6 M.
- 5 mL of urea 12 M (denaturing reagent) solution, Tris 100 mM, CaCl_2 20 mM.
- 1 mL of DTT 200 mM (reducing reagent) solution, Tris 50 mM, CaCl_2 10 mM.
- 1 mL of IAA 200 mM (alkylating reagent) solution, Tris 50 mM, CaCl_2 10 mM.
- 1 mL of NH_4HCO_3 100 mM solution.
- 100 μL of Trypsin 200 $\text{ng } \mu\text{L}^{-1}$ solution, Tris 50mM, CaCl_2 10 mM. The solution was kept in ice until use.

Digestion procedure-A.

A 30 μL aliquot of the extract fraction was placed in a 1.5 mL centrifuge tube and diluted 1:1 with 30 μL of the 12 M urea, 200 mM Tris buffer solution. A volume of 3.3 μL of the DTT solution was added, and the sample was mixed by gentle vortex. The reduction of the proteins was then carried out for 1 h at room temperature. A volume of 13.3 μL of IAA solution was added and the sample was mixed by gentle vortex. The alkylation of the proteins was carried out for 1 h at room temperature. A volume of 13.3 μL of DTT solution was added again to consume any unreacted IAA. The sample was mixed by gentle vortex and the reaction allowed to stand at room temperature for 1 h. The urea concentration was reduced by dilution with 517 μL of AcN, then the solution is mixed by gentle vortex. This dilution reduces the urea concentration to $\sim 0.6 \text{ M}$, at which the Trypsin retains its activity. For in-solution

proteins digestion, 67 μL of Trypsin solution (containing 20 μg of Trypsin) were added, the sample mixed by gentle vortex, and the digestion carried out overnight at 37° C. The reaction was stopped by reducing the pH < 6 with concentrated acetic acid.

Digestion procedure-B.

If this denaturant reagent is used, as is in the classical procedure, the resulting solution needs to be then diluted 4- to 8-fold with buffer to reduce the denaturant and maintain the enzymatic activity of trypsin. However, trypsin might be even then low-level inhibited or denaturated [27], and the presence of urea is not desirable for HPLC-MS analysis. Instead, a NH_4HCO_3 solution can be used. This buffer is unable to totally resuspend the proteins pellets generated by thermal denaturation, but some authors reported that they are likewise easily digested by trypsin in 3 h resulting in more specific cleavage and generation of sufficient numbers of tryptic peptides for protein identification [28,29]. This results suggest that enzymatic digestion can be efficiently carried out also without urea or detergents. Thus, in this alternative procedure urea was not employed.

A 30 μL aliquot of the extract fraction was placed in a 1.5 mL centrifuge tube and diluted 1:1 with 30 μL of the 100 mM NH_4HCO_3 solution. After buffer addition, 3.3 μL of the reducing reagent (DTT solution) were added and the sample was mixed by gentle vortex. Denaturation and reduction of the proteins was carried out for 5 min at 95° C. After cooling to room temperature, for in-solution proteins digestion 67 μL of trypsin solution were added, the sample mixed by gentle vortex, and the digestion carried out overnight at 37° C. The reaction was stopped by reducing the pH < 6 with concentrated acetic acid.

Digestion procedure-C.

In this protocol, the reduction by DTT is also avoided in order to simplify the procedure.

A 30 μL aliquot of the extract fraction was placed in a 1.5 mL centrifuge tube and diluted 1:1 with 30 μL of the 100 mM NH_4HCO_3 solution. A volume of 67 μL of trypsin solution was directly added, the sample mixed by gentle vortex, and the digestion carried out overnight at 37° C. The reaction was stopped by reducing the pH < 6 with concentrated acetic acid.

Identification of Se-species by MALDI-TOF. In order to identify the Se-species isolated by AE, fractions corresponding to each detected peak were collected and characterized by MALDI-TOF. Desalting and preconcentration of the fractions were carried out by washing with ultra-pure cold (4° C) water 50 volumes greater than that of the fractions and centrifugation (at 10,000 g, 4° C) in Amicon Ultra centrifuge filters (cutoff: 30,000 Da). Then, aliquots of this solution were mixed with saturated sinapinic acid, used as MALDI matrix, for the determination of intact proteins Mw. Linear acquisition mode was used, and mass calibration of the instrument was performed daily using a BSA standard.

Aliquots of the same desalted and preconcentrated fractions were also treated with the digestion protocols mentioned above. The digests were mixed with saturated HCCA as MALDI matrix for peptides characterization. Reflectron acquisition mode was used in this case. Mass calibration of the instrument was achieved by analysing daily the Calibration Mixture 2 from Applied Biosystems.

X.4. Results and Discussion

X.4.1. Total Se determination validation

Three replicates of bovine liver CRM were mixed with an appropriate amount of ⁷⁶Se spike solution (at concentration of 100 ng g⁻¹), digested in the microwave oven by the method mentioned above, and analyzed for total Se in order to validate the IDA methodology. The obtained result (0.70 ± 0.01 μg g⁻¹) was in agreement with the certified value (0.71 ± 0.07 μg g⁻¹).

X.4.2. Evaluation of extraction efficiency

In order to evaluate the extraction efficiency, total Se was determined in the extracts for each investigated procedure, and in the corresponding blanks. The concentration was then compared with the results obtained by microwave digestion of the same sample aliquots. The sum of total Se determined in the alkaline digested residues and Se level in the corresponding sequential extracts was compared with the total level of Se in the fresh tissue, as a form of quality control. The values were not

significantly different (*t*-test: *p*-value > 0.05) for all the extraction procedures. This demonstrated that there were no losses of the analyte during the sequential steps, and also that alkaline and microwave digestions are compatible methods. Table X-2 reports the results obtained for all the extraction procedures and different extraction times/number of cycles.

Table X-2 Total Se concentration (referred to the solid sample) in the extracts and the corresponding efficiencies calculated for the different extraction procedures investigated.

Extraction procedure	Time/cycles	Se [ng g ⁻¹]		Efficiency [%]	
		TB	TBD	TB	TBD
Stirring	30 min	50	57	30	33
	1 h	73	79	43	46
	2 h	92	99	55	59
	3 h	111	118	65	69
Ultrasonic bath	10 min	30	34	18	20
	20 min	49	53	29	31
	30 min	64	70	38	41
	40 min	79	87	47	51
Ultrasonic probe	3 cycles	54	92	32	54
	6 cycles	57	100	34	59
Ultra-Turrax	4 cycles	73	76	43	45

Only a little increase in efficiency was achieved by adding Triton-X to the buffer, most likely because all the methods present enough strength to break down the membranes and allow the complete release of cytoplasmatic proteins. The extractions with ultrasonic probe were an exception, where Triton-X addition increased significantly the efficiency, which otherwise was relatively low. The use of detergent was rejected because it did not allow significant improvements in extraction efficiency, and can be problematic for chromatographic separation of the extracts (further sample preparation steps are required to remove the detergent before separation). Increasing the extraction time/number of cycles improved efficiency for all the methods, but the use of ultrasonic probe or Ultra-Turrax allowed to significantly reduce the extraction time necessary to obtain the same efficiency, compared with ultrasonic bath and stirrer. On the other hand, with stirring in ice and

ultrasonication in cold water (4° C) it is difficult to control the temperature, while the use of ultrasonic probe can give problems of analytes/sample loss due by formation of bubbles, particularly when detergent is used. Ultra-Turrax was finally chosen as best device for total Se extraction from rat colon tissues, taking into account a balance among efficiency, reproducibility and speed.

X.4.3. Optimization of the extraction procedure for species preservation

Species integrity preservation during extraction is a mandatory requisite for Se speciation study. Extraction procedure and buffer composition needs to be carefully optimized by avoiding thermal and chemical degradation, particularly if the expected species are unstable. In order to evaluate the Se-species preservation, the Ultra-Turrax extracts were injected undiluted into the chromatographic system for separation by SEC. A typical chromatogram is showed in Figure X-1a.

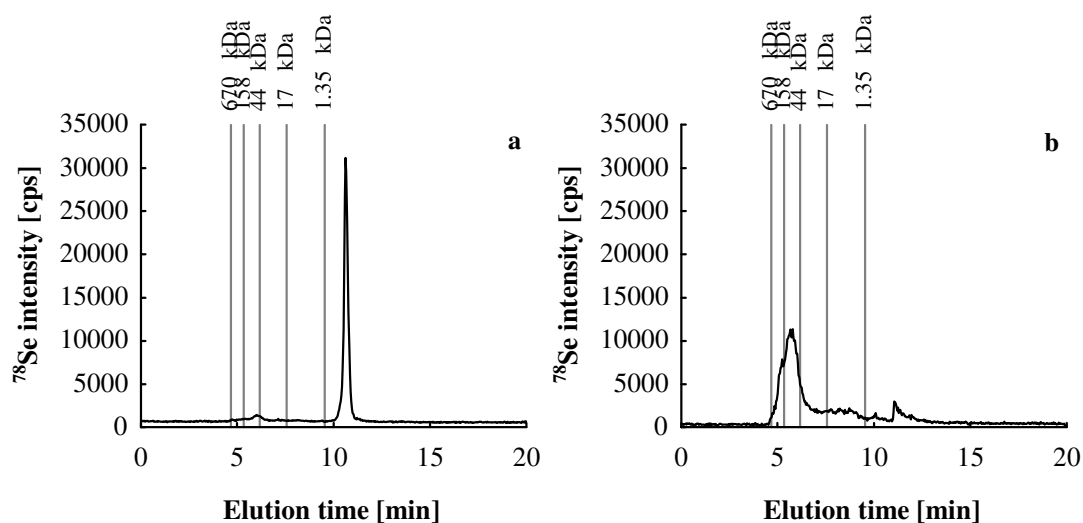


Figure X-1 Chromatograms for ^{78}Se obtained by SEC-HPLC of the same rat colon sample extract: (a) without bubbling N_2 in the buffer during the extraction and (b) by bubbling N_2 .

Only one peak was obtained, corresponding to non-proteic species. $\text{Se}(\text{Cys})_2$, SeMet, selenate and selenite standards were injected into the SEC column, and eluted with slightly different times (even if out of the linear range of the column), establishing that the observed extract peak consists in selenite. This observation agrees with the release of that specie by oxidation of the selenolic groups from Se-proteins [30]. To avoid such degradation, the extraction buffer was deoxygenated by

continuously bubbling N_2 during the procedure. The final instrumental set-up is shown in Figure X-2.

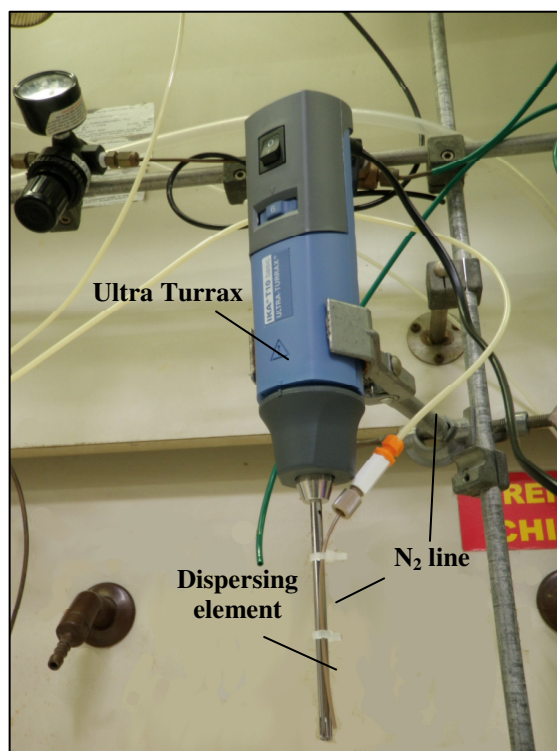


Figure X-2 Picture of extraction apparatus with Ultra-Turrax dispersion homogenizer.

The extract obtained and injected then into the SEC system, resulted in the chromatogram showed in Figure X-1b, where a high Mw species peak appeared, and the inorganic species peak was drastically reduced. Thus, bubbling N_2 is effective to protect the analyte from oxidative degradation, but further precludes the use of detergents during the extraction. SDS should be also avoided, because unfolds the proteins and then renders them more sensitive to oxidation. These aspects need to be taken into account in methodologies based on ICP-MS detection, since the loss of Se from the proteins implies the impossibility to detect them, but also in studies based on enzymatic assays because Se is part of the active site, and therefore its loss compromises the activity of Se-proteins. Degradation problem is scarcely discussed in the literature [8,31,32], and most of the studies directly recur to proteolytic digestion to detect individual amino acids [4,33-35]. However, by proteolytic digestion most of the information about the original speciation pattern of Se get lost, because all Se-proteins contain the same amino acid SeCys.

X.4.4. Separation of Se-species by SEC-HPLC

Rat colon extracts from the same pooled sample (3 replicates and a blank) were injected undiluted into each of the chromatographic systems equipped with one of the three SEC columns for a preliminary screening of the Se-species. Based on the literature, three Se-proteins are expected to be present in rat colon tissue: GPx1 (homotetrameric, Mw of the monomer 22,305 Da), GPx2 (homotetrameric, Mw of the monomer 22,014 Da) and TrxR1 (homodimeric, Mw of the monomer 54,386 Da) [36]. Therefore, a commercial pure standard of rat TrxR1 was also injected to estimate the elution time of the protein. Commercial standards of rat GPxs were unavailable, so their retention time was estimated by injecting a standard of GPx1 from bovine liver (Mw of the monomer 22,659 Da). The Mw of the expected proteins in the sample are very similar (GPx1: 89,220 Da, GPx2: 88,056 Da, TrxR1: 108,772 Da), hence none of the columns allowed the resolution of the proteins under investigation (see Figure X-3). The Superdex column resulted in a minor peak at 13.0 min, not completely resolved from the major peak at 14.9 min. Both the Shodex and the Bio-Rad columns presented a single major peak, at 10.2 min and 5.6 min, respectively. The Shodex column has a multimode separation mechanism (SEC/weak AE), while the Superdex column has semipreparative characteristics. In both cases the peak shape was not as good as that obtained using the Bio-Rad column. Even if the Shodex column was offered good resolution for low Mw compounds (i.e. Se-amino acids)[37,38], it was here unsuitable for the direct separation of high Mw protein mixtures. From the injection of rat TrxR1 and bovine GPx1 standards it was also observed that TrxR1 elutes after (but very close to) the bovine GPx1 (see Figure X-3) even if it has higher Mw, probably as effect of the complex quaternary structure of the protein.

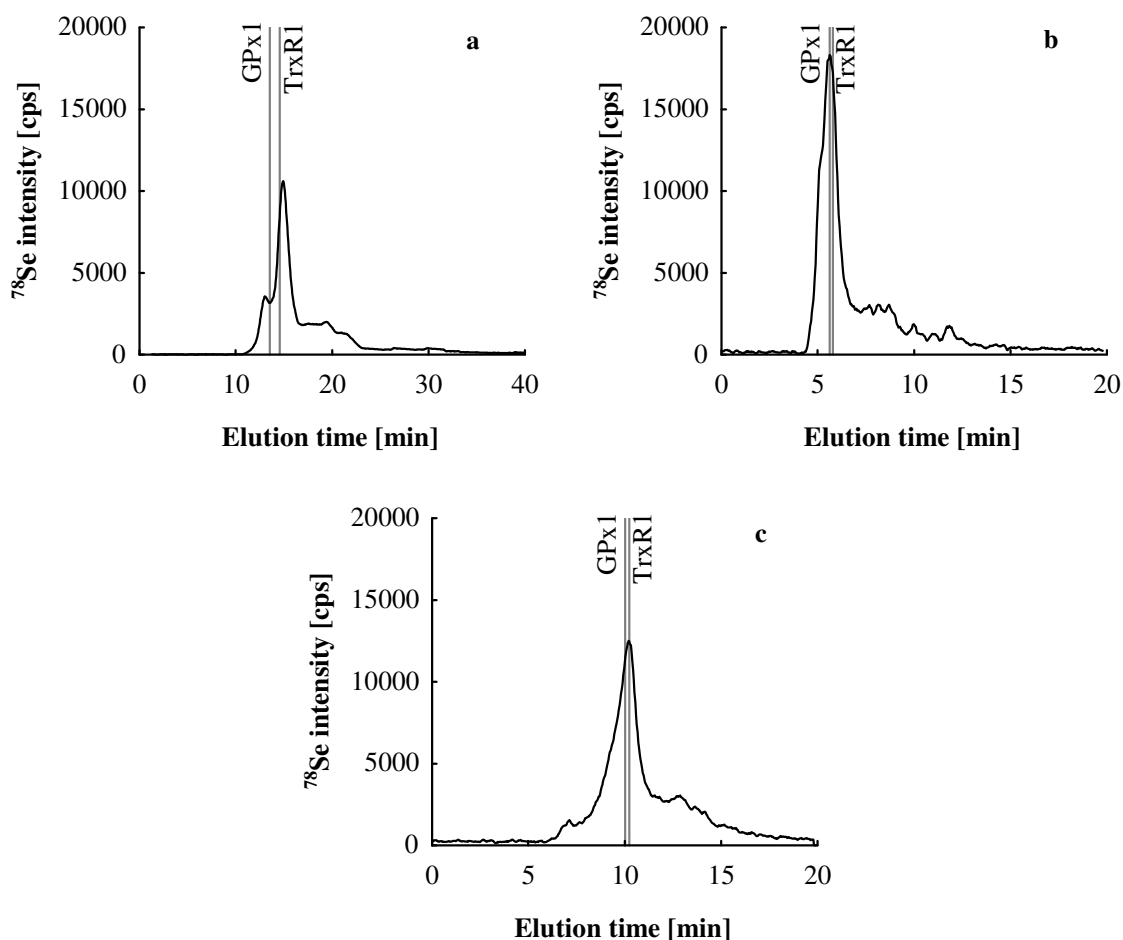


Figure X-3 ^{78}Se chromatograms obtained for the same rat colon extract by SEC-HPLC with columns: (a) Superdex 75 10/300 GL, (b) Shodex Asahipak GS-520 HQ SEC, (c) Bio-Rad Bio-Sil SEC 125-5. The elution times signed by gray lines were obtained by injecting TrxR1 from rat liver and GPx1 from bovine liver standards.

In order to possibly improve the chromatographic separation, the influence of mobile phase composition and pH was evaluated by injecting the protein SEC calibration mixture into the Superdex column, and by eluting it with different buffers. The following mobile phases were used for isocratic elution: (A) AmAc 50 mM at pH 5.0; (B) AmAc 50 mM at pH 7.0; (C) Tris-HCl 50 mM at pH 7.4; (D) Tris-HCl 50 mM + NaCl 150 mM at pH 7.4; (E) phosphate buffer (PhB) 50 mM + NaCl 150 mM at pH 7.2. The absorbance at 280 nm was monitored. No significant differences were observed in the chromatographic profiles by changing the mobile phase, as can be observed in Figure X-4. Peaks resolution was slightly affected by eluent salinity for low Mw proteins, but the region of interest (Mw > 20,000 Da) for this study showed highly uniform behaviour.

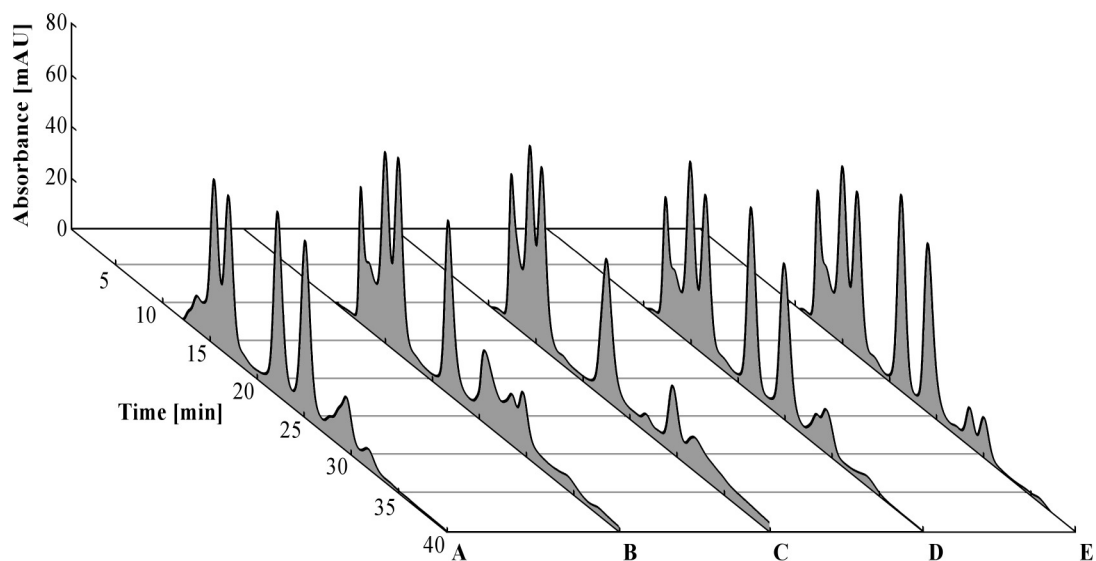


Figure X-4 UV Chromatograms ($\lambda = 280 \text{ nm}$) of the SEC calibration mixture obtained by using different mobile phases: (A) AmAc 50 mM pH 5.0; (B) AmAc 50 mM pH 7.0; (C) Tris-HCl 50 mM pH 7.4; (D) Tris-HCl 50 mM + NaCl 150 mM pH 7.4; (E) PhB 50 mM + NaCl 150 mM pH 7.2.

As marked previously, none of the SEC columns were able to isolate at least two peaks of Se-species, but a useful separation of the major Se peak from other species uncontained Se was achieved by using the Bio-Rad column, as showed by the UV chromatogram in Figure X-5.

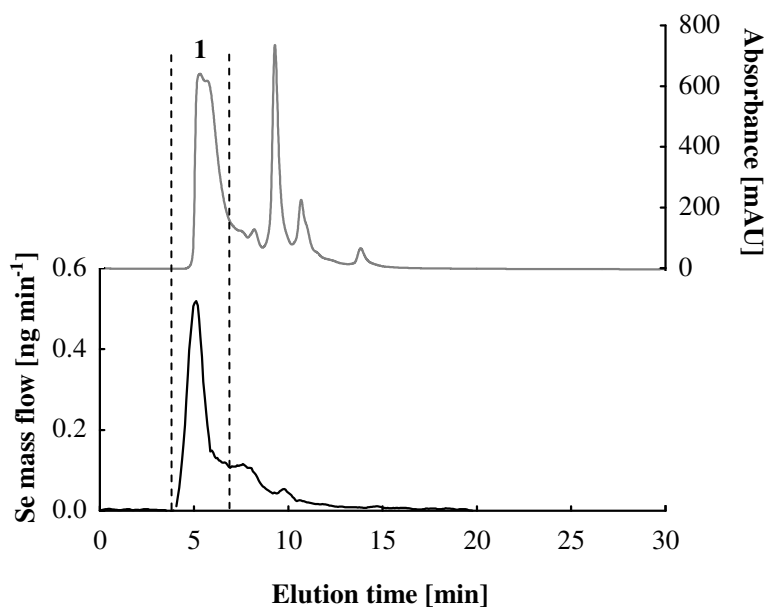


Figure X-5 UV (above) and Se mass flow (below) chromatograms obtained by SEC of the rat colon extract (fraction 1, collected for AE separation, is indicated).

Therefore, the SEC-HPLC system was selected as first purification step in order to reduce matrix complexity and to remove low MW proteins possibly interfering in other chromatographic separation mechanisms and/or MALDI characterization. Quantitative results obtained by post-column ON-IDA, and integration of the mass flow chromatograms in Figure X-5, showed that the Bio-Rad SEC column recovery was $94 \pm 2 \%$, and fraction 1 accounted $49 \pm 1 \%$ of the total Se in the extract.

X.4.5. Separation of Se-species by AE-HPLC

Anion exchange chromatography was the second separation mechanism investigated to isolate the Se-species present in the SEC fraction detected by ICP-MS. Fraction 1 from SEC (see Figure X-5), corresponding to the major peak ($M_w > \sim 30,000$ Da), was collected, preconcentrated twofold by ultracentrifugation in Amicones (cutoff: 10,000 Da), and subsequently analyzed by AE-HPLC-ICP-ORS-QMS (3 replicates and a blank). The obtained chromatogram is shown in Figure X-6.

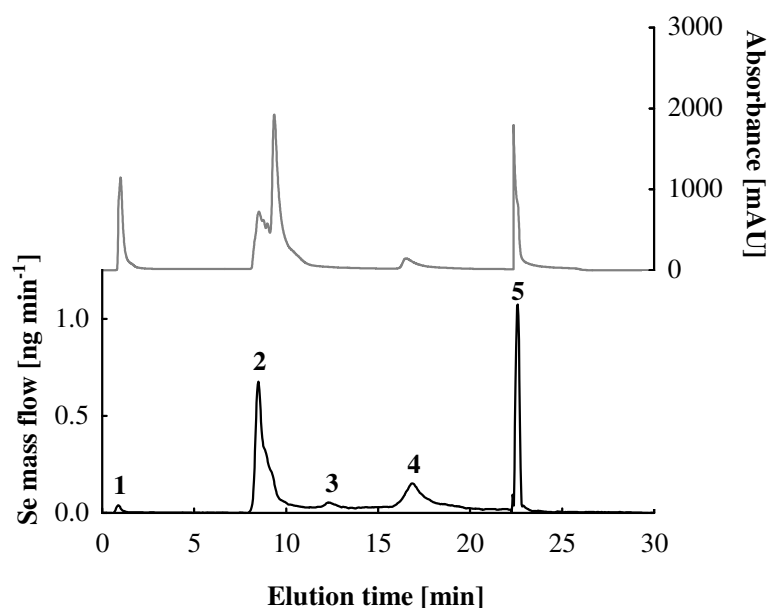


Figure X-6 UV (above) and Se mass flow (below) chromatograms obtained by AE-HPLC separation of the SEC fraction 1 from rat colon extract (peaks 1-5 are indicated).

Optimization of the HPLC elution program allowed the separation of 5 well-resolved peaks of Se-species. The theoretical pI of the expected Se-proteins (GPx1: 7.7, GPx2: 8.3, TrxR1: pI 5.9) was in agreement with the selected elution conditions, compatibly with the correspondence of GPx2 to peak n. 2, GPx1 to peak n. 4 and

TrxR1 to peak n. 5. Injection of a pure TrxR1 standard solution resulted in a single peak at elution time matching with the peak n. 5 of the extracts. Since the standards of rat GPxs were commercially unavailable, their possible identification cannot be allowed by matching of retention times with pure proteins samples. However, based on a chromatographic run obtained by linear gradient from 100 % of buffer B to 100 % of buffer C (5-25 min), and with an hypothesized resulting linear pH gradient, the experimental pI of the species corresponding to the fractions 2 and 4 were estimated as 8.1 and 7.6, respectively. The calculated values agree with the theoretical pI of GPx2 (8.3) and GPx1 (7.7), respectively. Quantitative results by ON-IDA showed that TrxR1 accounted 25.5 % of the total Se in the extract ($20.2 \pm 1.7 \text{ ng mL}^{-1}$ of Se). Considering a constant ratio Se/protein 1:1 (for the monomer) TrxR1 content in the extract can be estimated as $0.13 \text{ nmol mL}^{-1}$.

The fraction 1 elutes with the void volume of the column, at pH 8.8 (buffer A), a condition which is incompatible with the expected proteins in the extract (pH higher than their pI). Such peak could be due by the presence of other unexpected species, nevertheless new Se-proteins (possibly present at extremely low level) appears to be improbably detectable by this analytical method. On the other hand low Mw species (i.e. inorganic Se, as selenite, produced by analytes degradation) should be absent in the extract, and eventually removed by two phases of dimensional cutoff (SEC fraction collection for $M_w > 30,000 \text{ Da}$ and ultracentrifugation in $10,000 \text{ Da}$ cutoff membrane). A test was carried out without pH correction of the fraction, before injection into the AE system, where the pH of the SEC fraction is 7.4 while the starting eluent for AE is buffered at pH 8.8. The chromatogram obtained for the fraction injected at pH 7.4, shown in Figure X-7, presented a much more abundant fraction 1, while the fractions 2, 4 and 5 were reduced. Consequently, the presence of peak 1 is due by species (mainly GPxs) incompletely equilibrated with the new pH conditions. Even if this effect is negative for quantitative proposes, fraction 1 constitutes only 1.2 % of total Se in the extract ($1.0 \pm 0.3 \text{ ng mL}^{-1}$), and therefore is negligible. The presence of peak 4 is also incompatible with the expected Se-species. Since fraction 4 elutes in the same conditions of peak 2, but with higher retention time, it could be due by the GPx2 monomer, which is an other possible degradation product of the tetrameric protein [30]. The concentration of Se in peak 4 is $3.6 \pm 0.9 \text{ ng mL}^{-1}$, corresponding on 4.6 % of total Se, which is relatively low in respect to the other main proteins.

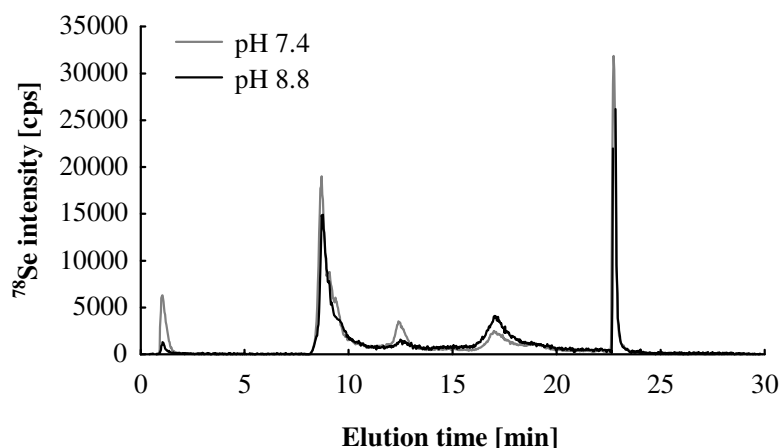


Figure X-7 ^{78}Se AE chromatograms obtained by injecting the same rat colon extract SEC fraction at pH 7.4 (gray line) and at corrected pH 8.8 (black line).

Quantification of peaks 2 and 4 showed that GPx2, a protein secreted into the gastrointestinal mucosa, is the major Se specie in rat colon tissue extract, accounting 42.7 % of the total Se ($33.5 \pm 0.9 \text{ ng mL}^{-1}$). The cytoplasmatic GPx1 represents 26.0 % of Se in the extract ($20.6 \pm 1.3 \text{ ng mL}^{-1}$). Considering also for GPxs monomer a constant ratio Se/protein 1:1, GPx1 and GPx2 content in the extract can be estimated as 0.11 and $0.07 \text{ nmol mL}^{-1}$, respectively.

X.4.6. Separation of Se containing species by AF-HPLC and AF-CE-HPLC

The 2AF chromatographic system adopted for serum/plasma Se-proteins speciation (see paragraph VII.3) resulted highly efficient and robust. No information in the literature are available about the possible binding capability of HEP or BLUE stationary phases in respect to the rat colon Se-proteins. The home-made stainless steel normalbore HEP and BLUE columns were used also in this study according to the instrumental set-up in Figure VII-6. Rat colon extract was injected into the system, and a chromatographic run was realized adopting the program: 0-5 min 100 % AmAc 50 mM, pH 7.0 (buffer A; BLUE column connected on-line); 5-10 min 100 % AmAc 1.5 M, pH 7.0 (buffer B; column BLUE excluded); 10-15 min buffer B (BLUE column connected on-line); 15-20 min 100 % buffer A (BLUE column connected on-line). A flow of 1.2 mL min^{-1} and a sample loop of $100 \mu\text{L}$ were used. In order to avoid overload of the nebulizer (nebulisation rate $400 \mu\text{L min}^{-1}$), and to reduce matrix

effects, the flow coming out of the HPLC system was splitted, directing only 0.5 mL min⁻¹ to the ICP-ORS-QMS.

The chromatogram obtained for rat colon extract is showed in Figure X-8a.

