Laboratorium voor Analytische Scheikunde
Instituut voor Nucleaire Wetenschappen

UNIVERSITEIT GENT

Gel Electrophoresis for Elemental Speciation Purposes

Cyrille Cédric Chéry

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Wetenschappen: Scheikunde

Promotor:	Prof. Dr. L. Moens
Copromotoren:	Prof. Dr. F. Vanhaecke
	Dr. R. Cornelis

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Dankwoord

"Ik spreek Spaans tegen God, Italiaans tegen vrouwen, Frans tegen mannen en Duits tegen mijn paard."
Keizer Karel de Vijfde

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Merci pour tout, m'n zonnetje.

CCC

Contents

List of Abbreviations

1DE	one-dimensional gel electrophoresis
2DE	two-dimensional high resolution gel electrophoresis
AAS	atomic absorption spectrometry
BN-PAGE	blue native – polyacrylamide gel electrophoresis
C	percentage of cross-linking
CB	Coomassie blue
CE	capillary electrophoresis
CGE	capillary gel electrophoresis
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CZE	capillary zone electrophoresis
DRC	dynamic reaction cell
DTT	dithiothreitol
ETV	electrothermal vaporisation
FFF	field flow fractionation
GC	gas chromatography
GE	gel electrophoresis
GF-AAS	graphite furnace – atomic absorption spectrometry
GSH-Px	glutathione peroxidase
HG-AFS	hydride generation – atomic fluorescence spectrometry
HPLC	high-precision liquid chromatography
ICP-MS	inductively coupled plasma – mass spectrometry
IE	ionisation energy
IEC	ion exchange chromatography
IEF	isoelectric focusing
IPG	immobilised pH gradient

LA	laser ablation
LC	liquid chromatography
LOD	limit of detection
MS	mass spectrometry
NAA	neutron activation analysis
NEM	*N*-ethylmorpholine
OES	optical emission spectrometry
PAGE	polyacrylamide gel electrophoresis
PIXE	particle induced X-ray emission
PN	pneumatic nebuliser
R	regression coefficient
RPC	reversed-phase chromatography
RPq	rejection parameter q
RSD	relative standard deviation
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography
Sec	selenocysteine
SECIS	Sec insertion sequence
SGE	slab gel electrophoresis
S / N	signal-to-noise ratio
T	mass of acrylamide per gel volume
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UC	ultracentrifugation

1. Introduction

1.1. Trace elements and analytical chemistry

The realisation that elements present at a very low concentration, in other words trace elements, could have a tremendous impact on life was only made possible by a drastic improvement in instrumentation in analytical chemistry, as well as in quality control of the processes including contamination-free sampling. By definition [Vandecasteele, 1993], trace elements have concentrations below the 10^{-4} g g^{-1} range, and ultratrace elements even below 10^{-8} g g^{-1}. The classification between major and trace elements may seem artificial but can be traced back to the early developments of analytical chemistry in the nineteenth century. The trace elements could be hardly measured then, or at least with huge difficulties. The second hardship was also contamination, a real danger during sampling or analysis for such low concentrations.

A striking example is selenium, which, in the nineteenth century, was thought to be solely toxic, then, from new experiments in 1957, it was found to be merely beneficial to mammals [Zachara, 1992]. The late realisation that it was essential and part of an amino-acid, selenocysteine, which can be traced back to the early developments of life on Earth, came as a shock to the biochemical world. Those various discoveries were only made possible by improvements in the quality of the analytical detection and sampling. It is relatively easy to detect selenium in poisonous soils at a 10^{-2} g g^{-1} level as it is present in the Northern Great Plains of the United States [Barceloux, 1999], but it has only been possible to detect selenium in human proteins at a 10^{-8} g g^{-1} level for the last few decades.

Much has been done in order to determine which elements, and at which concentration, are present in biological matrices, like serum, muscles, food and plants, to name only a few. Yet, those average values merely give a superficial idea about their bioavailability, toxicity and other mechanisms of action on living beings. The key to the comprehension of these mechanisms lies in fact in the form in which an element is present, among other things its oxidation state, the nature of the ligands around it and the degree to which it is methylated. This actually means its inherent chemistry. Let us take the example of arsenic, which is much more toxic in the form of arsenate and arsenite than methylated arsenic. The latter form is present in seafood in noticeable concentrations, but because this form of arsenic is harmless for the consumer, it is no major health concern. The opposite is true if all the arsenic is present under its inorganic form.

In fact, the assumption that the interaction between a trace element and a living system is fully described by its total concentration is as meaningless as assuming that the total concentration in carbon gives enough information about a chemical system as complex as a cell, consisting of sugars, proteins and DNA.

This explains why a branch of analytical chemistry has dedicated itself to the study of trace elements, stressing the research on their chemical forms, or in other words their speciation, to yield a better understanding of the specific role of these elements in living organisms.

This interest for trace element speciation has now reached a wider audience and is no longer limited to academic spheres. The industry and governments are getting involved, especially with the advent of new and more stringent regulations [Ebdon, 2001].

1.2. Definitions

1.2.1. Speciation

The IUPAC definition for speciation reads as follows [Templeton, 2000]:

i. Chemical species: specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure.
ii. Speciation analysis: analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample.
iii. Speciation of an element; speciation: distribution of an element amongst defined chemical species in a system.

This definition will be used in the frame of this work relatively to living systems for macromolecules, like proteins, in combination with metals. That is why point i. can be simplified into:

i. Chemical species: specific complex or molecular structure.

1.2.2. Fractionation

In order to get an insight into a system as complex as a living cell or a biological matrix, a process is necessary to classify its chemical compounds. For

elemental speciation, this means a, at least partial, separation of the species of interest from the rest of the matrix. This process can be defined as fractionation:

i. Fractionation: Process of classification of an analyte or a group of analytes from a certain sample according to physical (*e.g.*, size, solubility) or chemical (*e.g.*, bonding, reactivity) properties.

Many fractionation methods are available and will be reviewed more in detail in Chapter 2.

One point cannot be stressed enough in the case of fractionation of biological matrices, namely the stability of the species. It is one of the major pitfalls of speciation analysis. Out of their natural chemical environment, in a different pH, salinity or any other parameter of the sample, the risk is high that the species transform in new ones, or in more stable ones. The picture given by this fractionated sample is then no longer reliable. Careful studies are necessary to prevent this kind of problem.

1.3. Aim of this study

This study aims at proving that gel electrophoresis is a relevant fractionation method for speciation studies.

To fulfil this goal, two parallel strategies were followed. First, it has to be proved that the separation method is relevant for the two extremes of fractionation of macromolecules, namely for an element covalently bound to the macromolecule, like selenium in selenoproteins, and also for weak complexes, like vanadium in metal-protein complexes. Second, it has to be proved that detection of the trace elements in the gels can be shifted from nuclear detection methods, which remain up to now mainly used, to cold detection methods, namely electrothermal vaporisation – ICP-MS and, last but not least, laser ablation – ICP-MS.

1.4. Two elements in particular: selenium and vanadium

As mentioned, one of the aims of this work was to demonstrate that gel electrophoresis was relevant for both labile complexes and covalently bound, thus stable, elements in macromolecules. Vanadium and selenium were chosen to

illustrate those two cases. The relevance of this choice with regard to biochemistry will be explained in the following.

1.4.1. Vanadium

The speciation analysis of vanadium is of paramount importance because of the versatility of its occurrences and forms and of their respective properties. There is circumstantial evidence that vanadium is essential to higher organisms although this point has not yet been formally proved. Additionally, human beings come or will come more and more frequently in contact with higher doses of this element, either through the environment (combustion of vanadium-containing coal or oil, stainless steel) [Nriagu, 1998] or by medication.

1.4.1.1. Physical characteristics

Vanadium has two stable isotopes, ^{50}V and ^{51}V, with an isotopic abundance of respectively 0.25 % and 99.8 %, making it practically a monoisotopic element. Next to these stable isotopes, a variety of radiotracers of vanadium exist, under which ^{48}V and ^{52}V are mostly used.

1.4.1.2. Chemistry

This transition element can adopt various oxidation states, from -III to +V due to its electronic configuration [Ar]$3d^3 4s^2$. Its chemistry is very rich and in aqueous solution, it can show three oxidation states, III, IV and V depending on the redox potential of the solution. The various vanadium ions readily interconvert, making the speciation analysis of this element quite difficult.

1.4.1.3. Biochemistry of vanadium

Vanadium has a complex bio-inorganic chemistry, especially in ascidians, a sea organism [Fraústo da Silva, 1991]. This animal has actually raised the interest in vanadium among biologists and chemists, proving that this element is relevant for living beings. Just afterwards, various vanadium-enzymes were discovered in some bacteria and plants and a vanadium-organic compound was found in a mushroom, its role remaining unknown.

In mammals, the question of the essentiality of vanadium is still open. No active vanadium-compound could be isolated, but the absence of this element in the food seems to disturb growth. The precise mechanism of action of vanadium in this case is still unknown and this question of essentiality is still highly debated. In fact, an updated description of the role of this element seems more appropriate, namely beneficial instead of essential [Nielsen, 2000].

A very interesting development in the biochemistry of vanadium is its use as a pharmaceutical. Medication based on vanadium is very promising [Thompson, 2001], since some species of this element exhibit insulin-like properties, with the huge advantage that those molecules or at least the active centre of those molecules are stable in the gastric juice, allowing an oral administration [Thompson, 1999]. Furthermore, vanadium compounds may have anticancer effects [Evangelou, 2002]. However, vanadium and some of its derivatives are potentially toxic [Domingo, 2002]. This toxicity being dependent on the transport mechanism and the target organs explains why speciation of these compounds in various body fluids and tissues is of prime interest. The metabolic fate of vanadium has already been described at basal levels, where transferrin is responsible for its transport, as it is for many metals, but less is known about vanadium administrated at pharmacological levels.

1.4.2. Selenium

Selenium is a metalloid that has attracted much attention in the last years because of both its beneficial properties and toxicity [Barceloux, 1999]. It is beneficial because of its anti-oxidative properties, giving it a distinctive role in cancer prevention. It is not clear what the mechanism or even the species is which is responsible for this protection, be it even partial, against cancer. Nonetheless, it is well known and documented that, in mammals, this element is specifically incorporated to form selenocysteine which in turn is used to form selenoproteins. Thirteen such proteins have been discovered up to now, some playing a role in anti-oxidation processes. The question is still open whether additional selenoproteins might be discovered.

1.4.2.1. Physical characteristics

Selenium has six stables isotopes, summarised in Table 1.1 [Rosman, 1999].

The most widely used selenium radiotracer is ^{75}Se, a γ-emitter, with a half-life of 120 days.

Table 1.1: Selenium stable isotopes.

Isotope	Isotopic abundance (%)
^{74}Se	0.9
^{76}Se	9.0
^{77}Se	7.6
^{78}Se	23.6
^{80}Se	49.7
^{82}Se	9.2

1.4.2.2. Chemistry

This metalloid, right under sulphur in the periodic table, shares many characteristics with it. Its electronic structure is $[Ar]3d^{10}4s^24p^4$ and, as can be deduced from this, its oxidation states are –II, +IV and +VI.

The inorganic chemistry of selenium is comparable with that of sulphur, with a distinctive characteristic that it is slightly more electronegative.

1.4.2.3. Biochemistry of selenium

The bio-inorganic chemistry of selenium is determined by this latter feature. In a molecule like selenocysteine (see Table 1.3 for its structure), the selenium amino-acid in enzymes, selenium as selenol is negatively charged at physiological pH, where its sulphur counterpart, cysteine, is mainly neutral. This negative charge results in a higher reactivity of selenium in the active centre of enzymes, in comparison with a sulphur enzyme. If selenocysteine is substituted by cysteine, the activity of the enzyme falls by two or three orders of magnitude [Köhrle, 2000].

As aforementioned, selenium is selectively incorporated to form the 21st amino-acid, selenocysteine, abbreviated Sec. This incorporation is genetically coded in some species by the UGA codon, even if this codon is normally a stop codon. A genetic signal determines whether the polypeptide sequence will be stopped or whether Sec will be included. This signal is an insertion sequence, downstream of the UGA codon, known as the Sec insertion sequence (SECIS). In other words, the translation of UGA depends on the context.

So far, a dozen selenoproteins have been discovered in mammals. Insects, more precisely Drosophila, also have selenoproteins [Alsina, 1998; Martin-Romero, 2001]; one plant, *Chlamydomomas reinhardtii*, has got a selenoprotein encoded in its genome; yet, no selenoprotein has been found in yeast [Novoselov, 2002; Fu, 2002]. In fact, it seems that selenoproteins were lost during evolution in some plants and yeast [Novoselov, 2002].

The role of some of these proteins is not totally understood and a further question is the possibility of existence of other selenoproteins in higher organisms. Table 1.2 gives an overview of the selenoproteins known at present [Köhrle, 2000; Gladyshev, 1999].

Table 1.2: Selenoproteins identified at present.

Name	Function	Expression
Glutathione peroxidase 1	Glutathione-dependant Hydroperoxide removal	Ubiquitous
Glutathione peroxidase 2	Glutathione-dependant Hydroperoxide removal	Gastrointestinal tract
Glutathione peroxidase 3	Antioxidant Hydroperoxide removal	Plasma
Phospholipid hydroperoxide glutathione peroxidase	Phospholipid hydroperoxide removal	Ubiquitous
Thyroid hormone deiodinase 1	Conversion of T_4 to T_3 Inactivation of T_4 and T_3 (T_3:active 3,3'-5 triiodothyronine; T_4: inactive prohormone thyroxine)	Thyroid gland, liver, kidney, central nervous system
Thyroid hormone deiodinase 2	Conversion of T_4 to T_3	Pituitary and thyroid glands, placenta, heart and skeletal muscles, central nervous system, brown fat
Thyroid hormone deiodinase 3	Inactivation of T_4 and T_3	Placenta, central nervous system, skin
Thioredoxin reductase	NADPH-dependant reduction of thioredoxin	Ubiquitous
Selenophosphate synthetase 2	Synthesis of selenophosphate	Ubiquitous
Selenoprotein P	Antioxidant (?) Selenium storage (?)	Plasma
Selenoprotein W	Redox related process (?)	Ubiquitous
15-kd selenoprotein	Protein folding (?)	Ubiquitous

This table can be extended with a list of various selenoproteins, whose function remains undefined and about which very little is known: selenoprotein P12, R, T, X, N. These proteins have actually been identified *de novo*, by scanning nucleotide sequence databases for sequences presenting a potential SECIS (Sec insertion sequence), specific for selenoproteins. After they were expressed, it was checked whether they were selenoproteins, but their role, the conditions for their expression and the organ in which they are expressed remain unknown.

Selenium is also present in proteins where it is randomly incorporated as selenomethionine, because eukaryotes do not make the difference between methionine and its selenium counterpart. This is true for albumin in humans or when selenium concentrations are high enough to compete with sulphur; this is usually achieved in the toxic range of selenium [Stadtman, 1980]. Selenomethione does not exhibit any enzymatic activity. To distinguish between those two classes of proteins, the terminology selenoprotein (selenocysteine-specific insertion) and selenium-containing proteins (selenium fortuitously at the place of sulphur) has been coined.

Prokaryotic organisms also contain selenoenzymes, but the picture is more complicated than for eukaryotes, since selenium is present in these either as a selenocysteine residue or alternatively, in a few molybdoenzymes, as a component of a bound cofactor. These prokaryotic selenoproteins catalyse redox reactions and also lead to the formation of selenoethers [Köhrle, 2000].

Further, selenium has been isolated in various biological materials. They are either metabolites or detoxification molecules, depending on the amount of selenium in the surroundings of the organism. The major materials of interest for studies related to selenium are:

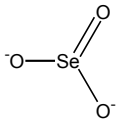
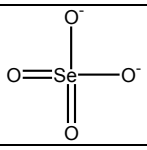
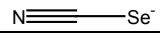
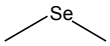
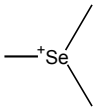
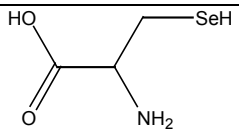
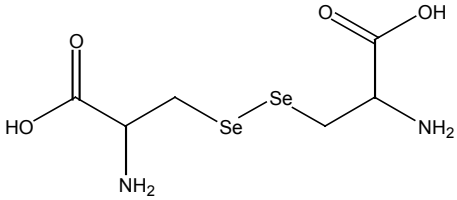
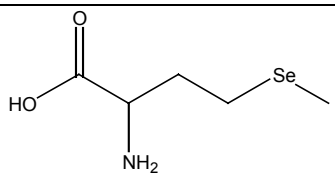
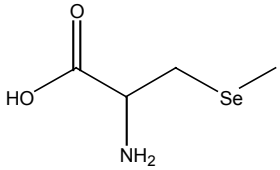
- urine, in order to elucidate the excretion mechanism of selenium,
- micro-organisms and plants, as potential source of selenium supplements and to elucidate the role of this element in those organisms,
- and, last but not least, samples of mammalian origin to characterise selenoproteins.

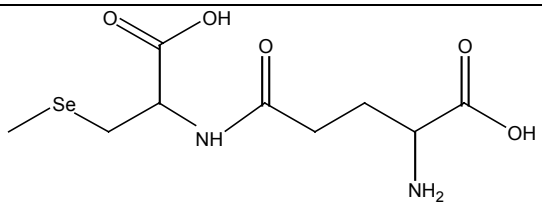
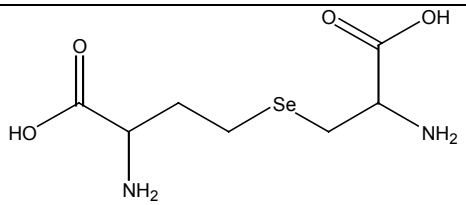
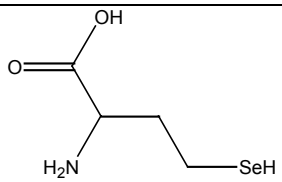
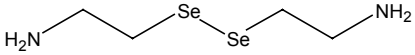
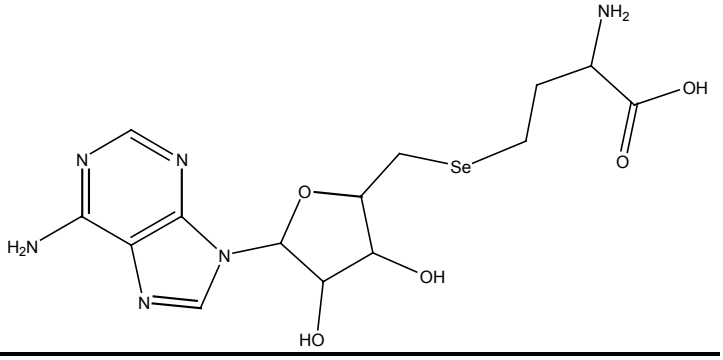
A non-exhaustive list of the analytes of interest is given in Table 1.3 [Łobiński, 2000; Uden, 2002]. Although they are small compounds, most of them are found in combination with proteins and other macromolecules, as a degradation product from

selenoproteins, metabolites or selenium source for the production of the same selenoproteins. They are mentioned here for that reason, even if fractionation with gel electrophoresis is not relevant for some of them.

The general biochemical cycle of selenium in mammals, including the production of selenoproteins, is given in Figure 1.1.

Table 1.3: Selenium compounds in biological samples.
The neutral form of the organic compounds is shown.

Name	Formula
Selenite	
Selenate	
Selenocyanate	
Methylselenol	
Dimethylselenide	
Trimethylselenonium cation	
Selenocysteine	
Selenocystine	
Selenomethionine	
Se-methylselenocysteine	

γ-glutamyl-Se-methylselenocysteine	
Selenocystathionine	
Selenohomocysteine	
Selenocystamine	
Se-adenosylselenohomocysteine	

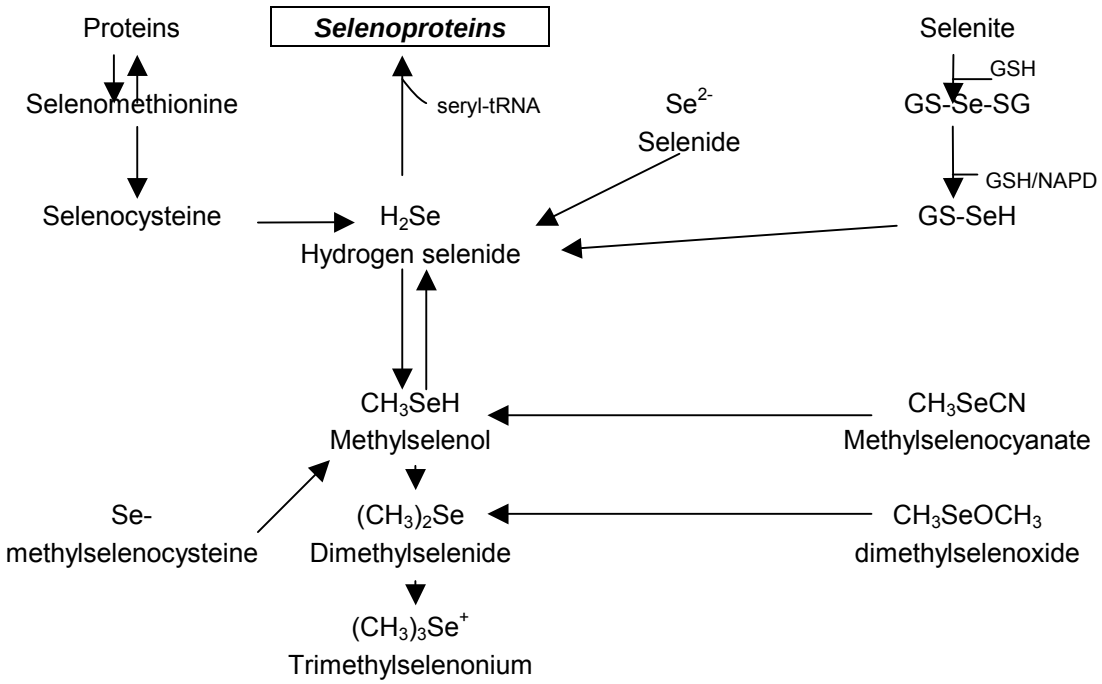


Figure 1.1: Biochemical cycle of selenium in mammals.

As can be seen in Figure 1.1, both inorganic and organic forms can be utilised by the body. Before incorportation into selenoproteins, the selenium sources have to be transformed into selenide (H_2Se). For example, selenite is reduced in the red blood cells. In the presence of an excess of selenium, the selenium compounds are methylated and readily excreted, exhaled as dimethylselenide or dimethyldiselenide, responsible for the typical garlic foul breath or excreted via the urine as the trimethylselenonium ion [Patching, 1999].

Similarly to many other trace elements, selenium exhibits both beneficial and toxic effects. Selenium is an essential element. Deficiency is the direct cause of the Keshan disease, a cardiomyopathy, found in areas of China with diets very low on selenium. The soil of this area, Keshan, contains hardly any selenium. The production of selenoproteins is then hardly possible and the glutathione enzymatic cycle is disturbed. The very interesting point about selenium is that selenium deficiency seems also linked to an increased susceptibility to certain types of cancer, vascular disorders, atherosclerosis, platelet hyperaggregability, anaemia and hypertension.