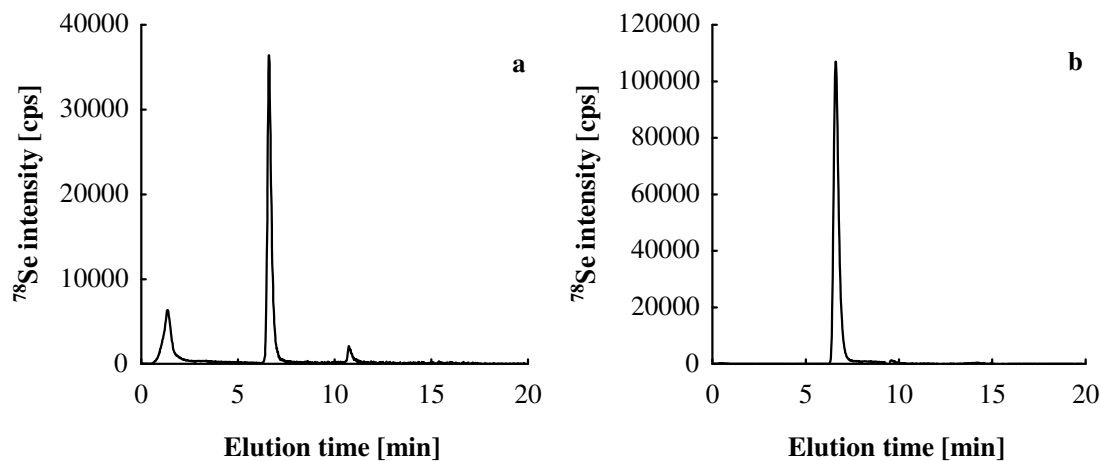


Figure X-8 ⁷⁸Se 2AF chromatograms obtained by injecting: a) rat colon extract; b) TrxR1 rat standard.

A significant part of the total Se in the extract (~56 %) was retained by the HEP column, while a minor fraction (~4 %) was retained by the BLUE column. This behaviour demonstrated that some of the expected Se-proteins in rat colon tissue bind to heparin. TrxR1, the only Se-protein available as pure standard among the species of interest, was then injected into the same 2AF system. The chromatogram, shown in Figure X-11b, confirmed that TrxR1 is one of the Se-proteins with the ability to bind heparin.

In order to explore the potentiality of HEP stationary phase for the separation of rat colon Se-proteins, a new HPLC system was employed by connecting on-line the previously used HEP column with an ion exchange column. As reported in the previous paragraphs, the Mono-Q AE column binds the Se-proteins of rat colon extract by using a starting buffer at pH 8.8. Therefore, the possible coupling of HEP and AE columns requires this pH for the starting buffer. The binding properties of HEP as function of the pH were tested by using the column alone with the program: 0-6 min 100 % starting buffer; 6-12 min 100 % buffer B; 12-18 min 100 % starting buffer. As starting buffer, AmAc 5 mM at pH 6.0, AmAc 50 mM at pH 7.0, and Tris-HCl 25 mM at pH 8.8 were employed. The chromatograms, in Figure X-9, showed

that at pH 8.8 the HEP column loses most of its binding ability for rat colon extract Se-proteins, therefore HEP-AE coupling resulted unsuitable.

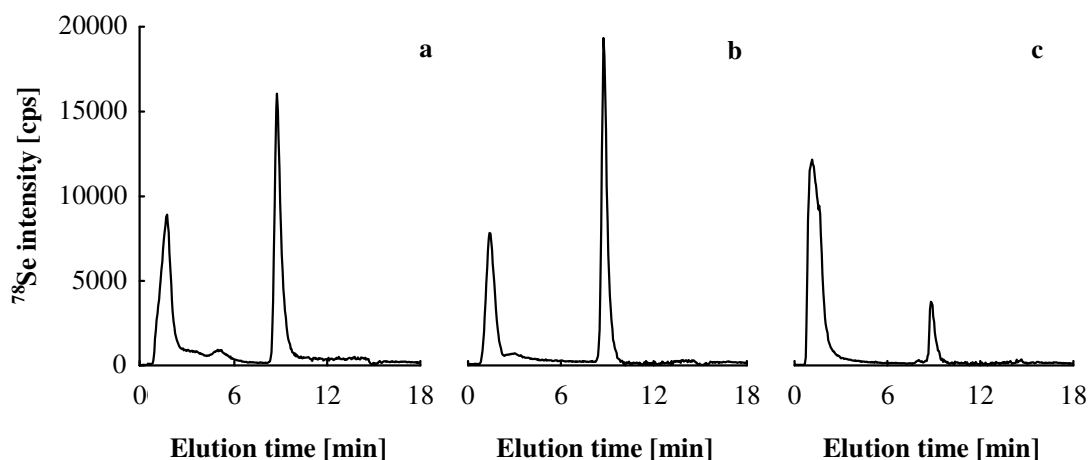


Figure X-9 ^{78}Se HEP chromatograms obtained by injecting rat colon extract and using as starting buffer: (a) AmAc 5 mM pH 6.0; (b) AmAc 50 mM pH 7.0; (c) Tris-HCl 25 mM pH 8.8.

Cation exchange was then selected as alternative separation mechanism for on-line coupling with HEP. The CE column was connected to the HPLC system according to the set-up presented in Figure X-10.

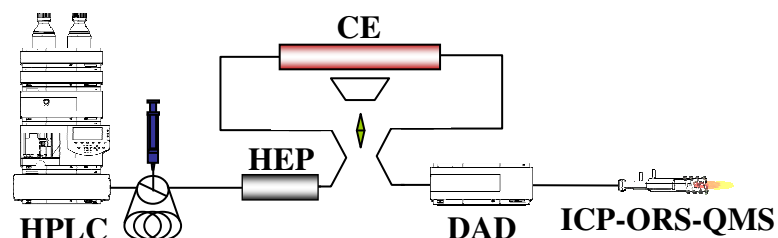


Figure X-10 Instrumental set-up for speciation of rat colon extract Se-proteins by HEP-CE-HPLC-DAD-ICP-ORS-QMS.

A DAD was connected on-line for the monitoring of absorbance chromatogram. A high salinity eluent was used for the elution of species retained by HEP, therefore a switching valve was employed to exclude the CE column from the system during this step. The used HPLC program was: 0-15 min 100 % AmAc 5 mM pH 6.0 (buffer A; CE column connected on-line); 15-25 min 100 % AmAc 1.5 M pH 6.0 (buffer B; CE column excluded); 25-35 min 100 % buffer A (CE column excluded); 35-45 min 67

% buffer A, 33 % buffer B (CE column connected on-line); 45-55 min 100 % buffer A (CE column connected on-line). The chromatograms are shown in Figure X-11.

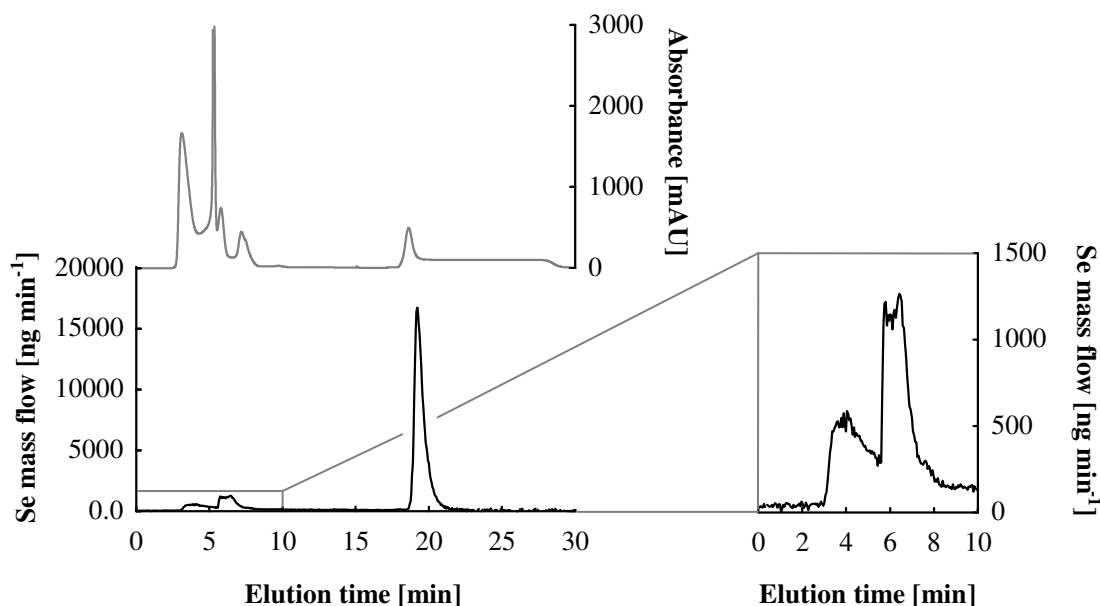


Figure X-11 UV at 280 nm (above) and ^{78}Se (below) HEP-CE chromatograms for rat colon extract.

Most of the total Se was retained by the HEP column. The non-heparine-binding fraction was relatively separated by CE, resulting in two partially resolved peaks. Even if the separation by CE of proteins uncontainig Se resulted effective, as shown by the UV chromatogram, for Se-species was not completely realized, most likely due to the insufficient time of interaction between analytes and stationary phase. In fact, the CE column used in this investigation is more adapt to peptides separation, since it presents a pore size (300 Å) at the lower limit for proteins. However, the results obtained by these tests remark the good potential of AF and CE, used both on-line and off-line, as an alternative strategy for Se-proteins separation in rat colon extracts.

X.4.7. Identification by MALDI-TOF

The standard solutions of bovine GPx1 and rat TrxR1 were directly analyzed by MALDI-TOF for intact proteins characterization. The spectra are shown in Figure X-12.

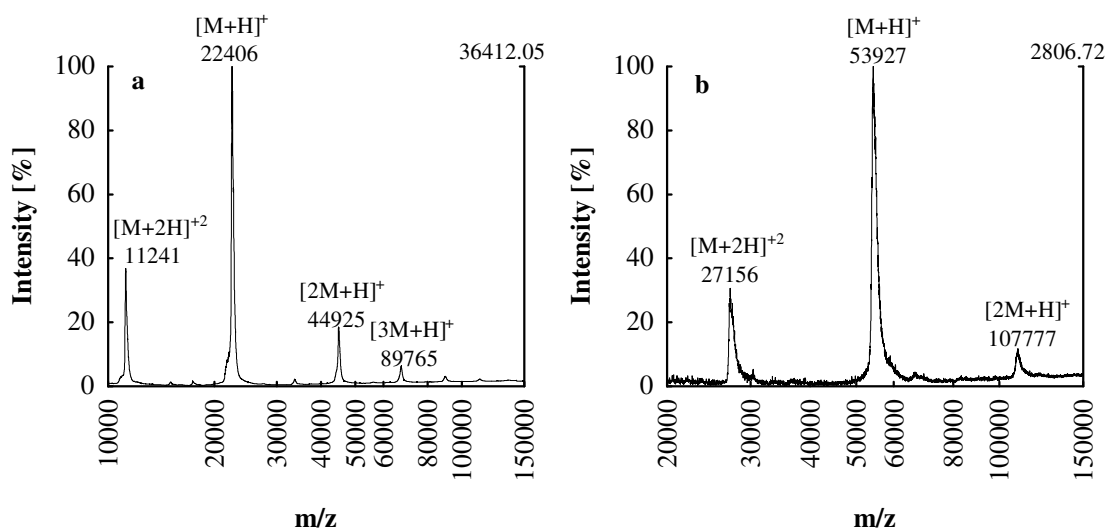


Figure X-12 MALDI-TOF m/z spectra of protein standards: (a) bovine GPx1; (b) rat TrxR1.

Molecular ions, double charges and dimers of the protein were detected. The experimental masses were 32,406 Da for bovine GPx1 and 53,927 Da for rat TrxR1, slightly lower in respect to the theoretical masses (22,659 Da and 54,386 Da, respectively). These bias could be imputable to instability of the instrumental mass calibration.

Fractions 2, 4 and 5 from AE chromatography (see Figure X-6) were collected and analyzed by MALDI-TOF for intact proteins identification. Figure X-13a shows the mass spectrum obtained for MALDI-TOF analysis of fraction 2.

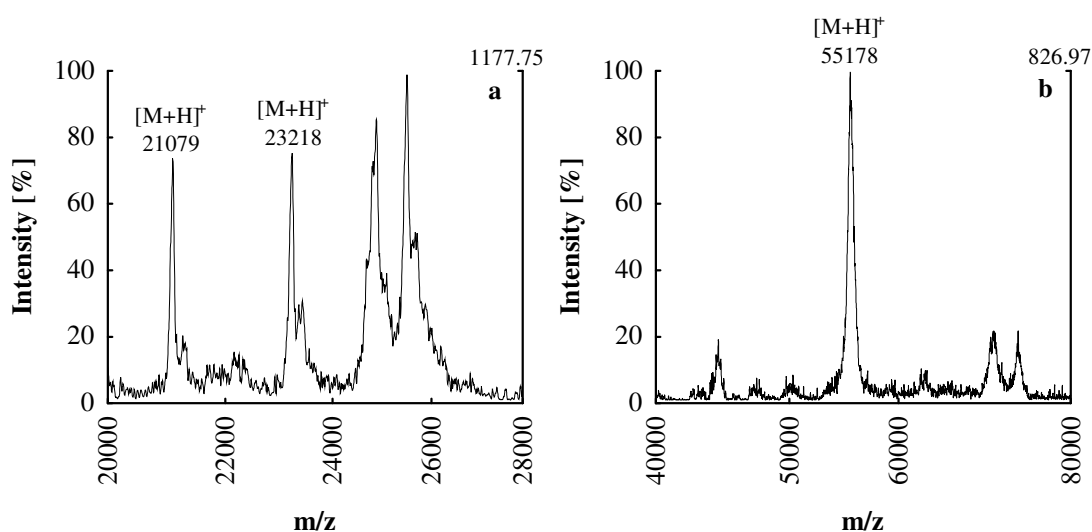


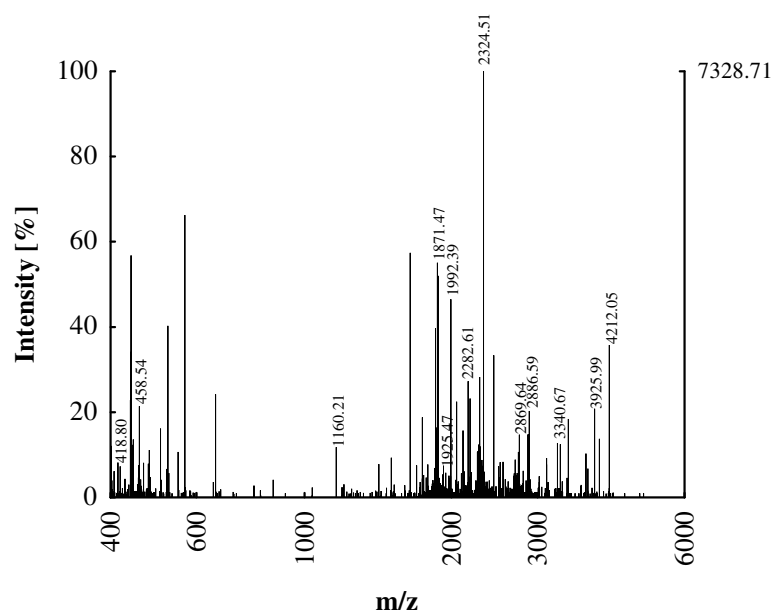
Figure X-13 MALDI-TOF m/z spectra for intact proteins characterization of rat colon extract AE fractions 2 (a) and 5 (b).

Two molecular ions $[M+H]^+$ were observed at m/z 21,079 Da and 23,218 Da which are both possible candidates for GPx2 identification (theoretical Mw 22,014 Da). Since a commercial standard of GPx2 was unavailable, a comparison with the analysis of a spiked sample was impossible. Both observed m/z values could correspond to the target protein, with a mass difference due by degradation processes which do not involve the selenolic group (for the lower mass) or by matrix effects, such as the formation of clusters in the ionization process.

Figure X-13b shows the spectrum obtained for fraction 5: a molecular ion $[M+H]^+$ was observed at m/z 55,178, which is ~800 Da higher than the predicted Mw of the candidate protein TrxR1 (54,386 Da). The same fraction was also collected after injection of an extract spiked with the standard of TrxR1, and again analyzed by MALDI-TOF. A similar molecular ion $[M+H]^+$ was observed at m/z 55,303, but more intense in respect to the background. On the other hand, as previously reported, the direct analysis of the TrxR1 standard gave a molecular ion $[M+H]^+$ at m/z 54,394. The bias observed between the Mw of TrxR1 predicted/measured as pure standard and spiked to the sample appears to be also due to matrix effects.

No candidate masses for GPx1 were observed in fraction 4, most probably due to the low concentration of the Se-species and the complexity of the matrix.

Tryptic digestion was carried out on the same fractions collected from AE, as well as on a standard solution of rat TrxR1. The MALDI-TOF spectrum obtained for peptides characterization by the analysis of the TrxR1 standard is shown in Figure X-14. The spectrum was processed by MASCOT peptide mass fingerprint in order to univocal identify the protein. Twenty sequence matching were obtained, corresponding to 52 % sequence coverage. The score was significant, therefore the TrxR1 standard was confirmed to contain this protein. On the other hand, Se-proteins identification by characterization of the tryptic digests resulted impossible for rat colon extract AE fractions. Four peptides, shown in Figure X-15, matching with theoretical fragments of GPx2 digestion were observed in fraction 2, but too few to render the protein identification statistically significant.



Match to: **TRXR1_RAT** Score: 113 Expect: 3.8e-08
Thioredoxin reductase 1, cytoplasmic OS=Rattus norvegicus GN=Txrd1 PE=1 SV=4

Nominal mass (M_r): 54352; Calculated pI value: 5.95
 NCBI BLAST search of [TRXR1_RAT](#) against nr
 Unformatted [sequence string](#) for pasting into other applications

Taxonomy: [Rattus norvegicus](#)

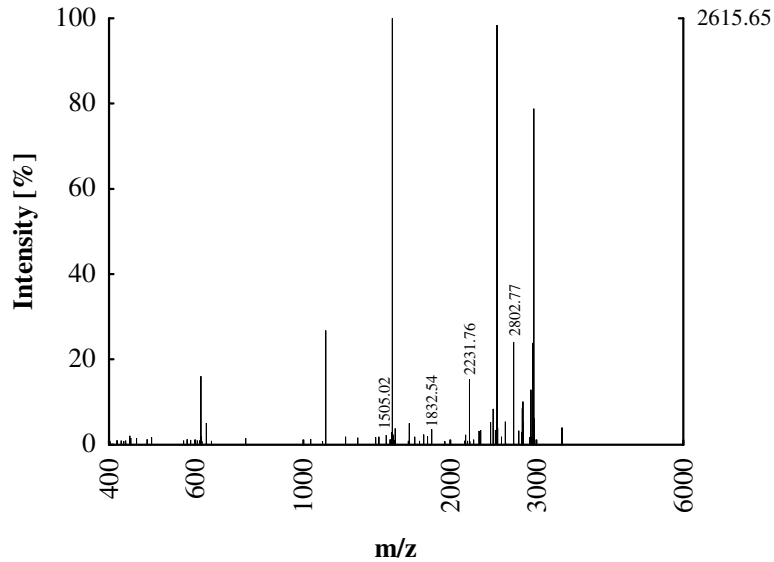
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
 Number of mass values searched: 84
 Number of mass values matched: 20
 Sequence Coverage: 52%

Matched peptides shown in **Red**

```

1  MNDSKDAPKS YFDLIIGG GSGGLAAAKE AAKFDKKVMV LDFVTPTPLG
51  TNGGLGTCV NVGCIPKKLM HQAALLGQAL KDSRNYGWL EDTVKDWEK
101 MTESVQNHIG SLNWGYRVAL REKKVVYENA YGKFIGPHKI MATNMGKKEK
151 VYSAERFLIA TGERPRYLGI PGDKEYCISS DDLFSLPYCP GKTLVVGASY
201 VALECAGFLA GIGLDVTVMV RSILLRGFDQ DMANKIGEHM EEHGKFIRO
251 FVPTKIEQIE AGTPGRLKVT AKSTNSEETI EDEFNTVLLA VGRDSCTRTI
301 GLETVGVKIN ETGKIPVTD EEQTNVPYIY AIGDILEGKL ELTPVAIQAG
351 RLLAQRLYGG STVKCDYDMV PTTVFTPLEY GCCGLSEEKA VEKTGEENIE
401 VYHSEFWPLE WTVPSRDNK CYAKVICNLK DNERVVGFHV LGPNAGEVTQ
451 GFAAAKCGLT KQOLDSTIGI HPVCAEIFTT LSVTKRSGGD ILQSGCUG
  
```

Figure X-14 MALDI-TOF m/z spectrum of rat TrxR1 standard tryptic digest (above) and screen plot of the MASCOT peptide mass fingerprint search for the corresponding peaks list compared to the theoretical sequence of the protein.



```

Match to: GPX2_RAT Score: 19 Expect: 95
Glutathione peroxidase 2 OS=Rattus norvegicus GN=Gpx2 PE=1 SV=3

Nominal mass (Mr): 22001; Calculated pI value: 8.32
NCBI BLAST search of GPX2\_RAT against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Rattus norvegicus

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 64
Number of mass values matched: 4
Sequence Coverage: 38%

Matched peptides shown in Red

  1 MAYIAKSFYD LSAIGLDGEK IDENTERGRA VLIENVASLI GTTRDYIQL
 51 NELQCRFPRR LVVLGFPCNQ FGHQENCQNE EILNSLKYVR HGGGFQPTFS
101 LTQKCDVNGO NOHPVFAYLK DKLPYPYDDP FSLMTDPKLI IWSPVRRSDV
151 SYNFEKFLIG PEGEPFRFRYS RTFQTINIEP DIKRLKVAI
    
```

Figure X-15 MALDI-TOF m/z spectrum of fraction 2 tryptic digest (above) and screen plot of the MASCOT peptide mass fingerprint search for the corresponding peaks list compared to the theoretical sequence of GPx2.

Conclusions

This study demonstrated that among several protocols for the extraction of Se-species from rat colon samples, Ultra-Turrax disperser is the most suitable device to obtain at the same time good extraction efficiency and Se-species preservation. Bubbling N₂ during the procedure allowed to prevent the most problematic, but often ignored aspect, that is oxidative degradation of selenolic groups, with consequent loss of Se as selenite. A method based on two-dimensional SEC and AE-HPLC-ICP-ORS-QMS allowed to efficiently separate and accurately quantify at least three Se-species present in the extracts, potentially identified as the proteins GPx1, GPx2 and TrxR1. Among them, GPx2 showed to be the most abundant Se protein in rat colon extracts in terms of Se, while TrxR1 was the most abundant at protein level. The developed method is suitable for future studies aimed to compare the Se-proteins pattern in the colon of rats affected by cancer and healthy individuals, in order to investigate the potential role of colon tissue Se-proteins as biomarkers of the disease.

A journal article, in attachment, was published regarding the study presented in this chapter.

X.5. References

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XI. DEVELOPMENT OF METHODOLOGIES FOR SE-PROTEINS SPECIATION IN HUMAN COLON TISSUE

XI.1. Introduction

Four glutathione peroxidase isoenzymes (GPx1, GPx2, GPx3, GPx4), SelP and TrxR1 are expressed in the human colon. Among these Se-proteins, GPx2 is specifically expressed in colon tissue, and it was proposed to constitute a barrier against hydroperoxide absorption. In colorectal adenoma and CRC tissues a decreased expression of GPx1, GPx3, GPx4, and SelP was observed, while expression of GPx2 was increased [1-3]. GPx2 is more resistant to Se deprivation than GPx1 and therefore may compensate for its loss [4-6]. However, GPx1 has a 3-fold higher specific activity than GPx2; this means that an increase in GPx2 at the expenses of GPx1 may result in lower total GPx activity [6]. GPx2 expression has been also shown to be depended on the stage of malignant transformation. In early stages, GPx2 expression increases and is pronouncedly associated with the vesicular structures. In progressed stages of malignancy, the structures disintegrate and GPx2 distribution became more diffuse. These observations support the hypothesis that GPx2 might be involved also in cell growth and differentiation [7].

The data suggest that the quantitative aberrant expression of Se-proteins is important for progression of colon carcinogenesis in humans. More specific information are needed to elucidate these tissue- and cell-specific patterns of Se-proteins as endpoints of Se supply and biological function of these proteins family. mRNA expression, protein expression, or enzyme activity of Se-proteins by Northern blot, Western blot or enzymatic tests are the methods employed until now, but they are poorly quantitative, and their results are not much comparable [2]. New analytical methods, based on quantitative, accurate and precise techniques, are strongly required to achieve unambiguous information regarding Se-proteins quantitative distribution in the cancerous human colon tissue.

XI.2. Goals of the study

This study was aimed to the development of an analytical methodology for the determination of Se-proteins in colon tissue. As second step of the project, after the method was optimized for healthy rat colon samples (see Chapter X), in this work it was transferred to healthy human tissues. Some modifications of the extraction procedure were tested, in order to increase the analytes preservation reproducibility. The separation of Se-species in the extracts was carried out by exploring the potentialities of SEC, AE and AF -HPLC methods. On-line coupling with ICP-ORS-QMS detector and ON-IDA employment, allowed to achieve the absolute quantification of the peaks were isolated. Some preliminary results are presented in this chapter.

XI.3. Experimental

XI.3.1. Instrumentation, reagents and materials

Details regarding the instrumental set-up and reagents used were already exposed in the paragraph X.3. The adopted variations or versions are reported below.

Tissue extractions were carried out by using only the Ultra-Turrax T-8 homogenizer. The SEC column was a TSK-GEL G2000SW with Mw range of 5,000-150,000 Da (Tosoh Bioscience, Stuttgart, Germany). The narrowbore home-made columns (see paragraphs VII.5.1 and X.4.6) were used for 2AF-HPLC.

A Tris-HCl 10 mM solution buffered at pH 7.4 was used for proteins extraction. The extraction buffer was prepared by adding NaCl 25 mM, β -mercaptoethanol (BME), 5 mM and phenylmethanesulfonylfluoride (PMSF) 0.1 % v/v. Protease inhibition cocktail 1% in weight was added just before the extractions. The buffers was store at 4° C and de-gassed with N₂ immediately prior to use.

The proteins: Thyroglobulin (670,000 Da), BSA (66,000 Da), Chicken ovalbumin (44,000 Da), Ribonuclease A (13,700 Da), and Aprotinin (6,500 Da), were purchased from Sigma-Aldrich (Milan, Italy), and mixed in the appropriate proportions to obtain a gel filtration mix for SEC column calibration. Standard pure solutions of TrxR1 from rat liver and GPx1 from human liver were also purchased from Sigma-Aldrich. Enriched ⁷⁷Se was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder, was dissolved in a minimum volume of sub-boiled nitric acid and diluted to volume with ultra-pure water. The concentration of this solution was established by reverse IDA.

XI.3.2. Samples

Human colon samples were obtained from the Bank of Tumor Tissues, Clinical Surgery II, Department of Oncologic and Surgical Sciences at the University Hospital of Padova (Italy), in collaboration with the research group of S. Pucciarelli, MD and M. Agostini, MD. The samples were collected during a biopsy from a portion of healthy mucosa in a patient affected by colon cancer at the right side.

XI.3.3. Procedures

Proteins extraction. Sample aliquots of 0.1 g were placed into a 1.5 mL Eppendorf tube, 0.4 g of extraction buffer (cooled at 4° C) were added and the tubes immediately placed in ice in order to prevent thermal degradation of the species. Extraction was carried out in ice by Ultra-Turrax following the same procedure selected in the study of rat samples (see paragraph X.3.4). After the extraction, samples were centrifuged at 10,000 g, 4° C, for 30 min, the supernatant was transferred to a clean Eppendorf tube and immediately stored at -20° C until analysis.

Separation of Se in the extracts. SEC was performed using the conditions reported in Table XI-1. The SEC column was calibrated by diluting in the mobile phase and

injecting 100 μL of the calibration mixture. The chromatographic profile was monitored by UV detector at 280 nm, and by coupling with the ICP-MS (acquisition of mass 78). The AE chromatographic separation was achieved by injecting 100 μL of the extract and by using the operative conditions also reported in Table XI-1. The elution sequence consists of the following steps: 0-5 min 100 % buffer A; 5-10 min 100 % buffer B; linear gradient to 95 % buffer B, 5 % buffer C at 20 min; linear gradient to 50 % buffer B, 50 % buffer C at 35 min; 35-45 min 100 % buffer C; 45-55 min 100 % buffer A.

Table XI-1 HPLC operating conditions for Se speciation in human colon extracts.

SEC	
Mobile phase	Tris-HCl 10 mM, pH 7.4 + BME 5 mM
Gradient	Isocratic
Flow rate	1 mL min ⁻¹
Injection volume	100 μL
AE	
Mobile phases	A - Tris-HCl 10 mM, pH 8.6 + BME 5 mM B - AmAc 10 mM, pH 7.0 + BME 5 mM C - AmAc 1.5 M, pH 7.0 + BME 5 mM
Flow rate	1 mL min ⁻¹
Injection volume	100 μL

Quantification of Se-species. Se-species concentration in the extract separated by AE was determined by ON-IDA-ICP-MS. For this purpose a ⁷⁷Se-enriched solution of the appropriate concentration was continuously added to the eluate (flow: 5 $\mu\text{L min}^{-1}$) through a T-connection. The ⁷⁸Se/⁷⁷Se isotopic ratio was calculated and corrected as previously mentioned for Se determination by IDA (see paragraph VI.5.1). Plasma conditions and acquisition parameters for HPLC-ICP-MS coupling were those used in the study regarding rat colon extracts (see Table X-1).

Chromatographic method characterization. Intrumental repeatability was estimated for each peak of the AE chromatogram as RSD % of average Se concentration, calculated from 6 consecutive injections of the same extract. Method repeatability was estimated for the same peaks as RSD % of average Se concentration calculated from 6

consecutive injections of independently extracted samples, collected from the same individual. Signal linearity for each isolated Se-species was also assessed. Since GPx1 is the only commercially available among the species of interest, and the peaks were not identified, linearity was assessed by injecting a pooled extract diluted with the extraction buffer (obtaining the 20 %, 60 % and 80 % of the original concentration of the species), the undiluted extract (100 % the of original concentration of the species) and the extract preconcentrated by ultracentrifugation (at 10,000 g, 4° C) in Microcone 10,000 Da cut-off filters (obtaining the 125 % and 150 % of the original concentration of the species). Method linearity was expressed as R^2 of the linear regression.

XI.4. Results and Discussion

XI.4.1. Separation of Se-species by SEC-HPLC

Healthy human colon extracts from the same pooled sample (3 replicates and a blank) were injected undiluted into the SEC systems. The UV chromatogram and the Se mass flow chromatogram obtained for the human extract are shown in Figure XI-1. Six Se-proteins are expected to be present in human colon tissue: GPx1 (homotetrameric, Mw of the monomer 21,946 Da), GPx2 (homotetrameric, Mw of the monomer 21,954 Da), GPx3 (homotetrameric, Mw of the monomer 23,464 Da), GPx4 (homotetrameric, Mw of the monomer 19,526 Da), SelP (monomeric, Mw 41,233 Da) and TrxR1 (homodimeric, Mw of the monomer 54,534 Da) [1]. Since the Mw of the expected proteins ranges in a narrow interval, the column did not allow to isolate different high molecular weight ($M_w > 60,000$ Da) fractions. The same effect was observed for rat colon extracts (see paragraph X.4.4).

Among the expected proteins, the only (human) GPx1 is commercially available as pure standard. Both human GPx1 and rat TrxR1 standard solutions were injected into the SEC-HPLC system, obtaining the Se mass flow chromatograms shown in Figure XI-2.

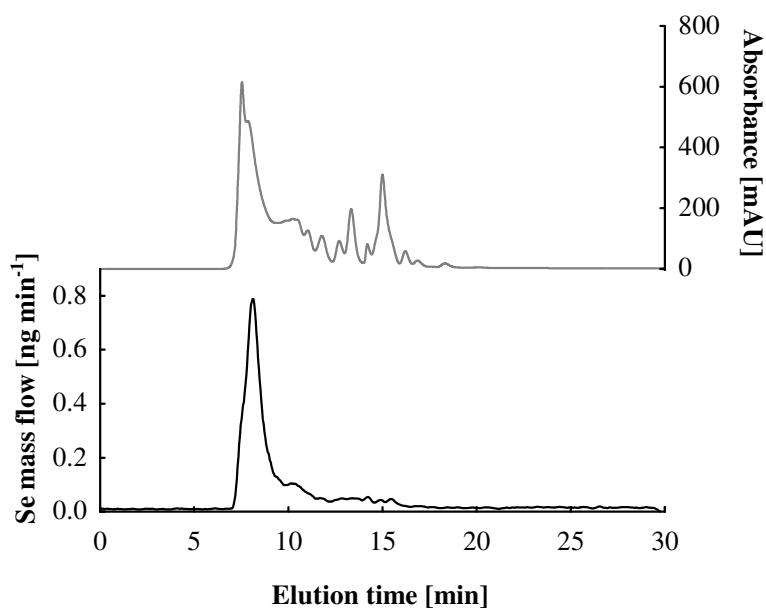


Figure XI-1 UV (at 280 nm) chromatogram (above) and Se mass flow chromatogram (below) of human colon extract obtained by SEC-HPLC-DAD hyphenated to ICP-ORS-QMS.

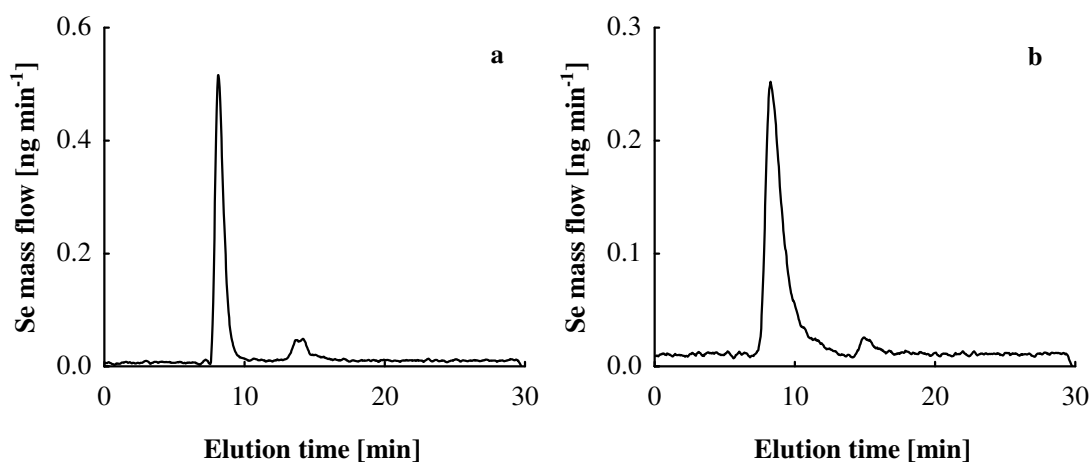


Figure XI-2 Se mass flow chromatograms of human GPx1 (a) and rat TrxR1 (b) obtained by SEC-HPLC-DAD hyphenated to ICP-ORS-QMS.

The SEC column was unable to separate the two standard proteins, despite the theoretical difference in Mw of about 20 kDa. The same behaviour was revealed also for rat colon extracts by using three different SEC columns (see X.4.4).

Similarly to the rat colon extracts separation, also for humans the SEC column allowed to separate the major Se peak from other species uncontainig Se, as shown by the UV chromatogram in Figure XI-1. However, the advantages in second dimension separation reproducibility entailed by the SEC step were compensated by the introduction of BME to extraction solution and mobile phases, therefore SEC was

rejected in this case as first dimension separation. The investigation was subsequently carried out by injecting the human colon extract directly into the AE column.

XI.4.2. Separation of Se-species by AE-HPLC

After optimization of the HPLC program, the injection of undiluted extracts in the AE column resulted in chromatograms like that shown in Figure XI-3.

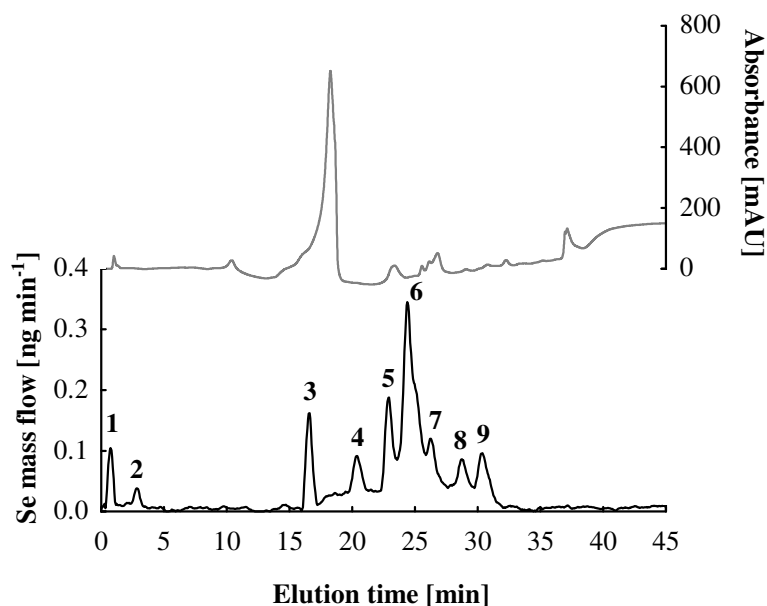


Figure XI-3 UV (at 280 nm) chromatogram (above) and Se mass flow chromatogram (below) of human colon extract obtained by AE-HPLC-DAD hyphenated to ICP-ORS-QMS. The Se species peaks are indicated.

At least 9 peaks of Se-species were separated, more than the expected species. The lack of pure standards did not allow a direct comparison of retention times for a preliminary species identification, a part of the only available standard (GPx1). However, human serum contains other three Se-proteins which are very well known and were broadly investigated also in this Ph.D. project (see Chapters VII-IX). A GPx1 standard solution and undiluted BCR-637 human serum were injected into the AE system, obtaining the mass flow chromatograms in Figure XI-4. The AE-HPLC set-up optimized for human colon extract showed to effectively separate also the Se-proteins in human serum matrix. The concentration of Se-proteins in human serum is known [8]. SeIP accounts ~65 % of total Se in this matrix, therefore the

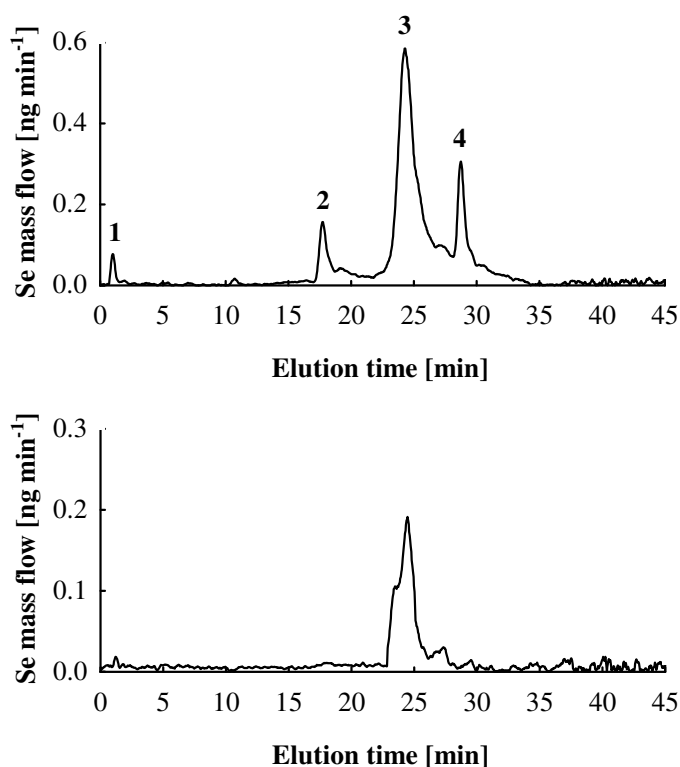


Figure XI-4 Mass flow chromatograms of: a) BCR-637 human serum and b) human GPx1 standard obtained by the AE-HPLC system optimized for human colon extracts, hyphenated to ICP-ORS-QMS. The Se species peaks are indicated.

peak n. 3 in the AE chromatogram was supposed to be this protein. Since GPx3 and SeAlb are present in comparable percentage (~22 %) their identification from quantitative information cannot be possible. Nevertheless, Alb is known to be a highly bounding protein for a large variety of trace metals. By monitoring Mn, Fe, Cu and Zn in the AE chromatographic run, large peaks of these elements were detected in correspondence of the peak n. 2 of Se, but did not at other elution times. Therefore, it can be reasonably supposed that Se peak n. 2 in human serum is due by SeAlb, and peak n. 4 by GPx3. Notably, all the three Se-proteins are retained by the AE stationary phase, while in the 2AF system GPx3 elutes in the void volume (see paragraph VII.3). The retention of GPx3 in AE allows to separate it from the other extremely low-concentration Se-species possibly present in serum. A fourth peak was detected in correspondence of the void volume (peak n. 1). At this stage, no hypothesis can be laid regarding the identity of Se-species present in this peak. Such species account for the ~2.5 % of total Se. An other study detected non-proteic Se-species in human plasma/serum [9], but they were demonstrated to be probably products (selenite) of Se-proteins degradation due to sample storage [10]. The chromatograms in Figure

XI-4 suggested that the peak n. 6 in the AE chromatogram of human colon extract (Figure XI-3) could be due to the co-elution of GPx1 and SelP. The peak n. 3 presents a retention time close to that of SeAlb, which could be due to the presence of this protein in blood residues on the fresh tissue used for the extraction.

XI.4.3. Separation of Se species by AF-HPLC

Similarly to the investigations carried out for rat colon extracts (see paragraph X.4.6), human colon extract was also injected into the 2AF-HPLC system in order to test the possible binding ability of HEP and BLUE stationary phases to the human proteins. Narrowbore 2AF columns were employed in this case, adopting the HPLC program exposed in the paragraph VII.5.1 at p. 171. The chromatogram, shown in Figure XI-5, presents two peaks corresponding to a fraction eluted in the void volume and a fraction retained by the HEP stationary phase.

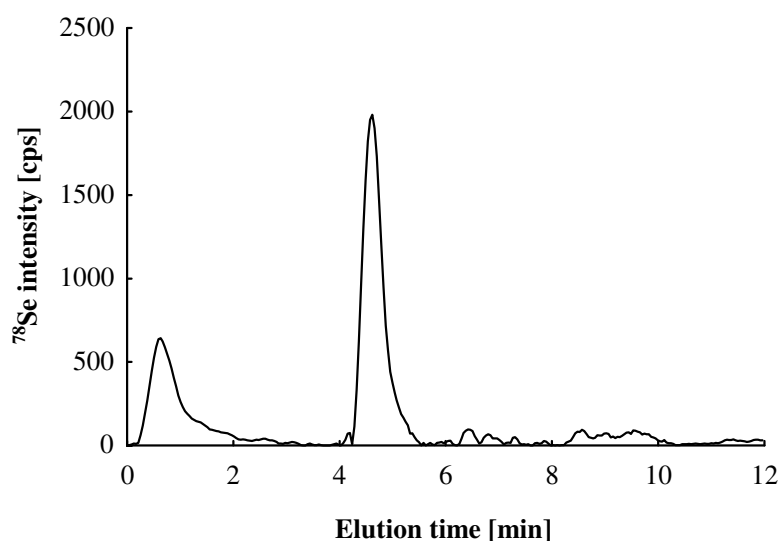


Figure XI-5 Chromatogram of human colon extract obtained by 2AF-HPLC hyphenated to ICP-ORS-QMS.

The only human Se-protein known to be heparine binding is SelP, present also in the human colon extract, but due to the large intensity of the second peak its is reasonable to suppose that other Se-proteins have this property. No other hypothesis can be proposed at this stage concerning the identity of Se-species separated by the HEP column.

XI.4.4. Se-species quantification

The Se peaks concentration in the AE chromatogram of human colon extract, estimated as average of 6 independently extracted samples collected from the same individual, were quantified by ON-IDA, resulting in the values reported in Table XI-2. The concentration ranged from $\sim 1 \text{ ng mL}^{-1}$ to $\sim 15 \text{ ng mL}^{-1}$, with a RSD lower than 12 % also for the less abundant. The major peak was the n. 6, according to the possible presence of SeIP, which is the only Se-protein containing 10 atoms of Se per molecule, instead of 1 like the others.

Table XI-2 Average \pm SE (95 % confidence interval) of Se concentration for the peaks obtained by AE chromatography of human colon extracts.

Peak n.	Se [ng mL^{-1}]
1	4.62 ± 0.38 (3.87 - 5.37)
2	1.16 ± 0.06 (1.28 - 1.03)
3	5.92 ± 0.61 (4.73 - 7.11)
4	2.64 ± 0.22 (3.07 - 2.22)
5	5.71 ± 0.33 (5.06 - 6.37)
6	14.76 ± 1.11 (12.59 - 16.93)
7	1.67 ± 0.10 (1.48 - 1.86)
8	3.33 ± 0.31 (3.93 - 2.73)
9	3.37 ± 0.41 (2.56 - 4.18)
sum	43.19 ± 2.35 (38.58 - 47.80)

XI.4.5. Chromatographic method characterization

The AE chromatographic method was characterized by determine the repeatability of retention times and peaks concentration (instrumental and method repeatability). The results are reported in Table XI-3. Very good repeatability was obtained for all retention times. The instrumental repeatability was $\leq 5 \%$ for peaks n. 1, 2, 3 and 6, and between 10 and 18 % for the other peaks. Method repeatability was comparable with instrumental repeatability for peaks n. 4, 5 and 7, while it was notably higher (from 10 to 20 % higher) for the others. Notably, method repeatability includes not only the sum of all deviation sources in sample preparation, but also possible differences of Se-proteins concentration in the original fresh samples, which were distinct portions of the same tissue. Further investigations should be carried out in order to elucidate the individual role of these two sources of bias.

Table XI-3 Average \pm SE (95 % confidence interval) of peaks retention time for AE chromatography of human colon extracts (n = 12). Instrumental repeatability (n = 6 consecutive injections of the same extracted sample) and method repeatability (n = 6 consecutive injections of independently extracted samples from the same individual) of the method are also reported.

Peak n.	Retention time [min]		Instrumental repeatability [RSD %]	Method repeatability [RSD %]
1	0.75 \pm 0.01	(0.74 - 0.77)	5	20
2	2.59 \pm 0.12	(2.36 - 2.81)	2	13
3	16.47 \pm 0.07	(16.33 - 16.60)	3	25
4	20.40 \pm 0.04	(20.33 - 20.47)	16	20
5	22.91 \pm 0.02	(22.87 - 22.94)	18	14
6	24.43 \pm 0.01	(24.40 - 24.46)	3	18
7	26.38 \pm 0.02	(26.34 - 26.42)	15	14
8	28.71 \pm 0.01	(28.68 - 29.73)	10	23
9	30.29 \pm 0.02	(30.24 - 30.33)	11	30
sum			4	13

Response linearity for peaks concentration was evaluated as exposed above, the graphs are represented in Figure XI-6. Good linearity ($R^2 > 0.98$) was obtained for peaks n. 3, 5, 6, 7, 9 and 10. The peaks n. 4 and 8 had higher variability ($R^2 = 0.91$ and 0.95 , respectively), but still with a clear linear trend. The first two peaks decreased in concentration for the preconcentrated samples, clearly meaning that the species crossed the microcone cut-off membrane, and thus were diluted instead of be preconcentrated. As a consequence, it is possible to establish that the corresponding Se-species have a Mw lower than 10,000 Da.

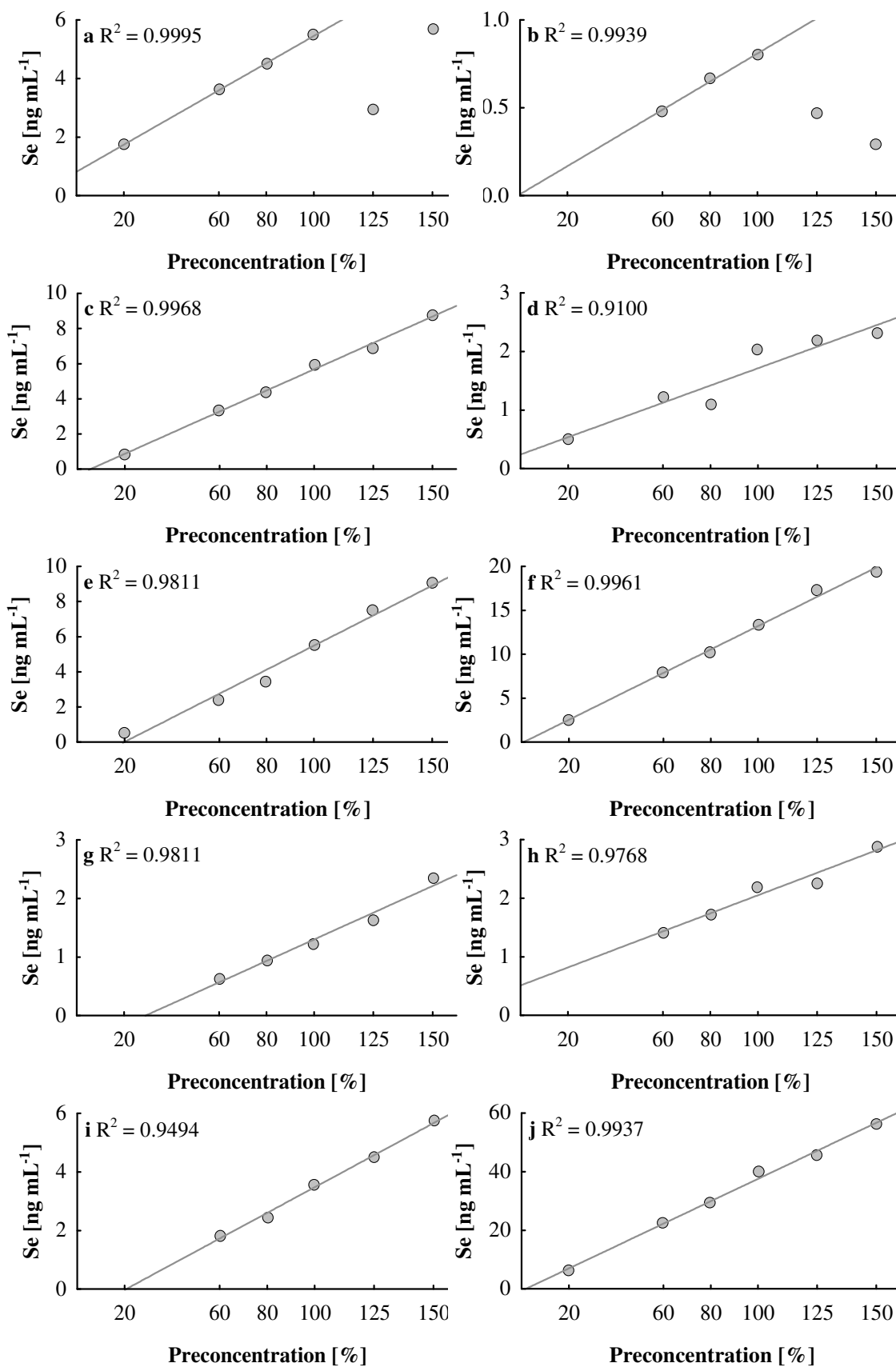


Figure XI-6 Response linearity for human colon extract peaks 1 - 9 (a - i) and their sum (j).

XI.5. Conclusions

In this study a methodology for extraction of Se-species from rat colon tissue was transferred to human samples. Chromatographic SEC, AE and 2AF mechanisms for the separation of Se-species in the extracts were explored. A method based on AE-HPLC, coupled on-line to ICP-ORS-QMS detection, allowed to separate at least nine different Se-species, and was demonstrated to be successfully applicable also for Se-proteins speciation in human serum. The chromatographic separation of colon extracts by 2AE-HPLC showed that in addition to SeIP, probably other Se-proteins present in this matrix are able to bind the HEP stationary phase. The AE chromatographic separation method was characterized in terms of retention times, instrumental and method repeatability. The employment of ON-IDA approach allowed to individually quantify the Se concentration in each peak detected in the AE chromatographic profile.

These preliminary results are very encouraging in the perspective of the future development of a method for the quantitative characterization of complete Se-proteins pattern in human colon samples. The application of the method could be useful to elucidate the role of Se in CRC onset, progression and care.

XI.6. References

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GENERAL CONCLUSIONS

A new analytical method was developed for the speciation of Se-proteins in human serum, based on microbore 2AF-HPLC hyphenated to ICP-SFMS in HR mode using ON-IDA and a high efficiency desolvation interface. The method allowed to perform the simultaneous determination of Se-proteins in a sample volume (5 μ L) twentyfold lower and a time for analysis (7 min) twofold shorter than those required by the previous state of the art. This method could be particularly useful for applications in which the amount of available serum is limited, such as in bio-medical studies based on mouse/rat models, where the amount of blood that can be sampled daily is commonly of a few microliters (≤ 20). An interlaboratory comparison was then carried out to compare the performances of 13 different methods for Se-proteins determination in human serum by 2AF-HPLC-ICP-MS. The results allowed to select the best analytical method available nowadays for this specific task, and provided the first indicative concentration of individual Se-proteins level in a commercially available human serum (BCR-637). Two journal articles, in attachment, were published regarding the studies carried out on method development for human serum samples.

One of the methods previously developed for Se speciation in human plasma/serum was then applied for the comparison of Se-proteins distribution in patients affected by type II diabetes mellitus and healthy controls. Patients with diabetes presented significantly lower levels of GPx3 and SeAlb. Significant negative

correlations were also revealed between these two Se-proteins and clinical parameters including FPG, HbA1c and ACR. A similar study was also performed to assess the serum Se-proteins status in colorectal cancer patients. Again, a significant correlation was observed between SeAlb concentration, early cancer stage (TNM I) and some clinical parameters. Promising information emerged from these results, regarding the association of Se-proteins and the considered diseases. From the case-control design of the studies, it cannot currently be ascertained which is the causal relation between altered Se-proteins status and the diseases. However, our studies demonstrated the high potential of the methods we developed for the investigation of the biochemical relationship between Se and human diseases, and mark the importance to move from total Se to individual Se species determination for the identification and the functional characterization of new biomarkers. A journal article was published regarding the study on type II diabetes.

An other method was subsequently developed for Se speciation in colon samples. Rat samples were used as models for humans in the first stage of the study. Particular attention was dedicated to the optimization of the extraction protocol. Ultra-Turrax disperser resulted the most suitable device to obtain at the same time good extraction efficiency and Se-species preservation. Bubbling N₂ during the procedure allowed to overcome the most problematic, but often ignored aspect, that is loss of Se by oxidative degradation of selenolic groups. A method based on two-dimensional SEC and AE-HPLC-ICP-ORS-QMS was developed to separate and quantify at least three Se-proteins of rat colon, potentially identified as GPx1, GPx2 and TrxR1. Among them, GPx2 resulted the most abundant in terms of Se, while TrxR1 was the most abundant at protein level. The methodology was then transferred to human samples, with opportune modifications. Chromatographic SEC, AE and 2AF mechanisms for the separation of Se-species in the extracts were further explored, and direct AE-HPLC coupled on-line to ICP-ORS-QMS detection was finally selected. The method allowed to separate at least nine different Se-species in the extracts, and resulted effective also for Se-proteins speciation in human serum. Other Se-proteins than SeIP, including rat TrxR1, showed to bind the HEP stationary phase. The study on rat samples, published in a journal article, as well as the preliminary results on the method transfer to human colon, proved that development of methods for the quantitative characterization of complete Se-proteins pattern in tissues is feasible. The

application of these methods could be extremely useful to elucidate the role of Se in the diagnosis, onset, progression and care of colorectal cancer.

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Speciation analysis of selenoproteins in human serum by microbore affinity-HPLC hyphenated to ICP-Sector field-MS using a high efficiency sample introduction system

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Abstract Microbore affinity-HPLC (AF-HPLC) hyphenated to inductively coupled plasma-sector field mass spectrometry (ICP-SFMS) was applied to the simultaneous speciation analysis of glutathione peroxidase (GPx), selenoprotein P (SeIP) and seleno-albumin (SeAlb) in 5 μ L of (human) serum. ICP-SFMS (high resolution mode, HR, $M/\Delta M=10,000$) was used for the quantification of selenium in GPx, SeIP and SeAlb after their separation by AF-HPLC, without prior sample preparation or mathematical correction of the spectral interferences. In order to compensate for the loss in sensitivity due to the detection in HR mode and the very low amount of sample taken for analysis, a high-efficiency sample introduction system was used as an interface between AF-HPLC and ICP-SFMS. This led to a signal-to-noise ratio enhancement of about one order of magnitude compared to the conventional experimental ICP-SFMS set-up. Apart from the very low sample consumption, another major advantage of the method described here is the significant reduction of the analysis

time (≤ 7 min). Quantification of GPx, SeIP and SeAlb was carried out using on-line (post column) isotope dilution; comparison with on-line external calibration by using Se-L-cystine standard is also addressed. The method accuracy for the determination of total protein bound Se was assessed by the analysis of a human serum reference material (BCR-637) certified for total Se content.

Keywords Speciation analysis · Human serum selenoproteins · Microbore affinity chromatography · Inductively coupled plasma-sector field mass spectrometry · On-line isotope dilution

Introduction

Selenium is one of the most widely investigated of all the essential trace elements in the last decades, mainly due to its role in cancer prevention [1]. Most biological functions of Se are mediated by the selenoproteins [2, 3] but compared to low molecular weight selenium species [4, 5], their accurate quantification in biological (human) samples is still an analytical challenge because of their low concentration and the very high complexity of such biological matrices, which interfere considerably in the analytical process.

Although a relatively high number (~25) of selenoproteins were identified in humans, the ones present in serum are commonly determined for the assessment of the selenium status in healthy individuals or in correlation with various health disorders. In (human) serum, selenium is incorporated (as selenocysteine, SeCys) into two selenoproteins, namely glutathione peroxidase (GPx, isoform 3) and selenoprotein P (SeIP). Selenoalbumin (SeAlb), which is a seleno-containing protein, is also formed in human

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serum/plasma by random replacement of methionine (which is a constituent of albumin, HSA) by selenomethionine (SeMet).

The mostly used methods for speciation analysis of GPx, SelP and SeAlb in human serum involve their separation by affinity-HPLC (AF-HPLC) coupled on-line with inductively coupled plasma-mass spectrometry (ICP-MS) [6–9] and to a less extent anion exchange [6, 10] or size exclusion-HPLC (SE-HPLC) [11, 12], which suffer from poor column recovery and/or low separation efficiency. Micro-affinity HPLC coupled with low flow ICP-MS in submicrolitre samples of human plasma has also been reported [8, 13] but this method is limited to the measurement of a single selenoprotein (SelP) only. Although generally, patient plasma/serum is available in a volume range of a few mL, hence analytical miniaturization is not necessary in this respect, the development of methods with a very low sample consumption is important for studies where the amount of sample is limited. This is the case of bio-clinical studies using mice models, where an amount of blood of only 20 μL can be sampled daily (<http://www.nc3rs.org.uk/bloodsamplingmicrosite/page.asp?id=391>), in order to avoid the perturbation of its haematological status.

The main drawback of AF-HPLC arise from the poor retention of GPx and hence its co-elution with non retained species, such as Cl^- and Br^- (present at high levels in serum), which lead to serious spectral interferences, such as $^{40}\text{Ar}^{37}\text{Cl}$ on ^{77}Se , $^{79}\text{Br}^1\text{H}$ on ^{80}Se and $^{81}\text{Br}^1\text{H}$ on ^{82}Se . Alleviation of these interferences can be performed by off-line serum clean-up using (anion exchange) solid phase extraction (SPE) [7] and multi-affinity media for removal of interferences [14], or on-line resolving of selenoproteins from Br/Cl by two dimensional HPLC separation employing anion exchange (AE) AF-HPLC (AE-AF-HPLC) [9] before ICP-MS detection. However, these approaches introduce additional steps in the analytical process hence increasing the analysis time and the number of uncertainty sources.

Apart from the interference of Br and/or Cl on GPx determination, a drawback of AF-HPLC is the co-elution (in the void fraction) of GPx with inorganic (or other low molecular-mass) Se species possibly present in the serum. Nevertheless, it is worth noting that in serum from healthy people (without selenium supplementation) only Se(IV) was detected apart from selenoproteins [12]. Additionally, the total level of Se species other than selenoproteins in control human serum does not exceed 5% of the total Se level [15]. Therefore, the determination of Se in the GPx fraction separated by AF-HPLC approximates well the genuine level of GPx.

Collision/reaction cell ICP-Quadrupole MS (CRC-ICP-QMS) has also been successfully applied to the quantifica-

tion of serum selenoproteins following AF-HPLC separation [6]. The main advantage of CRC is the alleviation of Ar_2^+ dimers, which interfere with ^{80}Se (the most abundant Se isotope) but addition of H_2 in the CRC ICP-QMS leads to the formation of hydrides that overlap with several Se isotopes, as following: $^{76}\text{Se}^1\text{H}$ on ^{77}Se , $^{77}\text{Se}^1\text{H}$ on ^{78}Se , $^{81}\text{Br}^1\text{H}$ on ^{82}Se and most critically, the formation of $^{79}\text{Br}^1\text{H}$, which interfere on the determination of ^{80}Se . Although the chromatographic step generally addresses the latter problem, in the particular case of AF-HPLC this is not possible because of the co-elution of GPx and Br (in the void fraction). Therefore, when applied to the analysis of human serum, which contains elevated amounts of Br (at $\mu\text{g mL}^{-1}$ level), the CRC cannot resolve the severe spectral interferences mentioned before. A common way to deal with these interferences is the use of correction equations, as described in detail elsewhere [7, 16] but this approach introduces inherently an additional uncertainty in the final result but also an additional step in the analysis (the assessment of the correction factor). The determination of selenium in serum can also be carried out by CRC ICP-QMS with Xe as collision gas [17], but this approach is limited for routine analysis because of the elevated cost of the high purity Xe.

The unique approach theoretically allowing interference-free quantification of selenoproteins without additional sample preparation and/or mathematical corrections relies on the spectral resolving of interferences by ICP-sector field-MS (ICP-SFMS) in high resolution (HR) mode ($M/\Delta M=10,000$). Nevertheless, this approach leads to a significant loss in sensitivity, which is a critical point in trace analysis, particularly when the sample availability is limited. Increase in sensitivity can be achieved by using micronebulizer/desolvator systems for the efficient introduction of the HPLC eluent into the ICP-MS, which also allows the analysis of very low amounts of sample [18].

This paper focuses on the development of a new analytical method for the simultaneous speciation analysis of GPx, SelP and SeAlb in human serum on the basis of microbore AF-HPLC hyphenated to ICP-SFMS. The method described here is a follow-up of the procedures developed previously for the simultaneous speciation analysis of GPx, SelP and SeAlb in human serum [7, 9]. Nevertheless, to authors' knowledge, this is the first application of microbore AF-HPLC and ICP-SFMS for the speciation analysis of selenoproteins in human serum. Compared to the previous work, the separation has been miniaturized in order to decrease the sample consumption and in addition, a sample preparation step was not necessary for removing the Cl and Br based interferences due to the use of ICP-SFMS. By increasing the linear velocity of the mobile phase, a significant reduction of the analysis time (<7 min) was achieved. In order to compen-

sate for the loss in sensitivity due to the high-resolution mode and the very low amount of sample taken for analysis, a high-efficiency sample nebulisation/desolvation system (APEXTM) was used as an interface between the AF-HPLC and ICP-SFMS.