Historically, the use of selenium as an anti-carcinogenic agent dates back to 1912 when it was used in France under the name of ‘Colloidal Selenium A’. Systematic epidemiological studies began in 1966 and are still taking place to determine which forms of cancer selenium can help to prevent, under which species it must be administered and in combination with which other antioxidant (vitamin, other trace elements, etc.). One of the latest materials used as nutritional supplement is selenised yeast, also known as selenium-yeast. It is now the most widespread food supplement, commercially available [Moesgaard, 2001]. The protein-bound fraction can be as high as 70 % of the total selenium content. Although selenium is not specifically incorporated in yeast proteins, the chemical form of the trace element is the same as in the selenoproteins.

Notwithstanding, there are huge discrepancies between various dietary supplements, casting some doubts about the quality of such dietary supplements and even the safety of their use. For this reason, reliable analytical methods are needed, together with certified reference materials, as the one currently prepared by the European Commission [Chassaigne, 2002], which has been analysed during this work (see 5.1).

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2. Fractionation Methods for Macromolecules

The goal of this chapter is to describe the various fractionation methods that were applied during this work. First in line comes gel electrophoresis, whose position among the other methods for the fractionation of macromolecules will be discussed.

2.1. Gel electrophoresis

The first description of the phenomenon of electrophoresis can be dated back to 1809, when Reuss noticed the migration of fine sand particles in suspension in water in a tube at which extremities a difference of potential was applied [Reuss, 1809, quoted in Ribeiro, 1961]. In two centuries, the technique evolved from this free migration in water to moving boundary electrophoresis, which earned Arne Tiselius his Nobel Prize in 1948. Electrophoresis has now become a mature fractionation method. Among the array of the electrophoretic separations, gel electrophoresis, or to be complete, slab gel electrophoresis, has grown into an essential part of the analytical tools in biochemistry, especially with its latest development, two-dimensional electrophoresis. Its application to elemental speciation is on the rise, especially the last few years [Chéry, 2003a].

2.1.1. Principle and definitions

In an electric field, charged molecules or complexes will migrate to the electrode bearing the charge opposite to their global charge, positively charged particles toward the cathode (-) and negatively charged particles to the anode (+). This global charge is not necessarily the charge of the molecule, but rather the charge of the particle that is formed during electrophoresis, for example the charge of the protein in a SDS (sodium dodecyl sulfate, see 2.1.5.2.2) micelle or the charge of a metal complex. If a voltage V is applied between two electrodes separated by a distance L, a field E appears according to Equation 2.1. The migration velocity v of a particle in this field is proportional to the mobility μ of the particle and the field strength E (Equation 2.2), where μ is an intrinsic parameter of the particle.

$$E = \frac{V}{L} \qquad \text{Equation 2.1}$$

$$v = \mu E \qquad \text{Equation 2.2}$$

The mobility is directly linked to the charge of the particle. According to Hückel, the mobility can be written as:

$$\mu = \frac{\varepsilon\varsigma}{6\pi\eta r} \qquad \text{Equation 2.3}$$

where ε is the dielectric constant, ς is the zeta potential of the particle, η is the viscosity of the solution and r is the hydrodynamic radius of the particle.

The product $\varepsilon\varsigma$ is the effective charge of the ion.

The unit mainly used in electrophoresis is the volt-hour (V h), since this value is proportional to the displacement d of the particle. Indeed, it can be proved that the velocity, v, rapidly becomes constant and by combining Equations 2.1 and 2.2:

$$d = vt = \left(\frac{\mu}{L}\right)(Vt) \qquad \text{Equation 2.4.}$$

Since μ is a characteristic of the particle and L of the system, the distance to the electrode d is related to a unit that has the dimension of a voltage multiplied by a time, which is traditionally expressed in V h.

The velocity at which the particle migrates also depends on the viscosity of the medium and the density of the gel, which acts as a sieving role.

Migration occurs in a liquid medium, namely the buffer, which is one of the key elements of the separation and especially of the stability of the metal bound to the macromolecule. This buffer is not only a pH buffer but is also a good solvent for the particles. A combination of two or three buffers may have to be used, namely for the cathode, anode and gel, if the separation process requires it (see 2.1.4.4).

The gel is the second key parameter for good separation of the particles. It determines as a first approximation which separation mechanism occurs. Various gels are available, agarose and polyacrylamide being the most common ones. Generally, the choice is made between the two according to the size of the particles to be analysed. For larger particles, typically over 10 nm in diameter, agarose gel is preferred, especially for the analysis of DNA or RNA. Polyacrylamide gel is the polymer of choice for most proteins and DNA or RNA fragments.

This polymer is obtained by copolymerisation of acrylamide and a cross-linking agent, usually *N,N'*-methylenebisacrylamide, which confers its three-dimensional structure to the gel. The pore size is defined by two parameters, C and T, both expressed in %. They are related to the polymerisation process and the quantities of monomer and cross-linking agent used, T being the mass of acrylamide per gel volume and C the percentage of cross-linking agent in the gel. For our purpose, it is

only necessary to know that if *T* increases, the pore size decreases (the more polymer per volume, the less free volume) [Andrews, 1988].

2.1.2. Apparatus

Basically, an electrophoresis experiment requires a high voltage generator, typically up to 2000 V, a set of electrodes and a temperature-controlled separation surface or chamber. The last point is critical since heat is produced through Joule effect, which can disturb the separation. For small gels, less than 10 cm^2, Peltier cooling is very efficient. For larger gels, water-cooling is the best solution. Several variations are available from this starting set, including submarine electrophoresis (*i.e.* electrophoresis with the gel lying in the buffer) and vertical or horizontal electrophoresis. Other accessories may be necessary if the method is used intensively, such as staining trays. Detection for elemental speciation will be discussed in 3.2.

2.1.3. Applicability to elemental speciation

The area covered by gel electrophoresis for speciation purposes is charged macromolecules to which any metal or metalloid is bound, covalently or not. This work deals with proteins, but other macromolecules can also be separated by this method, like DNA or humic acids.

From a literature survey, it appears that this method has been used in combination with numerous metals. The applicability is not limited to certain elements, but by practical considerations such as the limits of the detection method for the element of interest. The most representative applications of gel electrophoresis to speciation to this day are summarised in Table 2.1. The care taken to check the stability of the species of interest has been highlighted in the remarks of this table. A further possible pitfall is the contamination from the electrodes as illustrated in the case of the speciation of platinum with platinum electrodes.

Whereas applications of gel electrophoresis in general are numerous, they are just emerging in the field of elemental speciation. They range from separation of DNA / RNA, humic acids, proteins, to dyes.

Table 2.1: Applications of slab gel electrophoresis to speciation.

Element	Matrix	SGE separation	Detection	Remark	Reference
Co	Serum	Crossed immuno-electrophoresis	LA-ICP-MS	Stability of species?	[Neilsen, 1998]
Fe	Apotransferrin	Native	Autoradiography		[Vyoral, 1998]
	Bacterium	SDS-PAGE	PIXE		[Szökefalvi-Nagy, 1990]
P	Milk	SDS-PAGE	NAA		[Stone, 1987; Stone, 1991]
Pb	Humic acids	SDS-PAGE	LA-ICP-MS		[Evans, 2000]
Pt	Grass	SDS-PAGE	Voltammetry	Contamination from electrodes?	[Messerschmidt, 1995]
	Serum	Native 2DE	Autoradiography	Very promising method	[Lustig, 1999a]
Se	Soft tissues	2DE	Autoradiography		[Behne, 1996; Jamba, 1996]
	Yeast	2DE	Autoradiography	Stability checked	[Chéry, 2001]
			ETV-ICP-MS	Stability checked	[Chéry, 2002a]
			LA-ICP-MS	Stability checked	[Chéry, 2003b]
	Glutathione peroxidase	SDS-PAGE	Mineralisation: HPLC-fluorescence		[Vézina, 1990]
			GF-AAS		[Sidenius, 2000]
			ETV-ICP-MS	Stability checked	[Chéry, 2002a]
			LA-ICP-MS	Stability checked	[Chéry, 2003b]
	Soft tissues	SDS-PAGE	Liquid scintillation		[Qu, 2000]
			HG-AFS		[Chen, 2002]
			LA-ICP-MS		[Fan, 2002]
V	Serum	Native	Autoradiography	Stability checked	[Chéry, 2002b]
Multi-element	Kidney	IEF	NAA	Stability of species?	[Jayawickreme, 1988]
	Liver	IEF	X-ray fluorescence		[Gao, 2002]

A particular branch of gel electrophoresis is worth mentioning, two-dimensional gel electrophoresis (2DE). It is the most advanced and successful method, this success owing to the increasing importance of proteomics: the

combination of PROTEin analysis and genOMICS aims at unravelling the mysteries of the expression of the genome into proteins. Thus, proteomics needs to map all proteins present in a particular sample, *e.g.*, *Saccharomyces cerevisiae*, human serum, *Eschericia coli* to quote just a few. Two-dimensional electrophoresis has become a widespread and most reliable analytical method for this purpose [Rabilloud, 2000]. To give just one example, 2DE enables the separation of more than 10 000 proteins on a single gel [O'Farrell, 1975].

2.1.4. Techniques and procedures

2.1.4.1. Basics

Even before choosing the procedures, the following question has to be addressed: is the metal covalently bound to the protein? If so, as is the case for selenium in some proteins, the species are relatively stable during the separation and denaturing conditions can be used; this means conditions where only the primary structure is preserved. Otherwise, nondenaturing electrophoresis must be applied, even if this implies a loss in separation efficiency; nondenaturing is equivalent to native. The first decision is therefore whether a native or a denaturing procedure is to be used. The other questions to be answered are related to the kind of sample or the mixture of proteins to be separated: gradient or linear gel (see 2.1.4.3), with or without stacking (2.1.4.4). Any paired combination is feasible, giving eight theoretical associations.

2.1.4.2. Native or denaturing electrophoresis

The first method and the most evident one is native electrophoresis. It means that the proteins, without any modification in their secondary and tertiary structures, are submitted to electrophoresis. The buffer is chosen so that the protein is not denatured. Biochemists use this separation method when they are interested in the activity of the enzymes that are isolated, activity that would be lost if the proteins were denatured. However, this type of separation is subject to a major drawback, as no buffer system is suitable for all separations. Firstly, no universal buffer exists for the separation of all proteins. In a buffer with a pH below 10, proteins with a pI at 11, thus positively charged, migrate to the cathode, and proteins with a pI below 10, negatively charged, migrate to the anode; in other words, they migrate in opposite

directions and cannot be seen on a single gel. A solution would be to use extreme pHs but those are prohibited since they denature the proteins. Secondly, no buffer exists that allows the correct preservation of all metal-protein complexes, *e.g.*, some buffers may affect a vanadium-protein complex without affecting a platinum complex.

For speciation purposes, however, the native method is compulsory when the metal is not covalently bound to the protein. Should the protein be denatured, the complexing site would be destroyed and the metal would be set free.

Various non-denaturing buffers have been proposed [Chrambach, 1983] and one will be given as example in 2.1.5.1.

2.1.4.3. Restricting medium: gradient or linear gel.

Basically, separation takes place either in a restricting medium or a free medium. Restricting means that the particles interact with the gel, either physically, because of their size, or, with an even broader definition, chemically, because of interactions of the proteins with a pH gradient or with antibodies.

For the separation according to size, except during stacking, a restricting medium is preferred. There is still a question to be answered, namely whether the gel must be used with a constant density or a gradient. A gradient is a continuous change in density of the gel in the direction of migration, or in other words, a continuous gradient of the pore size. The gel begins with large pore sizes and ends with restricted sizes, so that the friction force constantly increases in the gel, up to a point where the velocity of the protein is very low. A gradient also ensures the separation of a broad mass range, typically from 10 kDa to 200 kDa, in comparison with smaller ranges for homogeneous gels. Thus, a gradient gel allows sharper and sometimes easier separations, especially when little is known about the range of molecular masses of interest. Homogeneous gels have still got advantages, especially when one is interested in a particular mass or family of proteins, since it offers a kind of zoom process. Further, a more precise mass determination is possible.

2.1.4.4. Stacking or sample concentration: discontinuous buffers

By stacking, one understands a process capable of concentrating the sample in the gel before the real separation occurs. This is particularly interesting if the analytes are present at low concentration. Stacking concentrates the proteins in

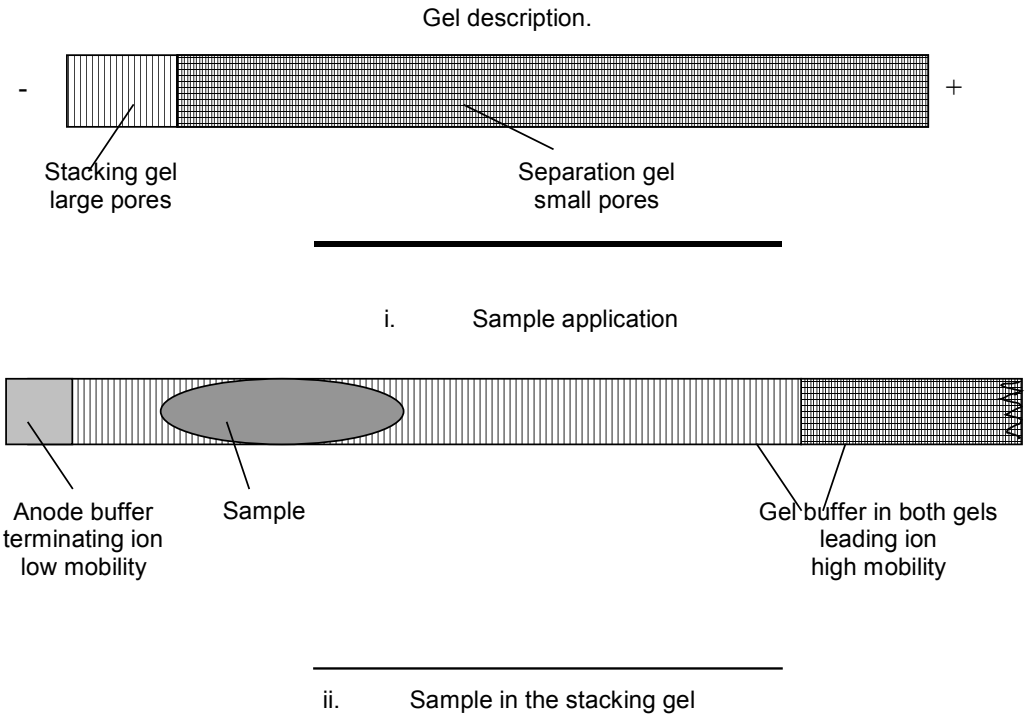
sharp bands, a prerequisite for *Rf* measurements, *i.e.* migration distance, and for speciation when the metal concentration is low. The principle is isotachophoresis, or more precisely moving-boundary electrophoresis, as is explained further. The method is also widely known as discontinuous or disc electrophoresis because of the discontinuity of properties between stacking and separating gels in buffer and pH.

For clarity, the basics of the method will be summarised here, for a system migrating from the cathode to the anode. The gel is physically made of two zones, the first one, where the sample is applied, being the stacking gel, where the gel is not restricting; the second is made of the resolving gel, where separation occurs. A set of three buffers must be chosen, but even if this task is very tedious, it is one of the best documented [Chrambach, 1983]. The goal is to get three types of ions with increasing mobilities from the cathode to the anode:

- a terminating ion, with a low mobility, at the cathode in this case;
- a leading ion, with the highest mobility of the ions, present in the gel and the anode;
- a common counter ion.

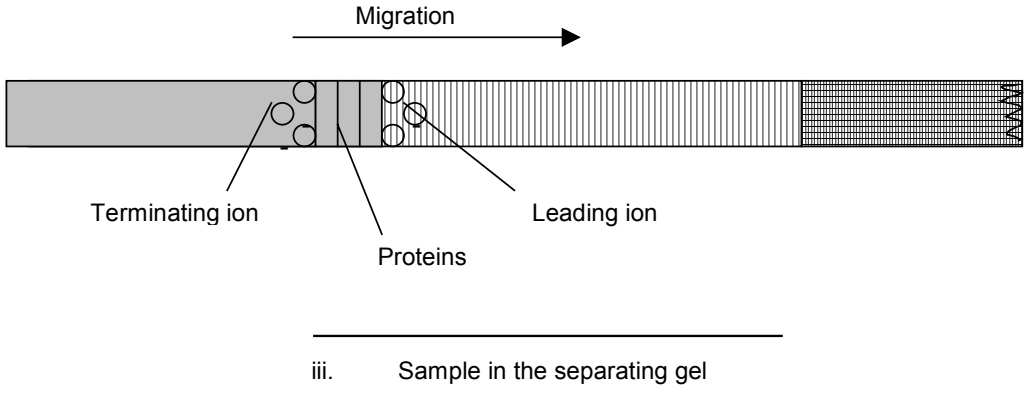
The proteins are applied at the cathode and, as a matter of speaking, are sandwiched between the two ions, the terminating and leading ions. Hardly any friction is encountered by the ions with the gel, the ions are in a free solution. When voltage is applied, molecules range according to their mobilities: from the cathode, the terminating ion, the proteins and the leading ion. The migration occurs with a most interesting characteristic, a constant concentration in one band, dependent of the concentration of the leading ion. The more concentrated the leading ion, the more concentrated all the bands are. Through this effect, the proteins are pre-separated in sharp bands (see Figure 2.1).

At the border between stacking and separating gel, a new force, the friction with the gel material, affects the macromolecules. The terminating ion does not interact with the gel, since its size is negligible in comparison with the pore size, and migrates further. At this stage, the proteins are surrounded by the terminating ion and migrate farther, but this time following the principles of zone electrophoresis.



Separation starts.

The proteins are separated according to the principles of isotachophoresis.
Sharp bands are obtained.



Separation occurs according to the sieving effect of the gel. Frictions with the polymer network are at the basis of this sieving effect.

In the separating gel, the terminating buffer goes farther but the proteins, which encounter a high friction, are separated according to their mass-to-charge ratio.

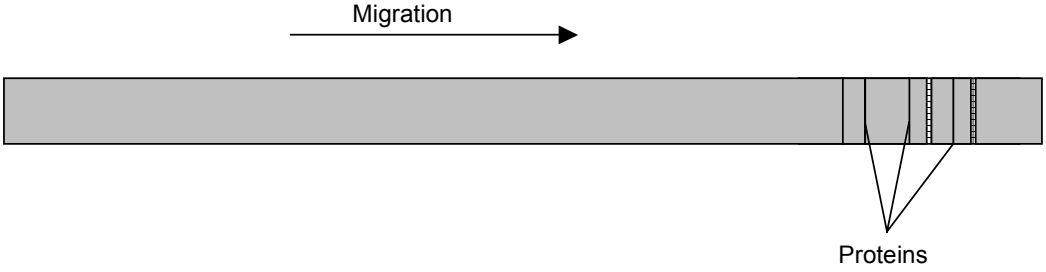


Figure 2.1: Principle of stacking.

2.1.5. Separations processes

In order to give a practical idea of a separation by gel electrophoresis, two methods have been chosen: one-dimensional native electrophoresis and two-dimensional high-resolution electrophoresis. Those separations illustrate the most extreme cases in trace element speciation with gel electrophoresis: 2DE is used when high separation capacity is needed but when the species can resist denaturation, whereas non-denaturing electrophoresis is used when the complex is fragile and would not resist a denaturing separation.

Common features can be recognised in both examples. First of all, the stability of the species is a point that cannot be stressed enough and must be checked by an independent method. Since the gels may trap oxygen during the polymerisation, if the species are sensitive to oxidation, reducing agents can be used like thioglycolate [Bansal, 1991].

Secondly, the proteins must be brought into solution, in a sample buffer compatible with the separation method. The quantity of protein necessary for an optimum detection of these proteins is given by rules of thumb; in fact, this quantity primarily depends upon the detection method: silver staining, rubies staining, Coomassie blue or ^{14}C. For example, a sensitive method like silver staining requires around 10 mg mL^{-1} as total concentration of proteins; for the visualisation of a specific protein, *ca.* 10 ng of protein are necessary, a quantity that is dependent not only on the protein itself, but also on the separation method. Indeed, in a one-dimensional experiment, the protein is spread over a whole band in comparison with a spot from 2DE, concentrated on 1 mm^2, therefore requiring less protein. One must also take into account the quantity of trace element in the sample and the detection of the metal. However, a limiting factor is that gel electrophoresis has also a maximum loading capacity above which no separation occurs. A compromise has to be found between the two parameters. If a large amount of protein has to be used to detect the trace element, the use of Coomassie blue for the proteins can be considered, which is *ca.* 50 times less sensitive than silver staining; should the latter be used, the risk is high that the whole gel would be darkened by the proteins.

2.1.5.1. Non-denaturing electrophoresis

Although one-dimensional electrophoresis does not imply that the method be non-denaturing, such a combination has been chosen for simplicity. The converse is true, non-denaturing electrophoresis being mostly one-dimensional, up to this date.

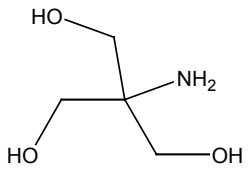
The choice of the buffer system is the first step.

A set of buffers optimum for the separation of proteins with acidic pI (below *ca.* pI 8), and widely used, is a slight modification of the set described by Laemmli [Laemmli, 1970]:

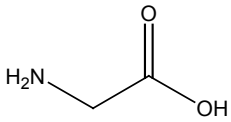
- electrode buffer: glycine / Tris base, pH 8.3;
- gel buffer: Tris base / HCl, pH 6.4;
- polarity: separation towards anode, the sample is applied at the cathode;
- 100 mL of each solution is sufficient. Buffer strips and gel are rehydrated with adequate solutions.

This set belongs to the type of discontinuous buffers, with Tris as the common ion, chloride the leading ion and glycine the trailing ion. These chemicals are presented in Structures 2.1 and 2.2.

Structure 2.1: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.



Structure 2.2: Glycine.



For other separations, such as separations of basic proteins (pI above 8), other sets have been optimised and can be found in authoritative reviews [Chrambach, 1983] or books [Hames, 1998].

The second step is the preparation of the sample. The sample, proteins in our example, must be dissolved in a buffer compatible with the method, they must be

kept in solution and, of course, the species must be stable. In order to be compatible with the electrophoresis procedure, the sample should not contain too much salt. In fact, a sample with too high an ionic strength is one of the main causes of failure of a separation, since a high charge concentration causes a drop in the resistance of the solution: small ions migrate more easily and in such a case the proteins stay at their point of application. A further requirement is a good solvent that allows a smooth penetration of the sample in the gel. This step may be critical, especially for large or hydrophobic molecules, for which it is difficult to go from a free solution to a solution in a gel. Therefore, an optimal sample is the combination of the following:

- the desalted original sample;
- diluted in the gel buffer, which is the first solution with which the proteins will be in contact in the gel;
- with 10 % glycerol, to mimic the gel concentration and therefore facilitate the penetration in the gel;
- mild detergents, useful to keep the proteins in solution and prevent aggregation; but there is a real danger that the proteins could be denatured or the species degraded.