Quantification of selenoproteins was carried out using on-line (post column) isotope dilution (ID) and its comparison with external calibration (EC) by using Se-L-cystine (SeCys) standards is also addressed. The method accuracy for the determination of total protein bound Se was assessed by the analysis of a human serum reference material (BCR-637) certified for total Se content.

Experimental

Reagents and samples

All reagents used were of at least analytical-reagent grade purity and the solutions were prepared gravimetrically in a class 1,000 clean room under a class 100 laminar flow bench. Details regarding the standards and reagents used in this work can be found elsewhere [9]. A control serum obtained from a single healthy volunteer was used throughout for the method optimization. Because of the low level of GPx and SeAlb but also to prevent the denaturation of selenoproteins, the serum was injected onto the HPLC columns without dilution.

Instrumentation

An 1100 Series HPLC system from Agilent Technologies Deutschland GmbH (Waldbronn, Germany, <http://www.home.agilent.com/agilent/home.jsp>) was used for the separation of the selenoproteins. The AF-HPLC system consisted of two affinity columns (5 cm length × 1.0 mm internal diameter, id), namely Heparin-Sepharose (HEP) and Blue-Sepharose (BLUE) in-house packed with the stationary phases of the corresponding commercial columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden, <http://www.gehealthcare.com/eucu/>). A switching valve module Microneb 2000 Direct Injection Nebulizer (Cetac Technologies Inc, Omaha, Nebraska, USA, <http://www.cetac.com/>) was used for the (automated) exclusion of the BLUE column from the AF-HPLC system.

An Element2 (Thermo Scientific, Bremen, Germany, <http://www.thermo.com/>) ICP-SFMS was used for the on-line determination of the content of Se in GPx, SeIP and SeAlb using the optimum parameters given in Table 1. The detector dead time was optimized using the ICP-MS manufacturer's recommendations (values around 20 ns were commonly obtained). Optimization of the APEX ICP-SFMS system was carried out daily using a solution

of 1 ng mL⁻¹ In and 5 ng mL⁻¹ Se; intensities up to 8–10 × 10⁶ and 8 × 10⁵ cps were usually obtained for ¹¹⁵In and ⁷⁸Se, respectively, in low resolution, whereas in HR mode, the sensitivity dropped to ~10⁵ (¹¹⁵In) and ~5,000 (⁷⁸Se) cps.

An APEXTM Q from Elemental Scientific Inc. (Omaha, USA, <http://www.icpms.com/>) was used for the introduction of the AF-HPLC eluent into the ICP-SFMS instrument. The APEXTM Q is a fully integrated inlet system for ICP-MS that connects directly to the torch injector and incorporates a standard MicroFlow PolyPro-ST nebulizer (Elemental Scientific Inc.).

A PEEK T was used to mix the AF-HPLC effluent with the ⁷⁷Se spike solution (2 ng g⁻¹) pumped by a solvent delivery system (S1100 type, Sykam GmbH, Eresing, Germany). In order to ensure equilibration of the AF-HPLC eluent with the ⁷⁷Se spike solution, a custom-made knotted reactor of 100 cm linear length of Teflon tubing (0.25 mm id) was used as a transfer line between AF-HPLC and the APEX ICP-SFMS.

Analytical procedures

Speciation analysis of GPx, SeIP and SeAlb in human serum by microbore AF-HPLC hyphenated to APEX ICP-SFMS

Separation of selenoproteins by AF-HPLC was carried out using an experimental set-up [6, 7] exploiting proteins' selectivity towards HEP and BLUE stationary phases, whose chemical composition/structure is described elsewhere [19]. Briefly, the HEP stationary phase retains selectively SeIP whereas the BLUE affinity media retains both SeIP and SeAlb. Given that GPx is retained neither by HEP nor by BLUE, separation of GPx, SeIP and SeAlb is therefore achieved. However, truly on-line use of HEP and BLUE columns for the separation of GPx, SeIP and SeAlb is not possible. If they are directly connected on-line (in this order), the elution of SeIP from the HEP column loads the BLUE column with elution buffer and hence SeIP is not retained anymore onto this column (it co-elutes with SeAlb). The only feasible approach to separate GPx, SeIP and SeAlb in the same analytical run using HEP and BLUE affinity media relies on the exclusion of the BLUE column from the chromatographic system (by means of a switching valve) during the elution of SeIP from the HEP column, followed by the re-insertion of the BLUE column back into the system for the consequent elution of SeAlb (retained on this column).

The elution sequence used in this work was as follows: for 0–1 min, solution of ammonium acetate 0.05 mol L⁻¹ (buffer A) was passed through the system (elution of GPx), while the switching valve was in the

Table 1 Optimum operating conditions for the microbore AF-HPLC-APEX ICP-SFMS system

<i>HPLC conditions</i>		
AF-HPLC columns	Hitrap Heparin-Sepharose (HEP) and Hitrap Blue-Sepharose (BLUE): 5 cm×1.0 mm id×34µm	
Injection volume	5 µL	
Flow rate	0.3 mL min ⁻¹	
Elution mode	Step gradient	
Mobile phase	Binding buffer (A): CH ₃ COONH ₄ 0.05 mol L ⁻¹ (pH~7) Elution buffer (B): CH ₃ COONH ₄ 1.5 mol L ⁻¹ (pH~7)	
<i>APEX ICP-SFMS parameters and data acquisition</i>		
RF power	1,550 W	
Nebulizer flow rate	1.0 ± 0.2 L min ⁻¹ (depending on daily optimization)	
Additional gas (Ar) (introduced just before the plasma torch by means of a T connector)	0.15 ± 0.05 L min ⁻¹ (depending on daily optimization)	
Auxiliary gas flow rate	1.0 L min ⁻¹	
Plasma gas flow	16 L min ⁻¹	
APEX spray chamber temperature	140 °C	
APEX cooler temperature	2 °C	
Detection mode	Counting	
Integration type	Average	
Isotopes monitored	⁷⁷ Se, ⁷⁸ Se, ⁸² Se	
	High resolution (M/ΔM=10,000)	Medium resolution (M/ΔM=4,000)
Run/passes	200/1	250/1
Mass window	125	125
Samples per peak	20	20
Search window	60	50
Integration window	60	60

load position (HEP and BLUE columns connected on-line). At 1 min the valve is switched to the inject position (the BLUE column is excluded from the system) and kept in this position up to 5 min while solution of ammonium acetate 1.5 mol L⁻¹ (buffer B) is passed through the microbore HEP column for the elution of SeIP. At 5 min the valve is switched back in the load position (the BLUE column is reintroduced into the AF-HPLC system) and during 1 min buffer B is passed through the system for the elution of SeAlb from the BLUE column. Finally, buffer A is pumped through both columns for one more minute for their re-conditioning before a new separation takes place.

For quantification by using EC, standards of SeCys with a concentration range between 5–100 ng mL⁻¹ (prepared in buffer A) were injected into the AF-HPLC system (with both HEP and BLUE columns connected).

Quantification of selenoproteins by on-line ID was carried out by adding post-column (using a T-connector) a solution of ⁷⁷Se spike (2 ng g⁻¹) at a constant flow rate of 0.15 mL min⁻¹ by means of a solvent delivery system. Before peaks integration, the chromatograms were smoothed by adjacent averaging ($n=5$).

Results and discussion

Microbore AF-HPLC separation of selenoproteins in human serum

The separation of GPx, SeIP and SeAlb was performed using a double column AF-HPLC system exploiting proteins' selectivity towards the HEP and BLUE stationary phases as described before. It is for the first time that in-house made microbore HEP and BLUE columns were used for this purpose and hence their chromatographic features such as peak shape and analytes' recovery was investigated, as described in the next paragraphs.

Determination of the columns capacity

The capacity of the microbore AF-HPLC columns used in this work was assessed by analyzing volumes of serum (or equivalent) in the range 1–15 µL. A sample loop of 5 µL was employed for this study and the volumes of 1 and 2.0 µL investigated here correspond to 5 and 2.5 fold, respectively, dilution of the serum just

before analysis (loops with volumes of 1 and 2.0 μL introduce a high uncertainty when a conventional manual injection valve is used). A linear response was obtained for volumes of serum up to 10 μL . A 5 μL sample loop was used for further experiments, as a compromise between the minimum sample consumption and ensuring sufficient sensitivity for accurate speciation analysis of GPx, SelP and SeAlb by microbore AF-HPLC-APEX ICP-(high resolution) SFMS.

Determination of the column recovery factor of selenoproteins

The chromatograms obtained by microbore HEP and BLUE AF-HPLC columns individually are shown in Fig. 1a and b, respectively. When using the HEP column alone two peaks are obtained, the first one is attributed to GPx+SeAlb (not retained, eluted with the void volume by means of the binding Buffer A) and the second for SelP (elution with buffer B). Two peaks are also obtained by using the BLUE column solely but one peak corresponds to GPx and one for SelP+SeAlb (Fig. 1b).

Because of the lack of selenoproteins standards (with known level of Se) and also due to the difference in properties of the existing molecular standards of GPx and SeAlb compared to the corresponding serum proteins, the assessment of the recovery of each individual selenoprotein from the HEP and BLUE columns was not possible in this study. Consequently, the overall column recovery of all selenoproteins was determined by comparison of the total AF-HPLC peak area (GPx+SelP+SeAlb) with that of the peak obtained by analysis of the same amount of serum in flow injection (FI) mode (no column used).

It is worth noting that enhanced sensitivity is commonly obtained when introducing elevated carbon concentration into the plasma [7, 9, 20] and hence, in order to avoid discrimination between signals obtained with different elution (Buffer A and B), the recovery factor was calculated using only buffer B, both in chromatographic and FI modes. A recovery of $\sim 100\%$ was obtained for GPx+SelP+SeAlb from HEP, whereas only $\sim 80\%$ of selenoproteins was recovered from the BLUE column. The latter result is most likely the consequence of the low recovery of SelP from the BLUE column, as explained further. As can be seen in Fig. 1b, the intensity of the second peak (SelP+SeAlb) is lower compared to the second peak in Fig. 1a (SelP only), which is an indication of a low recovery of SelP from the BLUE column. In order to prove this assumption, the recovery factor of SelP from the BLUE column was calculated by rationing the intensity of SelP obtained from the BLUE column to that obtained from the HEP column (assuming a

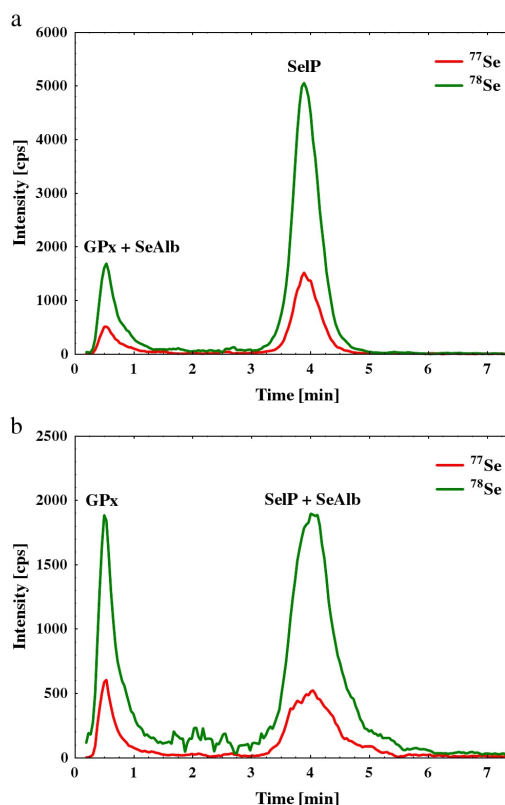


Fig. 1 Chromatograms obtained for the analysis of 5 μL of human serum by microbore HEP (a) and BLUE (b) AF-HPLC hyphenated to APEX ICP-SFMS in HR (the more intense peak corresponds to ^{78}Se)

quantitative recovery of SelP from the HEP column), as following:

$$R_{\text{SelP, BLUE}}(\%) = \frac{\text{SelP}_{\text{BLUE}}}{\text{SelP}_{\text{HEP}}} \times 100 \quad (1)$$

where SelP_{HEP} is the 2nd peak in Fig. 1a and $\text{SelP}_{\text{BLUE}}$ is calculated using Eq. (2) and assuming that $[\text{GPx}]_{\text{HEP}} = [\text{GPx}]_{\text{BLUE}}$ and $[\text{SeAlb}]_{\text{BLUE}} = [\text{SeAlb}]_{\text{HEP}}$.

$$\text{SelP}_{\text{BLUE}} = [\text{GPx}]_{\text{BLUE}} + [\text{SelP} + \text{SeAlb}]_{\text{BLUE}} - [\text{GPx} + \text{SeAlb}]_{\text{HEP}} \quad (2)$$

Please note that the symbols of selenoproteins (GPx, SelP and SeAlb) in Eq. 1–2 represent peaks area. A recovery of $\sim 65\%$ of SelP from the BLUE column was calculated using Eq. (1), which confirms the $\sim 80\%$ average recovery of SelP+SeAlb from the BLUE column. It should be underlined that in practice, the BLUE column cannot be used for the separation of SelP, because of the

low recovery provided for this protein. Nevertheless, in the particular case of double column AF-HPLC system, the BLUE column is set-off before the elution of the SeIP from the HEP column (SeIP does not pass through the BLUE column), hence allowing its use for the simultaneous determination of GPx, SeIP and SeAlb.

Optimization of nebulization/detection conditions of APEX ICP-SFMS

Effect of nitrogen make-up gas for the APEX system introduction

Preliminary experiments were performed using nitrogen as a make-up gas for the APEX device, as recommended by the manufacturer, but decrease in sensitivity more than 5 fold was noticed for Se, most likely due to the Ar-N₂ mixed plasma obtained in this case. Also, mass discrimination between isotopes was noticed e.g., the ratio ⁸²Se/⁷⁷Se varied from ~1.1 (theoretical value is 1.1435) up to ~5. Argon was also used instead of nitrogen as make-up for APEX and no signal suppression occurred; however slight increase in imprecision between consecutive replicates was noticed and hence further experiments were carried out without any make-up gas addition into the APEX device.

Influence of methanol addition in the HPLC eluents on the nebulization/detection efficiency

The experiments were first carried out by using ammonium acetate with concentrations of 0.05 (for binding) and 1.5 mol L⁻¹ (for proteins' elution), respectively, as used in previous studies [7, 9]. The effect of addition of methanol to both buffers was also investigated in order to improve the efficiency of nebulization/detection of the APEX ICP-SFMS system. For this purpose, buffers A and B were prepared in methanol with concentrations ranging from 1 to 20% (v/v) (introduction of methanol over 15% extinguished the plasma). Maximum sensitivity enhancement of ~10% was obtained by using binding/elution buffers prepared in 5% (v/v) methanol. Nevertheless, poor precision between consecutive replicates was noticed when using binding/elution buffers prepared in methanol and hence, for further experiments, buffers A and B prepared in pure water were used.

ICP-SFMS detection. Forward power optimization

Apart from the parameters that were daily optimized for optimum plasma conditions, the forward power (RF) was the main factor influencing the signal intensity of selenoproteins. No study regarding the optimum RF power for selenoproteins speciation analysis was reported so far in the

literature. In this work, the influence of the RF power on the intensity of selenoproteins signal was evaluated in the range between 1,100 and the maximum achievable (1,600 W). The ionization efficiency for GPx, SeIP and SeAlb increased over the entire range investigated; further experiments were carried out using an RF power of 1,550 W, in order to ensure maximum sensitivity while avoiding the use of the ICP-SFMS instrument at its maximum RF power settings.

A chromatogram obtained by microbore AF-HPLC hyphenated to ICP-SFMS (HR) by means of the APEX is shown in Fig. 2a; in addition, a chromatogram obtained by using a conventional hyphenation using a double-Scott spray chamber cooled at 2 °C is shown in Fig. 2b. As can be seen, an increase in sensitivity of more than one order of magnitude is obtained using the APEX as an interface between microbore AF-HPLC and ICP-SFMS.

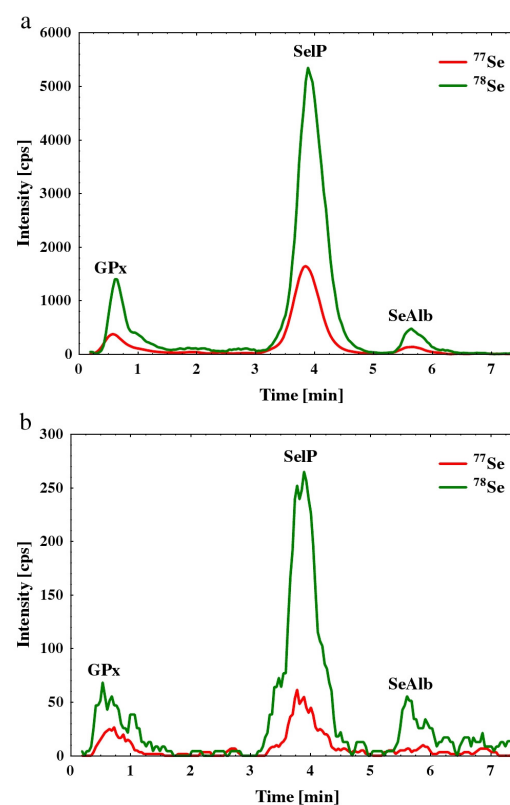


Fig. 2 Typical chromatograms obtained for ⁷⁷Se and ⁷⁸Se by microbore AF-HPLC hyphenated to ICP-SFMS (HR) using the APEX system (**a**) and using a conventional spray chamber system (**b**) (the more intense peak corresponds to ⁷⁸Se)

Table 3 Concentrations (average \pm standard deviation, $n=3$) of Se in GPx, SelP, SeAlb and total protein-bound Se (GPx + SelP + SeAlb) in BCR-637^a obtained by microbore AF-HPLC coupled to APEX ICP-

SFMS in medium resolution mode using on-line ID (⁸²Se/⁷⁷Se ratios) and EC (SeCys standard, ⁷⁷Se, ⁷⁸Se and ⁸²Se)

Protein	Se [ng mL ⁻¹]					
	On-line ID ^b		EC			
	AF-HPLC-ICP-SFMS	AE-AF-HPLC-ICP-QMS ^c	AF-HPLC-ICP-SFMS		AE-AF-HPLC-ICP-QMS ^c	
	[⁸² Se/ ⁷⁷ Se]	[⁸² Se/ ⁷⁷ Se]	[⁷⁷ Se]	[⁷⁸ Se]	[⁸² Se]	[⁸² Se]
GPx	18 \pm 1	13 \pm 1	18 \pm 1	19 \pm 3	18 \pm 1	13 \pm 1
SelP	61 \pm 3	65 \pm 5	62 \pm 9	62 \pm 8	61 \pm 7	59 \pm 1
SeAlb	13 \pm 1	14 \pm 1	9 \pm 1	10 \pm 1	8.7 \pm 0.3	16 \pm 1
Total protein bound Se	92 \pm 3	92 \pm 5	89 \pm 9	91 \pm 9	88 \pm 7	88 \pm 2

^a total Se certified: 81 \pm 7 ng mL⁻¹ (certified value \pm expanded uncertainty, coverage factor $k=2$)

^b measurement of ⁷⁸Se in MR was not possible because of the insufficient resolving power to separate ⁷⁸Se from the ⁴⁰Ar³⁸Ar interference

^c two dimensional HPLC separation employing anion exchange (AE) and AF-HPLC [9]

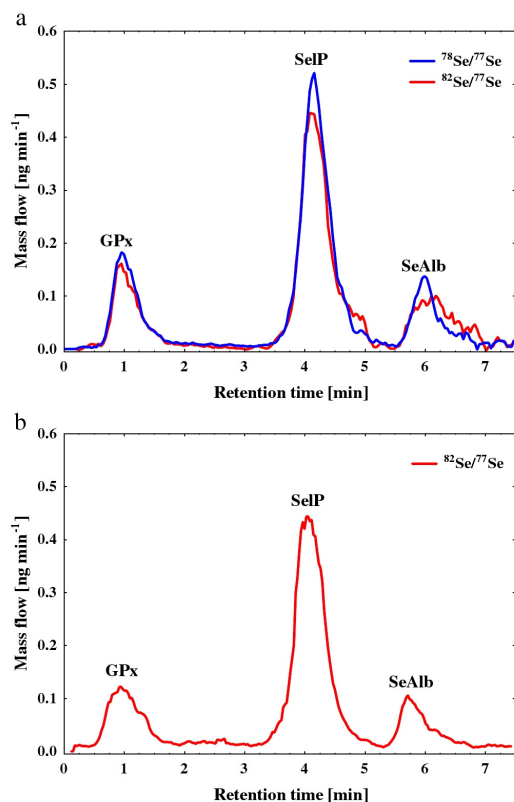


Fig. 3 Typical mass flow chromatograms (addition of 2.0 ng mL⁻¹ spike solution of ⁷⁷Se at a constant flow rate of 0.15 mL min⁻¹) obtained for the analysis of BCR-637 CRM by microbore AF-HPLC and APEX ICP-SFMS in HR (a) and MR (b) (the slightly more intense profile in graph a corresponds to ⁷⁸Se/⁷⁷Se ratio)

results obtained by a different laboratory could be found in the literature for methods intercomparison. Unless more data in terms of selenoproteins levels in the same commercially available serum materials are provided by different laboratories, evaluation of methods accuracy is extremely difficult.

EC led also to a significant positive bias for total protein bound Se, GPx and SeAlb (see Table 2) when the detection was carried out in HR. The latter results are not completely understood yet, but most likely EC fails in the accurate assessment of the response factor of Se in HR (with considerably lower sensitivity) because of the strong influence of the high carbon content of proteins in the ionization efficiency, whereas this effect is compensated by on-line ID. Conversely, the results obtained by EC in MR mode are comparable with those obtained by on-line ID (either in HR and MR), which indicates that the APEX could be capable to alleviate the spectral interferences originating from Cl and Br. Work is undergoing to test the efficiency of the APEX system as a sample introduction for the AF-HPLC eluent into a conventional ICP-QMS (without CRC), which is known to seriously suffer because of the Cl and Br spectral interferences on Se isotopes. This could be useful for clinical laboratories which only have an ICP-QMS without collision cell or lack an expensive ICP-SFMS (high resolution) instrument.

Analytical performance characteristics

Repeatability (relative standard deviation, R.S.D.,%) for 7 successive injections of 5 μ L un-diluted control serum was calculated either from the mass flow (⁷⁸Se/⁷⁷Se) and intensity (⁷⁸Se) chromatograms. R.S.D of 5% for GPx, 6% for SelP and 7% for SeAlb was obtained from the mass flow chromatogram measurements and it was comparable

with that calculated from the peak area of the intensity chromatograms (5% for SeIP and 7% for GPx and SeAlb).

The assessment of data correlation was carried out by measuring SeCys standards with concentration up to 250 ng mL⁻¹ (at 10 different levels); the calibration graph was linear with a correlation coefficients >0.999.

A method detection limit of 2.5 ng mL⁻¹ was calculated from the standard deviation of the y-intercept (divided for the slope) [22] of a calibration curve obtained by analyzing SeCys in the range 10–100 ng mL⁻¹ using the same experimental set-up as for the determination of selenoproteins.

Conclusions

This study demonstrates that microbore AF-HPLC hyphenated to ICP-SFMS using on-line ID is a promising tool for interference-free determination of selenoproteins in only 5 µL of human serum; it ensures the shortest analysis time reported so far for the simultaneous determination of GPx, SeIP and SeAlb. No correction equations or additional sample preparation steps were necessary but a high-efficiency sample desolvation/introduction system should be used to compensate for the loss in sensitivity when HR is employed. This method could be particularly useful when the amount of serum is limited, such as bio-medical studies using mice models, where the amount of blood that can be sampled daily is commonly of a few microliters (≤20).

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GENERAL PAPER

Challenges in the accurate speciation analysis of selenium in humans: first report on indicative levels of selenoproteins in a serum certified reference material for total selenium (BCR-637)

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Abstract Se is one of the most investigated essential trace elements in the past years, mostly due to its cancer prevention properties. Nevertheless, the accurate determination of its biologically active species, such as the selenoproteins (SeProt) in human serum, is currently a challenging task. This is because of the lack of appropriate quantification standards, certified reference materials (CRMs), and/or reference measurement methods. Additionally, most of the methods developed so far for the determination of SeProt were applied to the analysis of control (volunteers) serums, which are not available to other laboratories, therefore making methods inter-comparison virtually impossible. We present here for the first time indicative levels of SeProt in a commercially available human serum, namely the BCR-637 CRM with certified level of total Se. The concentrations of selenium associated with glutathione peroxidase (GPx), selenoprotein P (SeIP) and selenoalbumin (SeAlb) in this serum were calculated using the results obtained by 13 different analytical methods (literature and non-published data) on the basis of (affinity) high-performance liquid chromatography (AF-HPLC) coupled to inductively coupled plasma-mass

spectrometry (ICP-MS). The indicative levels of SeProt in the BCR-637 serum can be used for validation of methods dealing with the determination of these proteins in human serum.

Keywords Human serum · Certified reference material · BCR-637 · Selenoproteins · Indicative concentrations

Introduction

Selenium (Se) is an essential trace element, which plays a fundamental role for human health [1]. Although originally Se was regarded as toxic and even carcinogenic [2], the perception about this element changed radically in the past decades. The most important biomedical property of Se derives from its prevention effect against some forms of cancer [3], but it appears that adequate alimentary Se supply has also a beneficial role in prevention of several other diseases [4].

In the past years it has been revealed that most biochemical functions of Se in humans are mediated by selenoproteins (SeProt), where it is incorporated in the form of the selenocysteine (SeCys), which is now considered the 21st amino acid (required for life). The assessment of the Se status in humans is therefore important, and hence the development of reliable analytical methodologies for the determination of Se (bio)species, particularly SeProt, is currently an urgent demand.

The mostly used bio-indicator for the assessment of the Se status in humans is the serum/plasma, which contains two important SeProt, namely the glutathione peroxidase (GPx) and selenoprotein P (SeIP). The synthesis of GPx and SeIP is genetically encoded, and hence these Se species are regarded as 'real' SeProt. Apart from GPx and SeIP, a

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fraction of Se is incorporated into the serum albumin (HSA), forming selenoalbumin (SeAlb) by means of the replacement of the (sulfur) methionine (component of HSA) with selenomethionine (SeMet) commonly originating from the diet. SeAlb is not considered a 'real' SeProt because its production is not genetically encoded, as it is the case of GPx and SelP. Nevertheless, SeAlb could be used as a biomarker for the assessment of the Se status, particularly correlated with nutrition [5].

Accurate determination of SeProt in human serum is currently an analytical challenge. Because of their low concentration and the complexity of the serum matrix, the SeProt determination requires very sensitive and selective analytical approaches. It is worth underlining that given the biomedical importance of Se (as SeProt), the number of publications in terms of development of reliable analytical methods for SeProt determination is extremely scarce.

The most critical issue in terms of SeProt determination in human serum is the method validation. At present, this is virtually impossible because of the lack of 'speciated' serum materials with certified or at least indicative levels of SeProt. Furthermore, apart from the authors' work in this field [6–9], the other published methods dealing with the determination of SeProt have been applied to the analysis of control (volunteers) serum samples rather than serums commercially available. This limits drastically their usefulness from a metrological point of view because such (control) serums are rarely available to other laboratories and hence methods inter-comparison is impossible. Therefore, the development of reference measurement methods for SeProt determination in human serum and/or producing human serum reference materials with at least indicative levels of SeProt is currently a real demand.

We report here for the first time indicative concentrations for GPx, SelP, and SeAlb (in terms of Se) in the human serum BCR-637 (that is commercialized by the Institute for Reference Materials and Measurements, Geel, Belgium, total Se certified concentration = $81 \pm 7 \text{ ng mL}^{-1}$) by compiling the results obtained by using 13 different analytical methods, which were already published [6–9] or are part of unpublished works. This study could be particularly useful for the validation of newly developed methods for the determination of SeProt in human serum or as a basis for (preliminary) inter-laboratory comparisons.

Determination of selenoproteins in human serum: an analytical challenge

It is well known that the proteins are macromolecular compounds, synthesized by means of 21 amino acids only [10]. Two approaches are commonly used for the identification/quantification of proteins (including SeProt), either by

measuring the proteins with their macromolecular chain intact or by breaking them down up to the aminoacids level, followed by their quantification. Both approaches are discussed for the particular case of SeProt in the following paragraphs.

Determination of intact selenoproteins

Determination of intact SeProt (without breaking down the macromolecular chain) is commonly carried out by their separation using high-performance liquid chromatography (HPLC) and further detected (as Se) using inductively coupled plasma-mass spectrometry (ICP-MS) [11, 12]. More details regarding these techniques (HPLC and ICP-MS) and their application for the determination of bio-species can be found elsewhere [13–16].

The most efficient HPLC separation of intact serum SeProt is obtained using a double-column AF-HPLC system exploiting protein selectivity towards the heparin (HEP) and BLUE (commercial names) stationary phases [11], whose chemical composition/structure is described elsewhere [12]. Briefly, the HEP stationary phase retains selectively SelP, whereas the BLUE affinity media retains both SelP and SeAlb. Given that GPx is retained neither by HEP nor by the BLUE column, separation of GPx, SelP, and SeAlb is therefore achieved. The main drawback of AF-HPLC is the co-elution (in the void fraction) of GPx with inorganic (or other low molecular-mass) Se species possibly present in serum. Nevertheless, Se species other than SeProt in human serum does not usually exceed 5% of the total Se level [17] and hence the determination of Se in GPx approximates well the genuine level of Se in this protein [8].

Approaches for quantification of intact selenoproteins

In contrast to the classical trace chemical analysis, where commercial standards are available for most of the analytes, many species of interest in bioinorganic trace analysis have not yet been isolated in sufficient purity to be used as (external) quantification standards. In this context, two alternatives are commonly used, namely the species unspecific (on-line or post-column) isotope dilution-mass spectrometry (IDMS) and the external calibration (EC) using surrogate standards, as described below.

On-line isotope dilution (ICP) mass spectrometry

One of the most reliable approaches for the accurate quantification of biospecies for which standards are not available, is the on-line (species unspecific) IDMS [18]. In this case, the bio-species are separated by HPLC coupled online with a mass spectrometric detector (e.g. ICP-MS) and mixed post-column with an isotopically enriched material known also as 'spike'. This is commonly an

inorganic form of the analyte, which has the relative atomic abundances different from the natural values, meaning that an isotope is ‘artificially’ enriched compared with its natural state [19, 20]. In case of SeProt determination, the spike is usually an inorganic Se species (e.g. selenate) or an aminoacid (e.g. SeMet).

The quantification of bio-species by on-line IDMS is based on the detection of a hetero-element contained in the analyte (e.g. metal, semi-metal or non-metal), such as Se in SeProt and by converting the intensity chromatograms into mass flow chromatograms using Eqs. 1–3 [7, 21]; more details regarding the principle of on-line IDMS can be found elsewhere [22].

$$F_x(t) = F_{\text{spike}} \frac{M_x \cdot {}^1A_{\text{spike}} - {}^2A_{\text{spike}} \cdot R_m(t)}{M_{\text{spike}} \cdot {}^2A_x \cdot R_m(t) - {}^1A_x} \quad (1)$$

where

$$F_{\text{spike}} = F_{\text{standard}} \frac{M_{\text{spike}} \cdot {}^1A_{\text{standard}} - {}^2A_{\text{standard}} \cdot R_{\text{cal}}}{M_{\text{standard}} \cdot {}^2A_{\text{spike}} \cdot R_{\text{cal}} - {}^1A_{\text{spike}}} \quad (2)$$

and

$$F_{\text{standard}} = F_{\text{HPLC}} \cdot \gamma_{\text{standard}} \quad (3)$$

$F_x(t)$, mass flow of the unknown sample eluting from the column (ng min^{-1}).

F_{spike} , mass flow of the spike solution (ng min^{-1}).

$M_x = M_{\text{standard}}$, atomic mass of (natural) Se (g mol^{-1}).

M_{spike} , atomic mass of Se in the spike solution (g mol^{-1}).

${}^1A_{\text{spike}}$, ${}^2A_{\text{spike}}$, abundance (%) of the isotope ‘1’ (enriched) and of isotope ‘2’, respectively, in the spike. 1A_x , ${}^1A_{\text{standard}}$, 2A_x , ${}^2A_{\text{standard}}$, abundance of isotope ‘1’ and ‘2’ in the unknown sample and standard, respectively (tabulated natural abundances are commonly used).

F_{standard} , mass flow of the standard solution eluting from the column (ng min^{-1}).

F_{HPLC} , flow rate of the mobile phase for HPLC separation (mL min^{-1}).

γ_{standard} , concentration of the standard solution (ng mL^{-1}) used for the determination of F_{standard} .

$R_m(t)$, ratio between the isotopes 1 and 2 in the eluent mixed on-line with the spike solution (the isotope ratio varies with the time as a consequence of the chromatogram development).

R_{cal} is usually determined by injecting a standard solution of the analyte directly into the mobile phase without the HPLC columns and using a large sample loop (1–2 mL) so that a large enough temporary steady-state signal is obtained [18].

It should be noted that in contrast with species-specific IDMS (SSIDMS), the on-line IDMS is not considered a primary method of analysis. The accuracy of on-line IDMS

depends on the quantitative recovery of the analytes from the HPLC column(s) because the spike isotope is added post-column and hence it cannot compensate for the analyte loss and/or incomplete peaks resolving during the chromatographic separation.

Calibration using surrogate standards

Because the signal in ICP-MS is quasi-independent of the (organic) molecular moiety of the bio-species, it gives the possibility to use surrogate Se standards, such as inorganic Se or small molecular weight (Se) compounds (e.g. SeMet, Se-cystine, etc.) for the quantification of SeProt. When HPLC separation is involved, the use of surrogate standards is feasible only if they match the chromatographic behavior of the bio-species, particularly in terms of recovery from the column. Hence, if the Se standards and SeProt elute quantitatively from the HPLC column(s) and the influence of the organic moiety of the analyte is negligible or have the same effect on the analytical response for the standard and analyte(s), surrogate standards can be used for quantification purposes. Although this approach is not fully valid from a metrological point of view, it provides results comparable with on-line IDMS [7], whereas it simplifies significantly the overall analytical procedure, and it is more suitable for routine analyses.

A metrological approach for the quantification of Se species in human serum

A feasible alternative to the determination of intact SeProt consists of their enzymatic hydrolysis (in the whole serum) followed by the determination of the resulting selenoaminoacids (SeCys and SeMet) in the (enzymatic) extract by reversed phase HPLC (RP-HPLC) coupled on-line to ICP-MS [17, 23]. The major advantage of this approach consists of the availability of SeMet standards, thus allowing accurate quantification of at least SeAlb in human serum (SeMet is released from SeAlb as a result of the enzymatic hydrolysis). Additionally, isotopically enriched SeMet species is also available, thus making possible the application of SSIDMS, which has the potential of a primary method of chemical analysis. Nevertheless, the accuracy of SSIDMS depends on the yield of the hydrolysis process, as this approach cannot compensate for loss of SeMet caused by incomplete hydrolysis of SeAlb.

The main disadvantage of this approach (involving proteins’ hydrolysis) for SeProt quantification arises from the lack of SeCys standards because of its high instability (it is prone to oxidation). Consequently, accurate determination of GPx and SeIP (both releasing SeCys by enzymatic hydrolysis) is impossible; an alternative is the

use of SeMet as a surrogate standard for the quantification of SeCys [17].

Another issue regarding this approach for the quantification of SeProt arises from the fact that following enzymatic hydrolysis, both GPx and SelP will lead to the same species (SeCys) and hence the genuine speciation of serum Se is lost. This limits the usefulness of SeCys determination in the enzymatic extract of serum for biomedical studies, because in this case SeCys reflects the sum of two proteins (GPx + SelP) with different biochemical properties. Therefore, from a metrological point of view, when performing the enzymatic hydrolysis of the whole serum, focus is placed on the determination of SeMet by means of SSIDMS. Nevertheless, SeMet measured using this approach reflects the total SeMet level in serum and not only that incorporated in SeAlb. Hence, if SeMet originating only from SeAlb is of interest, SeMet possibly present in serum as free species will lead to positive bias. Therefore, the fraction of free SeMet should be assessed preliminarily and if necessary, separated from the serum before the enzymatic hydrolysis.

Indicative concentrations of selenoproteins (in terms of Se) in the BCR-637 human serum CRM

Analytical methods

As mentioned earlier, ‘speciated’ serum certified reference materials (CRMs) for SeProt determination have not yet been produced, thus making the method validation virtually impossible. An alternative is the analysis of a serum material certified for total Se, and assuming that Se is almost entirely bound to proteins, the agreement between the level of total protein bound Se and the certified value can be considered a valuable tool for method validation.

In the past years, we have dealt with the determination of SeProt in human serum, and each newly developed method was applied (also) to the analysis of the BCR-637 CRM, which is a human serum certified for total Se level. We present here the data in terms of Se levels in GPx, SelP, and SeAlb in the BCR-637 serum, obtained by using 13 different methods (M1 up to M13) [6–9]; a brief description of each method is provided below.

M1. Determination of SeProt by (normal bore) AF-HPLC coupled to ICP-quadrupole MS following serum clean-up by (anion exchange) solid-phase extraction

Separation of SeProt in human serum (100 μ L) was carried out by AF-HPLC (normal bore, 4.6 mm internal diameter columns) following the off-line serum clean-up by anion exchange-solid phase extraction; quantification was performed by using ICP-quadrupole MS (ICP-QMS)

coupled on line with AF-HPLC with EC and Se(VI) as external standard [6].

M2. Determination of SeProt by tandem anion exchange-AF-HPLC (normal bore) coupled to ICP-quadrupole MS

A method based on a tandem anion exchange (AE) and affinity AF-HPLC (AE-AF-HPLC) hyphenated to ICP-QMS was used for the determination of SeProt in human serum (100 μ L); quantification was carried out by on-line IDMS ($^{82}\text{Se}/^{77}\text{Se}$ ratio) [7].

M3. The same as M2 but the quantification of SeProt was carried out by EC using Se-L-cystine standard (monitoring ^{82}Se) [7].

M4. Determination of SeProt was carried out off-line by (high resolution) ICP-sector field MS analysis of the GPx, SelP, and SeAlb fractions separated by AE-AF-HPLC (as used in M2); quantification was carried out by EC using Se(VI) standard (monitoring ^{82}Se) [8].

M5. Determination of SeProt by microbore AF-HPLC coupled to ICP-Sector field-MS using a high-efficiency sample introduction system (APEX)

The determination of SeProt was carried out by using microbore (1.0 mm internal diameter columns) AF-HPLC (5 μ L of serum) coupled to ICP-sector field-MS in high-resolution mode (HR). In order to compensate for the loss in sensitivity when working in HR and also because of the very low amount of sample taken for analysis, a high-efficiency sample nebulization/desolvation system (APEX) was used as an interface between AF-HPLC and ICP-SFMS. Quantification of SeProt was carried out by on-line ID ($^{78}\text{Se}/^{77}\text{Se}$ ratio) [8]. Method detection limits (MDLs, criterion used was three times the standard deviation of the blank) were calculated for ten successive injections of the analytical reagent blank. MDLs obtained were 0.4 ng mL^{-1} for GPx, 2.0 ng mL^{-1} for SelP, and 1.0 ng mL^{-1} for SeAlb [8].

M6. The same as M5 but using the $^{82}\text{Se}/^{77}\text{Se}$ ratio [8].

M7. The same as M6 but performing the analyses in medium resolution (MR) [8].

M8. The same as M7 but performing the quantification by EC with Se-cystine standard (monitoring ^{77}Se) [8].

M9. The same as M8 but quantification was carried out using ^{78}Se [8].

M10. The same as M8 but the quantification was carried out using a different isotope (^{82}Se) [8].

M11. Determination of SeProt in human serum by (normal bore) AF-HPLC coupled to collision/reaction cell ICP-MS

The determination of SeProt was carried out by (normal bore) AF-HPLC coupled to ICP-quadrupole MS as described in the previous methods (M1–M10) with the difference that the ICP-MS was provided with a collision/reaction cell (CRC ICP-QMS). This instrument (PQ-Ex-Cell, Thermo Elemental, Bremen, Germany) was equipped with a (quartz) impact bead spray chamber thermostatted to 3 °C and fitted with a glass concentric type nebuliser. A mixture of He–H₂ (4.5:2.5 mL min⁻¹) was used as collision/reaction gases. Quantification was carried out by EC using SeMet standard (monitoring ⁸⁰Se) [9]. In this case, the correction of the ⁸¹Br¹H interference that overlaps with ⁸⁰Se was carried out as described elsewhere [6].

M12. The same as M11 with the difference that in-house-made microbore AF-HPLC (1.0 mm id, as described in Ref. [8]) columns were used. In this case, only 5 μL of serum was necessary for one analysis (monitoring ⁷⁸Se) (unpublished work).

M13. The same as M11, but the detection was carried out by ICP-quadrupole MS (Agilent 7500cx, Agilent Technologies, Tokyo, Japan) equipped with a collision/reaction cell using Ar as a collision gas (1 mL min⁻¹). Differently from the use of He–H₂ mixtures, Ar allows the removal of the spectral interference on ⁷⁸Se by breaking down the ⁴⁰Ar³⁸Ar dimers. This mode prevents the formation of a new interference on ⁷⁸Se, such as ⁷⁷Se¹H, commonly formed in the collision/reaction cell when H₂ is used a reaction gas. Therefore, this approach leads to accurate quantification of ⁷⁸Se without using any mathematical corrections for the elimination of the ⁴⁰Ar³⁸Ar interference. Quantification was performed by EC using SeCys standards (monitoring ⁷⁸Se) (unpublished work).

Assessment of the normal distribution of the SeProt levels in BCR-637 serum obtained by using the methods M1–M13

The SeProt data obtained by using methods M1–M13 for the analysis of the BCR-637 serum were tested for their normal distribution using the Kolmogorov–Smirnov (K–S) criterion [24], and they resulted normally distributed. This test was carried out by ordering the means x_{mi} obtained by each method (p , number of methods, in our case $p = 13$) for a given SeProt level and then calculating the K–S statistic parameters (D) using Eqs. 4–6. The null hypothesis of normality is rejected at a level of $\alpha = 0.05$ if D exceeds the correspondent critical value K_α [24].

$$D^+ = \max_{i=1, \dots, p} |i/p - \rho(i)| \quad (4)$$

$$D^- = \max_{i=1, \dots, p} |\rho(i) - (i-1)/p| \quad (5)$$

$$D = \max [D^+, D^-] \quad (6)$$

where $\rho(i)$ is the expected cumulative frequency (from normal distribution) of the value x_{mi} , calculated by means of its z -score (defined by Eq. 7).

$$z = \frac{x - \bar{x}}{s_p / \sqrt{p}} \quad (7)$$

where x , actual value of SeProt concentration, \bar{x} , mean of all the values, s_p , standard deviation of the values taken into study (all 13 methods).

Outliers identification and calculation of the indicative concentrations of SeProt (in terms of Se) in the BCR-637 CRM

The average concentration and the combined standard uncertainty (u_c) of GPx, SeIP, and SeAlb (as well as their sum) in the BCR-637 serum, obtained using the methods M1–M13 are summarized in Table 1. u_c was calculated taking into account the repeatability of each method (S_r) and the component reflecting the between-methods variability (S_{methods}), calculated as recommended by the ISO 5725-2 guide [25] using Eq. 8 (the correlation between methods was neglected):

$$u_c = \sqrt{S_r^2 + S_{\text{methods}}^2} \quad (8)$$

where

$$S_r = \sqrt{\frac{1}{p} \sum_{i=1}^p s_i^2} \quad (9)$$

where s_i , experimental standard deviation for the analysis of n replicates by using method i , p , number of methods (in our case $p = 13$).

$$S_{\text{methods}} = \sqrt{\frac{1}{p-1} \sum_{i=1}^p (\bar{\gamma} - \gamma_i)^2 + \frac{n-1}{n} S_r^2} \quad (10)$$

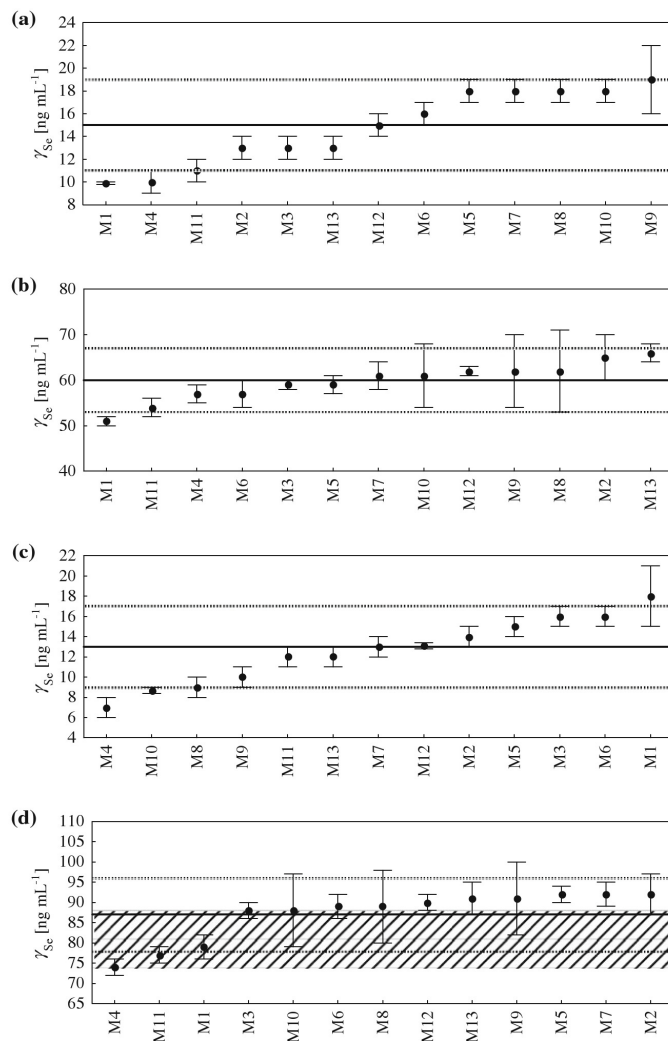
where $\bar{\gamma}$, mean of the results (concentrations) obtained by all methods ($p = 13$), γ_i , result (concentration) provided by

Table 1 Mean concentration \pm combined standard uncertainty ($p = 13$) of SeProt in the BCR-637 CRM human serum determined by using M1–M13

	γ_{Se} (ng mL ⁻¹)
GPx	15 \pm 4
SeIP	60 \pm 7
SeAlb	13 \pm 4
GPx + SeIP + SeAlb	87 \pm 9 (90 \pm 7) ^a

^a Recalculated after outlier elimination ($p = 10$)

Fig. 1 Selenium concentration (ng mL^{-1}) in GPx (a), SeIP (b), SeAlb (c), and GPx + SeIP + SeAlb (d) of the BCR-637 human serum CRM obtained using 13 different methods on the basis of (affinity) HPLC coupled to ICP-MS. The central line represents the average of all 13 results (M1–M13) whereas the dotted lines correspond to the combined standard uncertainty (u_c). The bars shown for each result correspond to the standard deviation calculated for three independent serum replicates. In (d), the central line in the gray area represents the certified value of total Se whereas the gray area represents the confidence interval (95%)



method i (average of n independent replicates), n , number of replicates analyzed by method i (in our case $n = 3$).

It is worth noting that the level of total protein bound Se [(87 ± 11) ng mL^{-1} , as Se] is not significantly different (t test, p value >0.05 , $\alpha = 0.05$) than the certified value (81 ± 7) ng mL^{-1} . This is an indication of the methods' accuracy and hence the indicative concentration values for SeProt calculated in this work could be tentatively used for validation in studies where 'speciated' CRMs are not available [15].

The distribution of the SeProt levels (in terms of Se) and their sum in BCR-637 determined by using the methods

M1–M13 is shown in Fig. 1a–d. The central line corresponds to the concentration of Se calculated as the average of the results obtained by M1–M13, whereas the dotted lines define the range corresponding to the combined standard uncertainty (u_c). The deviation associated with each concentration corresponds to the standard deviation (SD) of each method ($n = 3$). As can be seen in Fig. 1a–d, a relatively high spread of the results was obtained: the relative standard deviation (RSD, %) was 27% for GPx and SeIP and 35% for SeAlb, respectively. This indicates that the determination of SeProt in a complex matrix such as the human serum is a difficult analytical task.

Fig. 2 Values of Mandel’s h statistic parameter applied to the SeProt data obtained by means of the methods M1–M13. The bold horizontal lines mark the critical value at $\alpha = 0.01$, whereas the dotted horizontal lines indicate the critical value at $\alpha = 0.05$

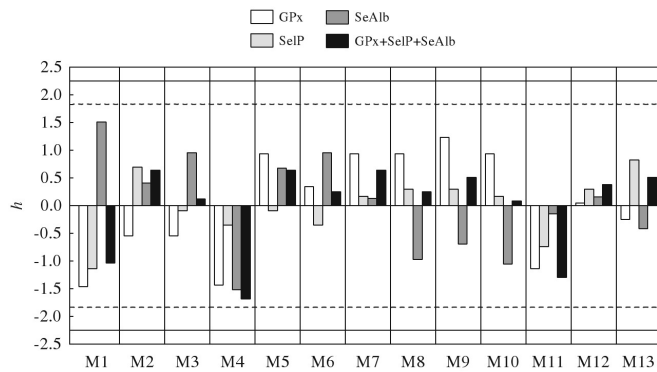
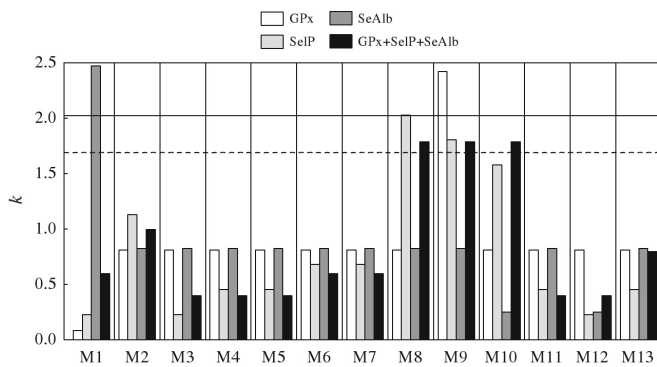


Fig. 3 Values of Mandel’s k statistic parameter applied to the SeProt data obtained by means of the methods M1–M13. The bold horizontal line marks the critical value at $\alpha = 0.01$ and the dotted horizontal line marks the critical value at $\alpha = 0.05$



The outliers were assessed using the Hempel statistical test (also called Huber’s test) [26]. This is based on the calculation of the median value (M_{mi}) of the means (x_{mi}), the absolute residuals (r_i) of each mean from M_{mi} , and the median of the absolute residuals (M_{ri}). A value (x_{mi}) is an outlier at a chosen level (in our case $\alpha = 0.05$) if $r_i > 4.5 M_{ri}$. The outliers found using this test were the following: SeIP concentration obtained by using M1 and the total SeProt level (GPx + SeIP + SeAlb) obtained with M1, M4, and M11. By eliminating these outliers, the data remained unchanged for SeIP, whereas for total SeProt, the mean value and u_c changed slightly (see Table 1). The data reported in Table 1 provide the first indicative concentrations of SeProt (in terms of Se) in a commercially human serum and hence they can be useful for method validation and/or methods inter-comparison.

Assessment of method accuracy and precision

In order to assess the most appropriate method in terms of accuracy and precision (e.g. repeatability), the Mandel’s h and k tests [25] were applied by considering the whole

pooled values of GPx, SeIP and SeAlb and their sum, respectively. h and k plots indicate which methods exhibit patterns of results that are markedly different from the others in the study [25]. This approach, which is commonly used for processing the data obtained in inter-laboratory comparisons, was applied here to evaluate the between-methods statistic consistency.

The h test (Eq. 11), which takes into account the mean values provided by each method, allows the evaluation of the overall accuracy of a result obtained by using all methods (in our case M1–M13).

$$h_i = \frac{x_{mi} - \bar{x}_m}{\sqrt{\frac{1}{p-1} \sum_{i=1}^n (x_{mi} - \bar{x}_m)^2}} \tag{11}$$

where x_{mi} , is the result obtained by using the method i , \bar{x}_m , is the mean of all the means taken in the study, and p , the number of methods/results (13).

If a method provides only negative or positive values for h , it is assumed that the method is affected by a systematic error; when h is in the extreme range, i.e., above the critical values of the statistic, it is assumed that the results are biased. Applying the h test to the SeProt data obtained by

methods M1–M13, we found that the best overall accuracy considering all four variables (GPx, SeIP, SeAlb and their sum) was obtained by M12 (see Fig. 2). This finding is interesting because M12 allows accurate determination of SeProt in only 5 μ L of human serum and hence it could be particularly useful for biomedical studies using mice models, where the amount of blood that can be sampled daily is commonly of a few microliters (≤ 20) [27].

Mandel's k test was also used here in order to evaluate the repeatability of each method with respect to the overall repeatability; the k_i parameter was calculated using Eq. 12.

$$k_i = \frac{s_i \sqrt{p}}{\sqrt{\sum_{i=1}^p s_i^2}} \quad (12)$$

where s_i is standard deviation corresponding to each method ($n = 3$) and p , the number of methods (13).

Based on Eq. 12, it can be deduced that the bigger is k_i , the poorer is the method repeatability. As shown in Fig. 3, the best repeatability was again achieved by method M12. It is worth noting that a repeatability exceeding the critical value for 5% level of significance was obtained by M8, M9, and M10 for the sum GPx + SeIP + SeAlb, and by M9 for SeIP; 1% level of significance was exceeded by M1 for SeAlb, M8 for SeIP, and M9 for GPx.

Mandel's h and k statistical tests showed that the methods M1–M13 provided satisfactory accuracy with respect to the overall mean, whereas the best method for SeProt determination in human serum amongst those considered in this study turned to be M12, which is based on microbore AF-HPLC coupled to CRC ICP-QMS (see description above).

Conclusions

This study reports for the first time indicative concentrations (in terms of Se) of GPx, SeIP, and SeAlb in a commercially available human serum, namely the BCR-637, certified for total Se. This is particularly important given the lack of CRMs for the determination of individual SeProt in human serum as well as the lack of reference measurement method for these important bio-species for human health. Additional research should be pursued to characterize more human serums in terms of SeProt levels by several laboratories using different methods, in order to produce 'speciated' CRMs or at least to provide indicative levels, due to the importance of such materials for method validation and for ensuring data traceability for the assessment of selenium status in humans.

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Plasma selenoproteins concentrations in type 2 diabetes mellitus—a pilot study

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The association between the concentration/activity of selenium/selenoproteins in plasma and type 2 diabetes mellitus is still a matter of debate. This cross-sectional pilot study evaluates whether patients with diabetes present a different plasma selenoproteins status than a healthy control group and examines whether the introduction of clinical parameters allows the detection of correlations and further grouping criteria. For this purpose, the levels of plasma glutathione peroxidase (GPx), selenoprotein P (SeP), and seleno-albumin (SeAlb) present in 40 patients affected by type 2 diabetes mellitus were determined simultaneously and accurately by a newly developed analytical method. The results show that patients with diabetes demonstrate significantly lower levels of GPx and SeAlb with respect to healthy subjects (11 ± 3 ng/mL and 9 ± 2 ng/mL vs 18 ± 8 ng/mL and 11 ± 2 ng/mL, respectively). Significant negative correlations were revealed among GPx, SeAlb, and clinical parameters including fasting plasma glucose, hemoglobin A1c, and the albumin-to-creatinine ratio. Our findings suggest an association between the individual selenoproteins concentration and the presence of diabetes, including associated clinical parameters. It currently cannot be ascertained whether the altered selenoproteins status is a consequence or a causative factor for diabetes. This study demonstrates the potential of a method for individual selenoproteins determination for investigating the biochemical relationship between selenium and diabetes. (Translational Research 2010;156:242–250)

Abbreviations: ACR = Albumin-to-creatinine ratio; ANCOVA = analysis of covariance; BMI = body mass index; CL = confidence limits; FA = factorial analysis; FPG = fasting plasma glucose; GPx = glutathione peroxidase; HbA1c = hemoglobin A1c; HDL = high-density lipoprotein; HPLC = high-performance liquid chromatography; ICP-MS = inductively coupled plasma-mass spectrometer; K-S = Kolmogorov-Smirnov; LDL = low-density lipoprotein; LRA = logistic regression analysis; OR = odds ratio; SD = standard deviation; Se = selenium; SeAlb = seleno-albumin; SeP = selenoprotein P; S-W = Shapiro-Wilk

A series of studies have shown recently that selenium (Se) has insulin-mimetic properties both *in vitro* and *in vivo*, including the stimulation of glucose uptake^{1,2} as well as the regulation of glycolysis, gluconeogenesis, fatty acid synthesis, and the

pentose phosphate pathway.^{3,4} Se also prevents or alleviates the adverse effects that diabetes has on cardiac,⁵⁻⁷ renal, and platelet functions^{8,9} as well as atherosclerosis progression.¹⁰

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AT A GLANCE COMMENTARY**Roman M, et al****Background**

This article reports a novel cross-sectional pilot study of complete serum selenoproteins status in patients affected by type 2 diabetes mellitus, compared with healthy control subjects and with a wide range of clinical parameters. For this purpose, a newly developed analytical method was applied for the simultaneous quantification of plasma glutathione peroxidase, selenoprotein P, and selenoalbumin.

Translational Significance

Our findings revealed an association among individual selenoproteins concentration, the presence of diabetes, and some of its main parameters. This study demonstrates the high potentiality of a method for individual selenoproteins determination in the investigation of the biochemical relationship between selenium and diabetes.

Mueller and Pallauf¹¹ performed an elegant study in type 2 diabetic db/db mice to show that selenate influences the following important mechanisms involved in the insulin resistance state characteristic of type 2 diabetes mellitus: (1) it reduces the activity of liver cytosolic protein tyrosine phosphatases as negative regulators of insulin signaling by about 50%, and (2) it increases the expression of the peroxisome proliferator-activated receptor gamma. These 2 mechanisms are responsible for the changes in the intermediary metabolism, particularly gluconeogenesis and lipid metabolism.

No definitive data are reported in the literature about the possible association between levels of Se in plasma/serum and the presence of type 2 diabetes mellitus. Studies have shown that mean plasma¹² or serum^{13,14} Se concentrations and glutathione peroxidase (GPx, type 3) activity¹⁵ are lower in patients with diabetes than in controls. Lower Se levels also have been found in gestational diabetic pregnancies with respect to normal pregnancies.¹⁶⁻¹⁹ However, a study by Bleyers et al²⁰ did not observe significant differences in serum Se even if subjects over the highest quintile had an increased prevalence of diabetes compared with those below the first quintile. Whiting et al¹⁵ observed lower plasma Se levels in patients with diabetes but only with a disease duration ≤ 2 years. Other works showed no significant difference in plasma Se levels of patients

with diabetes^{21,22} or even demonstrated higher serum Se levels in diabetic children with respect to healthy subjects.^{23,24}

Recent studies have revealed that the total Se level may be an unsuitable biomarker because of the different metabolic action of specific selenoproteins, and therefore, the speciation of Se has been proposed as a necessary requirement for a more thorough investigation of the relationship between Se status and diabetes.²⁵ For these reasons, new analytical methods based on the coupling of separation techniques, such as high-performance liquid chromatography (HPLC), with a Se-specific detector, such as inductively coupled plasma-mass spectrometer (ICP-MS), have been developed for the speciation of selenoproteins in body fluids. Few applicative studies have been published that obtained promising results.

In this pilot study, a newly developed method for the accurate and simultaneous determination of the plasma selenoproteins GPx, selenoprotein P (SeIP), and the (inactive) Se-containing protein selenoalbumin (SeAlb, for simplicity, also referred to as a selenoprotein)²⁶ was used to investigate for the first time the possible relationship among the complete plasma selenoproteins pattern, presence of type 2 diabetes mellitus, and clinical parameters in 40 patients with diabetes versus 15 healthy control subjects. A relatively low number of samples was analyzed to carry out a pilot study that can serve as a suitable design of broader investigations.

PATIENTS AND METHODS

Patients and study protocol. The study involved 40 patients with type 2 diabetes regularly attending the Diabetes Clinic of the Department of Medical and Surgical Sciences at the University of Padova (Italy). The control group included 15 healthy subjects with normal glucose tolerance as assessed by an oral glucose tolerance test according to American Diabetes Association recommendations.²⁷ All enrolled patients with type 2 diabetes and normal control subjects followed a dietary regimen of 50% carbohydrates, 30% fats, and 20% proteins. This dietary program was not modified for at least 6 months preceding the analysis. All patients with type 2 diabetes were treated with oral hypoglycemic drugs. Informed written consent was obtained from all subjects participating in the study according to the Helsinki Declaration.

Blood samples were collected in the morning, after a 12-h overnight fast and were centrifuged at 3000 rev/min for 10 min. Fasting plasma glucose (FPG), hemoglobin A1c (HbA1c), and the albumin excretion rate were measured immediately, and the remaining samples were divided into aliquots of 1.5 mL and stored at -20°C

until analysis for the determination of total cholesterol, triglycerides, azotemia, creatinine, and selenoproteins content. The albumin-to-creatinine ratio (ACR) also was evaluated in the spot urine collection in the morning.

Analytical methods. FPG was determined by a glucose-oxidase method.²⁸ HbA1c was measured by a liquid chromatography method (Bio-Rad Laboratories, Milan, Italy).²⁹ Total cholesterol, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol were measured by enzymatic analytical chemistry (CHOD-PAP method, Roche, Milan, Italy),³⁰ as were plasma triglycerides (GPO-PAP colorimetric enzyme test, Roche).³¹ Urinary albumin³² and creatinine³³ were determined in the morning spot urine samples, and the urinary albumin ($\mu\text{g/mL}$)-to-creatinine (mg/mL) ratio was calculated; microalbuminuria was defined as mg/g .

Determination (in terms of Se content) of GPx, SeIP, and SeAlb in human plasma was achieved simultaneously by HPLC coupled with ICP-MS. The method details including the standards/reagents and the optimum parameters were described by Jitaru et al.²⁶ The interferences Cl^- and Br^- were removed from the sample by anion exchange solid-phase extraction. The method allows the simultaneous separation of GPx, SeIP, and SeAlb by injecting $100\ \mu\text{L}$ of plasma onto a double-column affinity chromatographic system. A high-resolution selenoprotein separation can be obtained in less than 15 min per sample, and the custom affinity columns guarantee more than 150 consecutive injections without performance loss. The online coupling of HPLC with a Se-specific detector, such as an ICP-MS, allows the quantification of the plasma selenoproteins with high accuracy and precision despite their extremely low concentration and reaches detection limits of about $0.1\ \text{ng/mL}$ for GPx and $1.0\ \text{ng/mL}$ for SeIP and SeAlb. The sum of GPx + SeIP + SeAlb was considered to approximate rather accurately the total Se level in plasma.

Statistical data treatment. Mean values, standard deviation (SD), the principal distribution parameters of selenoproteins concentration, and the relative percentage of total plasma Se for diabetic and healthy groups were calculated. The normality of the data distribution for the 2 groups was tested by the Shapiro-Wilk (S-W) test³⁴ to apply the t test correctly for paired-groups comparison. The selenoprotein concentration and percentage also were tested for possible confounding effects by analysis of covariance (ANCOVA, calculating the variable coefficients β and corresponding P values)³⁵ and, if necessary, were adjusted for confounding factors.