Parallel to the choice of the buffer, the problem of the stability of the compounds has to be tackled. As already mentioned, the choice of a buffer system is the key not only to a good separation of proteins but also to speciation. In order to apply the separation method to speciation, it is advisable to first test the stability of the compounds to be separated in the buffers. For example, if the species vanadium-protein has to be separated in a given buffer, experiments ought to be performed with the species in the buffer to check whether free metal is produced, *i.e.*, if the equilibrium between free vanadium and complexed vanadium is disturbed. Such experiments can be ultrafiltration or size-exclusion chromatography, any method that is able to separate the metal-complexes from the free element.

An example of incompatibility of a buffer set and a trace element is vanadium: glycine can complex vanadium and strips proteins from the trace element to which it was bound [Lustig, 1999b]. Therefore, the use of buffers other than glycine or tricine has to be explored.

- Equipment:

No equipment is standard for one-dimensional electrophoresis (1DE). There is a large choice between flatbed, submarine or vertical systems, each of which has advantages over the other, but none influencing speciation.

- Gel:

Various gels sizes can be used, 5 cm x 5 cm or 25 cm x 10 cm gels, most of which are commercially available. If a specific gel is needed, preparation is possible in the laboratory, at low cost. Another possibility is to use a rehydrated gel, wash it thoroughly, dry and rehydrate it in the necessary buffer.

The buffers are laid directly under the respective electrode.
The sample is brought on the gel either:

- directly, if the sample volume is small (1 µL);
- onto a sample strip (1 µL to 10 µL);
- in a sample trough (up to 15 µL) if these were foreseen during polymerisation.

See Figure 2.2 for a typical set with a flatbed system.

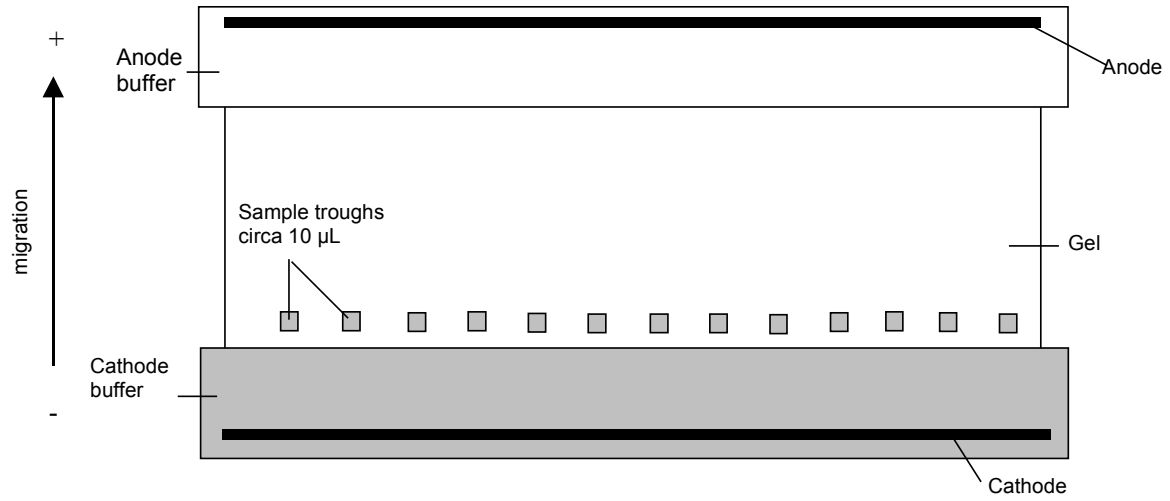


Figure 2.2: Typical set for flatbed separation.

The separation programme is also dependent on the apparatus but common features are always identifiable. A mild voltage (*ca*. 25 V cm^{-1}) is applied in the first step to let the proteins enter the gel material, *i.e.* the stacking gel. Afterwards the real separation begins.

For example, for a homogeneous gel, 25 cm (approximately 25 lanes) on 11 cm (separation length), stacking gel T = 5 % (thus wide pores for the stacking effect), C = 3 % (33 mm), resolving gel T = 10 %, C = 2 % (77 mm), the program can be written as [Westermeier, 1993]:

	Voltage (V)	Current (mA)	Power (W)	Duration (min)
1st step	500	10	10	10
2nd step	1200	28	28	50

The separation is stopped when the dye, indicating the front line, is at the anode.

The following steps are the detection of the trace elements and the visualisation of the proteins.

2.1.5.2. Two-dimensional gel electrophoresis (2DE)

Two-dimensional electrophoresis is the latest development of gel electrophoresis, as evident from the exponentially increasing number of articles about this method. As already mentioned, the reason of this success is due to proteomics, since a gel can map nearly all proteins present in a sample. For speciation analysis, 2DE is only applicable if the metal under study is covalently bound, since the method is denaturing in its most refined form, *i.e.* high-resolution. Nondenaturing two-dimensional electrophoresis has been described [Lustig, 1999a], but is based on different separation principles from those mentioned here and little has been published until now. That is why this part concentrates on denaturing electrophoresis.

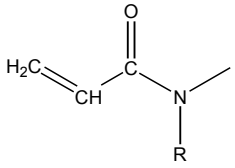
2DE unites two separation mechanisms that exist independently from one another: isoelectric focusing (IEF) and sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). It is thus a separation according to pI in the first dimension and size in the second. In most of the contemporary publications, IEF is used as first dimension and SDS-PAGE as the second dimension. Both methods can be used independently and what is mentioned here is true for both SDS-PAGE one-dimensional electrophoresis and IEF one-dimensional electrophoresis, with slight modifications for the latter. One should refer to books [Westermeier, 1993; Hames, 1998; Rabilloud, 2000] or articles [Görg, 2000; O'Farrell, 1975] for practical and state-of-the-art description of the method.

2.1.5.2.1. Isoelectric focusing (IEF)

This separation procedure relies on one of the major characteristics of proteins, their isoelectric point. Indeed, the charge of a protein is pH dependent and at a characteristic pH, this net charge is zero. It is possible to polymerise a gel with a pH gradient, named immobilised pH gradient (IPG), or to use a chemically created pH gradient, formed by free carrier ampholytes. They are both commercially available.

- In the case of an IPG, the pH gradient is already chemically present before electrophoresis. It is obtained by pouring a controlled amount of derivatised buffers, which become polymerised in the gel. Their general formula is:

Structure 2.3: General structure of the monomer of IPG buffer group.



where R contains either a carboxilic or an amino group. The pH at a certain position in the gel is a function of the relative amount of basic and acidic buffer groups that have been brought to the position considered. This amount is precisely controlled by high-precision pumps when the gel is poured.

- The second solution, although historically the first, is to use a mixture of ampholytes in the electrophoresis buffer. Ampholytes are bifunctional amphoteric buffer molecules, which thus exert both acidic and basic properties. Immediately before the sample is brought onto the gel, a voltage is applied at the extremities of the gel and is responsible for the migration of the ampholytes that form the pH gradient. This technology is still used for capillary electrophoresis IEF (CIEF, see 2.2.1.4).

The choice of the form of gradient is sometimes important for the quality of the separation. To begin with IEF separations, especially as the first dimension of 2DE, it is generally more secure to use the IPG technology, where the IPG strips, usually about 5 mm wide and *ca.* 5-20 cm long, are stored in a dehydrated form.

The principle of IEF, either with IPG or free ampholytes, is given in Figure 2.3.

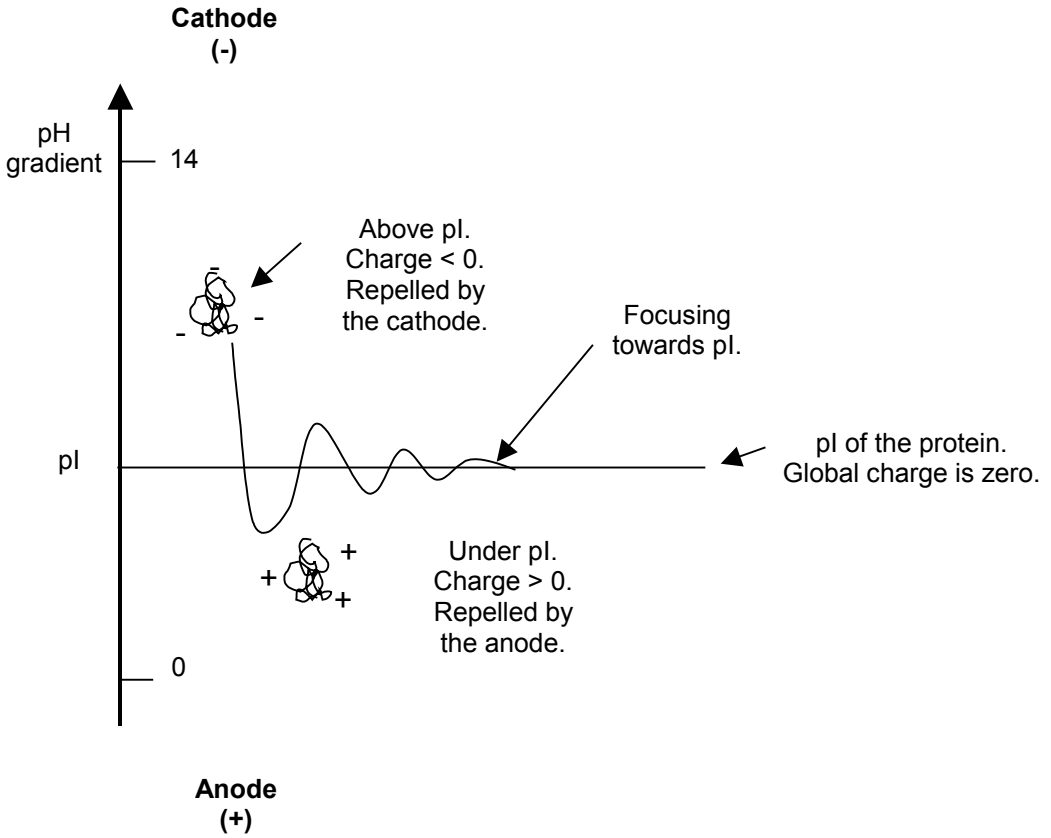


Figure 2.3: Principle of IEF.

The main groups responsible for the charge of a protein are the carboxylic (noted –COOH / $–COO^-$ according to their protonation) and the amino, imidazole and guanidine (noted $–NH_4^+$ / $–NH_3$) side-groups.

The basic part of the gel is pointed towards the cathode (-). Let us take the example of a protein initially above its pI in the gradient. Its side-chains are partly deprotonated. The protein is deprotonated as $–COO^-$ and $–NH_3$ above its pI and therefore negatively charged. Consequently, it migrates towards (+) and towards its pI. At the pI, the charge is zero (–COOH and $–NH_3$) and the field does not influence the particle any more.

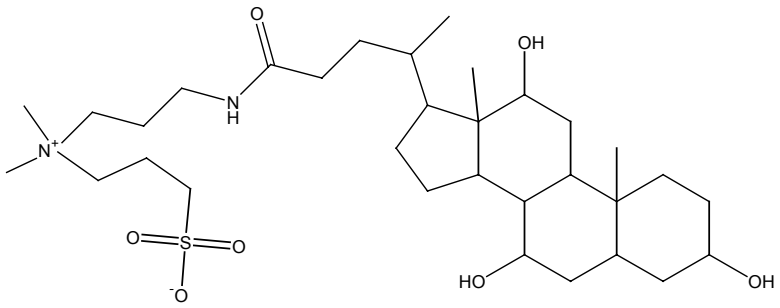
This reasoning is conversely valid for a protein initially present at the anode.

Should the protein diffuse, above its pI, the charge is negative ($–COO^-$ and $–NH_3$) and repelled by the cathode (-) and below it, the charge is positive (–COOH and $–NH_4^+$), repelled by the anode (+). That is why the separation is usually named focusing, stressing the fact that the protein comes to a definite spatial point in the gel by a ping-pong mechanism.

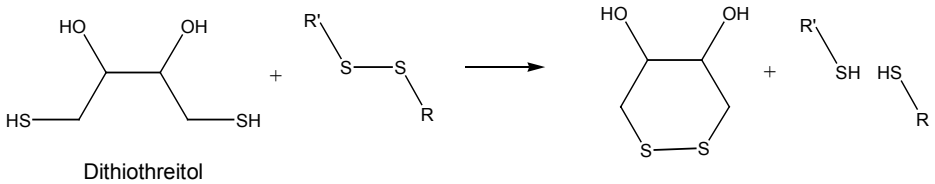
Although not a requirement for IEF, it is better to denature the proteins at this stage, firstly to be compatible with the second dimension and secondly to bring hydrophobic proteins in solution. The quantity of protein is also a determining factor for detection and must be adjusted to the detection method, as already mentioned, but should not exceed 200 µg in a narrow strip, about 20 cm long. This solubilisation takes place in a mixture, further referred to as sample solution, containing [Görg, 2000]:

- urea, a chaotropic agent; such an agent is, by definition, able to disrupt the structure of water and is thus used to break the hydrogen bonds in and between proteins and unfold them;
- a non-ionic detergent, such as CHAPS (see Structure 2.4), to bring the proteins in solutions without contributing to the ionic strength of the solution;
- a reducing agent, DTT (see Reaction 2.1), to break the disulfide bonds in proteins
- possibly a protease inhibitor, depending on the sample, in order to prevent any proteolysis.

Structure 2.4: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.



Reaction 2.1: Reduction of disulfide bonds in proteins by DTT, dithiothreitol.
R and R' are the two main chains of the protein.



Dithiothreitol

Once again, it must be checked whether the sample is stable in this solution. The same strategy as that mentioned in 2.1.5.1 is recommended. In particular, since the

separation occurs in a pH gradient, control of the stability of the species is necessary at the extreme pHs, usually 3 and 11.

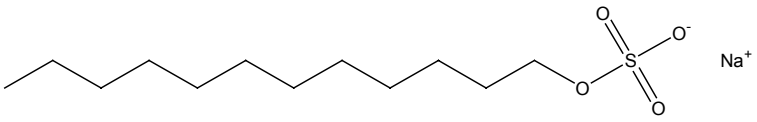
For our example, IPG, the strip must be rehydrated for at least 10 h, either in the sample solution or in a solution containing the same chemicals, except the analytes, the sample being added at the end of rehydration. After rehydration, a low voltage (in our example 300 V) is applied for 1 h to force the proteins into the gel and afterwards a high voltage is used to achieve the focusing (up to 8000 V, if it can be reached) for at least 6 h.

Between the two dimensions, an equilibration step is necessary to change the solution in which the proteins will be separated in the second dimension, namely SDS.

2.1.5.2.2. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE)

The separation principle is based on the constant affinity of SDS, sodium dodecyl sulfate (see Structure 2.5), for proteins, since 1.4 g SDS binds to 1 g protein. From then on, the intrinsic charge of the protein is masked by the charge of SDS; thus there is a constant charge per gram protein. Instead of a separation according to mass and charge, charge is here related to mass. This method has other advantages, when it can be applied to speciation, such as an enhanced solubility of the proteins or a real random coil of the chain, which allows an easier mass estimation of the particle.

Structure 2.5: SDS, sodium dodecyl sulfate.



For SDS-PAGE, the choice is once again left open between gradient or homogeneous; a gradient gel may be easier to begin with, since the mass range is broader, especially when little is known about the sample. Most of the time a stacking effect is used to enhance the resolution in the second dimension. This last point can be recognised in the set up of the buffers [Laemmli, 1970]:

- cathode buffer : glycine / Tris, 10 g L^{-1} SDS;
- gel buffer : Tris / HCl, 10 g L^{-1} SDS;
- anode buffer : Tris / HCl;
- glycine is the trailing and chlorine the leading ion.

The IPG strip is laid parallel and next to the cathode (-). Since the micelles are negatively charged, they migrate towards the anode.

The voltage programme is comparable with the others already mentioned, for a typical gel (25 cm x 11 cm):

i. 200 V (50 mA and 30 W maximum). The voltage is low for an optimum sample entry, until the sample front is 5 mm away from the IPG strip.
ii. The strip must be then removed and the cathode buffer put in on its place to avoid dehydration of the PA gel.
iii. 600 V (50 mA and 30 W maximum). The separation is stopped when bromophenol blue has reached the anode.

Afterwards, the gel is ready for the detection of the trace element and the proteins.

2.1.6. Detection of the proteins: staining procedures

In order to visualise the proteins in the gels after separation, various techniques are available. Following the classification of Rabilloud [Rabilloud, 2000], detection methods of proteins on gels belong to five types:

- Organic dyes. These dyes, historically from the textile industry, have a higher affinity for the proteins than for the gel background. One of the most used dyes is Brilliant Blue G. The bound dye is detected by optic densitometry.
- Fluorescence. This is achieved by using a dye that fluoresces, allowing a more sensitive detection than simple densitometry.
- Precipitation of salts. This case illustrates negative staining, where the salt has more affinity for the background than for the proteins. The gel is dipped in a solution containing the poorly soluble salt, which precipitates on the gel but less on the protein spots. A negative image is obtained, where the spots are clearer than the background.
- Radioactive isotopes. This method requires that the proteins be labelled, either in vivo or in vitro. It is an attractive alternative to staining procedures since it offers a detection limit comparable to silver staining but without any further chemical modification of the proteins.

- Metal ion reduction, namely reduction of silver. This is one of the most used methods since, in comparison with all mentioned methods, except radiolabelled proteins, it offers the lowest detection limit. The principle is the affinity of proteins for the silver ions. When a reductant is added, metallic silver is obtained at the location of the protein and not in the gel background. In that way, a positive image of the proteins is obtained. The type of the silver staining used in this work will be given in the following.

Various variations of the silver staining procedure exist. In the following procedure, silver nitrate is the silvering agent and the developer is formaldehyde in an alkaline carbonate solution [Heukeshoven, 1985]. This method has been chosen for this work since it offers one of the lowest detection limits, down to the nanogram protein, and because commercial kits are available, making the preparation of the staining less time-consuming and more reproducible. The gel is stained in a development tray allowing an automatic replacement of the solution after each step. In this way, the various solutions do not come in contact with each other.

i. The proteins are first fixated in the gel by an acidic precipitation. This step also allows a removal of all chemicals used for the electrophoretic separation, as SDS, which also show a high affinity for silver and could lead to a false visualisation.
ii. Rinsing.
iii. The gel is treated with a reductant, a thiosulfate solution, so that differences in affinity for the silver cations between proteins are erased. A sensitivity enhancer like glutardialdehyde can be added. It seems that such a sensitivity enhancer binds to the proteins and enhances the affinity of all the proteins for the silver cations.
iv. Rinsing.
v. The gel is brought in contact with the solution containing the silver cations. The silver cations concentrate where the proteins and the sensibility enhancer are present.
vi. Quick rinsing.
vii. The image is developed by soaking the gel in an alkali solution, *e.g.*, in a carbonate solution, containing a weak reductant, formaldehyde. Autocatalysis of the silver reduction is the driving force of this poorly

understood step. Silver is then reduced to metallic silver to form particles. Since more silver is originally present where the proteins are present and since the time dependence of the silver particles growth seems to be a third power of the time [Malygin, 1992], the development is faster at the protein location than in the gel background, where hardly any silver was present.

viii. The development is stopped by complexing the silver excess, *e.g.*, with EDTA.

ix. The gel is soaked in a glycerol solution to preserve it for storing.

x. The gel is left to dry for a couple of hours and preserved in, *e.g.*, a mylar foil. Such a gel and the proteins are stable for years, at least.

In most cases, staining can only be performed after the detection of the trace elements, since the gel is submitted to various chemical treatments that denature the protein and thus release the trace element from it. One major exception is the detection of covalently bound trace elements, as selenium, which can resist such a staining. Yet, if a radiotracer is used for the detection of the trace element, it is once again better to stain the gel after the autoradiography since silver can quench the radiation emitted by the radiotracer.

2.2. Other fractionation methods

The goal of this chapter is to critically assess the various methods available in the laboratory for the fractionation of trace elements in combination with macromolecules, as is the case for gel electrophoresis. Since the scope of this chapter is an introduction to those fractionation methods, reviews were given priority as the cited literature.

A growing trend is the development of hybrid approaches. In other words, two (or more) methods are used one after the other, on- or off-line. The methods described in the following may thus be combined for complex samples to form so-called multi-dimensional approaches.

These methods are summarised in Table 2.2.

Table 2.2: Fractionation methods relevant for macromolecules. See text for more details. (Peak capacity: number of peaks that can be resolved to the baseline [Wehr, 2003])

	Maximum theoretical peak capacity C or plate number N	Mechanisms available	Parameters driving the separation	Separation range	Reference
HPLC	N > 5,000; C = 200 In practice, C = 50 - 100	Reversed-phase Ion exchange	Hydrophobicity K_a		
SEC	N = 5,000 In practice, C = 10	Exclusion	Hydrodynamic radius	M_r > 10 kDa	
FFF	~SEC	Sedimentation Flow Thermal, etc.	Hydrodynamic radius	M_r > 1000 kDa M_r > 1000 kDa M_r > 10 kDa	
UC	In practice, C = 10	Sedimentation Archibald's principle	Mass density ρ, M_r	Subcellular fractions Mass distributions	
CE	N = 500,000	Electrophoretic mobility Sieving, pH gradient Isotachophoresis	μ M_r, pI		[Li, 1996]
SGE	C = 100	pH gradient sieving, with detergent sieving, without detergent	pI M_r Hydrodynamic radius		
LC^2 LC x CE on-line	N = 100,000; C = 200^2 In practice, C = 500 – 1,000	i. IEC i. SEC i. RPC ii. RPC ii. CZE ii. CZE	See the 1D methods		[Wehr, 2003] [Issaq, 2002]
2DE	C = 10,000 In practice, C = 500 – 1,000	i. pH gradient ii. sieving	i. pI ii. M_r	3 < pI < 9 M_r > 5 kDa	[O'Farrell, 1975]

2.2.1. Capillary electrophoresis (CE)

This method is of course close to gel electrophoresis and the basics of electrophoresis are given in paragraph 2.1.1. CE and gel electrophoresis share some separation methods, such as capillary gel electrophoresis (CGE) and isotachophoresis, even if the latter is mostly used in gel electrophoresis for sample stacking and not as a fractionation method. To use the most precise terminology, one should use the nomenclature slab gel electrophoresis (SGE) to differentiate it from CGE. Even among specialists, this terminology is hardly used.

Since SGE is mostly compared to CE, the various separation modes of the latter technique are presented here in more detail. Most CE separation modes are used in one form or another in slab gel electrophoresis [Li, 1993]. As for SGE, since the separation principles are quite different from one another, completely different characterisation mechanisms are available. All those modes are readily used for macromolecules, especially proteins [Wehr, 1998].

2.2.1.1. Capillary zone electrophoresis (CZE)

The separation is based on differences in the electrophoretic mobilities. The main difference with SGE is the presence of a significant electroosmotic flow. This flow originates from the presence of silanol groups on the capillary surface, which can be ionised in the electrophoretic medium. Cations are then present on the surface of the capillary and migrate towards the cathode: a concomitant migration of fluids towards the cathode is the result.

This flow is of main importance for the separation since in this way both anions and cations can be separated in the same run: anions can be detected at the cathode since the electroosmotic flow is significantly greater than the electrophoretic mobility: the sum of both migrations for an anion, towards the anode because of its mobility and towards the cathode because of the electroosmotic flow, is still to the advantage of a migration towards the cathode. A cation migrates anyway towards the cathode, so that detection of both ions is possible in one run at the cathode.