Unconditional logistic regression analysis (LRA, forward method)³⁴ was adopted to estimate the association between the plasma selenoproteins level as continuous variables and the correct classification of each subject

into healthy (negative) and diabetic (positive) groups. The LRA combines the information carried by every single variable into a linear multivariate model that uses the observed differences for classification purposes by probabilistic (quantitative) evaluation. Two alternative LRA models were selected and likelihood ratio tests were performed to assess which variables significantly changed the model. Confounding and interacting effects also were considered to evaluate associations between variables in which an association is the effect produced by each selenoprotein on the classification capability of the others. Model sensitivity (percentage of individuals diagnosed with diabetes that are classified correctly by the logistic model) and specificity (percentage of individuals in the control group who are assigned accurately to that group by the logistic model) were calculated. All sensitivities were set at maximum to obtain specificities to select the cut-off values. The input variables were characterized by their odds ratio (OR) and corresponding confidence limits (CL).

The possible presence of a significant correlation between the selenoprotein level and the quantitative clinical parameters was tested by calculating correlation coefficients. The parameters distribution was tested for normality by the S-W test. Linear correlations were used for the normally distributed data, whereas Spearman rank order correlations were used for the others. Possible interesting parameters then were selected as new grouping variables for the comparison of selenoproteins concentration between subjects in the first and fourth quarter of each clinical parameter via S-W and t tests. Factorial analysis (FA)³⁵ also was performed to combine the information carried by the selenoproteins level and the correlated clinical parameters to evaluate qualitatively the possible presence of distinct groups.

Results were considered significant at a 95% confidence level ($P < 0.05$). Statistica 8.0 (StatSoft Inc., Tulsa, Okla), Origin 7.5 (OriginLab Corp., Northampton, Mass), and Microsoft Office Excel 2003 (Microsoft, Redmond, Wash) were used for data analysis and graphical elaborations.

RESULTS

The patients' clinical characteristics and laboratory data are given in Table I. Patients with a mean duration of diabetes for 10 years show a poor metabolic control, as evidenced by the mean values of FPG and HbA1c. All patients had normal renal function as shown by the mean values of creatinine, azotemia, and ACR.

Comparison between patients with type 2 diabetes and healthy subjects based on their selenoproteins status. Selenoproteins level and percentage. The GPx, SeIP, SeAlb, and total Se mean concentration SD and P of the t test obtained by plasma analysis of patients

Table I. Clinical and metabolic parameters of the subjects under study (mean \pm SD)

	Type 2 diabetes mellitus (n = 40)	Healthy (n = 15)
Age (years)	69 \pm 12	46 \pm 3
Gender		
Female	16	12
Male	24	3
BMI (kg/m ²)	29.0 \pm 5.3	25.9 \pm 1.9
FPG (mg/dl)	179 \pm 59	81 \pm 6
HbA1c (%)	8.0 \pm 1.7	5.4 \pm 0.3
Triglycerides (mg/dl)	130 \pm 70	96 \pm 48
Total cholesterol (mg/dl)	191 \pm 38	189 \pm 16
HDL (mg/dl)	56 \pm 14	58 \pm 11
Azotemia (mmol/L)	217 \pm 90	—
Creatinine (mg/dl)	0.95 \pm 0.23	0.74 \pm 0.14
ACR (mg/g)	46.9 \pm 80.4	5.5 \pm 1.9

Table II. Concentration and percentage (mean \pm SD) of GPx, SelP, and SeAlb in plasma from type 2 diabetic patients and healthy control subjects; *P* and age-corrected *P* (in parentheses) for the paired groups *t* test

Protein	Healthy (n = 15)	Type 2 diabetes mellitus (n = 40)	<i>P</i> for <i>t</i> test
Se (ng/mL)			
GPx	11 \pm 3	9 \pm 2	< 0.01 (0.01)
SelP	56 \pm 8	58 \pm 9	0.65 (0.86)
SeAlb	18 \pm 8	11 \pm 2	< 0.01 (< 0.01)
GPx + SelP + SeAlb	85 \pm 16	78 \pm 12	0.06 (0.06)
Se (%)			
GPx	13 \pm 2	11 \pm 1	0.04 (0.19)
SelP	67 \pm 5	74 \pm 2	< 0.01 (< 0.01)
SeAlb	21 \pm 5	14 \pm 2	< 0.01 (< 0.01)

with diabetes and healthy subjects are reported in Table II. In addition, Fig 1, A shows the box plots of the selenoproteins absolute level. The percentage of each selenoprotein with respect to the total Se level in plasma also was calculated. Table II shows the selenoproteins percentage, SD, and *P* of the *t* test for patients with diabetes and healthy subjects, whereas Fig 1, B represents the corresponding box plots.

The S-W test was applied to evaluate the normality of both concentrations and percentages to confirm the normal distribution of all variables. Therefore, the *t* test was applied for group comparison. Lower levels and percentages of GPx and SeAlb (*P* < 0.01) were revealed in subjects with diabetes, whereas in the same subjects, the SelP percentage was significantly higher (*P* < 0.01).

The healthy control subjects presented a significantly (*P* < 0.01) lower mean age (46 \pm 3 years) in comparison with the group with diabetes (69 \pm 12 years). To test

the possible confounding effect of that factor on the selenoproteins level and on the percentage distributions, the ANCOVA method was adopted to express the age as a function of the observed variables. The coefficients of GPx ($\beta = -0.04 \pm 0.02$, *P* = 0.02) and SeAlb ($\beta = -0.13 \pm 0.05$, *P* < 0.01) concentrations were significant. The *P* values adjusted for age (Table II) were generally higher with respect to the uncorrected values, but the observed difference between GPx and SeAlb levels remained significant. All selenoprotein percentage was correlated significantly with age (GPx %: $\beta = -0.04 \pm 0.02$, *P* = 0.02; SelP %: $\beta = 0.16 \pm 0.04$, *P* < 0.01; SeAlb %: $\beta = -0.12 \pm 0.04$, *P* < 0.01). The age-adjusted *P* values are reported in Table II. Although SelP and SeAlb percentages were still significantly different between patients with diabetes and the healthy group, GPx was no longer statistically different (*P* = 0.19). The age-adjusted data have been adopted to increase the validity of all statistical elaborations subsequently applied.

The healthy-subjects group presented a relevant asymmetry in the distribution between genders (12 females and 3 males). Therefore, gender also was tested as a confounding factor by ANCOVA for both selenoprotein concentration and percentage. Nonsignificant differences for all β coefficients were observed in the data between females and males, signifying that adjustments for gender are unnecessary.

In general, percentages can be a useful tool for raw data interpretation but are calculated by introducing an artificial correlation between variables. For this reason, they do not satisfy the fundamental assumptions required by the more advanced statistical methods. Therefore, all subsequent data analyses are applied only to concentrations.

Logistic regression analysis. Logistic regression models based on selenoproteins concentration were developed to deepen the comparison between diabetes and healthy groups. Different input sets were tested by combining GPx, SelP, and SeAlb concentrations and their products to capture possible further effects (Table III). In the comparison of patients with diabetes and the healthy group, GPx, SelP, and SeAlb levels initially were tested individually and then combined. Only GPx (OR 3.61; CL 1.50–8.71) and SeAlb (OR 14.33; CL 2.78–73.92) were significant (*t* test, *P* < 0.01). The model based only on the SeAlb level gave a sensitivity of 100% and a specificity of 60%, whereas for the GPx model, the maximum specificity reached 23%. When combined, the 3 variables allow a sensitivity of 100%, and specificity increased to 67% or 73% (see Table III). The introduction of the products GPx \times SeAlb and SelP \times SeAlb generated a slight inhibition effect on model performances, suggesting that the

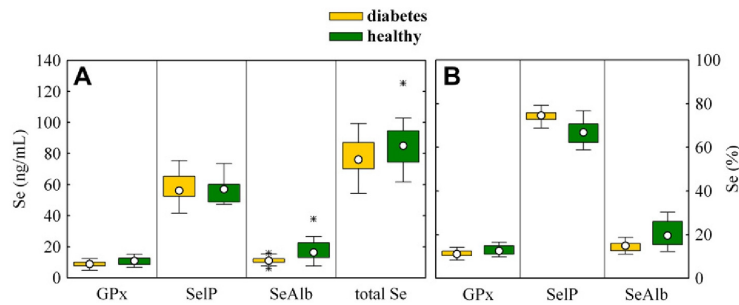


Fig 1. Box plots made by percentiles for selenoproteins concentration (A) and percentage (with respect to their sum) (B) in healthy subjects and patients with type 2 diabetes.

Table III. Classification performances of 2 logistic regression models

Model 1: sensitivity 100%, specificity 67%				
Variable	Estimate	Wald test	OR (CL 95%)	Concentration of risk twofold increase (ng/mL)
Intercept	0.91	3.85		
GPx	-0.96	2.21	0.38 (0.11-1.36)	-1
SelP	1.66	5.63	5.25 (1.36-20.66)	11
SeAlb	-3.22	6.60	0.04 (0.01-0.47)	-5
Model 2: sensitivity 100%, specificity 73%				
Variable	Estimate	Wald test	OR (CL 95%)	Concentration of risk twofold increase (ng/mL)
Intercept	0.89	0.63		
SelP	1.68	5.80	5.34 (1.37-20.90)	11
GPx + SeAlb	-3.56	11.33	0.03 (0.01-0.23)	-17

SeAlb level has a distinct relationship with the other selenoproteins.

Association between level of plasma selenoproteins and clinical parameters. Clinical parameters (see Table I) in the diabetes and healthy groups were analyzed with the S-W test to apply the *t*- or the Kolmogorov-Smirnov (K-S) tests appropriately. Body mass index (BMI) and creatinine were distributed normally in both groups, and consequently, the *t* test was applied. BMI was not statistically different, whereas creatinine was significantly higher in patients with diabetes (0.95 ± 0.23 vs 0.74 ± 0.14 mg/dL in healthy subjects). The K-S test applied to the other nonnormal parameters revealed significantly higher values in patients with diabetes for FPG (179 ± 59 vs 81 ± 6 mg/dL), HbA1c (8.0 ± 1.7 vs $5.4 \pm 0.3\%$), and ACR (46.9 ± 80.4 vs 5.5 ± 1.9 mg/g).

The possible association between plasma selenoproteins level and clinical parameters was investigated by the calculation of correlation coefficients within all

subjects. The S-W test showed that the parameters were not distributed normally with the exception of the total cholesterol. The linear correlation coefficient was calculated for this parameter, whereas the Spearman rank order correlation was calculated for the others (Table IV). GPx was correlated negatively with BMI, FPG, HbA1c, and ACR. A negative correlation also was observed between SeAlb and FPG, HbA1c, creatinine, and ACR. The total Se was correlated with HbA1c and ACR. The correlation coefficients between clinical parameters and selenoproteins level also were calculated independently within the diabetes and the healthy groups. The S-W test showed normal distributions of BMI and total cholesterol for patients with diabetes, whereas for healthy subjects, all parameters were distributed normally except the total cholesterol. A significant correlation exists between GPx and ACR for patients with diabetes ($r = -0.38$), whereas in healthy subjects, GPx was correlated positively with HbA1c ($r = 0.54$), and SelP as well as the total Se were correlated

Table IV. Correlation coefficients between plasma selenoproteins levels and clinical parameters in the whole sample. In parentheses, the *P* of the *t* test for plasma selenoproteins levels between subjects in the first and the fourth quarters of the group for each clinical parameter

	BMI*	FPG*	HbA1c*	Triglycerides*	Total cholesterol**	HDL*	Creatinine*	ACR*
GPx	-0.42 (0.11) [†]	-0.34 (0.80) [†]	-0.41 (0.14) [†]	-0.11 (0.37)	0.14 (0.31)	0.14 (0.14)	-0.27 (0.22)	-0.50 (0.05) [†]
SeIP	-0.06 (0.82)	0.02 (0.77)	-0.08 (0.55)	0.10 (0.54)	0.07 (0.75)	0.07 (0.03) [†]	-0.08 (0.22)	-0.05 (0.44)
SeAlb	-0.23 (0.67)	-0.38 (0.52) [†]	-0.45 (0.18) [†]	-0.07 (0.44)	0.09 (0.28)	0.06 (0.17)	-0.30 (0.94) [†]	-0.32 (0.86) [†]
Total Se	-0.23 (0.65)	-0.18 (0.93)	-0.28 (0.36) [†]	0.01 (0.46)	0.09 (0.56)	0.09 (0.03) [†]	-0.24 (0.27)	-0.23 (0.39) [†]

*Spearman rank correlation.

**Linear correlation.

[†]Significant (*P* < 0.05).

positively with triglycerides ($r = 0.59$, and $r = 0.52$, respectively). More couples of groups were taken for each clinical parameter considering the subjects who presented values in the first and fourth quarters of its distribution in the whole sample. The S-W test was applied to the selenoproteins level for patients in each group, obtaining normal distributions in all cases. The *t* test then was applied for the comparison of selenoproteins concentration between the 2 groups for each clinical parameter. All observed correlations were confirmed by a significant difference in the selenoprotein level for subjects in the first and fourth quarter (Table IV). The same analysis then was applied to consider only the patients with diabetes. In this case, only significantly lower concentrations of SeIP and total Se (53 ± 8 and 72 ± 12 ng/mL, respectively) were found in patients with HDL <47 with respect to patients with HDL >61 (62 ± 7 and 84 ± 8 ng/mL, respectively). Because of the low sample size, it was inappropriate to carry out the same comparison between quarters within the healthy subjects group. Most significant correlations or differences revealed when considering the sample as a whole disappeared when healthy subjects and patients with diabetes were investigated separately. This effect supports the hypothesis that many observed correlations for the whole sample are mostly the result of a bimodal distribution rather than a continuous relationship among variables.

Multivariate analysis. FA was performed to check the possible qualitative classification of healthy and diabetic groups by means of selenoproteins level coupled with their correlated clinical parameters. Two factors were selected based on the cumulative explained variance (66%). FPG, HbA1c, and ACR were the significant variables (loading >0.70) in the first factor, whereas creatinine, GPx, and SeAlb were significant in the second factor. Figure 2 shows the scores (Fig 2, A) and loadings (Fig 2, B) plots in which healthy subjects and patients with type 2 diabetes are separated into 2 distinct areas. This analysis provides only indicative results because of the low number of subjects, particularly for the healthy group ($n = 15$). Nevertheless, it depicts the

association among GPx, SeAlb, and some of the most important clinical parameters used in the diagnosis of type 2 diabetes.

DISCUSSION

The importance of collecting information not only on the presence/absence of proteins as specific biomarkers of disease but also on their concentration/activity in tissues and body fluids has emerged in recent years. The determination of proteins present at very low levels in such complex matrices is currently a challenging task. A large amount of work has been done in the last few years to develop new, accurate, precise, and fast methods for protein analysis. However, at present, few of these methods have been applied to real biomedical studies. As an ICP-MS is a specific detector for metals (or metalloids) at an extremely low level, its use coupled with a high-resolution separation technique, such as affinity-HPLC, offers a very powerful analytical tool for the study of metallo (or metalloid)-protein function in human diseases.³⁶ One example of this application is the investigation of plasma selenoproteins in patients with type 2 diabetes. Other detection systems such as atomic fluorescence spectrometry and atomic absorption spectrometry can be used for Se quantification in biological samples. Both of these methods allow elemental specificity, but the complicated online coupling with a liquid separation technique, such as HPLC, makes speciation analysis very difficult. Consequently, these methods have been used only for the total element determination of plasma Se in patients with type 2 diabetes.^{12-14,16-21} Alternatively, the activity of plasma GPx in patients with diabetes has been determined by enzyme-linked immunosorbent assay¹⁴ or other enzymatic assays.¹⁸ However, these techniques are difficult to handle, time consuming, as well as imprecise, and SeAlb cannot be assayed because it has no enzymatic activity (from the point of view of Se). ICP-MS allows easy coupling with HPLC, resulting in high specificity and sensitivity. In this study, the coupled HPLC-ICP-MS system was applied for the first time for the

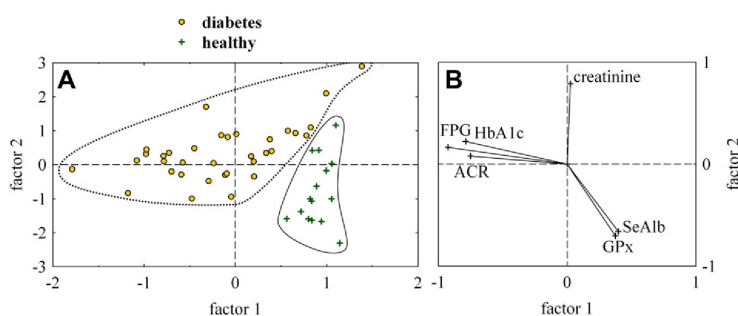


Fig 2. Scores plot (A) and loadings plot (B) for the first 2 components of the FA obtained for patients with diabetes and healthy subjects. Ovals indicate the boundary of the areas where patients with type 2 diabetes (broken line) and healthy subjects (unbroken line) are distributed.

cross-sectional investigation of the relationship between plasma selenoproteins level and diabetes. The method combines high-resolution protein separation with high accuracy and sensitivity in a procedure that requires only filtration as a sample-preparation step. GPx, SelP, and SeAlb were determined simultaneously in an analytical run of only 15 min per sample without any performance degradation for more than 150 injections.

In our study, GPx and SeAlb levels were significantly different ($P < 0.05$) in patients with type 2 diabetes compared with healthy subjects. Because SelP plays the fundamental role of Se carrier through the body³⁷ and because SeAlb is not an active protein, the total available Se from dietary intake should be incorporated preferentially into SelP, incorporated subsequently into GPx, and incorporated only as a last option in SeAlb. As a consequence, under low Se intake or higher oxidative stress conditions, the 3 selenoproteins in theory are ordered hierarchically in Se incorporation. SeAlb and GPx should be the most sensitive proteins to decrease in such altered conditions. The observed significantly ($P < 0.05$) lower level and percentage of SeAlb and GPx in patients with type 2 diabetes, as well as the correlations among SeAlb, FPG, and HbA1c, seem to indicate that altered plasma selenoproteins status could be a mirror of bad metabolic control. Because the plasma selenoproteins level could be a potential biomarker for oxidative stress, this hypothesis is in agreement with the association between oxidative stress and glycemic control reported in some other studies. Such a relationship is still controversial in the literature, probably because not all adopted oxidative stress biomarkers biosynthesizes via pathways that are related directly to glycemic control.³⁸ Many of our patients with type 2 diabetes are overweight or obese and therefore may be affected by insulin resistance. Lower concentrations of SelP and total Se were found in patients with diabetes having HDL <47 mg/dL compared with patients with

HDL >61 mg/dL. Studies carried out on rats have shown a beneficial effect of Se supplementation on lipid abnormalities in plasma, aorta, and adipose tissue, suggesting that Se potentially also can reduce the risk of cardiovascular disease in patients with diabetes.³⁹ Natella et al⁴⁰ have shown that Se supplementation can cause a reduction of atherogenic negative LDL in the postprandial phase in healthy subjects. The positive correlation found in this study between Se and HDL, a well-known marker of high-risk atherosclerosis development, supports the previous observations.

A recent study by Kornhauser et al¹⁴ revealed an inverse association between ACR and serum GPx as well as the total Se level in patients with type 2 diabetes, suggesting that a lower serum Se concentration in these patients may be implicated in diabetic nephropathy. Our study confirmed this negative relationship for GPx but not for total Se. However, the K-S test applied to compare the plasma GPx levels in patients with microalbuminuria (ACR >30) showed no significant difference. Most likely, the possible biochemical relationship is relatively weak, and it cannot be revealed by a t test because of its low power, which is related to the relatively few cases we evaluated. In fact, for the t test, we obtained a P value close to 0.05, even if higher (0.08), as well as Kornhauser et al¹⁴ considering 114 individuals had a P value close to 0.05, even if lower (0.04).

GPx and SeAlb levels were correlated significantly with the age of the subjects. Other studies observed the same association, which may be from the less-efficient absorption or increased elimination of Se in older persons.⁴¹ However, in this study, the differences in plasma selenoproteins concentration between patients with diabetes and healthy subjects were unaffected by the potential confounding effect of age and gender. It is important to state that a further potential confounding factor for the selenoproteins distribution, particularly for SeAlb, is the Se dietary intake.

Nevertheless, the relationship between individual plasma selenoproteins and the dietary intake is very difficult to assess and is still controversial in the literature.^{42,43} Even if in this study, the dietary Se intake was not monitored, all recruited subjects were treated with the same controlled diet for at least 6 months prior to the analysis and, moreover, were resident in the same (nondeficient) Se region.⁴⁴ Therefore, we reasonably can assume that the significant observed differences are only a result of the health of the subjects.

Few data have been available until now on the effect of Se on insulin resistance in patients with type 2 diabetes. Experimental evidences from *in vitro* and *in vivo* studies in animals suggested that Se may mediate many insulin-like actions and thus enhance insulin sensitivity,^{3,11} but this finding is still unconfirmed for patients with type 2 diabetes. The biochemical mechanism through which Se carries out its action is still unknown. The logistic model performances in our study have shown the potential of plasma selenoproteins status as a combined biomarker for type 2 diabetes in contrast with the misleading picture given by the total Se concentration. This study provides the first evidence for the possible role of Se in diabetes under unsupplemented conditions, which is a requisite that makes the results closer to the real behavior of Se in diabetes biochemistry.

Study limitations. The main limitation of the study is the relatively low sample size, particularly for the control group ($n = 40$ patients with diabetes and $n = 15$ controls). This aspect affects the confidence range of the statistical results and limits their interpretation, which is more relevant for the elaborations that required a further subgrouping of the subjects (ie, the comparison of selenoproteins concentration between quarters of the clinical parameters). In addition, significant differences in age and gender exist between the 2 populations studied. These parameters were analyzed carefully as potential confounding factors. Finally, because of the cross-sectional nature of the study, it is not yet possible to establish whether the revealed altered selenoproteins concentration in patients with diabetes can be considered a causative factor or a consequence of the disease.

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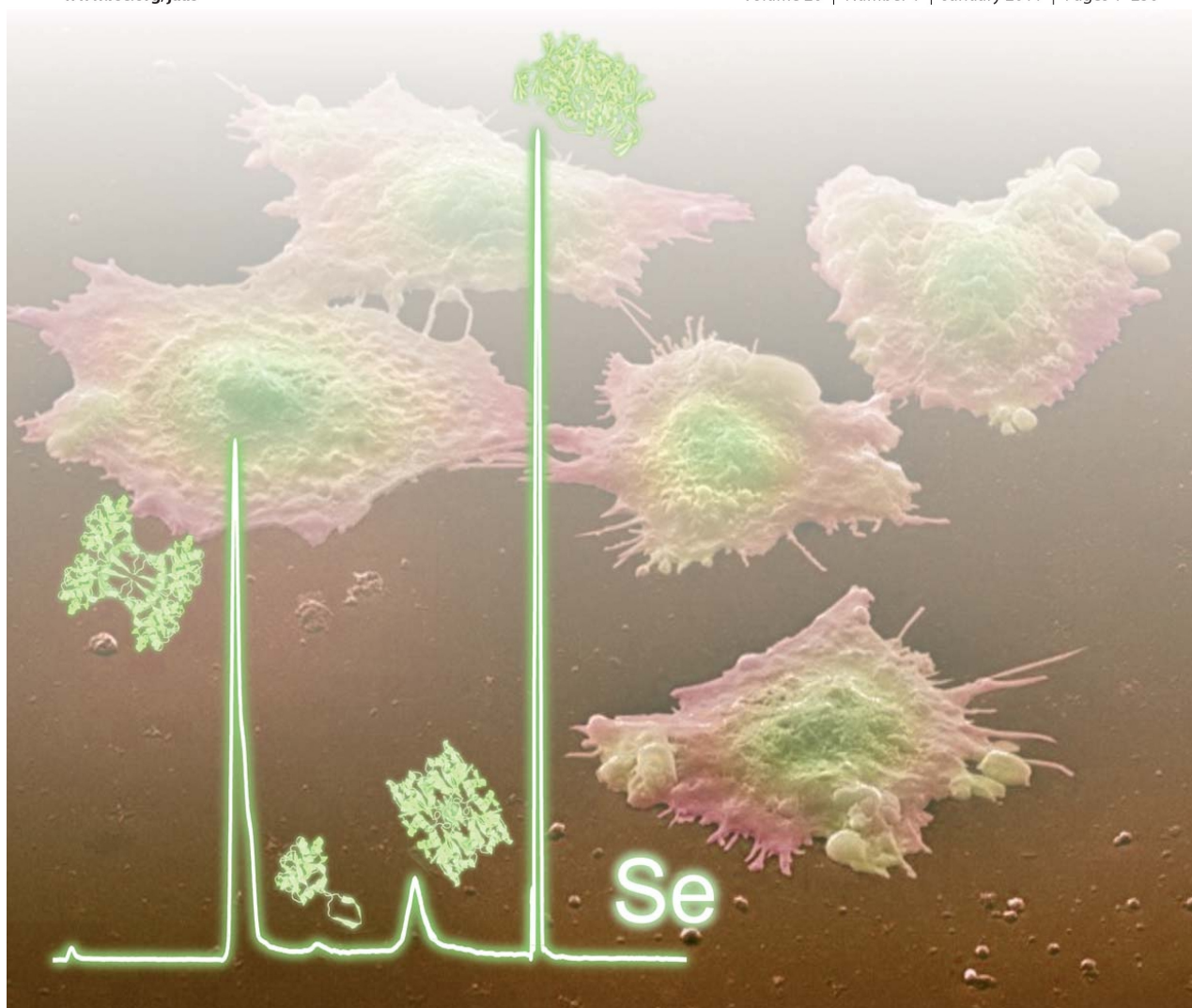
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PAPER

Selenium speciation in rat colon tissues†

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A study of selenium (Se) speciation in rat colon tissues is presented. Four different procedures for the extraction of Se compounds were evaluated in terms of recovery and species preservation. Total Se in tissue and extracts was determined by ICP-MS and isotope dilution analysis. The selected and optimized protocol allowed an extraction of 43% of Se, while continuously bubbling nitrogen in the solution during the procedure was mandatory to prevent the oxidative degradation of selenoproteins. Speciation analysis was then performed on the extracts using size exclusion- and anion exchange-HPLC for species separation. A number of Se compounds were detected in rat colon extracts, and individually quantified by coupling HPLC-ICP-MS and species-unspecific on-line (post-column) isotope dilution analysis. Among the isolated selenospecies, the two major proteins glutathione peroxidase type 2 and thioredoxin reductase type 1 have been potentially identified by their molecular weight using MALDI-TOF-MS.

Introduction

Selenium (Se) is an essential trace element in animals and humans, due to its important role in many biological functions including oxidative stress control, thyroid hormone regulation,¹ insulin mimesis,² and anticarcinogenic action.³ The biological action of Se in humans and animals is mediated by the selenoproteins, where Se is incorporated in the form of the amino acid selenocysteine. About 25 selenoproteins have been identified in human proteome⁴ and 24 in rat and mice proteome,⁵ but few of them have been fully characterized. The most interesting selenoproteins showed to carry out antioxidant action, as the glutathione peroxidases (GPxs), or cellular redox signaling, as the thioredoxin reductases (TrxRs). According to their specific function/location in the organism, such proteins can constitute promising biomarkers for global oxidative stress status as is the case for serum/plasma selenoproteins, but also may be important for organ-specific diseases. In this field the early detection and progression of cancer constitutes a major challenge. Many forms of cancer are associated with Se status,⁶ and among them colorectal cancer showed one of the most interesting associations.^{7–9} Despite the increasing need to investigate the relationship

between individual selenospecies concentration/distribution and specific diseases, so far only a few analytical methodologies have been developed for Se speciation in body fluids such as plasma, serum and urine,^{10,11} and even fewer methodologies address tissues. Most of the published studies regarding solid samples are focused on Se speciation in foodstuff and supplements such as yeast and wheat flour,¹² rice¹³ and fish.^{14–16} Therefore, accurate and precise analytical methodologies for the quantitative speciation analysis of Se in animal tissues are a necessity.

In order to investigate chemical speciation studies in solid samples, the first required step is the extraction of selenospecies to a liquid phase, without any alteration of their chemical form. Many approaches have been proposed in the literature to extract Se compounds from biological samples. Leaching by aqueous solutions and aqueous-organic mixtures allows the extraction of free selenoamino acids as well as soluble and weakly-bound selenospecies from several biological matrices, but results in low recoveries.^{17–24} The addition of sodium dodecyl sulfate (SDS) increases the extraction efficiency for the recovery of Se-containing protein fraction.^{17,25,26} This reagent degrades the proteins and renders them water soluble by forming ion pairs. Proteolytic enzyme mixtures have been also widely used for the extraction of Se in a variety of solid biological matrices.^{17,18,22,24,26–30} In this case, quantitative recovery of Se can be obtained, but since proteases break the peptidic bonds, all of the information concerning the original selenoproteins is lost.

A system based on an HPLC coupled on-line with a Se-specific and sensitive detector such as an ICP-MS constitutes the best option for the separation and detection of selenospecies in extracts.³¹ Such instrument must be equipped with a collision/reaction cell since the most abundant Se isotopes are affected by

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† This article is part of a themed issue highlighting outstanding and emerging work in the area of speciation.

spectral interferences generated in the ICP source. This solution has been successfully applied to both total Se determination and Se speciation.^{12,32} Quantification of Se in the chromatographic mode might be a critical issue because often commercially available standards for the detected compounds do not exist, and matrix effects can invalidate the use of other external calibrants. To overcome these drawbacks, a new powerful approach for reliable determinations in biological material speciation analysis consists of the application of species-unspecific isotope dilution techniques carried out on-line with HPLC-ICP-MS.³² This method allows accurate quantification of the heteroatom measured in the compound (in this case Se), even if its structure and composition are unknown. In this approach the species separation is performed first, and the enriched isotope is then added post-column.³¹ This methodology has been recently applied for Se speciation in yeast and wheat flour enzymatic extracts,¹² cod muscle certified reference material³³ and human serum.¹⁰

The aim of this study consists of the development of a methodology for the speciation of Se in colon tissues. Healthy rat colon samples were used as a model for humans in the first stage of the investigation, presented here. We studied different extraction procedures in order to optimize the balance between efficiency maximization and species preservation, developing a method for the separation of selenospecies based on two-dimensional (size exclusion and anion exchange) HPLC coupled on-line with ICP-MS detector. We then carried out the quantification of both total Se and individual selenospecies by isotope dilution analysis (IDA), identifying the isolated species by MALDI-TOF-MS analysis of the HPLC fractions.

Experimental

Instrumentation

Solid sample digestions were carried out with a Milestone microwave oven model Ethos-1 (Microwave Laboratory Systems, Socisole, Italy) with an EM-457(A) extractor module and an AC-100 open/close module with medium pressure PTFE vessels. Tissue extractions were carried out by using a Reax2 Stirrer (Heidolph, Kelheim, Germany), an ultrasonic bath Ultrasons (JP Selecta, Barcelona, Spain), a Sonicator up200s (Hielscher, Teltow, Germany) and an Ultra-Turrax T-8 homogenizer (Ika, Staufen, Germany). Alkaline digestion of the extraction residues was carried out in a controlled thermostatic bath Digiterm 100 (JP Selecta, Barcelona, Spain).

A HPLC system Agilent 1100 series (Agilent Technologies, Tokyo, Japan) equipped with autosampler and diode array detector (DAD) was used as solvent delivery system for method development, while a Shimadzu HPLC pump (Shimadzu LC-10AD, Shimadzu Corporation, Kyoto, Japan) was used for coupling with the ICP-MS. Injections were made using a model 7725 Rheodyne valve (Rheodyne, Cotati, CA, USA) fitted with a 100 μ L loop. The size-exclusion (SEC) columns were a Superdex 75 10/300 GL with MW range of 3000–70 000 Da (Amersham Biosciences, Uppsala, Sweden); a Shodex Asahipak GS-520 HQ SEC with MW range of 10 000–300 000 Da (Showa Deko, Tokyo, Japan); and a Bio-Rad Bio-Sil SEC 125-5 (Bio-Rad, Richmond, California, USA) with a MW range of 5000–

Table 1 Isotopic abundances (atom, %) of natural selenium and the ⁷⁶Se enriched spike solution (measured)^a

Mass	Natural	⁷⁶ Se enriched
74	0.89 ± 0.04	0.170 ± 0.001
76	9.37 ± 0.29	99.599 ± 0.005
77	7.63 ± 0.16	0.177 ± 0.008
78	23.77 ± 0.28	0.026 ± 0.005
80	49.61 ± 0.41	0.022 ± 0.006
82	8.73 ± 0.22	0.006 ± 0.001

^a Determined after corrections for dead time, SeH⁺ formation, BrH⁺ interference and mass bias discrimination, by using natural selenium (*n* = 5).