Such a separation is not achievable in the case of SGE, unless a surfactant like sodium dodecyl sulfate (SDS) is added.

2.2.1.2. Capillary gel electrophoresis

This method is totally comparable with SGE. The fractionation mechanism is based on a difference in analyte size as the molecules migrate through the pores of a sieving medium, the capillary filled with gel. Most separations rely on SDS as surfactant to form aggregates with the analytes, proteins, polynucleotides or DNA fragments and use polyacrylamide as the sieving medium. This latter form is simply a capillary SDS-PAGE.

2.2.1.3. Micellar electrokinetic capillary chromatography (MEKC or MECC)

Here a surfactant like SDS is added at a concentration above its critical micellar concentration. In that way, a biphasic solution is obtained, or in other words an emulsion. The analytes, as a function of their hydrophobicity, reside in the micelles, with a hydrophobic core, or in the polar electrophoretic medium. Since SDS is negatively charged, it migrates to the anode. The longer the analyte resides in the micelles, the higher its velocity towards the anode.

2.2.1.4. Capillary isoelectric focusing (CIEF)

Using ampholytes, a mixture of anolytes and catholytes, in other words of bases and acids, a pH gradient is created along the capillary. The proteins migrate then toward their pI as described in 2.1.5.2.1. After focusing, various zones are obtained, which are pushed towards the detector by using, *e.g.*, pressure.

2.2.1.5. Capillary isotachophoresis (CITP)

This mode differs the most from the other in its principle. It is used here as full fractionation method and not only as stacking, or pre-concentration procedure. An overview of the mechanism is given in 2.1.4.4.

For speciation purposes, CE and SGE have some drawbacks in common. The stability of the species can be impaired if complexing electrolytes or a wrong pH are used. A problem, more serious for CE than for SGE, is analyte loss on the capillary wall. The analytes can even be released after a certain time, giving ghost peaks.

However, the main difference between the two techniques regarding speciation is actually detection. Radiotracers cannot be used with capillary

electrophoresis due to the low amount of material injected on the capillary, typically a few nanoliters. On the other hand, CE can be hyphenated on-line with ICP-MS or ESI-MS (electrospray ionisation – mass spectrometry), whereas GE requires an off-line detection. Since the limit of detection of ESI-MS is poorer than that of ICP-MS, the general strategy followed up to now is to use ICP-MS as specific, elemental detector and afterwards ESI-MS to gain information about the identity of the species of interest.

Limits of detection for various elements can be in the 0.05 – 1 µg L^{-1} range with ICP-MS as detector [Michalke, 2003], even if the interface between CE and ICP is still troublesome. The main problem of the interface is the closing of the electrical circuit from the end of the capillary to the nebuliser (i). Further problems are (ii) the discrepancy in flow rates between the low flow rate of CE and the higher one necessary for an efficient nebulisation and (iii) the suction through the capillary due to the Venturi effect of the nebulisation gas. In order to solve problem (ii), either a sheath flow has to be added to the flow from CE, resulting in a considerable dilution of the analyte, or the flow rate of the separation itself has to be increased, resulting in a drastic change in the separation characteristics.

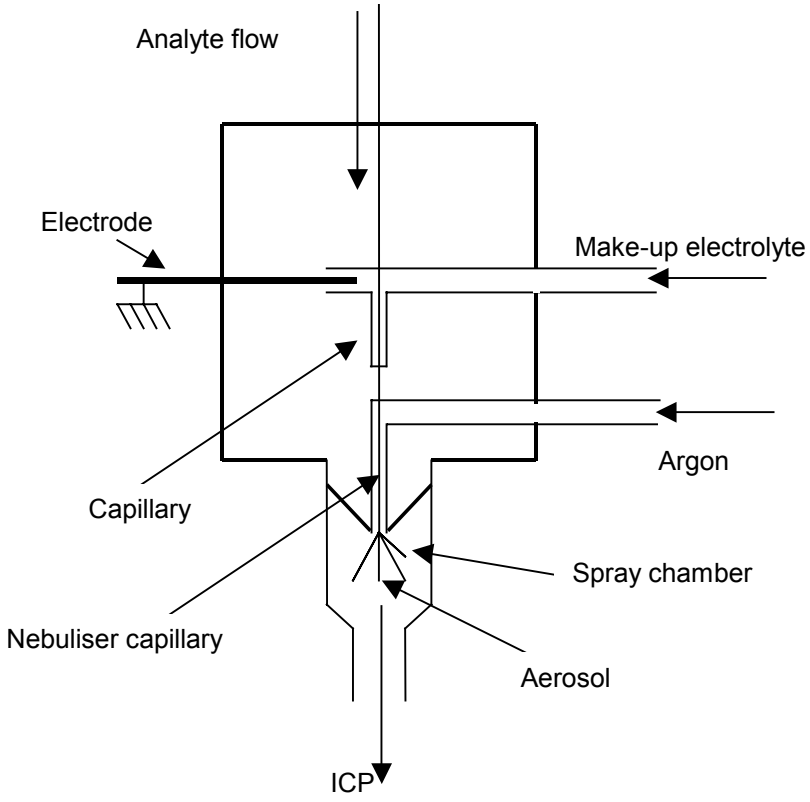


Figure 2.4: Interface between CE and ICP [Schaumlöffel, 1999].

The most widely used interface is based on the addition of a sheath flow, as in one of the latest interfaces shown in Figure 2.4. It is up to now the only one commercially available.

In this figure, both first problems (i and ii), grounding and too low a flow rate, are solved by the sheath flow itself, which is grounded. The suction effect of the nebulisation gas is solved by using a narrow capillary at the nebuliser, whose internal diameter is small enough (25 µm) to create a capillary force superior to the suction force.

CE has a higher sample throughput than GE, since a separation with detection only requires one hour, but for proteins especially, CE is not able to give a panoramic analysis of all proteins present in a sample, as SGE is able to do.

2.2.2. Chromatography

2.2.2.1. Gas chromatography (GC)

The application of gas chromatography to macromolecules is very limited, due to their negligible volatility. However, it is widely used for the determination of the building bricks of the proteins, amino acids, because of its high resolution. The analysis takes place after degradation of the original proteins and derivatisation of the amino acids to make them volatile. This method is especially appropriate to the determination of selenoamino acids, where the hyphenation is particularly efficient with a sensitive detector like ICP-MS or a detector which gives structural information like tandem MS. [Łobiński, 2000].

2.2.2.2. High-Performance Liquid chromatography (HPLC)

Liquid chromatography offers a wide panel of separation mechanisms, both based on physical principles, like size-exclusion, and chemical principles.

For the purposes of speciation of macromolecules, the main criterion of classification of the various methods is whether or not the structure (secondary, tertiary or quaternary) of the molecules may be disturbed, especially by using a polar modifier.

To briefly summarise the most widely applied methods, it can be seen that reversed-phase chromatography (RPC) is the method of choice when the structure of the molecule may be disturbed. Indeed, since retention is governed by polarity,

organic solvents are used to change the ratio of polarities between the stationary phase and eluent. Labile complexes or weakly associated compounds are then prone to denaturation in such a medium. Care must be taken in choosing a packing material with pores large enough not to exclude the macromolecules, but when this condition is fulfilled, a fractionation with a high resolution is obtained.

On the other hand, a method that is more respectful of the higher structure of macromolecules is ion exchange chromatography (IEC). The separation is mainly based on ionic interaction with the stationary phase, where ionic groups are bound. In the case of macromolecules, all other types of interactions are possible (Van der Waals, hydrogen bonding, etc.). Elution gradients in terms of ionic strength are used to control retention, normally with a constant pH and a certain amount of polar modifier when adsorption on the stationary phase is problematic.

A method that lies at the border between RPC and IEC is reversed-phase – ion pair chromatography (IPC). A counter ion is added to the eluent which pairs with the analyte and is chosen so that it is hydrophobic. Because of this last feature, it interacts with the apolar and hydrophobic stationary phase as for RPC. In the frame of speciation with macromolecules, this method has hardly been used.

[González, 2000]

2.2.2.3. Size exclusion liquid chromatography (SEC)

The parameter of fractionation is as a first approximation the size of the particles, more exactly the hydrodynamic diameter, thus the average size of the particle in the eluent. This fractionation takes place by sieving on a packing material with pores of a particular mean diameter. The analytes are in solution in the eluent, circulated by a classical liquid chromatography system. Size-exclusion chromatography is further subdivided into gel filtration chromatography if the mobile phase is aqueous and gel permeation chromatography if the mobile phase is an organic solvent [Meyer, 1999].

The first advantage of this technique is the rapid result obtained, where the maximum retention time is known, the total permeation time, on condition that no adsorption on the column occurs. No overlapping between two runs is thus possible. The separation takes place in *ca*. half an hour, a timescale that may be short enough when working with systems of which the kinetics are of the same scale. The second advantage is the principle of fractionation itself, yielding a molecular parameter, M_r, on the particle, when the separation is calibrated. However, this information should

be critically evaluated since further interactions between the analyte and the packing material are possible and may be predominant for particles with a high charge-to-volume ratio. Furthermore, the hydrodynamic diameter of biomolecules is known to be dependent on the ionic strength of the solution; the ionic strength of the eluent should be high in order to decrease polar interaction between the analytes and the packing material. The third advantage is the nature of the eluent in the case of gel filtration, mostly without organic solvent that could denature the analytes. This is of prime interest when working with weak metal-macromolecules complexes, where a slight addition of organic solvent is able to change the structure of the macromolecule and thus the nature of the complex.

The main disadvantage of size-exclusion is its low resolution. It is impossible to resolve two analytes of which the molecular masses differ only by 10 %.

2.2.2.4. Field flow fractionation (FFF)

This technique, developed in the 1960s [Giddings, 1966] has emerged from polymer and colloid sciences [Cölfen, 2000] and is widening its application field in the life sciences for the fractionation of cells [Chianéa, 2000] and biological macromolecules [Giddings, 1992]. Nonetheless, it has hardly been applied to speciation: up to now, the only publications related to both subjects were the speciation of various metals bound to protein standards [Siripinyanond, 1999] or bound to organic matter in river [Siripinyanond, 2002] in Barnes' group and sea waters [Hassellöv, 1999] in Lyven's group. Its figures of merit and soft separation principles make it very attractive for speciation with particles and macromolecules.

The separation mechanism and apparatus is related to chromatography, with also a wide variety of separation mechanisms. Although this method relies on both separation classes, physical and chemical, since the former is historically the first and the most widespread, this paragraph will concentrate on this one. The principle of the method is the superposition of a transversal force field to the chromatographic flow. This principle is schematised in Figure 2.5.

This figure illustrates the case where particles are smaller than 1 µm, where actually the separation factor is Brownian movement. Smaller particles, with a higher Brownian agitation, are prone to diffuse to the centre of the flow profile, a parabolic profile, and therefore move more rapidly than bigger ones.

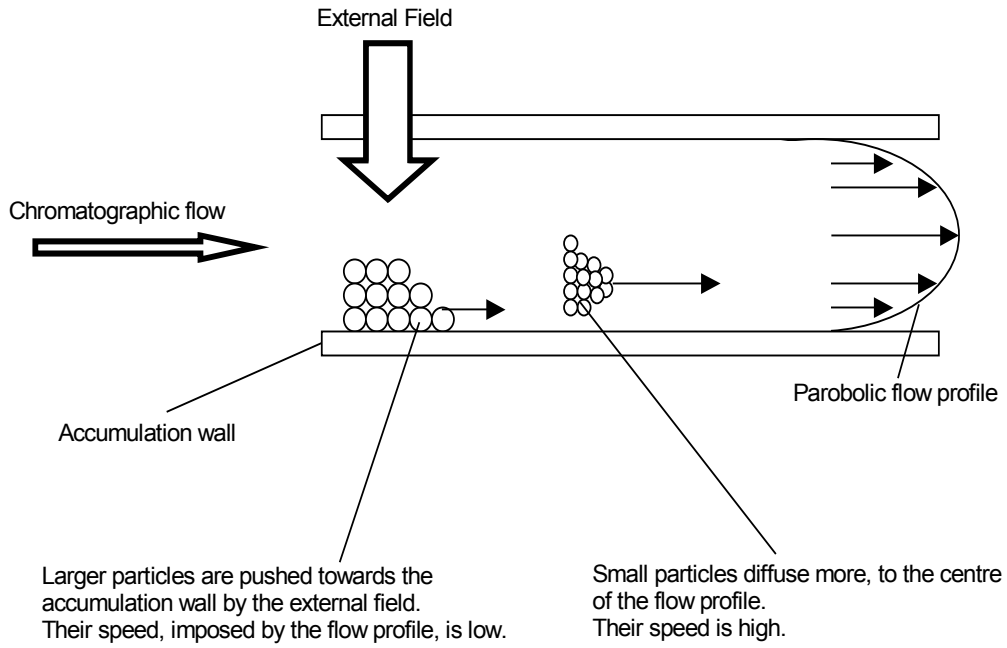


Figure 2.5: Principle of FFF.

These physical transversal fields are of diverse natures: sedimentation, thermal and flow in a first instance, but also electrical, magnetic, fluid cross-flow. That is why the theory of this method shares many features with centrifugation, thermal analysis and filtration. The main advantage of the method is its soft separation principle, which does not require a stationary phase. Non-specific interactions, which are common in chromatography, are thus avoided. The range of separation is also very wide, particle diameters typically from 1 nm to 100 µm. For macromolecules, this range goes from 10^3 to 10^{18} g mol^{-1} as molecular mass. Therefore, this method is relevant for the separation of macromolecules, colloids and even cells. A wide array of information is also gained from the separation, from hydrodynamic diameter to average molecular masses.

In fact, this method can be seen as complementary to size-exclusion chromatography, for particles of ultra-high-molecular-mass compounds, such as protein aggregates or cells. For those masses, SEC develops shear forces that are too high and detrimental to the stability of the compounds [Cölfen, 2000]. The resolution of FFF is once again comparable to SEC

2.2.3. Centrifugation, ultracentrifugation

These fractionation techniques are widely used as sample preparation techniques or simple separation techniques for speciation, *e.g.*, between a liquid and

colloids, like the separation of whole milk into skimmed milk and whey for example [Martino, 2001] or cell fractionation [Bibow, 1990; Vandervoet, 1992]. Yet, no attempt was ever made to use the full resolution power of ultracentrifugation, with which a high resolution can be obtained, for speciation purposes. The application field of this technique is macromolecules or association of macromolecules up to cells.

Ultracentrifugation (UC) offers two major mechanisms, sedimentation and isopycnic, with the advantage that the separation takes place once again without stationary phase in soft buffers [Williams, 1972]. A sedimentation experiment is simply the measurement of the migration distance of the analyte after centrifugation in a viscous solution. Among other things, the migration velocity is a function of the mass of the analyte. An isopycnic separation (see Figure 2.6), or density equilibrium sedimentation, is performed in a gradient of density, created for example by the centrifugation of a sugar solution; the solution at the bottom of the tube is more concentrated than at the top. The solution to be analysed can be put at the top of the tube, on the upper layer depleted in sugar and submitted to the centrifugal force. The analytes will migrate towards the zone where the density is equal to theirs. This analytical gradient method is actually comparable in its theory to isoelectric focusing, with a centrifugal force instead of a difference of potential and a density gradient instead of a pH gradient [Wang, 2002].

For speciation purposes, the main limitation of the method is the availability of chemicals of high purity in order to create the viscosity buffer.

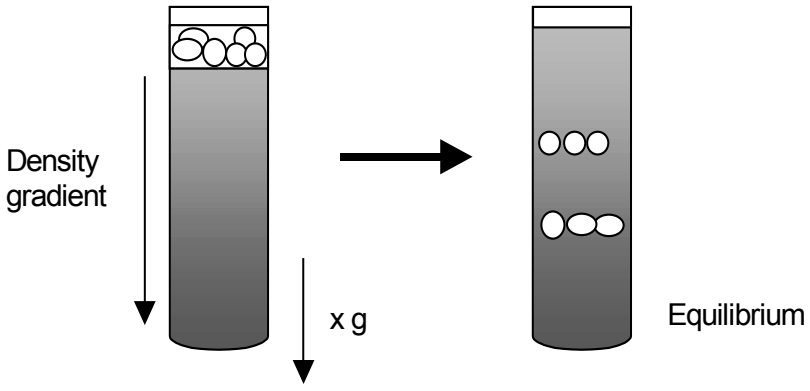


Figure 2.6: Principle of isopynic separation or density equilibrium sedimentation.

2.2.4. Ultrafiltration

Ultrafiltration shares the same principle as SEC, a fractionation according to size, once again on a membrane. The driving force of the separation is the centrifugal force and the result obtained is binary: is this compound above or under the cut-off of the filter. Yet, this technique is very valuable for speciation purposes since it yields rapid results and is useful for method development, when one is interested in the influence of a buffer on the stability of a compound for example [De Cremer, 1999].

One of the major pitfalls of this technique is the eventual affinity of the compounds for the membrane material [Van Landeghem, 1998].

2.2.5. Dialysis

Dialysis is routinely used for fractionation of marine and river samples, where the main purpose is to determine the amount of readily exchangeable (or labile) elements [Bufflap, 1995]. The simplicity of the principle, namely diffusion through a membrane towards a solution of lower concentration (chemical activity), is efficient but also limits the information given by the separation. In fact, the only information given by this method is the amount of readily exchangeable trace element in the sample and the kinetics of this exchange if measurements are done as a function of time.

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3. Elemental Detection

This chapter gives a general introduction to the methods used for the elemental analysis of solid samples. The second part is a review of the applications of these methods in the context of gel electrophoresis. The role of this chapter is also to give a description of the various detection techniques used for this research.

3.1. Detection of trace elements in solid samples

The detection methods used in this work are described here. They are also the most common detection methods for trace elements that allow the determination of very low concentrations. Other methods exist for the analysis of solid samples, but they are much less sensitive, like X-ray fluorescence spectrometry (XRF), or less available. Methods that have already been used in combination with gel electrophoresis in previous works, even if less widespread, like PIXE, are reviewed in 3.2.

First, a general comment on the advantages of solid sampling has to be given. Labour-intensive and time-consuming dissolution procedures are not necessary in such a case. This also limits contamination, coming from the chemicals used for the solutions, as well as analyte loss. Since the analyte is not diluted, the detection limits are improved.

3.1.1. Nuclear techniques

When the radioactivity of a sample is measured, either because a radiotracer has been used or because the sample has been submitted to activation by means of a cyclotron or reactor, various techniques are available. γ-radiation measurements with NaI(Tl) and Ge(Li) detectors are the best suited for solid samples. Other types of counting like liquid scintillation are used with samples brought into solution, by definition.

3.1.1.1. NaI(Tl) detector

A sodium iodide detector consists of a sodium iodide monocrystal, doped with thallium. This crystal has the property of commuting photons from the γ range to UV-VIS range of the spectrum. The latter photons are transformed into electrons, which

are multiplied in an electron multiplier. This electric signal is once again linearly amplified. The signal obtained is proportional to the energy of the γ-radiation.

3.1.1.2. Ge(Li) detector

The second class of γ detectors is the Ge(Li) detector, based on the semi-conductor Ge(Li). When an interaction takes place between the γ electromagnetic pulse and the semi-conductor, electrons are sent from the valence band to the conduction band. A difference of potential is then measurable, proportional to the energy of the γ-radiation. The signal is recorded and amplified. The resolution given by this technique is higher than the one of a NaI(Tl) detector, at the cost of sensitivity.

Both methods were used during this work: detection with NaI(Tl) of ^{75}Se in liquid chromatography fractions; purity control of ^{75}Se and ^{48}V after their production by Ge(Li).

3.1.1.3. Autoradiography

Autoradiography is the method of choice when the material from which an image must be obtained is thin, as an electrophoretic gel is. A complete and precise image of the distribution of radioactivity is obtained when the thin material is laid on the autoradiography screen, recording a kind of photograph of the distribution of the radioactive material.

Formerly, X-ray films were used, but the contemporary technology relies on phosphor screens [Johnson, 1990], which allow a more rapid detection with higher resolution. Further, a phosphor screen is theoretically infinitely reusable, as long as it has not been contaminated. After the signal has been read, the screen is erased by exposing it to visible light for a couple of minutes and is ready for use again.

The phosphorescent material is a suspension of BaFBr:Eu^{2+} crystals in a polymer. This crystal, when excited by radiation, sends an electron to the conduction bands, resulting in a different chemical structure, $BaFBr^{-}$ and Eu^{3+}. When the excited crystal is exposed to light, typically from a HeNe laser (633 nm), this energy is enough to destabilise the excited electron, which falls back to its ground state, emitting a photon at 390 nm. A laser is used because it allows an excellent spatial resolution. The luminescence is recorded in combination with the position of the laser and finally, when the whole screen has been scanned, a precise image of the

position and intensity of the original radioactivity is obtained, in the form of a digitised picture with the optical density as a function of the position.

Although both X-ray and phosphor screens primarily allow the detection of ^{14}C, ^{32}P and ^{35}S, pure beta-emitters, they may also be applied to other types of radioisotopes:

- isotopes emitting a β particle,
- isotopes that show electron capture,
- isotopes emitting γ radiation.

Various examples can be found in the literature: ^{75}Se [Lecocq, 1982; Behne, 1996; Jamba, 1996; Chéry, 2001], ^{63}Ni [Nielsen, 1994], ^{109}Cd [Scott, 1983a], ^{59}Fe [Vyoral, 1998], ^{65}Zn, ^{45}Ca [Scott, 1983b]. The only drawback is that the detection efficiency is lower for some of these latter isotopes. This means that a higher specific activity has to be used to get the same optical density as with the same activity of, *e.g.*, ^{35}S, or that a longer exposure time is required.

From the last remark, it is obvious that both parameters for detection by autoradiography are of importance, *i.e.* specific activity and exposure time. To get an idea if an element can be detected by this method, a simple test is required: a dry gel can be rehydrated with a solution of known activity of the radioisotope; by a simple weighing before and after rehydration, the amount of activity is known in the gel; the gel is left to dry, packed in plastic and exposed to the phosphor screen. Scanning of the gel will reveal if the original activity was high enough.

The procedure to use a phosphor screen is as follows. Just before use, the screen has to be erased since a background builds up after a while, even if the screen remains unused. After separation, on condition that the species remain stable with treatment, the proteins are fixed in the gel by an acidic solution and the gel is left to dry. This acidic precipitation of the proteins in the gel is actually the first step of the visualisation of proteins (see 2.1.6). The staining should stop at this point, since a metal-based staining (*e.g.*, silver staining) can quench the radiation emitted from the gel. The visualisation of the proteins can be resumed after the radiography is performed. In order to avoid contamination of the phosphor screen by the tracers in the gel, the gel is wrapped in a plastic foil, for example Mylar, the thinnest possible and only carbon-based to avoid loss of radiation through absorption of the material. A phosphor screen may also be stored at low temperature (less than -20°C, temperature at which the diffusion of the species in the gel is negligible) while

exposed to a gel, without apparent detriment to the quality of the picture. Thus, radiography also allows the detection of species that cannot be fixed in acid.