100 000 Da. The anion-exchange (AE) column was a Mono Q™ 5/50 GL (Amersham Biosciences, Uppsala, Sweden). All the HPLC columns were connected to the ICP-MS nebulizer with PEEK® tubing (30 cm × 0.25 mm i.d.). A syringe pump model Pump 11 (Harvard Apparatus, Edenbridge, UK) was used for spike addition in on-line post-column isotope dilution analysis.

An inductively coupled plasma mass spectrometer (ICP-MS) equipped with a collision/reaction cell system, model 7500cx from Agilent Technologies (Agilent, Tokyo, Japan) was used for Se determinations. The instrument consists of an ICP source with plasma-shield torch, an enclosed octapole ion guide operated in the RF mode and a quadrupole mass analyser with a SEM detector. A flow of 4.0 ml min⁻¹ of hydrogen was used to pressurise the octapole chamber. The sample introduction system consisted of a Meinhard nebuliser with double-pass glass spray chamber cooled down to 2 °C. Plasma operating conditions and acquisition parameters are presented in Table 1.

The selenospecies identification was carried out by a matrix assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) model Voyager-DE™ STR Workstation (Applied Biosystem, Langen, Germany) equipped with a nitrogen pulsed laser (337 nm) and operating in positive mode.

Reagents and materials

Ultra-pure water was obtained from a Milli-Q System (Millipore Co., Bedford, MA, USA). All reagents used were of analytical grade. Nitric acid (additionally purified by sub-boiling distillation), hydrogen chloride and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (TRIS) base, ammonium chloride, 25% (w/v) tetramethylammonium hydroxide (TMAH) solution in water were purchased from Sigma-Aldrich (Milan, Italy).

All the TRIS-buffered solutions were prepared in ultra-pure water, and the pH was adjusted dropwise by HCl. TritonX-100 and protease inhibitor cocktails (both from Sigma-Aldrich) containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin, E-64, bestatin, leupeptin, and aprotinin, were used for extraction buffer preparation. Protease inhibition cocktail (1% in weight) was added just before the extractions to the extraction buffer (TB) TRIS-HCl 50 mM, pH 7.4. For the preparation of extraction buffer with detergent (TBD), an aliquot of the TB solution was transferred in a plastic tube and Triton X-100 was added in 1% (v/v). All the buffers were stored at 4 °C and degassed immediately prior to use. Amicon Ultra centrifuge filter

devices (cutoff: 30 000 Da) were purchased from Millipore (Billerica, MA, USA).

Gel filtration standard mix for SEC column calibration containing the proteins: thyroglobulin (670 000 Da), bovine gamma-globulin (158 000 Da), chicken ovalbumin (44 000 Da), equine myoglobin (17 000 Da), and vitamin B₁₂ (1350 Da), was purchased from Bio-Rad (Richmond, California, USA). Standard pure solutions of thioredoxin reductase from rat liver and glutathione peroxidase from bovine liver were purchased from Sigma-Aldrich (Milan, Italy). Peptide calibration mixture 2 containing angiotensin I, ACTH 1–17, 18–39, 7–38 clips and bovine insulin was purchased from Applied Biosystems. Sinapic acid, α -cyano-4-hydroxycinnamic acid (HCCA) and TPCK-treated trypsin from bovin pancreas were also purchased from Sigma. A standard solution of 1000 mg L⁻¹ of SeO₃²⁻ stabilized in 2% (v/v) nitric acid Suprapur was purchased from Merck. Enriched ⁷⁶Se was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder, was dissolved in a minimum volume of sub-boiled nitric acid and diluted to volume with ultra-pure water. The concentration of this solution was determined by reverse isotope dilution analysis using a natural abundance standard. Table 1 reports the isotopic composition of natural Se and the measured isotopic composition of the enriched ⁷⁶Se spike. The isotopic composition of the Se standard solution was considered to be of natural isotopic abundance, as reported by Rosman and Taylor.³⁴

Samples

Rat colon samples (from healthy individuals) were obtained from the Animal Unit Laboratory of the University of Oviedo. The biological reference materials Bovine Liver NIST SRM 1577a, from the National Institute of Standards and Technology (Gaithersburg, MD, USA) was used as quality control for microwave digestion procedure.

Procedures

Sampling. Animals were killed by cervical dislocation. Colons were dissected into portions, opened longitudinally and the faeces were removed. The tissue was washed with cold (4 °C) TB solution, dried gently with paper and immediately stored at -20 °C until extraction.

Microwave digestion. Approximately 0.1 g of each sample was digested with 9 mL of HNO₃ diluted 1 : 3 in water and 1.5 mL of H₂O₂ in a microwave oven. An appropriate amount of ⁷⁶Se spike (calculated by the error magnification theory)³⁵ was added directly into the vessels before the digestion. A previously developed program for digestion of biological samples was used.³²

Extraction procedure study. A pooled sample (obtained from three individuals) was frozen in liquid N₂ and homogenized by grinding with a mortar and pestle. Three aliquots of 0.1 g of the homogenized sample were used for total Se determination by microwave digestion. Aliquots of 0.1 g of the homogenized sample were placed into a 1.5 mL Eppendorf tube, 0.4 g of extraction buffer (TB or TBD, at 4 °C) were added and kept at

0 °C in order to prevent thermal degradation of the species. Extraction was carried out on different aliquots of the sample by stirring (in ice), ultrasonic bath (in 4 °C water), ultrasonic probe (in ice) and Ultra-Turrax (in ice). Extractions were performed sequentially for 30 min, 1 h, 2 h, 3 h by stirring and by placing the samples in the ultrasonic bath. The ultrasonic probe was used in the continuous pulse mode (power 100%) for 10 s of extraction followed by a pause of 50 s, where the cycle was repeated three and six times. The Ultra-Turrax homogenizer was used at speed 6 for 10 s of extraction followed by a pause of 50 s. The cycle was repeated four times. After the extractions, samples were centrifuged at 10 000 g, 4 °C for 30 min and the supernatant was transferred to a clean Eppendorf tube for immediate storage at -20 °C until analysis. For all the sequential extractions, each step was performed by adding new 0.4 g of buffer to the residue of the previous extractions. The residues of the last sequential extraction were digested by direct addition of 5 mL of 25% TMAH solution and incubation at 60 °C for 4 h.

Speciation of Se in the extracts. Two-dimensional chromatographic separation of selenospecies in the extracts was performed by size exclusion (SEC) followed by anion exchange (AE) HPLC, both of them coupled on-line to the ICP-MS. The chromatographic conditions are reported in Table 2.

Three different SEC columns were calibrated by injecting 100 μ L of the diluted calibration mixture in the mobile phase. The chromatographic profile was monitored by UV detector at

Table 2 Operating conditions

SEC	
Columns	Bio-Rad Bio-Sil SEC 125-5 Shodex Asahipak GS-520 HQ SEC
Mobile phase	Tris-HCl (50 mM) pH 7.4
Gradient	Isocratic
Flow rate	1 mL min ⁻¹
Injection volume	100 μ L
AE	
Column	Mono Q™ 5/50
Mobile phases	A – Tris-HCl (25 mM) pH 8.8 B – Tris-HCl (25 mM) pH 7.4 C – Tris-HCl (25 mM), CH ₃ COONH ₄ (1M) pH 7.4
Flow rate	1 mL min ⁻¹
Injection volume	100 μ L
ICP-MS	
RF power	1500 W
Plasma gas flow rate	15 L min ⁻¹
Auxiliary gas flow rate	1.1 mL min ⁻¹
Ion lens setting	Optimized daily for best sensitivity of 10 mg L ⁻¹ Li, Co, Y and Tl, 1% (w/w) HNO ₃ solution
Reaction/collision cell H ₂ gas flow	4 mL min ⁻¹
Total Se determination	
Monitored masses	76, 77, 78, 79, 80, 81, 82 and 83
Points per peak	3
Acquisition time per point	4 s
Replicates	5
HPLC coupling	
Monitored isotopes	76, 77, 78
Points per peak	1
Acquisition time per point	0.5 s

280 nm. After selection of the best column, the main SEC fraction was collected in ice, centrifuged in Amicore at 10 000 g, 4 °C and washed with ~8 mL of 25 mM TRIS-HCl buffer at pH 8.8 (previously stored at 4 °C and saturated with N₂ for 10 min) until reaching a concentration two times greater than the originally injected volume.

The AE chromatographic separation was achieved by injecting 100 µL of the SEC fraction and using the operative conditions reported in Table 2. The elution sequence consists of the following steps: 1) 0–5 min 100% A; 2) 5–13 min 35% A, 65% B; 3) 13–21 min 90% B, 10% C; 4) 21–25 min 80% B, 20% C; 5) 25–30 min 100% C; 6) 30–35 min 100% A.

Determination of total Se in digested samples and extracts.

Microwave digested samples were diluted by weight to ~20 g with ultra-pure water, while the alkaline digested residues and the extracts were spiked with the appropriate amount of ⁷⁶Se enriched solution, and diluted 1 : 2 with ultra-pure water. All of the samples were directly analyzed by ICP-MS and total Se was determined by IDA. The ⁷⁸Se/⁷⁶Se isotopic ratio was preferred to ⁸⁰Se/⁷⁶Se to avoid further corrections due to the BrH⁺ interference on the ⁸⁰Se signal.³² Signal intensities were corrected for SeH⁺ formation and detector dead time, and the ratio was corrected for mass bias. Plasma conditions and acquisition parameters are reported in Table 2.

Quantification of selenospecies. Selenospecies concentration in the fraction collected from SEC and chromatographically separated by AE was determined by on-line post-column IDA-ICP-MS. For this purpose a ⁷⁶Se-enriched solution of the appropriate concentration was continuously added to the eluate (flow 0.01 mL min⁻¹) through a T-connection. The ⁷⁸Se/⁷⁶Se isotopic ratio was calculated and corrected as previously mentioned for total Se determination. Plasma conditions and acquisition parameters for HPLC-ICP-MS coupling are also reported in Table 2.

Identification of selenospecies by MALDI-TOF-MS. In order to identify the selenospecies isolated by AE, fractions corresponding to each detected peak were collected and analyzed by MALDI-TOF-MS. Desalting and preconcentration of the fractions were carried out by washing with of ultra-pure cold (4 °C) water with a volume 50 times greater than that of the sample, and centrifugation (at 10 000g, 4 °C) in Amicon Ultra centrifuge filters (cutoff: 30 000 Da). Then, aliquots of this solution were mixed with saturated sinapinic acid, used as MALDI matrix for the determination of intact proteins MW. Linear mode was used, and external calibration of the instrument was performed daily using a bovine albumin (MW 66 433 Da) standard.

The same desalted and preconcentrated fractions were also solubilized in 8 M urea, which was reduced by adding dithiothreitol to 1 mM, carboxyamidomethylated in 10 mM iodoacetamide and digested with trypsin overnight at 37 °C. The digests were mixed with saturated HCCA as MALDI matrix for peptide characterization and the reflector mode was used. External calibration of the instrument was achieved by analysing the calibration mixture 2 from Applied Biosystems.

Results and discussion

Total Se determination

Three rat colon samples collected from different healthy individuals were analyzed. The average concentration in the fresh tissue was 213 ± 48 ng g⁻¹. For method validation, three replicates of bovine liver CRM were mixed with an appropriate amount of ⁷⁶Se spike solution (at concentration of 100 ng g⁻¹), digested in the microwave oven and analyzed for total Se in order to validate the IDA methodology. The obtained result (0.70 ± 0.01 µg g⁻¹) was in agreement with the certified value (0.71 ± 0.07 µg g⁻¹).

Evaluation of extraction efficiency

In order to evaluate the extraction efficiency, total Se was determined in three aliquots of the pooled fresh sample, obtaining an average concentration of 169 ± 9 ng g⁻¹. Total Se was then determined in the extracts for each investigated procedure and in the corresponding blanks and residues. The concentration was compared with the results obtained by microwave digestion of the original fresh tissue in order to calculate the extraction efficiency. The sum of total Se determined in the alkaline digested residues and Se level in the corresponding sequential extracts was compared with the total level of Se in the fresh tissue as a form of quality control. The values were not significantly different (*t*-test: *p*-value > 0.05) for all the extraction procedures. This demonstrated that there were no losses of the analyte during the sequential steps, and also that alkaline and microwave digestions are compatible methods. Table 3 reports the results obtained for all the extraction procedures and different extraction times/number of cycles. Only a small increase in efficiency was achieved by adding Triton-X to the buffer, which may be because all of the methods present enough strength to break down the membranes and allow the complete release of cytoplasmatic proteins. The ultrasonic probe extractions are an exception, where Triton-X addition increased significantly the efficiency, which otherwise was relatively low.

Table 3 Total Se concentration (referred to the solid sample) in the extracts and the corresponding efficiencies calculated for the different extraction procedures investigated

Extraction procedure	Time/cycles	Se/ng g ⁻¹		Efficiency (%)	
		TB	TBD	TB	TBD
Stirring	30 min	50	57	30	33
	1 h	73	79	43	46
	2 h	92	99	55	59
	3 h	111	118	65	69
Ultrasonic bath	10 min	30	34	18	20
	20 min	49	53	29	31
	30 min	64	70	38	41
	40 min	79	87	47	51
Ultrasonic probe	3 cycles	54	92	32	54
	6 cycles	57	100	34	59
Ultra-Turrax	4 cycles	73	76	43	45

The use of the detergent addition was rejected because it did not significantly increase extraction efficiency, and was problematic for chromatographic separation of the extracts where further sample preparation steps are required to remove the detergent before separation. Increasing the extraction time/number of cycles improves efficiency for all the methods. The use of an ultrasonic probe or Ultra-Turrax significantly reduces the extraction time necessary to obtain the same efficiency compared with ultrasonic bath and stirring. It is difficult to control the temperature when stirring in ice and ultrasonating in cold (4 °C) water. The use of an ultrasonic probe can cause problems of analytes/sample loss due to formation of bubbles, particularly when detergent is used. Ultra-Turrax was chosen as best device for total Se extraction from rat colon tissues when taking into account a balance among efficiency, reproducibility and speed. Three replicates of extraction by this method were carried out on aliquots of the same sample to estimate the reproducibility, and an efficiency of $43 \pm 2\%$ was obtained.

Evaluation of species preservation and optimization of extraction procedure

Species integrity preservation during extraction is a mandatory requisite for Se speciation study. Extraction procedure and buffer composition needs to be carefully optimized by avoiding

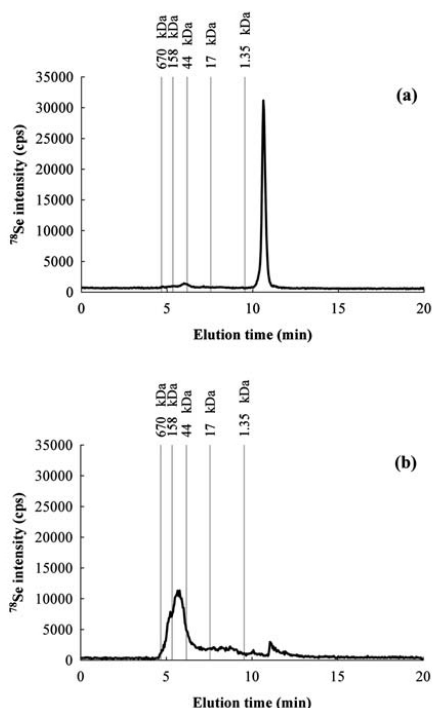


Fig. 1 Chromatograms for ^{76}Se obtained by SEC-HPLC of the same rat colon sample extract: (a) without bubbling N_2 in the buffer during the extraction and (b) by bubbling N_2 .

thermal and chemical degradation, particularly if the expected species are unstable. In order to evaluate the species preservation, the Ultra-Turrax extracts were injected undiluted into the chromatographic system for separation by SEC. A typical chromatogram is shown in Fig. 1a. Only one peak was obtained, which corresponds to non-proteic species. Selenocysteine, selenomethionine, selenate and selenite standards were injected into the SEC column and eluted with slightly different times. From the elution of the standards, it is not possible to clearly establish the identity of the peak observed for the extract, but relying on the literature we can assume that most likely it consists of selenite released by oxidation of the selenolic groups from selenoproteins.³⁶ To avoid such degradation, the extraction buffer was deoxygenated by continuously bubbling N_2 during the procedure. The extract obtained and injected into the SEC system resulted in the chromatogram shown in Fig. 1b, where a high MW species peak appeared, and the inorganic species peak was drastically reduced. Thus, bubbling nitrogen is effective to protect the analyte from oxidative degradation, but further precludes the use of detergents during the extraction. SDS should be also avoided, because it unfolds the proteins and then renders them more sensitive to oxidation. These aspects need to be taken into account in methodologies based on ICP-MS detection, since the loss of Se from the proteins implies the impossibility to detect them. Studies based on enzymatic assays should also be aware of these aspects because Se is part of the active site, and therefore its loss compromises the activity of selenoproteins. Degradation is scarcely discussed in the literature,^{17,37,38} and most of the studies directly address proteolytic digestion to detect individual amino acids.^{13,39–41} Proteolytic digestion results in the loss of most of the information about the original speciation pattern of Se because all selenoproteins contain the same amino acid selenocysteine.

Separation of selenospecies by SEC-HPLC

Rat colon extracts from the same pooled sample (three replicates and a blank) were injected undiluted into each of the chromatographic systems equipped with one of the three SEC columns for a preliminary screening of the Se-containing species. Based on the literature, three selenoproteins are expected to be present in rat colon tissue: GPx type 1 (GPx1, homotetrameric, MW of the monomer 22 305 Da), GPx type 2 (GPx2, homotetrameric, MW of the monomer 22 014 Da) and TrxR type 1 (TrxR1, homodimeric, MW of the monomer 54 386 Da).⁴² Therefore, a commercial pure standard of rat TrxR1 was also injected to estimate the elution time of the protein. Commercial standards of rat GPxs were not available, so their retention time was estimated by injecting a GPx1 from bovine liver (MW of the monomer 22 659 Da). The MW of the expected proteins in the sample are very similar (GPx1: 89 220 Da, GPx2: 88 056 Da, TrxR1: 108 772 Da), hence none of the columns allow the resolution of the proteins under investigation (see Fig. 2). The Superdex column results in a minor peak at 13.0 min which is not completely resolved from the major peak at 14.9 min. Both the Shodex and the Bio-Rad columns presented a single major peak, at 10.2 min and 5.6 min, respectively. The Shodex column presents a multimode separation mechanism (size exclusion/weak anion exchange), while the Superdex column has semi-preparative characteristics. In both cases the peak shape was not

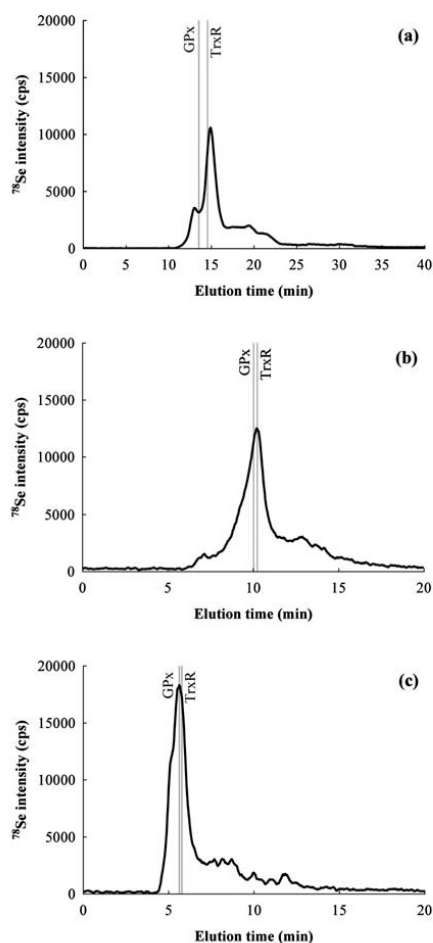


Fig. 2 Chromatograms for ^{76}Se obtained for the same rat colon extract by SEC-HPLC with different columns: (a) Superdex 75 10/300 GL, (b) Shodex Asahipak GS-520 HQ SEC, (c) Bio-Rad Bio-Sil SEC 125-5. The elution times signalled by gray lines were obtained by injecting TrxR1 from rat liver and GPx1 from bovine liver standards.

as good as that obtained using the Bio-Rad column. Even if the Shodex column offers good resolution for low MW compounds (*i.e.* selenoamino acids),^{43,44} it resulted here unsuitable for the direct separation of high MW protein mixtures. From the injection of rat TrxR1 and bovine GPx1 standards it was also observed that TrxR1 elutes after (but very close to) the bovine GPx1 even if it has higher MW, which may be an effect of the complex quaternary shape of the protein.

The Bio-Rad column did not allow the isolation of at least two peaks of selenospecies, but a useful separation of the major Se peak from other species without Se can be achieved, as showed by the UV chromatogram in Fig. 3a. Therefore, the SEC-HPLC system was selected as the first purification step in order to reduce

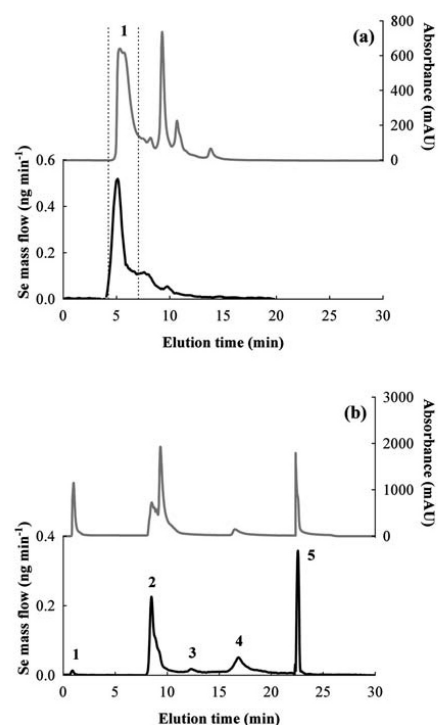


Fig. 3 UV and mass flow (Se) chromatograms obtained by: (a) SEC (Biorad column) of the rat colon extract (fraction 1 is indicated); (b) AE of the selected SEC fraction 1 (peaks 1–5 are indicated). The hypothesized identities of selenospecies corresponding to the peaks in the AE chromatogram are: 1) pH unequilibrated species; 2) GPx2; 3) GPx2 monomer; 4) GPx1; 5) TrxR1.

matrix complexity and to remove low MW proteins which possibly interfere with other chromatographic separation mechanisms and/or MALDI characterization. Quantitative results obtained by post-column on-line IDA and integration of the mass flow chromatograms (see Fig. 3a) show that the Bio-Rad SEC column recovery was $94 \pm 2\%$, and fraction 1 accounted $49 \pm 1\%$ of the total Se in the extract.

Separation of selenospecies by AE-HPLC

Anion exchange chromatography was the second separation mechanism investigated to isolate the selenospecies present in the SEC fraction detected by ICP-MS.

Fraction 1 from SEC (Fig. 3a) corresponds to the major peak (MW $> \sim 30\,000$ Da) which was collected and preconcentrated two times by ultracentrifugation in Amicones, and subsequently analyzed by AE-HPLC-ICP-MS (three replicates and a blank). The obtained chromatograms are shown in Fig. 3b. Optimization of the HPLC elution program finally allowed the separation of five well-resolved peaks of selenospecies. The theoretical pI of the expected selenoproteins (GPx1: 7.7, GPx2: 8.3, TrxR1: pI

Table 4 Concentration of Se in the fractions of the AE chromatogram (referred to the solid sample, average \pm SD, $n = 3$), and corresponding percentage referred to their sum

Peak no.	Se	
	/ng g ⁻¹	(%)
1	1.0 \pm 0.3	1.2
2	33.5 \pm 0.9	42.7
3	3.6 \pm 0.9	4.6
4	20.6 \pm 1.3	26.0
5	20.2 \pm 1.7	25.5
Sum	78.9 \pm 2.5	

5.9) are in agreement with the selected elution conditions and demonstrate compatibly with the correspondence of GPx2 to peak no. 2, GPx1 to peak no. 4 and TrxR1 to peak no. 5.

Injection of a pure TrxR1 standard solution resulted in single peak at elution time matching with the fifth peak of the extracts. Since the standards of rat GPxs were not commercially available, their possible identification cannot be obtained by matching retention times with pure proteins samples. However, based on a chromatographic run (not shown) obtained by a linear gradient from 100% of buffer B to 100% of buffer C (5–25 min), and with a hypothesized corresponding linear pH gradient, the experimental pH of the species corresponding to the fractions 2 and 4 were estimated as 8.1 and 7.6, respectively. The measured values agree with the theoretical pI of GPx2 (8.3) and GPx1 (7.7), respectively. Quantitative determinations by post-column IDA, reported in Table 4, showed that TrxR1 accounts \sim 25% of the total Se in the extract. Considering a constant ratio Se/protein 1 : 1 (for the monomer), TrxR1 extracted from the tissue can be estimated as 0.13 nmol g⁻¹.

The fraction 1 elutes with the void volume of the column, at pH 8.8 (buffer A), a condition which is not compatible with the expected proteins in the extract, where pH is higher than their pI. Such peaks could be due to the presence of other unexpected species, nevertheless new selenoproteins which are possibly present at extremely low levels appear to be improbably detectable by this analytical method. On the other hand, low MW species (*i.e.* selenite or free selenoamino acids, produced by analytes degradation) should be absent in the extract, and eventually removed by two phases of dimensional cutoff (SEC fraction collection for MW > 30 000 Da and ultracentrifugation in 10 000 Da cutoff membrane). A test was conducted without pH correction of the fraction before injection into the AE system, where the pH of the SEC fraction is 7.4 while the starting eluent for AE is buffered at pH 8.8. The chromatogram obtained for the fraction injected at pH 7.4 presented a much more abundant fraction 1, while the fractions 2, 4 and 5 were reduced. Consequently, the presence of fraction 1 is due by species (mainly GPxs) incompletely equilibrated with the new pH conditions. Even if this effect is negative for quantitative purposes, fraction 1 constitutes only \sim 1% of total Se in the extract (Table 4), and therefore is negligible. Fraction 3 elutes in the same conditions of fraction 2, but with higher retention time. Its presence could be due to the GPx2 monomer, which is another possible degradation product of the tetrameric protein.³⁶ The level of fraction 3

species correspond to \sim 5% of total Se, which is relatively low in respect to the other main proteins.

Quantification of fractions 2 and 4 (Table 4) showed that GPx2, a protein secreted into the gastrointestinal mucosa, is the major selenospecies in rat colon tissue extract, accounting for \sim 43% of the total Se. The cytoplasmatic GPx1 represents 26% of Se in the extract, comparable to the level of TrxR1. Considering a constant ratio Se/protein 1 : 1 for the GPxs monomer, GPx1 and GPx2 extracted from the tissue can be estimated as 0.11 and 0.07 nmol g⁻¹, respectively.

Identification by MALDI-TOF-MS

Fractions 2, 4 and 5 from AE chromatography (see Fig. 3b) were collected and analyzed by MALDI-TOF-MS for intact proteins identification. Fig. 4b shows a typical spectrum obtained for fraction 5: a molecular ion (M + H)⁺ was observed at m/z 55 178, \sim 800 Da higher than the predicted MW of the candidate protein TrxR1 (54 386 Da). The same fraction was also collected after injection of an extract spiked with the standard of TrxR1, and again analyzed by MALDI-TOF-MS. A similar molecular ion (M + H)⁺ was observed at m/z 55 303, and was more intense with respect to the background. On the other hand, the direct analysis of the TrxR1 standard gave a molecular ion (M + H)⁺ at m/z 54 394. The observed bias between the MW of TrxR1 predicted/measured as pure standard and spiked to the sample appears to be due to matrix effects such as the formation of clusters in the ionization process.

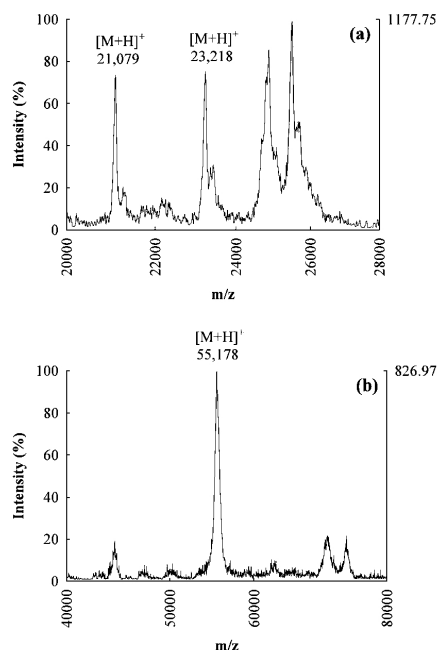


Fig. 4 MALDI-TOF-MS mass spectra of intact proteins for the AE fractions 2 (a) and 5 (b), the candidate molecular ions for GPx2 and TrxR1 are respectively reported.

Fig. 4a shows the mass spectrum obtained for MALDI-TOF-MS characterization of fraction 2. In this case two molecular ions (M + H)⁺ were observed at *m/z* 21 079 and 23 218 which are both possible candidates for GPx2 identification (theoretical MW 22 014 Da). Since no commercial standard of GPx2 was available, a comparison with the analysis of a spiked sample was impossible. Both observed *m/z* values could correspond to the target protein, with a mass difference due to degradation processes which do not involve the selenolic group (for the lower mass) or again by matrix effects (for the higher mass).

No candidate masses for GPx1 were observed in fraction 4, most probably due to the low concentration of the selenospecies and the complexity of the matrix. For these reasons, it was also impossible to identify selenoproteins by characterization of the tryptic digested fractions. Four peptides matching with theoretical fragments of GPx2 digestion were observed in fraction 2, but they were not sufficiently numerous to render the protein identification statistically significant.

Conclusions

This study demonstrates that among several protocols for the extraction of selenospecies from rat colon samples, Ultra-Turrax homogenizer is the most suitable device to obtain good extraction efficiency and species preservation. Bubbling nitrogen during the procedure prevents the problematic but often ignored aspect of oxidative degradation of selenolic groups, which result in the loss of Se as selenite. A method based on two-dimensional SEC and AE-HPLC-ICP-MS efficiently separates and accurately quantifies at least three selenospecies present in the extracts, potentially identified as the proteins GPx1, GPx2 and TrxR1. Among them, GPx2 was the most abundant Se protein in rat colon extracts in terms of Se, while TrxR1 was the most abundant at protein level. This study demonstrates that using a method based on HPLC-ICP-MS and post-column IDA, it is possible to simultaneously quantify all the three selenoproteins present in rat colon tissue at extremely low concentration. This method is suitable for future studies aimed to comparisons of the selenoproteins pattern in the colons of rats affected by cancer and healthy individuals, in order to investigate the potential role of colon tissue selenoproteins as cancer biomarkers.