The phosphor screen is left typically for one day for ^{35}S and for two days for ^{75}Se at similar specific activities. For longer periods, laying the screen in a lead coffer can improve the signal to background ratio by lowering the natural surrounding activity. The screen is then read by laser densitometry, erased and stored. Standard software is available for the image treatment of the gel.

3.1.2. Inductively coupled plasma – mass spectrometry (ICP-MS)

ICP-MS is one of the most powerful detection techniques for trace elements (< 10^{-4} g g^{-1}) or ultratrace elements (< 10^{-8} g g^{-1}). The excellent detection limits it offers, typically at the ng kg^{-1} level, made of this method a *sine qua non* for this work. Other detection methods, as atom absorption spectrometry (AAS) or inductively coupled plasma – optical emission spectrometry (ICP-OES) do not have comparable figures of merit, although they are plausible for other kinds of analysis. A further advantage of this detection method is the variety of the hyphenations that are possible: chromatography, laser ablation and electrothermal vaporisation will be given as illustrations in this work.

3.1.2.1. Principle

Various set-ups of the mass spectrometer are available, depending on the resolution that has to be achieved, the number of elements that have to be detected in a short period of time, etc. In this work, a quadrupole-based instrument was used; when necessary, in order to reach a higher resolution, the dynamic reaction cell technology was used, in combination once again with a quadrupole filter. That is why the description given in this chapter will be focused on this type of instrument.

The principle of this technique is illustrated in Figure 3.1. First, the aerosol formed by the introduction system is desolvated in the plasma, the molecules are then atomised and the atoms ionised. This mechanism is not totally understood, but a good model is that ionisation occurs through interactions, namely collisions, between the argon atoms and its electrons, partly free in this plasma, and the analyte.

As for all mass spectrometers, the crucial point is the interface between the atmospheric pressure and the low pressure, lower than 1 mPa, which is necessary to ensure a collision-free transmission of the ions, in other words a long enough free

path. This interface consists of two coaxial cones with a tiny opening at their vertex, with their axis in the direction of the plasma plume. Only a part of the gas coming out of the plasma is thus allowed to go farther, gas consisting of ions, neutrals and electrons. At each step of the ion transmission a vacuum pump is installed. The pump has to pump away all neutrals.

After those two cones, when the sampling of the plasma gas is achieved, the ionised atoms are focused by an electrostatic lens, which also deflects anions and electrons. Only cations are thus farther transported to the filtering device of the instrument, a joined magnetic and electric sector field or, as in our case, a quadrupole. The dynamic electromagnetic field that is present in the analysis device has the capacity to bring at the exit of the device only the ion with a selected mass-to-charge ratio; other ratios are simply destabilised and expulsed from a stable trajectory. The selected ions then leave the quadrupole to migrate towards the detector, most of the time an electron multiplier.

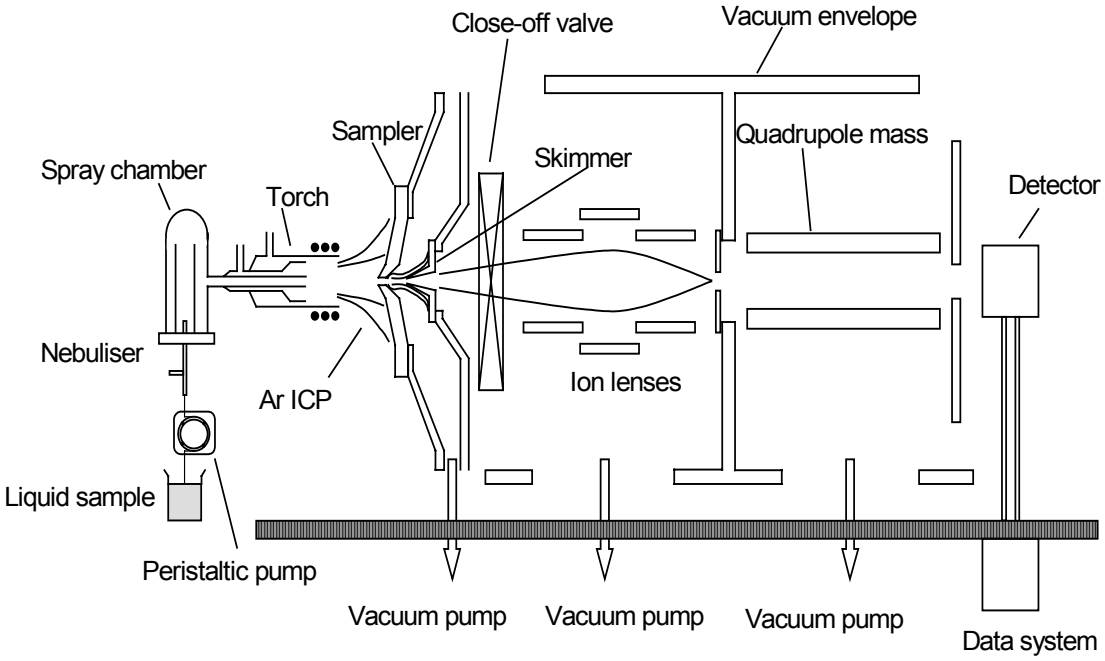


Figure 3.1: Principal components of a quadrupole ICP-MS. From [Saverwijns, 2000], originally from [Turner, 1998].

This technique suffers from certain disadvantages, limited in comparison with other detection methods, which can partly compromise its low detection limit. Firstly, spectral interference, *i.e.* the formation of ions with the same nominal mass as the analyte, can hamper the detection of the latter. This is the case in this work for the detection of Se, for which the detection of the most abundant isotope, $^{80}Se^{+}$, is made

difficult by the presence of the molecular ion $^{40}Ar^{40}Ar^{+}$, or the detection of vanadium through $^{51}V^{+}$ by $^{35}Cl^{16}O^{+}$. Such problems can be solved by using either a physical (high-resolution ICP-MS) or chemical (dynamic reaction cell) resolution.

Furthermore, the matrix, in which an element is present, plays an important role in, among other things, the position of the plasma plume, the ionisation equilibrium and the charge distribution, so that a calibration with the same matrix is preferable. The instrument is not stable over a long period of time, which makes the use of internal reference or periodical external standardisation mandatory.

3.1.2.2. Physical and chemical resolution with the ICP-MS

In order to prevent spectral interferences, various approaches have been followed. These approaches can be chronologically summarised, from the sample introduction to the mass analyser. The most drastic one is to separate the analyte from the species at the origin of the interfering ion, by any fractionation method. This is a sensible approach for analytes as $^{87}Sr^{+}$, interfered by $^{87}Rb^{+}$. Yet, this is not suitable for interferences originating from, *e.g.*, argon, which is present from the nebulisation on. The following step is to prevent the formation of the interference in the plasma itself. The instrument can then be used under “cold” plasma conditions or with plasmas relying on gases other than argon, like helium. Further in the instrument, between the plasma and the mass analyser, a cell can be installed to provide a chemical reactor in which the interference is chemically suppressed. Last but not least, the mass analyser itself can be of such a design that the resolving power is high enough to distinguish the interfering ion from the analyte.

3.1.2.2.1. “Cold” Plasma conditions

These conditions are achieved by operating the plasma at low power and high injector flow rate. The distribution of the ionic species after the plasma is thus totally changed, suppressing in particular the interfering species originating from argon [Douglas, 1998]. Of particular interest is the suppression of the $^{40}Ar^{40}Ar^{+}$, which could be ideal for the determination of $^{80}Se^{+}$. However, selenium has a relatively high ionisation potential and thus is hardly ionised in the “cold” plasma conditions. That is the reason why these conditions were not applied in this work.

3.1.2.2.2. Reaction / collision cell technology

3.1.2.2.2.1. Principle

As a method designed to suppress spectral interferences, the reaction / collision cell technology has gained success since it was commercially available in 1997. The terminology about this technology is rather new and has given rise to controversy, especially in what concerns the difference between reaction and collision. [Latino, 2001; Tanner, 2002].

The principle of this technology is the presence of a cell filled with a gas between the plasma and the quadrupole filter used for mass analysis. In the first version of this technology, known as collision cells, the interference should be suppressed by collisional induced dissociation according to its manufacturers: the gas present in the cell collides with the interfering ions and breaks them down. The real mechanism seems more complicated and is subject to controversy.

The latter version, known as reaction cell, relies on a chemical reaction either between the interfering ion and the reaction gas or between the analyte and the reaction gas. This case is illustrated below:

$(\text{interferent})^{+}$ + reaction gas →
$(\text{product of the interferent})^{n+}$ + $(\text{product of the reaction gas})^{n'+}$

where $n + n' = 1$.

e.g.:

$ArAr^{+} + CO \rightarrow ArAr + CO^{+}$

$ArAr \rightarrow 2\ Ar$

(see Figure 3.2 and 5.2.2).

The mass-to-charge ratio is thus modified and the product does not interfere any more with the analyte.

In the case of a reaction with the analyte, the product acquires a higher mass, at a mass-to-charge ratio that can be measured without suffering from interference.

$(\text{analyte})^{+}$ + reaction gas → $(\text{product of the analyte})^{+}$ + (product of the reaction gas)

e.g.: $V^{+} + O_2 \rightarrow VO^{+} + O$ (see 5.2.1.3.1.2).

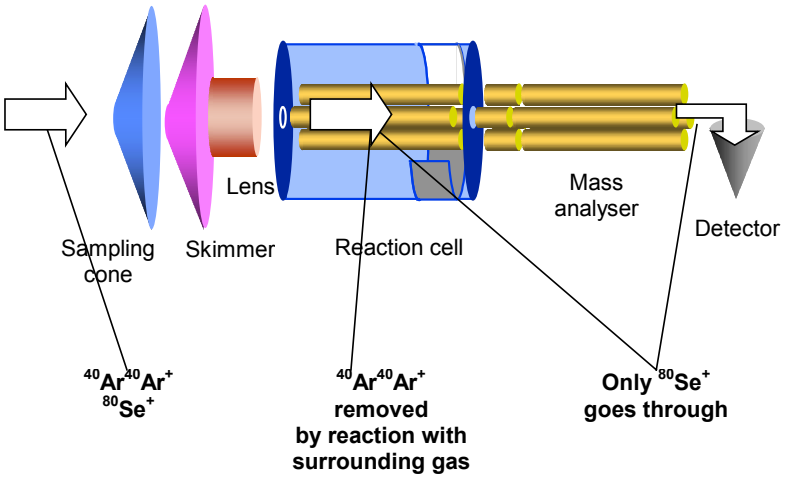


Figure 3.2: Working principle of the DRC technology.

3.1.2.2.2.2. Thermochemistry and kinetics

As for any chemical reactions, these reactions only take place when they are first thermodynamically allowed and when the reaction kinetics are favourable. Notwithstanding, the system is dynamic and cannot in fact reach its equilibrium, since there is a constant flow originating from the plasma through the reaction cell. However, the only practical approach is to determine whether the Gibbs energy of reaction after the equilibrium is reached, $\Delta_r G = \Delta_r H - T\Delta_r S$, is negative, in other words if the reaction is spontaneous. $\Delta_r G$ is indeed the right energy to describe such a system since the reaction pressure is maintained constant. In this complete work, the conventions for the use of symbols given by the IUPAC have been followed [Mills, 1993].

Some approximations are necessary in order to easily calculate this energy. First, since these reactions are bimolecular, between small particles such as oxygen or hydrogen, and mostly without condensation, in other words since there are two particles at the beginning and two at the end, the relative disorder after and before the reaction has taken place is more or less the same. Consequently, the entropy factor $\Delta_r S$ is negligible. In this way, the thermochemistry of the reactions is correctly described by $\Delta_r H$. This approximation is of course wrong in the case of a condensation (A + B → C), where the entropy drops, or when the number of reaction products is higher than the parents (A + B → C + D + E), where the entropy increases and could 'win' over the $\Delta_r H$ factor.

Further, $\Delta_r G$ (or $\Delta_r H$) is not available as such. It has to be calculated from $\Delta_r G°$. In the case of gases without phase change:

$$\Delta_r G = \Delta_r G^\circ + RT \ln \prod_i P_i^{\nu_i}$$

where P_i is the partial pressure of gas i and where:

$$\prod_i P_i^{\nu_i} = \frac{P_c . P_d}{P_a . P_b}$$

for a reaction noted:

$$A + B \rightarrow C + D$$

Yet, in practical cases, where A is the interfering ion, B the reaction gas, as in:

$$ArAr^+ + CO \rightarrow ArAr + CO^+$$

a steady state is reached for all species, especially if the kinetics of the reaction are high. Then, the partial pressures of the products are of the same order as the partial pressures of the precursors. This is not true for the reaction gas whose pressure is maintained high by a constant flow. Consequently:

$$\prod_i P_i^{\nu_i} = \frac{P_c . P_d}{P_a . P_b} \leq 1$$

$$\Delta_r G \leq \Delta_r G^\circ = \Delta_r H^\circ$$

This is in fact Le Chatelier's principle. In other words, the real case, at the cell pressure, is favourable to the reaction. That is why care should be taken in the case a reaction is apparently forbidden, as given by $\Delta_r G°$. It could in fact take place at the cell pressure if the difference with spontaneity is only a couple of RT (2.5 kJ mol^{-1}).

The determination of $\Delta_r H°$ is a classical calculation. For the aforementioned reaction, the reaction enthalpy is:

$$\Delta_r H° = \Delta_f H°(C) + \Delta_f H°(D) - \Delta_f H°(A) + \Delta_f H°(B)$$

where $\Delta_f H°(C)$ is the enthalpy formation of compound C.

By convention, the values of $\Delta_f H°$ are tabulated under standard conditions, at 298 K and 10^5 Pa. Those conditions are far from being the pressure and temperature in the reaction cell. Let us first examine the dependence of $\Delta_f H°$ on temperature. Pressure and volume are thus constant in the cell and temperature is hypothetically changed.

$$\left(\frac{\partial \Delta_f H^\circ}{\partial T}\right)_{P,V} = T\left(\frac{\partial \Delta_f S^\circ}{\partial T}\right)_{P,V}$$

according to third equation of Gibbs-Helmholtz. Further, $\Delta_f S$ is close to 0. Thus:

$$\left(\frac{\partial \Delta_f H^\circ}{\partial T}\right)_{P,V} = 0$$

$\Delta_f H°$ is thus independent of temperature.

The second factor that is drastically different from the standard conditions is the pressure in the cell. $\Delta_r H$, calculated with the various $\Delta_f H°$, is known at the standard pressure, $P° = 10^5$ Pa and should be known at the actual working pressure of the cell, *ca.* P_a = 1 Pa. One knows that:

$$\Delta_r H(P_a) = \Delta_r H(P°) + RT \ln \prod_i P_i^{\nu_i}$$

As already mentioned, in the case of a reaction such as:

A + B → C + D

$$\prod_i P_i^{\nu_i} = \frac{P_c . P_d}{P_a . P_b} \leq 1$$

$$\Delta_r H(P_a) \leq \Delta_r H(P°)$$

The reaction enthalpy is more favourable in this case. However, the difference is only of a couple of *RT* and is not important for very exothermic reactions like charge transfer reactions.

As a conclusion, values obtained from databases under standard conditions even if they are indicative, are very useful. If the calculated $\Delta_r H$ is exothermic, the reaction is thermodynamically allowed in the reaction cell. If it is endothermic and close to zero under standard conditions, the endothermicity can only be countered by using a high reaction flow. If the endothermicity at standard conditions is well above a couple of *RT*, it is not allowed.

Many formation enthalpies are available in databases, *e.g.*, [Linstrom, 2001], under one form or another. For instance, ionisation potential or standard potentials can be translated into reaction or formation energies. Practical examples of such calculations are given in 5.2.1.3.1.2.

The second *sine qua non* condition for the reaction to take place are favourable kinetics. The higher the reaction rate of a particular reaction, the more efficient it is. Once again, various databases have specialised in such data, although they are less comprehensive than thermochemistry databases. A comprehensive review about all these aspects and the various databases is to be found in [Tanner, 2002].

3.1.2.2.2.3. Secondary chemistry: dynamic aspect of the DRC technology

The presence of the reaction gas in the cell can be at the origin of a new interference on the analyte. An example is given by ammonia as the reaction gas [Latino, 2001]. In a chlorine rich medium, Cl^+ is present in the reaction cell and adducts such as $^{35}Cl^{14}NH_2^+$ can be formed. This adduct has the same mass-to-charge ratio as $^{51}V^+$. Ammonia is indeed frequently used to suppress the $^{35}Cl^{16}O^+$ interference on $^{51}V^+$. In order to destabilise species that can lead to a cell-based interference, a quadrupole is placed in the reaction cell allowing a filtering of the species. By adjusting the quadrupole parameters, a low- or high-mass bandpass is achieved. These are commonly under the terms of, respectively, rejection parameter q (RPq) and rejection parameter a (RPa). In our former example, by installing a low-mass filter, a high bandpass above 36, $^{35}Cl^+$ is destabilised and is ejected out of the reaction cell before it can react with ammonia. All further reactions, or sequential chemistry, are then impossible. This feature of the reaction cell is known as dynamic bandpass tuning, giving its full name to this technology, the dynamic reaction cell.

3.1.2.2.3. High-resolution ICP-MS

This is historically the first design to overcome the problem of spectral interferences. The principle is the combination of magnetic and electrostatic mass analysers instead of a quadrupole filter for mass analysis. The deflection of the ion in those fields is directly related to the mass-to-charge ratio of the particles. Such a static deflection, in opposition to the dynamic trajectories of a quadrupole, consequently offers a better separation between adjacent masses and thus a higher resolution.

3.1.2.2.4. Physical vs. chemical resolution

There are heavy discussions in the scientific community, or at least among representatives of ICP-MS producing firms, about the respective values of high-resolution and dynamic reaction cell technologies. No simple answer can be given, except that each instrument has its typical applications and limitations.

High-resolution spectrometers are incapable of resolving isobaric nuclides, while the DRC technology may be able to do so. An example is given by the ^{87}Rb / ^{87}Sr isobaric interference [Moens, 2001], which can be eliminated after selective reaction of strontium with CH_3F by measuring the intensities of SrF^+. A

second example is the spectral interference from $^{40}Ar^{40}Ar^{+}$ on $^{80}Se^{+}$ that requires a minimum resolution of *ca.* 9500 to be resolved; such a resolution could not be reached with most commercial double focusing instruments, only with the latest instruments such as the Element 2 (Finnigan). The DRC technology can suppress the $^{40}Ar^{40}Ar^{+}$ interfering ion, using, *e.g.* carbon monoxide as the reaction gas. Some other particular applications seem whithin easy reach of the DRC technology, like the resolution of various elements from oxides (*e.g.*, $^{96}Mo^{+}$ from $^{80}Se^{16}O^{+}$, requiring a resolution of 14189).

A high-resolution instrument also shows a slower scanning of the mass range, because of the hysterisis of the magnet. A practical disadvantage of high-resolution ICP-MS is its high cost.

On the other hand, high-resolution spectrometers offer precisions that are most of the time superior to that obtained by quadrupole-based instruments.

3.1.2.3. Solid sampling analysis with ICP-MS

The two main introduction systems for the analysis of solid sampling are electrothermal vaporisation and laser ablation. Spark ablation is limited to conductive materials and in the case of direct sample introduction, the amount of material brought to the plasma is very low. For these reasons, the former methods were not taken into consideration for this work on gels.

3.1.2.3.1. Electrothermal vaporisation (ETV)

The core of this introduction system is an oven. The sample is deposited into a graphite tube or onto a platform. This recipient is brought in the oven. The temperature can be raised stepwise, up to 3000°C, ensuring a thermal screening of the sample. The analyte, depending on its evaporation temperature, can evaporate before or after the matrix. At the instant of the formation of the aerosol containing the analyte, the aerosol is transported to the ICP-MS by an argon stream. This allows a sensitive detection, because the transport efficiency is high, and a mostly matrix-free detection, because the analyte is transported without the matrix [Grégoire, 2000; Kurfürst, 1998].

A general description of the device used in this work, a PerkinElmer HGA-600MS, is given in Figure 3.3 [Verstraete, 2003] as well as a picture of the oven and the graphite sealing probe. A graphite tube contains a cup, in which the sample is brought. A liquid can be added by automated pipetting through the hole of the

graphite cup; the liquid can contain a modifier, an internal reference or a calibration standard. As long as the sample is thermally treated without the formation of the analyte vapour, in other words in the pre-vaporisation step or pyrolysis, an internal gas flows through the graphite tube and transports the waste vapours out of the instrument. Just before the analyte vapours are formed, in the vaporisation step, the cup is sealed with a graphite probe and the vapours are swept into the ICP. The elemental analysis of this transient signal can take place from this moment on.

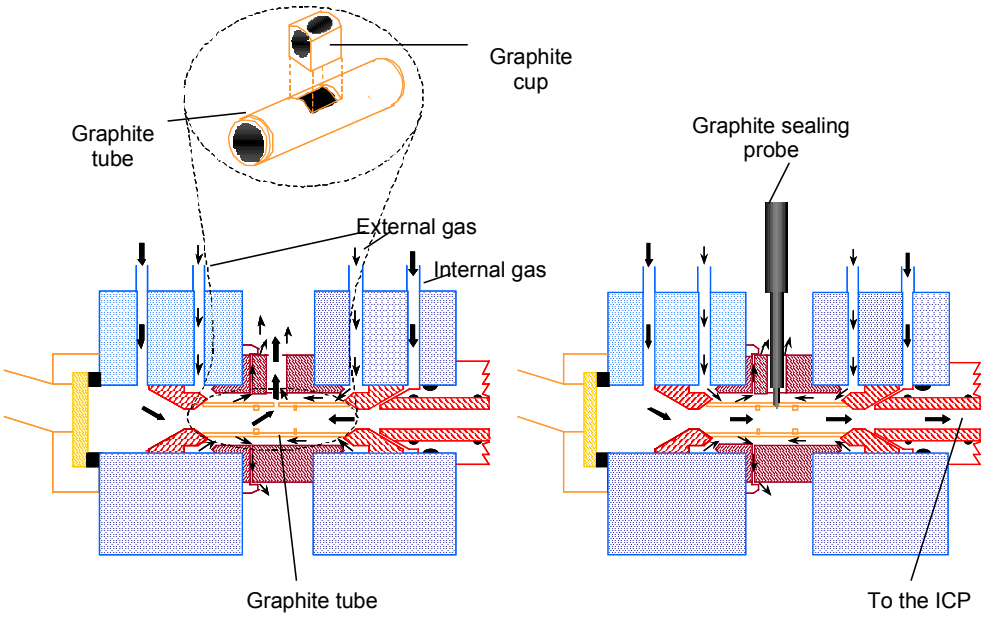
A general optimisation strategy can be found in Figure 3.4 in the case only a limited number of measurements can be performed on a sample, as is the case with gels after electrophoresis. This figure also mentions all relevant parameters for ETV-ICP-MS.

The main optimisation in ETV-ICP-MS is, quite logically, the temperature programme. Generally speaking, this programme consists of four steps:

1. Drying: at *ca.* 120°C, the water from the sample, plus the possible modifier and reference solutions, evaporates.
2. Pyrolysis: the matrix is removed.
3. Vaporisation: the compounds containing the analyte are decomposed leading to the formation of the vapour of the analyte. If the temperature of formation of the analyte is lower than the pyrolysis temperature of the matrix, as it may be possible for volatile elements, this step is shifted in second position.
4. Cleaning: to get rid of residues in the graphite tube.

When the vaporisation temperature of an analyte is too close to the pyrolysis temperature, a part of the analyte can be lost during the pyrolysis step. A better separation between the matrix and the analyte is thus necessary. A modifier can be added, whose role is to alter the thermal properties of the analyte. In that way, the analyte can either be thermally stabilised, so that it evaporates further away from the matrix, or made more volatile, so that the vaporisation is promoted before the removal of the matrix.

The vaporisation of the analyte and its transport can be promoted by the use of a physical carrier. The boundary between chemical and physical modifiers is sometimes vague and a chemical may fulfil both roles in ETV.



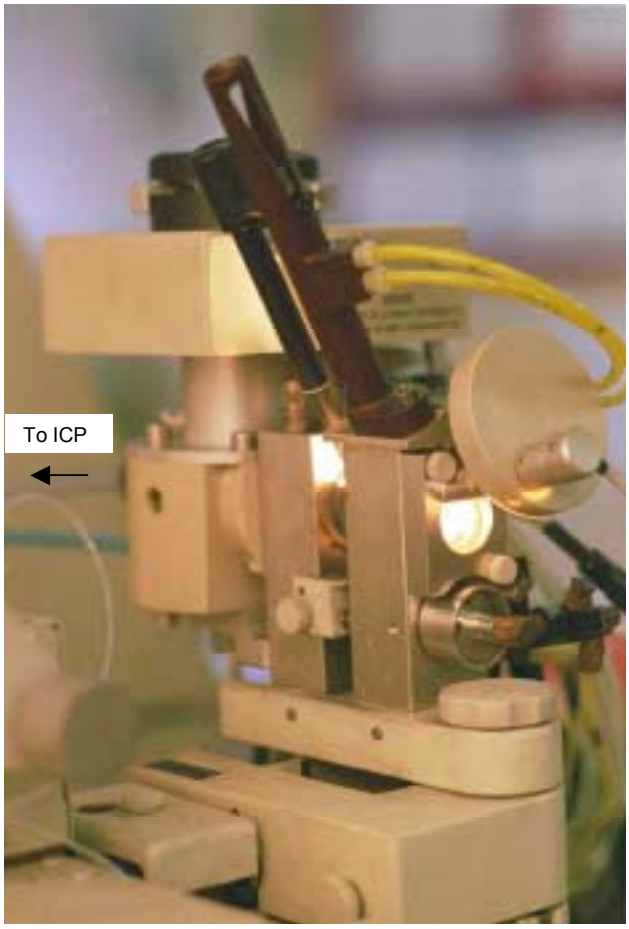


Figure 3.3: PerkinElmer HGA-600MS electrothermal vaporisation system. The white tubing at the left side of the picture is the transport tube to the ICP. Picture taken during the vaporisation step (the graphite probe is sealing the graphite tube).

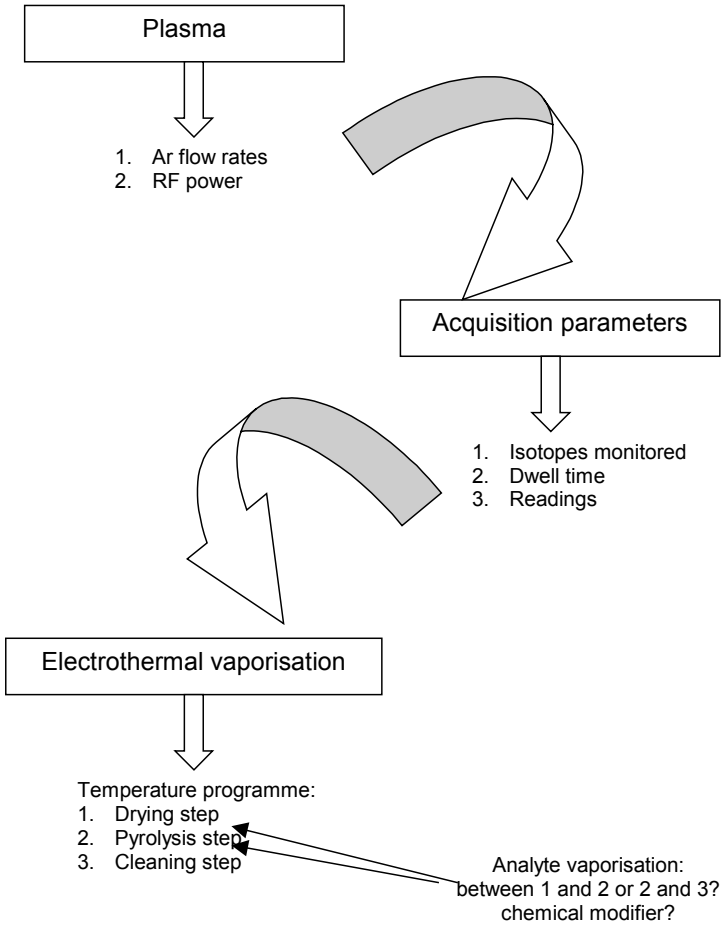


Figure 3.4: General optimisation procedure for ETV-ICP-MS.

Palladium is one of the most popular modifiers, and even of carriers, used in ETV. It has several advantages: it has a moderate vaporisation temperature and readily forms amalgams with nearly all elements.

ETV shows some limitations. The most important in the frame of this work are:

- First, the low precision of the method, typically 5-20 % RSD. This is due to variations in aerosol formation and transport, as well as plasma instability. Yet, this instability is partly due to sample inhomogeneity at mg levels. In the case of the analysis of gels, as later described, the precise response to the inhomogeneity of the sample is what is sought after.
- Even if the detection is mostly matrix-free, the little matrix that co-vaporises with the analyte can still have some influence. Calibration is, to a certain extent, matrix-dependent, so that matrix-matching may be preferable.

3.1.2.3.2. Laser ablation (LA)

The principle of this introduction system is that, during the interaction between a material and a highly energetic electromagnetic wave, part of the sample is ablated, in other words directly brought in the form of an aerosol. This is achieved by using a quasi-laser, namely a powerful focused electromagnetic wave, which is not necessarily temporarily or spatially coherent as a real laser is.

The beam directly excites electrons from the upper layer of the material, at such an energy scale that an explosion results. The higher the energy, the more this is true [Russo, 2002]. This explosion is spatially defined, forming a crater that is ideally cylindrical. This form is dependent on the optics used to homogenise the laser beam as well as the wavelength of the electromagnetic radiation. Various laser types are commercially available, from ArF to Nd:YAG lasers, with wavelengths respectively from 193 nm to 1064 nm, each wavelength offering different advantages [Günther, 2000].

The core of the device is the laser, which is described by the following parameters:

- Wavelength. The wavelength plays a key role in the interaction between the laser beam and the material. Infrared lasers exhibit an ablation mechanism related to thermal processes such as melting and vaporisation while UV radiation directly leads to an explosion because of the interaction with the chemical bond of the sample [Günther, 2000]. It influences the particle size distribution, among other things. The distribution seems to be more favourable when using a 193-nm laser because the explosion produces a finer aerosol, especially in combination with helium as the transport gas [Horn, 2003]. The smaller the particles, the higher the ICP-MS sensitivity is, because the atomisation of such particles is more efficient.
- Energy and fluence. The energy and energy density (fluence, in J m^{-2}) determines the amount of material ablated as a first approximation.
- Pulse duration. Up to now, most lasers produce pulses in the nanosecond range for analytical purposes. Shorter pulses, in the pico- and femtosecond ranges, may be advantageous to increase the ablation ‘shock’ and overcome problems such as non-stoechiometric ablation.

- The repetition rate. The higher the repetition rate, the more material is ablated by time unit.
- The laser beam diameter is, as a first approximation, the diameter of the crater.
- The energy distribution across the beam (*e.g.*, flat or Gaussian) determines the profile of the bottom of the crater, flat or spherical.

The optical set-up as depicted in Figure 3.5 in combination with a 193-nm laser seems to offer the best ablation characteristics. This device was used in this work.

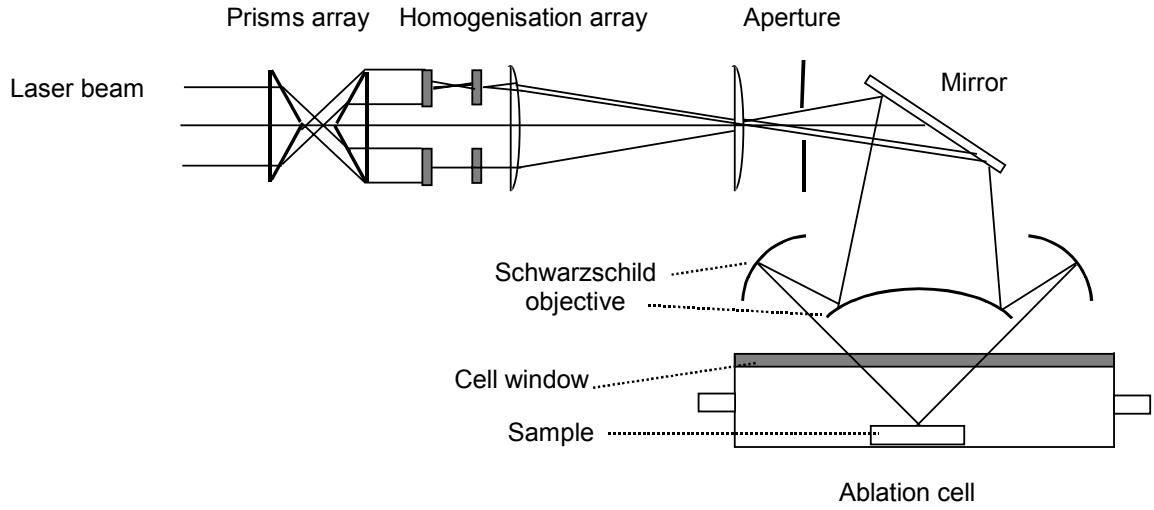


Figure 3.5: Optical beam path for a 193-nm laser. The beam is homogenised to obtain a flat energy distribution, the aperture is used to filter the edges of the beam and the Schwarzschild objective is used to focus the beam without any aberration [Günther, 2000].

The rest of the device also plays an important role in the quality of the ablation and the transport of the aerosol formed to the ICP. Figure 3.6 illustrates the set-up of such an introduction device. Ablation takes place in a so-called ablation cell, hermetically closed, except for the transport gas inlet and outlet, with the upper side transparent for the electromagnetic radiation. Similar to ETV, a gas is used to constantly transport the aerosol to the ICP-MS and two openings are made in the cell. Two transport gases are mainly used, argon or helium; it seems that helium offers a better transport because of the combination of its low viscosity and high thermal conductivity [Günther, 2001], especially when a 193-nm laser is used [Horn, 2003].

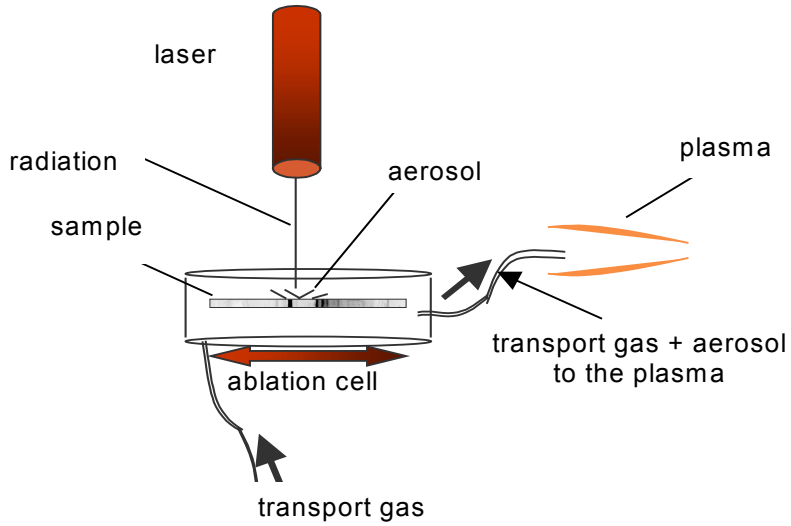


Figure 3.6: Principle of operation of laser ablation.

An interesting technological feature about the available instruments is that the ablation cell is mounted on a stage that allows a computer-controlled translation in all three spatial directions, as well as various displacement modes, such as a translation with a constant speed following a line, allowing the precise ablation of a sample on a line at its surface.

The advantages of this technique are numerous, without mentioning the general features of solid sampling analysis already given in the introduction of this chapter. First, it is not as labour-intensive as, *e.g.*, ETV, the displacement of the ablation being automated and computer-controlled. Second, it offers an excellent spatial resolution, in all three directions: a sample can be ablated in depth as well as on its surface.

Calibration is still problematic, and an internal reference is preferable when available to correct for differences in ablation efficiencies. Second, because differences exist between elements in ablation and transport efficiencies, the measured signals may not be representative of the composition of the sample or non-stoechiometric.

3.1.2.4. Acquisition of a constant or of a transient signal

An important feature of the acquisition of a signal given by ICP-MS is the strict difference that has to be made between the recording of a constant signal, as obtained from a pneumatic nebuliser (PN) and a transient signal, as obtained from

HPLC, ETV or LA. Indeed, the nature of the signal determines whether a long time can be spent for the measurement of one nuclide or not. By definition, for a constant signal, no information is necessary about the signal as a function of the time; in this case, several acquisitions can be averaged in order to achieve a higher precision. In the case of a transient signal the shape of the signal is of prime importance. A compromise has to be reached between precision, given by a longer acquisition time on one point, and exactitude in the real shape of the signal as a function of time, as given by a large number of points. This difference is reflected in the acquisition software of the instruments that were used during this work, namely the PerkinElmer SCIEX Elan quadrupole-based ICP-MS instruments. The various terms are defined in Table 3.1.

Table 3.1: Definition of the data acquisition settings for the Elan series.

Term	Definition	Command	Value for a constant signal (PN)	Value for a transient signal (HPLC, ETV, LA)
Spectral peak	One mass-to-charge ratio.	Points per spectral peak	1-5	1
Dwell time	Time spent on one point for its actual measurement.	Dwell time per acquisition point	150 ms	30 ms As short as possible
Sweep	Scanning of the instrument over the entire selected mass range.	Sweeps per reading	100	1
Reading	Sum of the signals given by the sweeps.	Readings per replicate	1	100 It is the actual number of points of the transient signal
Replicate	Sum of the signals given by the readings. A standard deviation can be calculated on the various replicates.	Number of replicates	5 Number large enough to be statistically significant	1 The entire transient signal

3.2. Detection of trace elements in gels after gel electrophoresis

3.2.1. General

In the case of the detection of trace elements after gel electrophoresis, two strategies are possible:

- either the use of a method that is able to determine the distribution of the trace elements in an entire gel, in other words that is able to scan the gel;
- or the use of a method that requires that the gel be cut in subsamples, to give a bulk information about the piece of gel or the band analysed; the entire gel has to be reconstructed after all pieces of gel have been analysed.

The former is more convenient but the latter allows quantitative analysis. Thus, the choice between the two sets of methods is dictated by strategy and need. One has to know which question is to be addressed: where the trace element is or in which quantity it is present.

3.2.2. Detection of the trace elements in subsamples of the gel

Although this procedure is more time consuming, since an additional manipulation is required before detection, analysis of subsamples is more reliable for quantitative analysis and more sensitive. Up to now, no method is able to scan a sample and give reliable quantitative information about the distribution of an element at very low concentration. Furthermore, it suffices when only a rough image of the distribution of trace elements is necessary. From the analytical point of view, all methods used for the detection of trace elements can in fact be considered; however, for simplicity, only the methods for which applications have already been published are presented here.

3.2.2.1. Nuclear analytical chemistry: scintillation counting, neutron activation analysis (NAA)

Scintillation counting can be used with samples radioactively labelled. According to the nuclear characteristics of the radiolabel, the method of choice will be either liquid scintillation counting or well-type NaI(Tl) detection. The former requires that the subsample be brought into solution, whereas the latter is directly suitable for the solid piece of gel. Liquid scintillation has already been successfully applied to the detection of ^{75}Se after PAGE [Qu, 2000]. The advantage of the method is a higher sensitivity than the phosphor screen technology, but at the expense of a good resolution of the gel.

NAA has been one of the first detection methods used for trace elements in gels [Stone, 1987, 1990, 1991; Jayawickreme, 1988]. Even if this method implies a large investment and a heavy infrastructure, the method is interesting because it relies on a totally different principle from the spectrometric methods mentioned previously. After the gel is sliced, the elements it contains are activated in a nuclear reactor through neutron bombardment. After a cooling time, the elements are detected by recording the whole gamma spectrum with a Ge(Li) detector and by assigning the peaks specific to each element. Quantification is once again possible. Theoretically, this method can be applied to a whole gel and an autoradiogram recorded (see later). However, the high neutron fluxes produce a high temperature and a high radiation that damage the gel; furthermore, a high background is obtained since any matrix element impurity and traces of the reagents in the gel might also be activated.

3.2.2.2. Liquid introduction system: inductively coupled plasma – mass spectrometry (ICP-MS), atomic absorption spectrometry (AAS) and optical emission spectrometry (OES)

In order to get a first image of the distribution of trace elements in gels, ICP-MS with a liquid introduction system is a method of choice. The gel must be excised and the pieces mineralised by microwave-assisted digestion. Each fraction is then quantified by ICP-MS. This method is particularly tedious, but gives detection limits that are excellent for most elements. Any other method that relies on dissolved sub-samples is, however, also applicable, such as AAS, OES, voltammetry [Messerschmidt, 1995] or fluorescence [Vézina, 1990].

3.2.2.3. Solid sample analysis: electrothermal vaporisation – ICP-MS; graphite furnace – atomic absorption spectrometry (GF-AAS)

These methods can be seen as improvements of the introduction system of the sample. Indeed, as solid sampling analysis, they do not require the destruction of the pieces of gel and still offer excellent detection limits. The sample is brought in the oven, the organic material removed and the trace elements brought to the plasma in the case of ETV [Wróbel, 1995] or detected in the furnace for GF-AAS [Sidenius, 2000]. The disadvantages of the method are that a precise optimisation of the temperature programme and of the use of chemical modifiers is necessary and that the optimisation is valid for only one element. A further limitation is the lack of precision of these methods. To correct for it, the unique solution is to analyse a sample a number of times high enough, but in the case of a gel this is hardly possible. Once the band is analysed, nothing is left for further analysis, unless several lanes have been prepared.

Still, the sample preparation is simpler than with a liquid introduction system and can be quantitative if the system is adequately calibrated.

3.2.3. Detection of the trace elements in a whole gel

The methods presented here rely on concepts already seen in 3.2.2, with the exception of autoradiography, and are refinements of the sample introduction system.

3.2.3.1. Autoradiography

Autoradiography is the method of choice when the material submitted to electrophoresis is labelled with a radiotracer. The image of the distribution of radioactivity is obtained when the autoradiography screen is laid on the gel. More details are given in 3.1.1.3 on this method.

3.2.3.2. Laser ablation – ICP-MS (LA-ICP-MS)

This method is a further step toward a reliable direct introduction of the sample into the spectrometer. Other combinations are plausible, like LA-OES, but the most powerful is presented here. LA-ICP-MS is a much promising technique for the detection of metals in gels after separation.

The sample, the gel in other words, lies in an ablation cell; the laser ablates a spot and the fumes from the ablation are brought from the cell to the plasma in a tube by a continuous gas flow, argon or helium. ICP-MS then gives the elemental composition of the protein present at the ablation site.

The ablation crater ranges from the µm range up to 400 µm, and is thus ideal to achieve a high spatial resolution in the case of gel electrophoresis. Furthermore, the sample can be moved in two dimensions, as the latest equipment is computer controlled, allowing a precise screening of the gel. This last point is much advantageous for 2DE.

For a material like polyacrylamide and the minimum protein spot size expected (about 200 nm in diameter), a low wavelength and a very energetic beam are not necessary; fractionation, which means a different composition of the ablation composition from the original sample, is probably no problem when only qualitative information is necessary, allowing the use of widespread and affordable lasers, such as Nd:YAG lasers.

Up to now, this method has only been used for few elements in gels, (Co in serum [Neilsen, 1998], Pb in humic acids [Evans, 2000], Se in soft tissues [Fan, 2002] and yeast [Chéry, 2003]) but can be extended to any element detectable by ICP-MS [Chéry, 2003].

3.2.3.3. Particle induced X-ray emission (PIXE)

This technique relies on the excitation of the electron of the inner shell by a collimated beam of energetic charged particles, protons. After excitation, the atoms emit characteristic X-ray spectra that allow their detection, mostly by an energy dispersive analysis. However, this method relies on a heavy investment since, *e.g.*, a cyclotron is necessary to produce protons of a few MeV.

With an appropriate apparatus to translate the gel, the former can be scanned; the method has already been applied to one-dimensional gels, where a scanning in the direction of migration is the easiest [Szökefalvi-Nagy, 1990].

3.2.3.4. Mass spectrometry (MS)

The applicability of MS to gel electrophoresis is well known and widespread, especially in the field of proteomics with matrix-assisted laser desorption / ionisation (MALDI). The combination of the two for speciation purposes has not yet been

reported but no hindrance really exists, except the low specificity of the method for metals, in other words its low sensitivity. MS may be thought of as a detection method dedicated to organic molecules, in our case protein identification, but it has already been successfully applied to elemental speciation, for example to the speciation of arsenic or selenium [McSheehy, 2001]. The same can be done with other elements, provided they give a typical loss, for example as for Se, or a recognisable isotopic envelope. Two examples to explain respectively the notions of typical loss and isotopic envelope: the loss of m/z 386 can only be attributed to Glu-Cys-(^{80}Se)-Gly, and not to its sulphur analogue in the work of McSheehy [McSheehy, 2001]; if sub-peaks can be recognised in a major peak and if these peaks are separated by the same m/z as between isotopes of an element and with the same height ratio as in the natural abundance, the major peak can be attributed to a species containing this element.

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4. Optimisation of the Separation Technique, Gel Electrophoresis

As mentioned in Chapter 2, the protocols for gel electrophoresis, as fractionation technique for speciation purposes, can be divided in two classes, according to the type of trace element to be dealt with: metal-protein complexes or covalently bound trace elements in the protein. The type of sample actually determines the separation mechanism. The two approaches are exemplified in the following, respectively through the speciation of vanadium among serum proteins [Chéry, 2002] and through speciation of selenium among yeast proteins [Chéry, 2001].

4.1. Gel electrophoresis of metal-protein complexes

4.1.1. Introduction

Vanadium is exemplary of the case of a weak association between the trace element and the protein. To prove this assertion, the formation constants of its complexes with proteins can be compared with those of iron. One of its strongest complexes with proteins is formed with transferrin; the value of the formation constant is $10^{6.5}$ whereas the formation constant of the iron-transferrin complex is $10^{22.7}$. This implies that the equilibrium between free vanadium (or readily exchangeable vanadium) and the vanadium-protein complex is easily disturbed.

Hence a low recovery of the complexes, or the instability of the species during separation, is often reported [Sabbioni, 1978; Chasteen, 1986a], because of competitive binding of vanadium with the separation phase or with the chemicals used for the separation [Crans, 1994]. Experimental conditions must be extremely well defined, checked and kept between strict boundaries to prevent the denaturation of the species.