Abbreviations

AE	anion-exchange
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
DAD	diode array detector
GPx	glutathione peroxidase
HCCA	α -cyano-4-hydroxycinnamic acid
HPLC	high performance liquid chromatography
ICP-MS	inductively coupled plasma-mass spectrometry
IDA	isotope dilution analysis
MALDI-TOF-MS	matrix assisted laser desorption-time of flight-mass spectrometry
MW	molecular weight
PTFE	polytetrafluoroethylene
RF	radio frequencies
SEC	size-exclusion chromatography

SEM	secondary electron multiplier
SDS	sodium dodecyl sulphate
TB	(TRIS-HCl) extraction buffer
TBD	(TRIS-HCl) extraction buffer with detergent
TMAH	tetramethylammonium hydroxide
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
TRIS	tris(hydroxymethyl)aminomethane
TrxR	thioredoxin reductase

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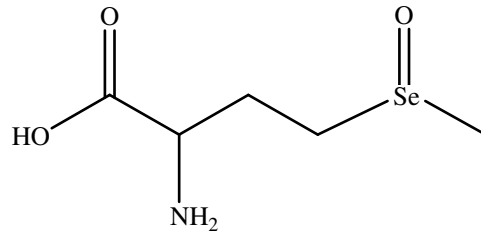
ATTACHMENTS

Table A-1	Se compounds of interest in proteomics.	p. 320
Table A-2	Human Se-proteins.	p. 324
Table A-3	Summary of epidemiologic studies of selenium and cancer risk.	p. 329

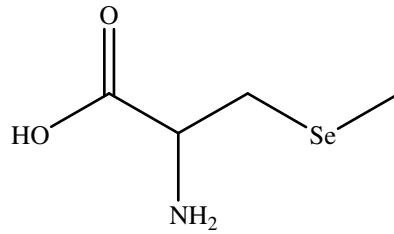
Table A - 1 Se compounds of interest in proteomics.

Name	Structural formula
methylselenide (MSe)	
dimethyldiselenide (DMSe)	
trimethylselenonium ion (TMSe)	
selenourea	
selenate (selenic acid)	
selenite	
SeCysteine (SeCys)	
SeCystine (Se-(Cys)2)	
SeMethionine (SeMet)	

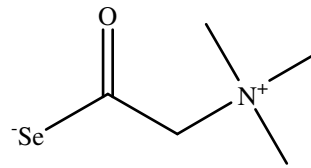
Se-methionine Se-oxide
(SeOMet)



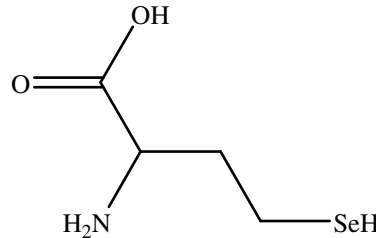
SeMethylSeCysteine
(Se-MCys)



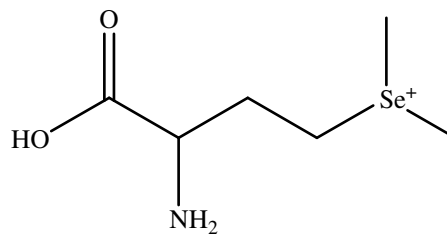
Selenobetaine
(Se-Bet)



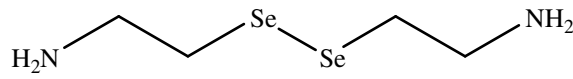
Se-Homocysteine
(Se-hoCys)



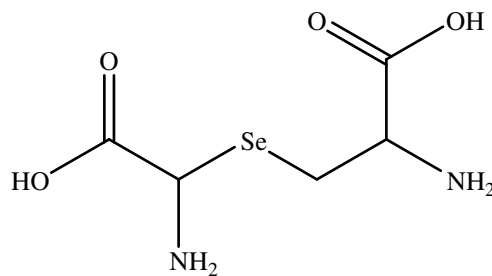
SeMethylSeMethionine
(Se-MM)



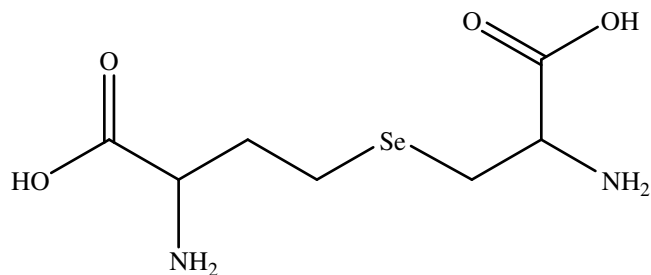
SeCystamine
(Se-Cya)



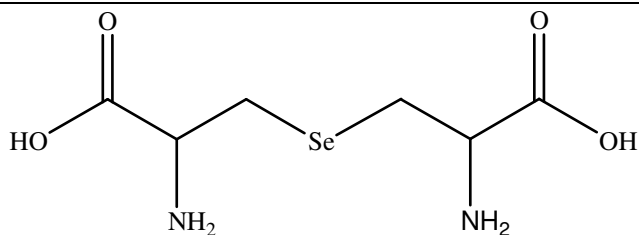
SeCystathione



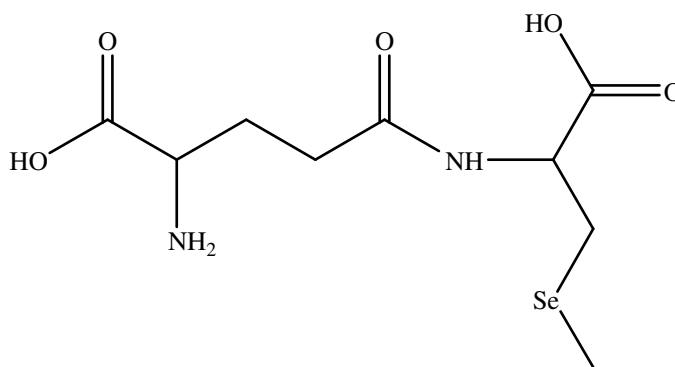
SeCystathionine
(SeCysth)



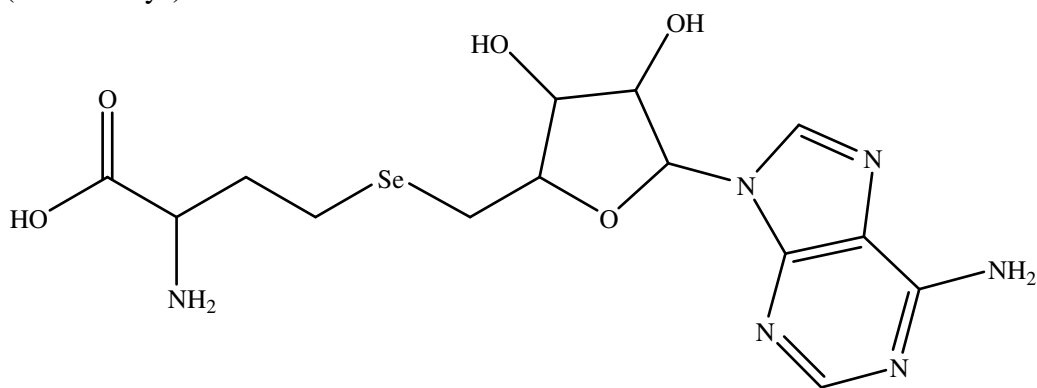
Se-Lanthionine
(Se-Lth)



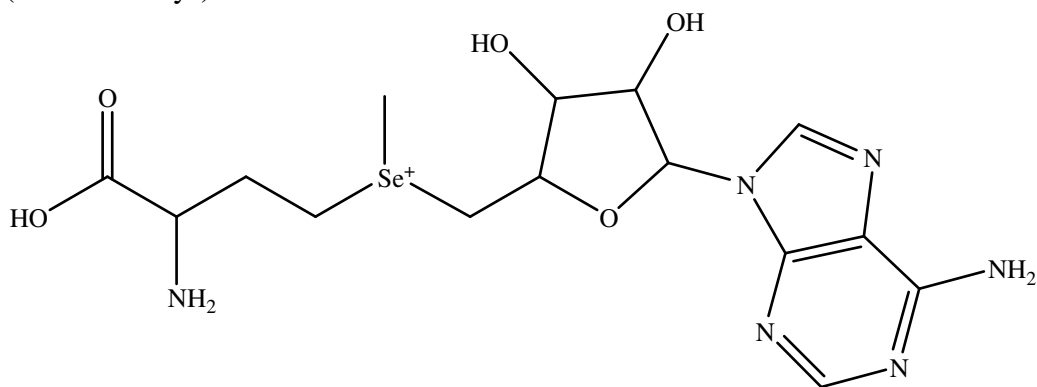
γ -glutamyl-
SeMethylSeCysteine
(GGMSC)

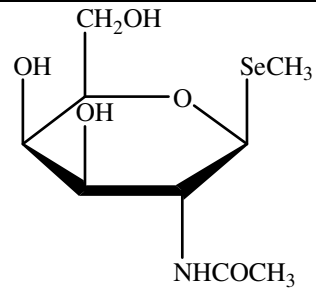


Se-adenosyl-homocysteine
(Se-A-hoCys)



metil-Se-adenosyl-homocysteine
(MSe-A-hoCys)



SeMethyl-*N*-acetylgalactosamine

Selenodiglutathione

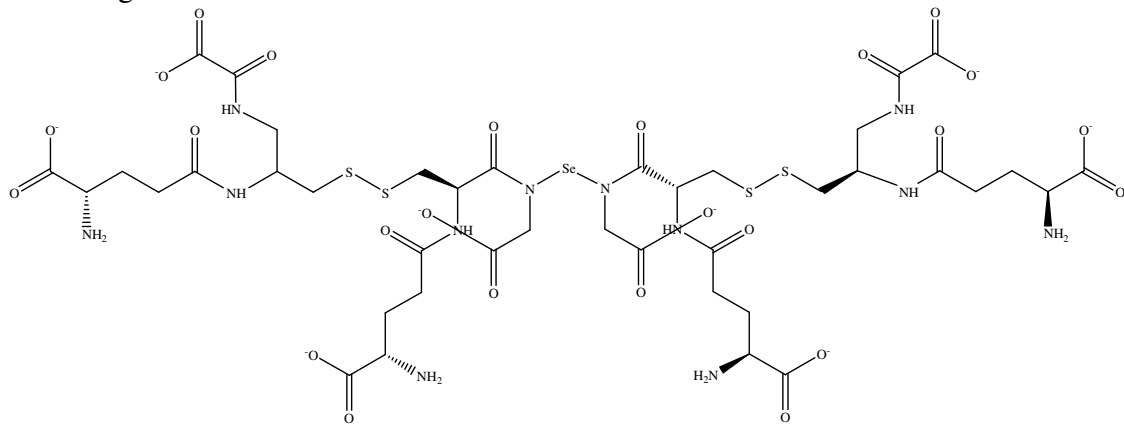


Table A - 2 Human Se-proteins.

Protein	Tissue distribution	Subcellular location	Function	Mass [kDa]	References
Methionine-R-sulfoxide reductase B1 (MrsB1, SelX, SelR)	ubiquitous	cytoplasm, nucleus	reduction of methyl-sulfoxide compounds to methionine	5-14 (2 isoforms)	[1-6]
Selenide, water dikinase 2 (Selenophosphate synthetase 2, SPS2)	liver	cytoplasm	synthesis of monoselenophosphate from selenide and ATP	47	[7-10]
Ethanolaminephosphotransferase I (Se-protein I, SelI, hEPTI)	various tissues; abundant in brain, placenta, liver, pancreas	membrane; multi-pass membrane protein	catalysis of phosphatidylethanolamine biosynthesis from CDP-ethanolamine ('Kennedy' pathway); formation and maintenance of vesicular membranes	45	[11-13]
Se-protein K (SelK)	various tissues; abundant in hearth	endoplasmic reticulum membrane	protection of cardiomyocytes from oxidative stress-induced cytotoxicity through the reduction of reactive oxygen species production	10	[14]
Se-protein N (SelN)	ubiquitous; abundant in skeletal muscle, brain, lung, placenta	endoplasmic reticulum membrane	reactive oxygen species reduction and redox-related calcium homeostasis	61-62 (2 isoforms, glycosylated)	[15,16]
Se-protein O	various tissues	unknown	unknown	73	[17-19]
Se-protein P (SeIP, SEPP1)	produced in the liver and heart, secreted into the plasma. Found also in the kidney	secreted	selenium transport from the liver to peripheral tissues; extracellular antioxidant defence	50-60 (2 isoforms)	[20-26]

Table A - 2 (continuation)

Se-protein S (SeIS, VIMP)	plasma, various tissues	endoplasmic reticulum membrane; single-pass membrane protein	cell protection from oxidative damage and endoplasmic reticulum stress-induced apoptosis; participation in the retro-translocation of misfolded proteins from the endoplasmic reticulum lumen to cytosol for degradation. Involved in spermatogenesis, lipoprotein metabolism and regulation of inflammatory response	21	[17,27,28]
Rdx proteins family					
Se-protein H (SeH)	various tissues, mainly expressed in embryonic or tumor cells	nucleus: redox-responsive DNA-binding protein	regulation of expression levels of genes involved in glutathione synthesis and phase II detoxification in response to redox status	13	[17,29,30]
Se-protein T (SeT)	ubiquitous	golgi and endoplasmic reticulum	redox regulation, cell adhesion, Ca ²⁺ homeostasis and neuroendocrine secretion	20	[17,31,32]
Se-protein V (SeV)	testes	unknown	redox regulation	37	[33]
Se-protein W (SeW, SEPW1)	various tissues, abundant in muscles	cytoplasm	antioxidant function in muscle and heart cells	9	[33-36]

Table A - 2 (continuation)

Se-protein M/SEP15 family			
Se-protein M (SelM)	mainly brain; kidney, lung and other tissues	cytoplasm; perinuclear region; endoplasmic reticulum, golgi apparatus	reduction of reactive oxygen specie by upregulation of GPx and SOD; calcium regulation; activation of the ERK pathway to attenuate α/γ -secretase-mediated proteolysis and Tau phosphorylation; may function as a thiol-disulfide oxidoreductase that participates in disulfide bond formation
15 kDa Se-protein (Sep15)	high levels in prostate and thyroid gland; lung, brain, kidney, H9 T cells	endoplasmic reticulum lumen	may be involved in redox reactions associated with the formation of disulfide bonds. May contribute to the quality control of protein folding in the endoplasmic reticulum
iodothyronine deiodinases family			
Type I iodothyronine deiodinases (DIO I)	liver, kidney, thyroid, pituitary gland	endoplasmic reticulum; single-pass membrane protein	deiodination of T4 (3,5,3',5'-tetraiodothyronine) into T3 (3,5,3'-triiodothyronine) and of T3 into T2 (3,3',5'-diiodothyronine)
Type II iodothyronine deiodinases (DIO II)	thyroid, heart, brain, spinal cord, skeletal muscle, placenta kidney, pancreas	membrane; single-pass membrane protein	deiodination of T4 into T3; essential for providing the brain with appropriate levels of T3 during the critical period of embryological development
Type III iodothyronine deiodinases (DIO III)	placenta, fetal liver	cell and endosome membrane; single-pass type II membrane protein	deiodination of T4 into T3 and of T3 into T2; regulation of thyroid hormone inactivation during embryological development; may play a role in preventing premature exposure of developing fetal tissues to adult levels of thyroid hormones

Table A - 2 (continuation)

glutathione peroxidases family					
cytosolic glutathione peroxidase (GPx 1, cGPx)	ubiquitous, highly expressed in erythrocytes, liver, kidney, lung	cytoplasm	reduction of soluble hydroperoxides (H ₂ O ₂) and some organic hydroperoxides, (hydroperoxy fatty acids, cumene hydroperoxide and tert-butyl hydroperoxide)	87 (homo-tetrameric)	[64-68]
Gastrointestinal glutathione peroxidase (GPx 2, GPx-GI)	liver, epithelium of the gastrointestinal tract	cytoplasm	reduction of soluble hydroperoxides and some organic hydroperoxides	93 (homo-tetrameric)	[69-71]
Plasma glutathione hydroperoxidase (GPx 3, pGPx)	plasma, milk, lung, aqueous humor; produced into the kidney	secreted	reduction of soluble hydroperoxides, organic hydroperoxides and some hydroperoxides of more complex lipids like phosphatidylcholine hydroperoxide	93 (homo-tetrameric)	[72-78]
Phospholipid glutathione hydroperoxidase (GPx 4, PHGPx)	primarily in testes	mitochondrion capsule (inactive structural), cytoplasm	reduction of soluble hydroperoxides, organic hydroperoxides, hydroperoxides of complex lipids, hydroperoxo groups of thymine, lipoproteins, cholesterol esters, hydroperoxides integrated in membranes; plays a role in leukotriene biosynthesis, regulation of cytokine signaling, reduction of hydroperoxides in HDL and LDL	22	[64,79-83]
Epididymal secretory glutathione peroxidase (GPx 5)	epididymis	secreted	protection against peroxide damage in sperm membrane lipids	23	[84-87]
Glutathione peroxidase (GPx 6)	olfactory epithelium, embryos	secreted	odorant-metabolizing protein	23	[17,88]

Table A - 2 (continuation)

class-I pyridine nucleotide-disulfide oxidoreductase family			
Thioredoxin reductase 1 (TrxR1, TxnRd1)	ubiquitous	cytoplasm	60-108 (homo-dimeric; 4 isoforms) [89-93]
			cellular redox regulation through reduction and thereby activation of thioredoxin, which serves as reducing equivalent and catalyzes many redox reactions; immunomodulation, embryogenesis. Isoform 1 induces actin and tubulin polymerization, leading to formation of cell membrane protrusions. Isoforms 4 and 5 enhance the transcriptional activity of estrogen receptors. Isoform 5 also mediates cell death induced by a combination of interferon-beta and retinoic acid
Thioredoxin reductase 2 (TrxR2, TxnRd2)	ubiquitous; highly expressed in the prostate, ovary, liver, testes, uterus, colon, small intestine	cytoplasm	60-106 (homo-dimeric; 4 isoforms) [94-97]
			maintenance of thioredoxin in a reduced state, blood cells and hearth tissue development. May play a role in redox-regulated cell signalling
Thioredoxin reductase 3 (TrxR3, TxnRd2, TGR)	testes	cytoplasm, nucleus, microsome, endoplasmic reticulum	75 [93,98,99]
			hioredoxin reductase, glutaredoxin and glutathione reductase activities; catalysis of disulfide bond isomerisation; promotion of disulfide bond formation between GPx4 and various sperm proteins; may play a role in sperm maturation by promoting formation of structural components

Table A - 3 Summary of epidemiologic studies of total Se level and cancer risk. Randomized trials have been excluded. RR or OR \geq 1 means statistically significant association.

reference	cancer	matrix	study design	cases/controls	population	risk estimate
Comstock <i>et al.</i> [100]	lung	serum	NCC	258/515	USA	RR=0.65 (0.41-1.02)
Kabuto <i>et al.</i> [101]	lung	serum	NCC	77/120	Japan	RR=0.56 (0.20-1.43)
Knekt <i>et al.</i> [102]	lung	serum	NCC	153/153	Finland	RR=0.66 (0.37-1.19)
Ratnasinghe <i>et al.</i> [103]	lung	serum	NCC	108/216	China	RR=1.20 (0.60-2.40)
Garland <i>et al.</i> [104]	lung	toenail	NCC	47/47	USA	RR=4.33 (0.54-34.60)
Hartman <i>et al.</i> [105]	lung	toenail	NCC	250/250	Finland	RR=0.20 (0.09-0.44)
van den Brandt <i>et al.</i> [106]	lung	toenail	Cohort	317/2459	The Netherlands	RR=0.50 (0.30-0.81)
Goodman <i>et al.</i> [107]	lung	serum	NCC	77/120	USA	RR=1.20 (0.77-1.88)
Jablonska <i>et al.</i> [108]	lung	plasma	CC	325/287	Poland	OR=1.21 (0.67-2.20)
Epplein <i>et al.</i> [109]	lung	serum	Cohort	207/414	USA	OR=0.70 (0.37-1.33)
Sattar <i>et al.</i> [110]	lung	plasma	CC	22/13	UK	no difference ^a
Hu <i>et al.</i> [111]	lung	diet	CC	227/227	China	RR=1.30 (0.70-2.20)
Zhou <i>et al.</i> [112]	lung	diet	CC	290/290	China	RR=0.76 (0.47-1.15)
Mahabir <i>et al.</i> [113]	lung	diet	CC	1676/1676	USA	OR=0.86 (0.64-1.15)
Nomura <i>et al.</i> [114]	lung	serum	CC	71/293	Hawaii	OR=1.1
Yoshizawa <i>et al.</i> [115]	prostate	toenail	NCC	181/181	USA	RR=0.39 (0.18-0.84)
Helzsoer <i>et al.</i> [116]	prostate	toenail	NCC	117/233	USA	RR=0.38 (0.17-0.85)
van den Brandt <i>et al.</i> [117]	prostate	toenail	CCH	540/1211	The Netherlands	RR=0.69 (0.48-0.99)
Allen <i>et al.</i> [118]	prostate	nails	CC	300/300	UK	RR=1.24 (0.73-2.10)
Gill <i>et al.</i> [119]	prostate	serum	Cohort	467/936	USA	OR= 0.99 (0.46-2.15)
West <i>et al.</i> [120]	prostate	diet	CC	358/679	USA	OR=1.6 (1.0-2.8)
Chan <i>et al.</i> [121]	prostate	serum	CS	489	USA	RR=1.35 (0.99-1.84)

Table A - 3 (continuation)

reference	cancer	matrix	study design	cases/controls	population	risk estimate
Peters <i>et al.</i> [122]	prostate	serum	CC	724/879	USA	RR < 1.00
Vogt <i>et al.</i> [123]	prostate	serum	CC	212/233	USA	OR=0.71 (0.39-1.28)
Ghadirian <i>et al.</i> [124]	prostate	toenail	CC	83/202	Canada	OR=1.14 (0.46-2.83)
Coates <i>et al.</i> [125]	prostate	serum	NCC	13/24	USA	RR=0.3
Knekt <i>et al.</i> [126]	prostate	serum	NCC	77/145	Finland	RR=0.41 (0.17-0.94)
Li <i>et al.</i> [127]	prostate	plasma	NCC	586/577	USA	RR=0.78 (0.54-1.13)
Goodman <i>et al.</i> [107]	prostate	serum	NCC	77/120	USA	RR=1.20 (0.77-1.88)
Brooks <i>et al.</i> [128]	prostate	plasma	NCC	52/96	USA	OR=0.24 (0.08-0.77)
Nomura <i>et al.</i> [129]	prostate	serum	CC	249/249	USA	RR=0.5 (0.3-0.9)
Van't Veer <i>et al.</i> [130]	breast	diet	CC	238/133	The Netherlands	OR=1.6 (0.8-3.4)
		plasma				OR=2.0 (0.9-4.4)
		erythrocyte				OR=0.9 (0.4-1.9)
		toenail				OR=1.1 (0.6-2.1)
Strain <i>et al.</i> [131]	breast	toenail	CC	99/105	Northern Ireland	OR=1.11 (0.55-1.27)
Ghadirian <i>et al.</i> [124]	breast	toenail	CC	327/688	Canada	OR=0.72 (0.40-1.31)
Coates <i>et al.</i> [125]	breast	serum	NCC	20/37	USA	RR=3.4
Hunter <i>et al.</i> [132]	breast	toenail	Cohort	434/436	USA	RR=1.10 (0.70-1.72)
Suzana <i>et al.</i> [133]	breast	toenail	CC	57/139	Malaysia	OR=1.71 (0.84-3.52)
		hair				OR=0.20 (0.05-0.84)
Suzana <i>et al.</i> [134]	breast	diet	CC	64/127	Malaysia	OR=1.71 (0.84-2.52)
		toenail				OR=1.92 (0.36-10.36)
		serum				OR=0.20 (0.05-0.84)
Met'er <i>et al.</i> [135]	breast	erythrocyte	CC	38/116	USA	OR=0.5 (0.2-1.3)

Table A - 3 (continuation)

reference	cancer	matrix	study design	cases/controls	population	risk estimate
van den Brandt <i>et al.</i> [136]	breast	toenail	Cohort	355/1248	The Netherlands	RR=0.84 (0.55-1.27)
Moradi <i>et al.</i> [137]	breast	plasma	CC	45/45	Iran	no difference ^a
Willet <i>et al.</i> [138]	breast	serum	CC	16/16	USA	no difference ^a
Joo <i>et al.</i> [139]	breast	hair	CC	40/144	Korea	no difference ^a
Arinola <i>et al.</i> [140]	breast	plasma	CC	29/30	Nigeria	no difference ^a
Cui <i>et al.</i> [141]	breast	tissue	CC	252/252	USA	OR=1.10 (0.72-1.68)
Van Noord <i>et al.</i> [142]	breast	toenail	Cohort	61/392	The Netherlands	OR=1.1 (0.5-2.9)
Rejali <i>et al.</i> [143]	breast	serum	CC	62/62	Malaysia	OR=1.92 (0.36-10.4)
Willet <i>et al.</i> [138]	breast	serum	CC	11/11	USA	no difference ^a
Schrauzer <i>et al.</i> [144]	breast	whole blood	CC	79/25	Japan	lower Se level ^a
Van't Veer <i>et al.</i> [145]	breast	toenail	CC	347/347	5 european counties	OR=0.96 (0.63-1.47)
Chen <i>et al.</i> [146]	stomach	plasma	CC	35/312	Taiwan	OR=0.84 (0.40-1.76)
Kabuto <i>et al.</i> [101]	stomach	serum	CC	202/226	Japan	OR=1.0 (0.5-1.9)
Zhang <i>et al.</i> [147]	stomach	serum	NCC	88/521	USA	OR=0.9 (0.6-1.4)
Nomura <i>et al.</i> [114]	stomach	serum	NCC	66/295	Hawaii	OR=0.9
Knekt <i>et al.</i> [126]	stomach	serum	CC	58/58	Finland	OR _M =0.26 OR _F =0.59 ^b
van den Brandt <i>et al.</i> [148]	stomach	toenail	CC	1550/1950	The Netherlands	RR=0.61 (0.33-1.11)
Wei <i>et al.</i> [149]	stomach	serum	CC	516/587	China	RR=0.75 (0.59-0.95)
Mark <i>et al.</i> [150]	stomach	serum	SCCH	402/1067	China	RR=0.47 (0.33-0.45)
Nomura <i>et al.</i> [114]	bladder	serum	NCC	29/293	Hawaii	OR=1.9
Wallace <i>et al.</i> [151]	bladder	toenail	CC	857/1191	USA	OR=0.90 (0.68-1.19)
Helzlsouer <i>et al.</i> [152]	bladder	serum	NCC	35/35	USA	OR=2.06 (0.67-6.35)
Zeeegers <i>et al.</i> [153]	bladder	toenail	CCH	431/2028	The Netherlands	OR=0.67 (0.46-0.97)

Table A - 3 (continuation)

reference	cancer	matrix	study design	cases/controls	population	risk estimate
Michaud <i>et al.</i> [154]	bladder	toenail	NCC	133/133	Finland	OR=0.90 (0.45-1.78)
Lin <i>et al.</i> [155]	bladder	urine	CC	30/30	Taiwan	higher Se level ^a
Kellen <i>et al.</i> [156]	bladder	serum	CC	178/362	Belgium	OR=0.48 (0.29-0.79)
Michaud <i>et al.</i> [157]	bladder	toenail	NCC	338/341	USA	OR _M =1.17 OR _F =0.36 ^b
Clark <i>et al.</i> [158]	colorectum	plasma	CS	28/20	USA	OR=3.79 (1.02-15.71)
Fernandez-Banares <i>et al.</i> [159]	colorectum	serum	CC	28/35	Spain	OR=0.17 (0.03-0.84)
Ghadirian <i>et al.</i> [124]	colorectum	toenail	CC	92/202	Canada	OR=0.42 (0.19-0.93)
Alimonti <i>et al.</i> [160]	colorectum	tissue	CC	15/15	Italy	higher Se level
Wallas <i>et al.</i> [161]	colorectum	serum	NCC	276/276	USA	OR=0.76 (0.44-1.30)
Männistö <i>et al.</i> [162]	colorectum	toenail	CC	289/433	Finland	OR=1.1
van den Brandt <i>et al.</i> [148]	colon	toenail	CCH	313/3187	The Netherlands	RR=0.77 (0.49-1.19)
.	rectum			166/3334		RR=1.01 (0.55-1.84)
Nomura <i>et al.</i> [114]	colon	serum	NCC	82/293	Hawaii	OR=1.8
	rectum			32/293		OR=1.6
Clark <i>et al.</i> [163]	skin	plasma	CC	190/103	USA	OR=4.39
Karagas <i>et al.</i> [164]	skin	plasma	NCC	132/264	USA	OR=0.86 (0.47-1.58)
Bresbow <i>et al.</i> [165]	skin	serum	CC	30/60	USA	OR=0.9 (0.3-2.5)
Sakoda <i>et al.</i> [166]	liver	toenail	NCC	166/394	China	OR=0.50 (0.28-0.90)
Yu <i>et al.</i> [167]	liver	plasma	Cohort	69/138	Taiwan	OR=0.62
Lu <i>et al.</i> [168]	esophagus	diet	CC	215/415	China	OR=0.30 (0.13-0.67)
Shukla <i>et al.</i> [169]	gallbladder	serum	CC	30/30	India	lower Se level ^a
		bile				lower Se level ^a
		tissue				lower Se level ^a

Table A - 3 (continuation)

reference	cancer	matrix	study design	cases/controls	population	risk estimate
Kucharzewski <i>et al.</i> [170]	thyroid	whole blood	CC	21/50	Poland	lower Se level ^a
Carrigan <i>et al.</i> [171]	pancreas	pancreatic juice	CC	35/35	USA	OR=5.8 (1.55-22)
Borawska <i>et al.</i> [172]	larynx	serum	CC	-	-	lower Se level ^a
Lu <i>et al.</i> [168]	esophagus	diet	CC	215/415	China	OR=0.30 (0.13-0.67)

OR: odds ratio, calculated between the lower and the higher tertile (or quartile or quintile or decile); RR: relative risk; NCC: nested case-control; CC: case-control; CCH: case-cohort; SCCH: stratified case-cohort; CS: cross-sectional; ^a difference between means ($\alpha = 0.05$; $p < 0.05$); ^b OR_M: odds ratio for males, OR_F: odds ratio for females.

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Estratto per riassunto della tesi di dottorato

Studente: _____Marco Roman_____matricola: 955499_____

Dottorato: _____Scienze Chimiche_____

Ciclo: __XXIII°_

Titolo della tesi: Development and applications of new analytical methodologies based on HPLC-ICP-MS for trace speciation analysis of selenium in biological samples.

Abstract:

New methods for the determination of seleno-proteins in human plasma/serum were developed by coupling miniaturized affinity HPLC systems to ORS-ICP-MS and ICP-SFMS detectors. A methods interlaboratory comparison allowed to provide for the first time the indicative reference concentration of individual seleno-proteins in the commercially available BCR-637 human serum. Two methods for seleno-proteins speciation in plasma/serum were applied to investigate their distribution in patients affected by type II diabetes and colorectal cancer, compared to healthy individuals. A new method was then developed for the speciation analysis of Se in rat colon tissues, based on two-dimensional HPLC coupled to ICP-QMS. Five species of Se were isolated, among which glutathione peroxidases 1 and 2, and thioredoxin reductase 1 were potentially identified by MALDI-TOF-MS. The method was transferred to human samples obtaining promising preliminary results.

Sono stati sviluppati nuovi metodi per la determinazione delle seleno-proteine in plasma/siero umani mediante accoppiamento di sistemi miniaturizzati di HPLC di affinità e detector ORS-ICP-MS e ICP-SFMS. Un confronto interlaboratorio tra metodi ha consentito di stimare per la prima volta la concentrazione di riferimento di seleno-proteine nel siero umano commerciale BCR-637. Due metodi per la speciazione di seleno-proteine in plasma/siero sono stati applicati per investigare la loro distribuzione in pazienti affetto da diabete di tipo II e cancro colonrettale, in confronto a soggetti sani. Un nuovo metodo è stato poi sviluppato per la speciazione delle seleno-proteine in tessuti di colon di ratto, basati su HPLC bidimensionale accoppiata con ICP-QMS. Cinque specie del selenio sono state isolate, tra cui glutathione perossidasi 1 e 2, e tioredoxina reductasi 1 sono state potenzialmente identificate mediante MALDI-TOF-MS. Il metodo è stato trasferito a campioni umani ottenendo promettenti risultati preliminari.