Up to now, few reliable analytical methods for the fractionation of vanadium species in biological samples have been reported. Size-exclusion chromatography and ion-exchange chromatography are mostly used [De Cremer, 2001]. Even with these protocols, the species are never totally stable, as demonstrated in [De Cremer, 2002a].

Further, classical native PAGE and IEF have already been applied to the speciation of vanadium in serum, but were proved to be inefficient [Lustig, 1999]. Indeed, the complex is not stable outside physiological pH, excluding the use of IEF; the chemicals used for native PAGE (*e.g.*, tricine, phosphate and Tris) destabilise the

vanadium-protein complex. It will, however, be shown that blue native electrophoresis is the native PAGE procedure that allows the analysis of the vanadium species. In order to develop a separation, it is mandatory to use chemicals, ionic strength, pH and organic solvents [De Cremer, 1999] that ensure the preservation of the species. Ideally, the effect of the various buffers and chemicals on the species should be tested by independent methods to prove that they do not affect them. This was done in this work by using ultrafiltration.

4.1.2. Experimental

4.1.2.1. Radiotracer

^{48}V carrier-free (main γ energies: 511, 983, 1312 keV) was produced as described in [De Cremer, 1999]. Vanadium was produced as vanadate (vanadium(V), $H_2VO_4^-$ at neutral pH), in water at pH 7.

4.1.2.2. Incubated transferrin and human serum

Freshly sampled human blood was separated into serum and blood cells as described elsewhere [Borguet, 1995]. The serum obtained and transferrin, purchased as a lyophilisate, were incubated with either ^{48}V or 'cold' vanadium for at last 16 h, at a constant temperature of 37°C.

For the 'cold' experiments, a 2 g L^{-1} transferrin solution was prepared, in 23.2 µM vanadate, 25 mM bicarbonate buffer, pH 7.5. A quantity low enough of vanadium (one vanadate ion for one transferrin molecule, although transferrin is able to complex two vanadate ions) was used to ensure that all vanadium was bound to either transferrin or serum proteins at this stage of the experiment. For the experiments with vanadium radiotracer, 12 kBq ^{48}V (or 41 fmol carrier-free isotope) were added to 1 mL of a 1 g L^{-1} transferrin and 16 kBq ^{48}V (or 54 fmol) to 1 mL serum.

4.1.2.3. Ultrafiltration experiments

To confirm the stability of the species in presence of the chemicals used for electrophoresis, an aliquot of the vanadium-transferrin solution, containing 0.5 mg L^{-1} Coomassie blue, was added to those chemicals, as summarised in Table 4.1. If

unbound vanadium was detected, *i.e.* if vanadium was measured in the filtrate, the chemical in question had to be rejected for further use with the species. These experiments were carried out in triplicate.

Table 4.1: Ultrafiltration experiment.
Composition of the solutions and result of the filtration.
CB: Coomassie blue
NEM: *N*-ethylmorpholine

Solution centrifuged: 210 µL transferrin solution plus 190 µL:			
Cathode buffer	Gel buffer	Anode buffer	Reference
2.5 g L^{-1} taurine	12.1 g L^{-1} NEM	2.3 g L^{-1} NEM	Water
20 mg L^{-1} CB	4.2 g L^{-1} acetic acid		
	0.2 g L^{-1} CB		
pH 9	pH 7.5	pH 7.7	pH 7.7
Free vanadium (%)			
5.4 ± 0.8	9.3 ± 1.8	8.6 ± 3.0	8.8 ± 0.8

Filters with a 5 kDa molecular mass cut-off were used throughout (Centrisart C4G Centrifugal Concentrators, Supelco). 400 µL solution were submitted to filtration for 2 h 20 min at 10,000 x *g*. 150 µL of the filtrate were diluted 200 times in 0.14 M HNO_3, with addition of cobalt (100 µg L^{-1}) as internal reference for the ICP-MS measurements. The solutions were measured by pneumatic nebulisation – inductively coupled plasma – mass spectrometry (PN-ICP-MS), with parameters summarised in Table 4.2.

The instrument was a PerkinElmer SCIEX Elan 5000 quadrupole-based ICP-MS. The amount of vanadium present was assessed by external calibration, using cobalt as internal reference. Interferences from $^{35}Cl^{16}O^{+}$ on $^{51}V^{+}$ were taken into account by measuring blank solutions, containing no V, and by subtracting the signal on m/z 51 given by the blank from the measurement. This approach was allowed since the amount of chlorine in these solutions was low. Otherwise, techniques such as the dynamic reaction cell technology would have had to be applied (see 5.2.1). In order to correct for vanadium adsorbed on the filter, the same experiment was carried out with a solution of pure vanadium. If in this case, the yield was less than 100 %, (which means adsorption on the filter), the percentage formerly obtained was corrected for this yield.

Table 4.2: Ultrafiltration experiment.
PN-ICP-MS settings for the measurement of vanadium.

ICP-MS instrument	*Elan 5000*
Sample introduction flow	1 mL min^{-1}
RF Power	1000 W
Ar gas flow rates	
Plasma	12 L min^{-1}
Auxiliary	1.2 L min^{-1}
Carrier	0.95 L min^{-1}
Data acquisition	
Scanning mode	Peak hop
Points per spectral peak	1
Dwell time per acquisition point	17 ms
Sweeps per reading	300
Readings per replicate	1
Replicates	3
Signals monitored	$^{51}V^{+}$, $^{59}Co^{+}$
Total measurement time	3 min

4.1.2.4. Electrophoresis material and apparatus

The electrophoresis separations were performed on a horizontal electrophoresis unit (Multiphor II electrophoresis system). The polyacrylamide gels were commercially available polyacrylamide gels, CleanGel 25S, purchased in a dry form, which means without buffers in the gel. This allowed the hydration of the gel with the native buffers developped in this work. The total acrylamide concentration, *T* (see 2.1.1), was chosen so that the gel would have its highest resolution in the 50-200 kDa range with a native separation (*T* = 10 % and *C* = 2 % in the separation zone). The anode and cathode buffers were brought in contact with the electrode with paper wicks. The staining unit was an automated Hoefer gel stainer. All these items were from Amersham Pharmacia Biotech.

The phosphor screen used for the detection of radioactivity in the gel (see 3.1.1.3) and the laser scanner used to scan the gel after the silver staining (Molecular Imager FX) were from Bio-Rad.

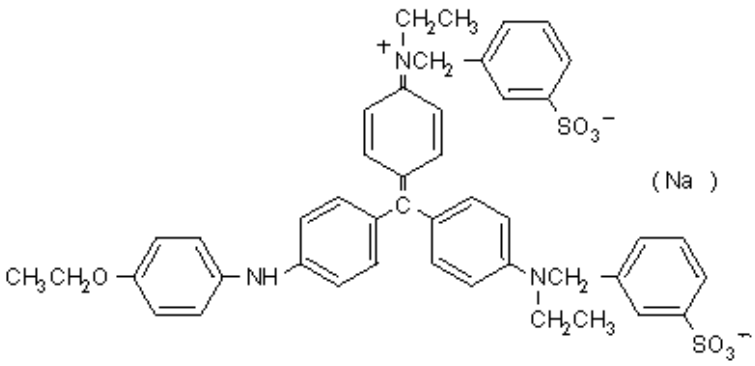
4.1.2.5. Blue native electrophoresis

In this experiment, a native electrophoresis was a prerequisite. Indeed, any alteration of the tertiary structure of the proteins could displace the protein ligands and thus destroy the species. Second, since it was proved that even classical native

electrophoresis gave no successful separation, either because of the electrical field itself or the ionic strength of the electrophoresis solution, a way to protect the complexes from the electrophoresis environment was sought after.

Because of its charge shift mechanism, it was thought that the blue native protocol [Schägger, 1991] would be the solution. This mechanism relies on an apparent charge given to the protein by Coomassie blue without disturbing the charge distribution in the protein itself. This chemical, given in Formule 4.1, is widely used in biochemistry, especially to stain proteins after gel electrophoresis. To better understand the staining mechanism, the interactions between Coomassie blue and the protein have been studied [Wei, 1997]. They are weak and are contributed to by nearly all known mechanisms (ionic, van der Waals, hydrogen bonding). This ensures that no type of interaction between Coomassie blue and the protein is dominant and strong enough to significantly disturb the metal-protein equilibrium.

Structure 4.1: Coomassie blue (also known as Acid Blue 83, Brilliant indocyanin 6B, Coomassie® Brilliant Blue R 250)
CAS Number: 6104-59-2.



The buffer systems were selected from the list compiled by Chrambach and Jovin [Chrambach, 1983] to get a moving boundary electrophoresis fulfilling the prerequisites of compatibility with the vanadium complexes and high-resolution separation. The composition of the buffer is given in Table 4.3.

Table 4.3: Composition of the electrophoresis buffers.
CB: Coomassie blue
NEM: *N*-ethylmorpholine

	Cathode buffer	Gel buffer	Anode buffer	Application buffer
	Taurine 2.50 g L^{-1}	NEM 12.1 g L^{-1} Acetic acid 4.2 g L^{-1}	NEM 2.30 g L^{-1}	Sucrose 250 g L^{-1} gel buffer
pH	to 9.0 with KOH	7.5	to 7.7 with HCl	
CB	20 mg L^{-1}	5 mg L^{-1}	0	100 mg L^{-1}

After incubation, ^{48}V-serum and ^{48}V-transferrin were diluted with the application buffer by a factor 100 and 10 respectively. 10 µL of the diluted solutions were applied onto the sample wells, in other words 1 µg transferrin and 0.1 µL serum. Each gel was run in duplicate.

Running buffers were applied by means of paper wicks that were replaced every 30 min. Temperature was kept constant at 20°C. Electrophoresis was started at 100 V (20 mA maximum) for 30 min, a voltage low enough to ensure that the proteins would enter the stacking gel and that no shear force on the proteins would denature the species. The voltage was subsequently raised to 600 V (50 mA maximum) until the end of the separation, *i.e.*, when the front was *ca.* 1 cm from the anode buffer. This 1 cm margin was necessary to keep any free vanadium from migrating into the buffer wicks; thus, free vanadium could be detected in the gel. A separation typically took 1500 V h or 2.5 h.

4.1.2.6. Autoradiography: detection of ^{48}V

Immediately after separation, the gel was wrapped in a mylar foil and deposited on the phosphor screen. The screen was exposed at –20°C for at least one week. The gel had to be kept frozen to prevent diffusion of the proteins. Any other fixation method, such as acid fixation, was prohibited since the species would not have outlasted such a treatment.

4.1.3. Results

4.1.3.1. Ultrafiltration

None of the three solutions, representative for the buffers used for electrophoresis, gave significant loss of vanadium in comparison with the transferrin-vanadium complex in water, taken as a reference medium (t-test at 95 % confidence level between water and each other series of results). Hence, it was decided to use those chemicals for BN.

4.1.3.2. Resolution of the separation

In human serum, two vanadium oxidation states have been detected: vanadyl (IV) and vanadate (V). The transport proteins for vanadyl are both albumin and

transferrin and for vanadate only transferrin [Chasteen, 1986b]. Hence, the gels were chosen to get a maximum resolution in the mass range of those proteins. With T = 10 %, the resolution was optimum for masses between *ca.* 40 and 600 kDa, as shown by the molecular mass markers in Figure 4.1. Transferrin and albumin were especially well resolved from each other.

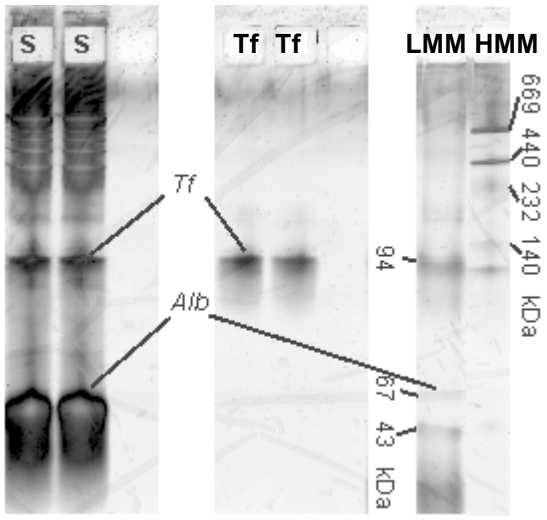


Figure 4.1: Silver staining after BN-PAGE of serum (lanes S), transferrin (Tf), low- (LMM) and high-molecular-mass (HMM) markers.
Tf, transferrin band; Alb, albumin band.

4.1.3.3. Stability of the complexes

The selective detection by autoradiography of ^{48}V, present in the same gel as depicted in Figure 4.1, is given in Figure 4.2. Hardly any ^{48}V is detected at the front of the ^{48}V-transferrin lane. Should a major amount of free vanadium be released from the transferrin, it would be present as vanadate, an anion ($H_2VO_4^-$), migrating at the front toward the anode. Hence, no free vanadium leached from the complex during electrophoresis, showing that the species are stable during separation. Further, no radioactivity is detected in the sample wells, which proves that the species are kept in solution and do enter the gel during electrophoresis.

The importance of Coomassie blue cannot be stressed enough. If the former separation is repeated, but this time without CB in the gel buffer, the equilibrium between complex and free vanadium is nearly entirely shifted toward the production of free vanadium. This is illustrated by the separation given in Figure 4.3. Since the gel buffer is replaced by the cathode buffer in a matter of minutes, the complex must

have been destabilised in this first stage. Coomassie blue is therefore mandatory for the stability of the complex during electrophoresis.

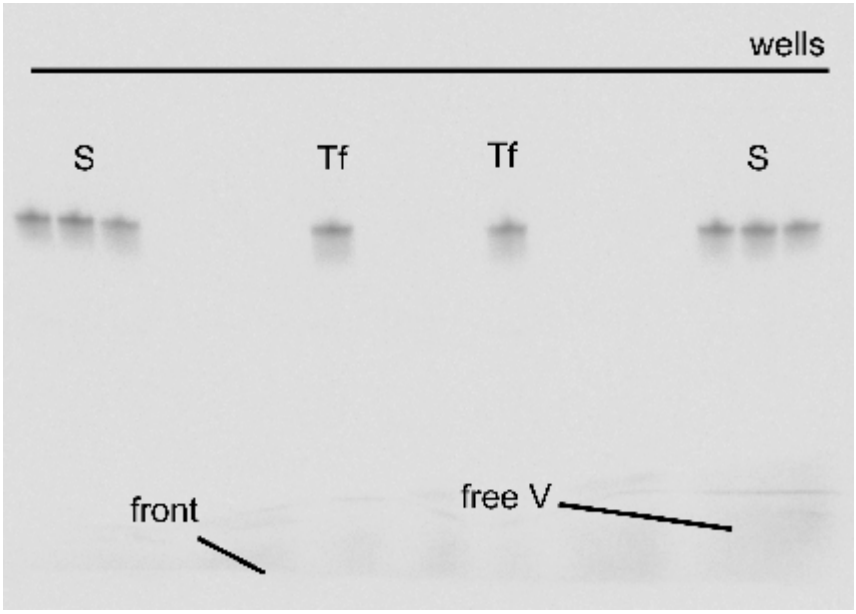


Figure 4.2: Autoradiogram of the gel depicted in Figure 4.1. Detection of ^{48}V.
The position of the sample wells is shown with a bold line.
The gel was not yet stained as in Figure 4.1 but used directly for autoradiography.
Lanes S, electrophoresis of serum; lanes Tf, electrophoresis of transferrin.

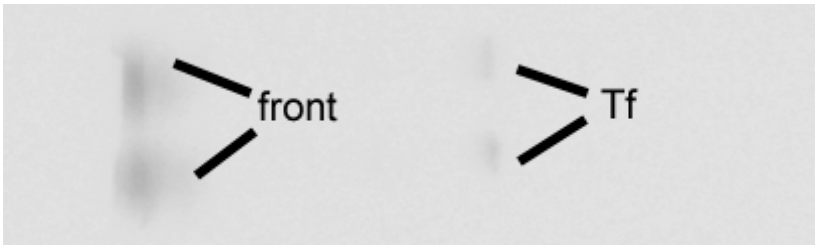


Figure 4.3: Autoradiogram of a gel containing no Coomassie Blue in the gel buffer.
All other parameters are as previously described.

4.1.4. Conclusions

By comparing the migration distance of transferrin with the ^{48}V-protein complex, one can conclude that this complex is the ^{48}V-transferrin complex. Further, since no other radioactivity is detected in a lane, no other complex is thought to be present. More particularly, albumin does not transport any vanadium in our experiment.

These results are consistent with the literature [Chasteen, 1986b]. This result is valid only under the experimental set described here, *i.e.* serum incubated with

vanadate. If vanadium is added under another form, *e.g.*, vanadyl, to serum or if administered in vivo, binding to albumin is possible, as described elsewhere [Takino, 2001; Yasui, 2000; Kiss, 2000].

The method described here can be further applied to the speciation of vanadium in tissues. Less is known about the identity of the vanadium compounds present there, apart from the fact that transferrin and ferritin complexes are present in liver, spleen and kidney [Sabbioni, 1981]. Other compounds are possibly present, for example in urine [De Cremer, 2002b]. For these samples, a simple comparison of the electrophoresis migration distances, as described here for transferrin, will be insufficient to identify the complexes and tandem mass spectrometry will have to be applied.

4.2. Gel electrophoresis of selenium-containing proteins

4.2.1. Introduction

Selenium is in this chapter studied as selenium covalently bound in proteins, in the form of selenomethionine or selenocysteine. A denaturing, high-resolution fractionation can thus be applied, as 2DE is.

Selenium is known to be essential to higher organisms. Some selenium species, although not yet identified, have been proved beneficial as a cancer chemopreventive agent and since then numerous selenium based food-supplements have been available, mostly in the form of yeast enriched with selenium. In order to better understand the action of the selenium species, much has been done to speciate selenium in yeast, in which it is present in low molecular mass compounds (*e.g.*, selenate, selenite, selenomethionine) as well as in macromolecules (proteins) [Korhola, 1986]. This work is an attempt to understand better how selenium is distributed among high-molecular-mass compounds, especially proteins.

Further, as already aluded to in 1.4.2.3, in yeast, selenium is not incorporated into proteins at specific sites, because no biochemical path leads to the formation of true selenoprotein in this eukariotic cell; selenium is incorporated into the proteins but instead of sulphur, to which it is chemically similar. In that way, a large amount of selenium-containing proteins can be produced. This property is routinely used for the production of the dietary supplement selenised yeast.

The strategy of this work was, after production of the 75-selenised yeast, to determine the mass distribution of selenium in proteins by means of size-exclusion chromatography and then to compare it with the distribution of selenium on a 2DE gel. Matching of results would pave the way to their validation.

4.2.2. Experimental

4.2.2.1. Sample preparation

4.2.2.1.1. Production of 75-selenium tracer

^{75}Se (half-life: 120.4 days; main gamma energies: 121, 136, 265 and 280 keV) was prepared by the nuclear reaction ^{74}Se (n,γ) ^{75}Se by irradiation of ultra-pure selenium (99.999 %) at the BR2 nuclear reactor of SCK.CEN (Mol, Belgium), for one week at 10^{14} neutron cm^{-2} s^{-1}. The specific activity at the time of the experiment was 8.44 MBq mg^{-1}. Selenium was dissolved in 14 M HNO_3, at a concentration of 5 mg mL^{-1}, and the pH adjusted to 7 with sodium hydrogen carbonate. Under these conditions, selenium should be oxidised to selenite, Se (IV); much stronger oxidation is needed to obtain selenate, Se(VI).

4.2.2.1.2. Selenium yeast growth

Saccharomyces cerevisiae Hansen (IHEM 6036) was obtained from the IHEM culture collection (Brussels, Belgium) on Sabouraud agar. It was transferred into a Sabouraud growth medium [Spencer, 1996] (3.3 g glucose and 0.5 g mycological peptone in 50 mL water), in a 250-mL Erlenmeyer flask, with constant agitation for a good aeration of the solution, at 25°C. All solutions and utensils were previously sterilised to prevent the growth of other microorganisms, which could disturb the growth of yeast or give artifacts on the 2DE gel. This experiment was run in three different modes:

i. 7.5 mg Se was added to the growth medium, *i.e.* 63.3 MBq ^{75}Se;
ii. same selenium concentration, but cold selenium;
iii. without selenium.

Such an amount of selenium may seem high in comparison with other procedures [Suhajda, 2000] but was necessary because of the relatively low specific

activity of the 75-selenium sample. At the end of the exponential growth, the cells were harvested by centrifugation and washed three times with water. The pellet thus obtained was lyophilised and stored.

4.2.2.1.3. Yeast lysis

Yeast cell lysis was performed in accordance with the method presented in [Harder, 1999]. In brief, the cells were disrupted by addition of hot SDS and sonication. Sonication gives enough vibration energy to disrup the cell membrane and SDS, a detergent, promotes the solubilisation of the hydrophobic compounds present in the cell (see 2.1.5.2.2 for the structure). The proteins were then brought into solution by addition of detergent (CHAPS), chaotropic agent (thiourea and urea) and a reducing agent (dithiothreitol) (see 2.1.5.2.1). The lysate was centrifuged (in this experiment: 10 000 x *g*, 10 min) and the supernatant frozen.

It should be mentioned that the lysate finally contains all chemicals needed for 2DE, even if further dilution is required to lower the proteins concentration to a concentration compatible with the capacity of 2DE. The chemical composition of the lysate is therefore representative of both lysis and 2DE solutions. Stability tests were performed by submitting this solution to ultrafiltration.

4.2.2.1.4. Derivatisation of selenocysteine

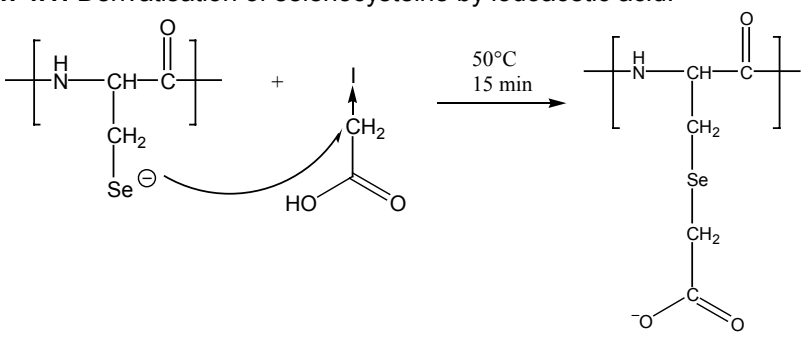
In order to protect selenocysteine from oxidation, a derivatisation with iodoacetic acid has been proposed [Takatera, 1994; Stadtman, 1984]. The reaction is represented formally in Reaction 4.1.

- A 1 M iodoacetic acid solution was prepared and its pH adjusted to 7.
- 70 µL were added to 500 µL yeast lysate and incubated at 50°C for 15 min.
- The derivatised lysate was filtered by means of ultrafiltration, using Centrisart C4G Centrifugal Concentrators from Sartorius with a 5 kDa cut-off. This was done until the volume decreased to 50 µL, to free the lysate from most of the reagents. This step is critical, because an ionic strength which is too high (due to iodine) can seriously disturb the first dimension of 2DE.

- Immediatly after filtration, the lysate was reconstituted to 875 µL with the buffer. This solution is subsequently referred to as the rehydration solution of the IPG strip.

The same derivatisation was done with iodoacetamide instead of iodoacetic acid.

Reaction 4.1: Derivatisation of selenocysteine by iodoacetic acid.



4.2.2.2. Ultrafiltration

In order to establish whether selenium was incorporated in yeast in high-molecular-mass compounds and to check the stability of the 75-selenium compounds during electrophoresis, the lysis product was submitted to ultrafiltration. It is worth noticing that this test was performed to assess the impact of the buffers but gives no information on the influence of the pH gradient of the first dimension – should the pH play a key role in the oxidation, its effect would be in this first dimension.

A variety of solvents or other chemicals were added to the lysis sample, which thus contained also the chemicals required for lysis / electrophoresis but without derivatisation. The sample was centrifuged for *ca.* 50 min at 2900 x *g* on 1 kDa cut-off filter (Microsep 1K Omega, Pall Gelman). The radioactivity in the filter (M_r > 1 kDa) and filtrate (M_r < 1 kDa) were measured with a NaI(Tl), 3'' x 3'' well type, scintillation detector coupled to a single-channel analyser (γ-spectrum range from 50 to 250 keV, measured for 10 min). To avoid differences in counting efficiency of ^{75}Se, because of varying geometries, 10 µL of the filtrate were transferred into a PET vial.

Because selenite is the main oxidation product expected from yeast lysate, the affinity of the filter material for selenite was estimated from the radioactivity lost on the filter when a pure solution of selenite in water was filtered. Since 27 % adhered to the filter, this factor was taken into account by multiplying the radioactivities of the filtrate by 1.37 = 1 / (1 – 0.27). This factor represents the average loss of selenite on

the filter and might be an overestimate in case of other solvents, *e.g.*, the detergents used here. The corrected value will give the maximum amount of selenite possibly present in the yeast lysate but also an average for other small (< 1 kDa) selenium compounds.

The volumes after centrifugation on the filter and in the filtrate were not necessarily equal among a series of replicates, because of different filtration efficiencies. For these reasons these discrepancies were taken into account by weighing all solutions. The percentage of selenium bound to compounds was calculated, 100 % being the radioactivity of a non-filtered solution of the same weight.

4.2.2.3. Size exclusion gel chromatography (SEC)

Analysis by SEC enabled establishment of a qualitative image of the mass distribution of selenium in the lysate.

These experiments were performed on an Åkta Purifier 10 system (Amersham Pharmacia Biotech) equipped with a UV detector and a sample collector. The buffers and the volume injected were filtered through 0.22-µm filters to prevent any clogging of the column with insoluble particles. The buffers were filtered on a surfactant-free cellulose acetate filter (Nalgene); the samples on Millex GV_{13} (Millipore). A crosslinked agarose column Superose 12 HR 10/30 (Amersham Pharmacia Biotech) was used, mainly because of its separation range (M_r between 1 kDa and 300 kDa), which corresponds to the separation range of 2DE in our experiments. Its total volume is V_t = 20 mL and exclusion volume V_0 = 7 mL. A buffer consisting of 20 mM Tris HCl, 100 mM NaCl, pH 6.86, was used; 100 µL lysate plus 100 µL buffer were injected. The column was calibrated by injection of the same low-molecular-mass markers as for electrophoresis. Fractions of 1 mL were collected and were measured off-line for ^{75}Se activity with the NaI(Tl) detector already mentioned.

4.2.2.4. Gel electrophoresis

All products and material were from Amersham Pharmacia Biotech. The first dimension, iso-electric focusing (IEF), was done on an IPGphor with immobilised pH gradient strips (IPG) (Immobiline Dry Strips). The pH gradient was a linear function of the distance, from pH 3 to pH 10. The IPG was rehydrated with 350 µL of the rehydration solution. The second dimension was performed by means of flatbed electrophoresis on a Multiphor II with SDS polyacrylamide gels (SDS-PAGE)

(Homogeneous 12.5). In the separating gel, these gels had as characteristics T = 12.5 %, C = 2 %, which gave a separation range of 20 – 300 kDa. Separation was achieved in accordance with the method of Görg *et al.* [Görg, 2000].

Masses were estimated by spotting low- and high-molecular-mass markers at the edges of the SDS-PAGE gel. The proteins in a gel were fixed by acid precipitation. The gel was dried and then exposed to a phosphor screen. After exposure, the gels were silver stained, in accordance with the method of Heukeshofen and Dernick [Heukeshoven, 1985], using a Hoefer Autostainer.

4.2.2.5. Autoradiography and image treatment

For the detection of ^{75}Se in proteins after 2DE separation, the gels were exposed to a phosphor screen (Bio-Rad) for one week at ambient temperature. The radiogram was obtained by laser scanning (Molecular Imager FX, Bio-Rad) of the phosphor screen.

The radiogram was further filtered by the software Adobe Photoshop (Adobe Systems) and analysed with the software Melanie II (Bio-Rad). Filtering was necessary because the images obtained had a very high background, probably because of the long exposure time. Basically, pixels representing low optical density were discarded. Detection by Melanie II was performed on this gel and a 'Gaussian gel' was created from those data. The Gaussian gel is an artificial picture of the original gel in which the spots have the same area and mean optical density as in the actual gel but where a spot is idealised as having an elliptical form with a Gaussian distribution of the optical density within the spot.

4.2.3. Results

4.2.3.1. Yeast growth

In the two growth media containing selenium, only a very small amount of yeast was harvested, 5 mg dry weight, as a consequence of the toxicity of selenium at such a high concentration. Despite this, a sample containing a relatively large amount of selenium was produced, with an activity of *ca.* 100 kBq ^{75}Se mg^{-1} dry weight of yeast. In comparison, production of selenium yeast under the same conditions, but with a selenium concentration 15 times lower, yielded 164 mg dry weight of yeast, but only with 3.8 kBq ^{75}Se mg^{-1} dry weight of yeast. The yield in

terms of the production of yeast may be higher, but not necessarily in terms of the production of 75-selenium yeast. Elemental selenium, apart from the proteins, was also formed; this was apparent from the reddish colour of the yeast cells.

4.2.3.2. SEC

SEC gives the general pattern of how selenium is distributed among proteins (Figure 4.4). The fractions between the exclusion and total volume of the column ($7 < V < 20$ mL) are most probably proteins. The peaks eluting after the total volume of the column ($V > 20$ mL) are chemicals from the lysis buffer; this was shown by injection of pure lysis buffer. Selenium-containing compounds eluting after V_t, showing therefore a non-ideal behaviour, were not identified.

Although the separation method was not optimised, as can be deduced from the poor recovery of the radioactivity (typically *ca.* 50 %, the rest being adsorbed on the packing material), the results show that selenium is present in every fraction, from masses higher than 97 kDa down to 5 kDa. All fractions contain roughly comparable ^{75}Se radioactivity. All molecular masses above 5 kDa are most probably proteins and the whole radioactivity after chromatography can be found back at masses above 5 kDa; one can therefore conclude that at least 50 % of the selenium compounds produced here are proteins.

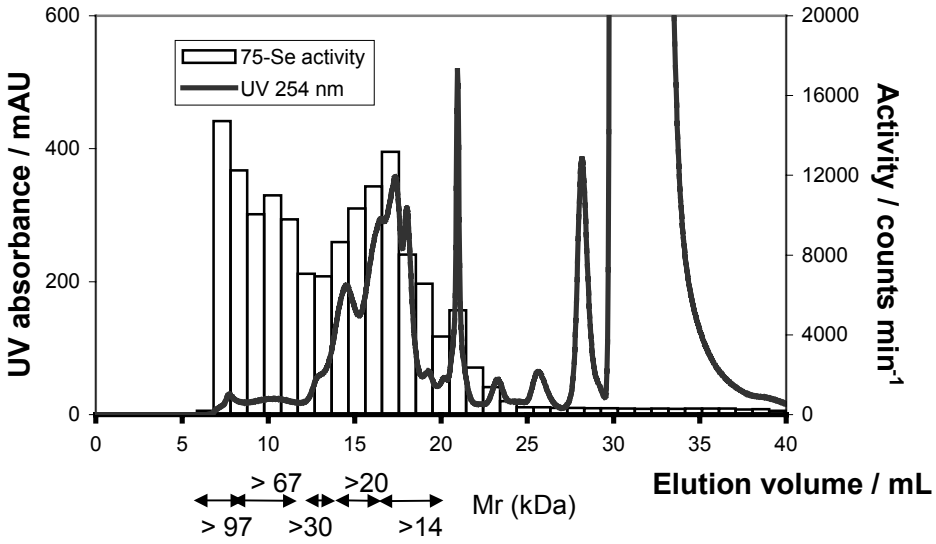


Figure 4.4: Size exclusion chromatogram of yeast lysate.

4.2.3.3. Ultrafiltration

The ultrafiltration experiments presented in Table 4.4 show, first, that no major quantities of water-soluble selenium compounds with masses below 1 kDa were produced during the fermentation process (lysate directly measured, 5 % of the radioactivity lower than 1 kDa). The most obvious compound in this category is inorganic selenium.

Table 4.4: Ultrafiltration experiments.
Percentage of 75-selenium detected in the filtrate.
Cut-off: 1 kDa. DTT: dithiothreitol.

Sample composition	Incubation time	Number of filtrations	% ^{75}Se < 1 kDa ± SD
Water (90 µL) + sample (10 µL)	0	5	5 ± 1
	23 h	5	7 ± 2
	46 h	5	6 ± 2
Water (50 µL) + sample (50 µL) 0.7 g L^{-1} DTT	-	2	9
Water (50 µL) + sample (50 µL) 24.7 g L^{-1} DTT	-	2	11
Ethanol (30 µL) + water (20 µL) + sample (50 µL)	-	2	28

Yet, it would be premature to conclude that proteins or high-molecular-mass compounds amount to 95 % in the lysate. When solvents with higher solvatation power for hydrophobic compounds are used for ultrafiltration, more selenium is detected in the filtrate (Table 4.4). This selenium is probably incorporated in hydrophobic compounds that are adsorbed on the filter membrane when dissolved in water but can pass the membrane in a better solvent. These hydrophobic compounds are not proteins, because they are too small, but might be selenium-containing molecules previously bound to proteins or simply free low molecular mass selenium-containing molecules. The low recovery observed for SEC may be explained by adsorbtion of these selenium species on the packing material. Indeed, production of hydrophobic degradation products has been reported [McSheehy, 2001] when DTT was used as reducing agent. DTT releases glutathione (γ-Glu-Cys-Gly, 307.3 g mol^{-1}), to which selenium can be bound on cysteine (γ-Glu-(Cys-Se)-Gly, 386 g mol^{-1}), from proteins. Glutathione is bound to proteins by a disulphide bridge with its cysteine and released when the disulphide bridge is reduced by DTT. Even if the concentration of DTT is drastically increased to 24.7 g L^{-1} before filtration (Table 4.4), the activity remains the same in the filtrate – either the reduction by DTT is not noticeable in our experiment, or the original DTT concentration was already above the critical concentration and increasing it has no effect any more.

The combined results from the two sets of experiments, ultrafiltration and SEC, imply that selenium-containing compounds with higher molecular masses have been produced – *ca.* 50 % of the selenium compounds present in the lysis solution.

4.2.3.4. 2DE

4.2.3.4.1. Oxidation of the selenium-containing proteins

A major problem observed during the first 2D electrophoretic separations without derivatisation was the lability of the selenium compounds – all the 75-selenium was detected at (+,+) (*i.e.* at the anode of IPG and the anode of SDS-PAGE), which shows that selenium was totally oxidised to a free anionic form (selenate, selenite). Oxidation of selenocysteine has already been reported, but mostly during acid hydrolysis [Stadtman, 1989; Whanger, 1994; Edmonds, 2000]. 2DE is quite a long separation procedure (*ca.* 20 h) during which selenium compounds are exposed to oxygen; this is why further ultrafiltration experiments were performed, with solutions exposed to air for 23 h and 46 h (Table 4.4). They show that no further oxidation occurs, because the percentage of free selenium does not increase significantly. Oxidation must, therefore, be attributed to the procedure itself; this occurs especially in the rehydratation and separation steps of the first dimension, since oxygen is trapped during the production of IPG strips, in a medium in which the pH varies from 3 to 11.

Methods have been proposed to prevent this effect, *e.g.* addition of an antioxidant, thioglycolate, in the electrophoresis buffer [Bansal, 1991]. In this work, derivatisation was applied.

Two derivatisation agents were used: iodoacetamide and iodoacetic acid. Iodoacetamide proved inadequate, because all the radioactivity was detected at (+,+), similar to what happened when no derivatisation was applied; iodoacetamide might not be strong enough to alkylate the selenium moiety [Ganther, 1979]. When iodoacetic acid was used, radioactivity spots were detected, spread over the whole gel both according to mass and pI. This map is further referred to as selenium map.

Doubts could be expressed about the oxidation of seleno-amino acids during 2DE when results of other groups are considered. Indeed, Behne's [Behne, 1996] and Bansal's [Jamba, 1996] groups, among others [Lecocq, 1982], have worked with 2DE of selenium compounds in animal tissues or cells, without apparent loss of selenium. The material they studied is different from yeast but the problem of oxidation of selenium from proteins is the same. The key point is that their starting

selenium samples had a specific activity approximately 2000 times higher than in this experiment. They obtained such a high specific acitivity for ^{75}Se by neutron irradiation of selenium enriched in ^{74}Se. Bansal et al. protected proteins from oxidation with thioglycolate, as already mentioned [Jamba, 1996]. In the other works, even if oxidation occurred, *e.g*. up to 90 %, there was still enough radioactivity to be detected on the gel, the remaining 10 % being *ca*. 20 times as active as our sample; the oxidation products (90 % in our example) may be on the front (+,+), but the radioactivity of the unoxidised proteins (10 %) is enough for detection.

One could, furthermore, argue that this derivatisation step does not in fact protect the selenium moiety from oxidation but rather inactivates the various enzymes present in the lysate, protease among others, by alkylating their active site. There are reasons why this might not be true. First, the separation occurs in a highly denaturing environment where the secondary structure of the proteins is lost and hence, most probably, also their enzymatic activity. Secondly, because a lysate left to room temperature for 23 h or even 46 h shows no evidence of oxidation, no protease activity can be linked to the oxidation of selenium-containing amino-acids; the selenium compounds must have been oxidised during the analytical process itself, 2DE.

One possible problem with alkylation of the selenium moiety is the shift it could induce in the characteristics of the protein (pI and M_r) and therefore the false protein map it could give; for example, an increase in M_r for proteins alkylated with iodoacetamide is known to occur when this derivatisation method is used between the first and second dimensions to prevent oxidation of the thiol groups. Nonetheless, no major difference was seen between the protein maps of derivatised and underivatised samples. Furthermore, no difference was seen between yeast cultivated with radiotracer or without, indicating that radioactivity is not a critical factor either.

4.2.3.4.2. Selenium map

The selenium map is shown in Figure 4.5. Two spots, a and b, were marked on the three maps to facilitate comparison.

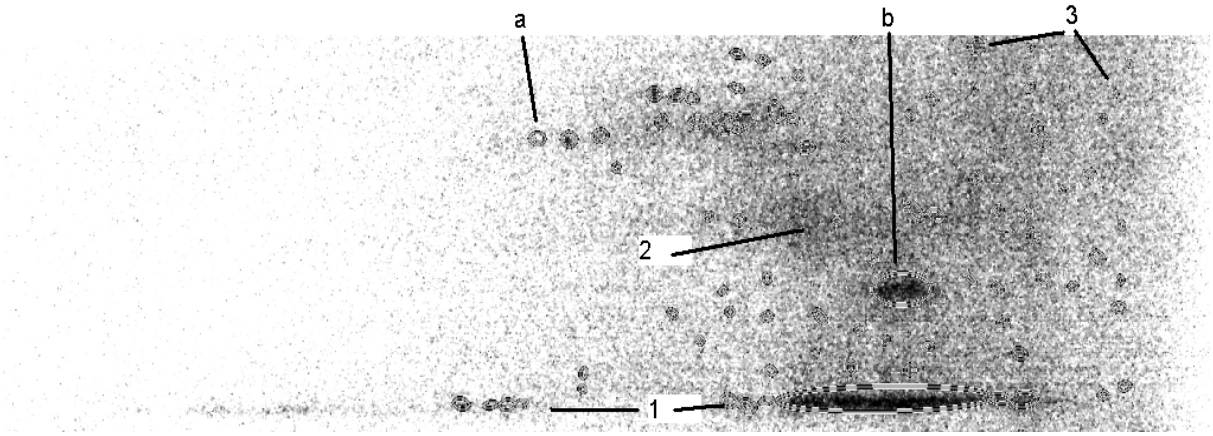


Figure 4.5: Autoradiogram of ^{75}Se-containing proteins in yeast: selenium map.
The main radioactive spots are circled by ellipses which are derived from the results shown in Figure 4.7 and added to the original picture.

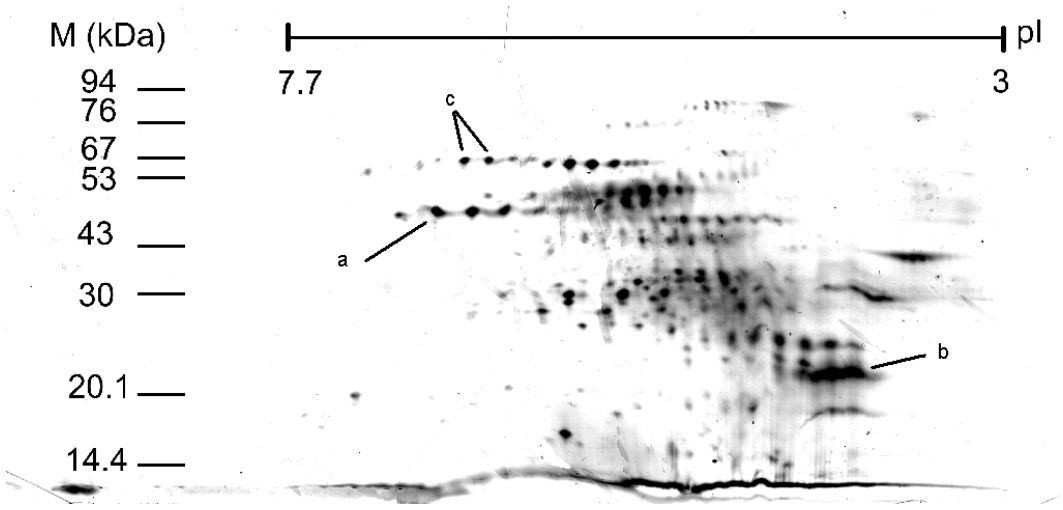


Figure 4.6: 2DE of yeast, protein map.
The most abundant proteins were detected by silver staining.
Only the region of pH 3- 7.7 is shown.

Two main facts are worth noticing about this separation.

First, the selenium map (Figure 4.5) does not totally match with the whole protein map given by silver staining (Figure 4.6). Some proteins are missing in the selenium map, such as proteins c. That probably means either that only the most abundant proteins are detected by autoradiography or that the selenium map is in fact a picture of the cysteine-rich proteins, which are known to occur in soybean for example [Sathe, 1992]. A further step should be the identification of the selenium-containing proteins, *e.g*. by means of trypsin digestion followed by TOF-MS (time of flight – mass spectrometry) of the fragments.

Second, as oxidation is critical with selenocysteine but not with selenomethionine, the two forms of selenium expected in proteins, this experiment tends to prove that the most abundant species of selenium in proteins of yeast is selenocysteine. This may be because of the growth process of yeast used here, since the presence of both selenocysteine and selenomethionine in yeast has been reported [Blau, 1961; Haas, 1992; Bird, 1997]. Further, not all proteins or macromolecules are present on the 2DE gel and autoradiogram. This is proved by the autoradiograms of the IPGs after separation: noticeable radioactivity is trapped in the IPG, radioactivity arising from molecules which do not leave the first dimension for the second. This implies that selenium-containing proteins are little soluble or that they are too big to enter the stacking gel, both of which features are typical of membrane proteins; membranes do, indeed, contain a noticeable quantity of selenium in selenised yeast, as shown by Zhil'tsova et al. [Zhil'tsova, 1998]. Another possibility is that other macromolecules, *e.g.* RNA or DNA, also contain selenium.

As already mentioned, the quality of the autoradiogram (Figure 4.5) is poor because of the high background. The image can be improved by treatment of the picture with standard software (Figure 4.7); the result is the so-called "Gaussian gel". Although the selenium map is still far from perfect, it shows the most intense spots. Some very diffuse spots are not detected, *e.g.* the numerous proteins denoted '2' on Figure 4.5. Others spots, *e.g.* spots '3', are artifacts from the background. Although it is not a spot as such, the frontline of the second dimension, noted '1', has also been shown; these are molecules of approximately 5 kDa.

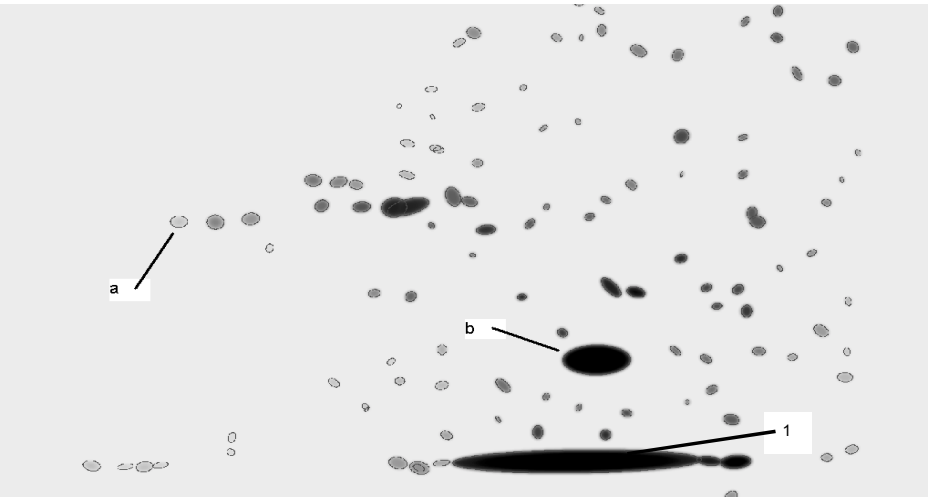


Figure 4.7: "Gaussian" autoradiogram of ^{75}Se-containing proteins in yeast.
The gel is physically the same as in Figure 4.5. This autoradiogram was obtained after treatment of the original data and a "Gaussian gel" was created.

Since the 2DE protein-mapping presented here (Figure 4.6) depicts only the most abundant proteins in yeast, the quality of the gel of the total protein cannot match with that of gels obtained in order to establish the proteome of yeast [Expasy, 2003; IBGC, 2003; GMM, 2003]. The method is, nevertheless, sufficiently reliable to furnish a map of selenium-containing proteins in selenised yeasts.

4.2.4. Conclusions

Gel electrophoresis, and especially two-dimensional gel electrophoresis, enabled the speciation of selenium among proteins. The main hardship encountered during these experiments is the oxidation of the selenium compounds, prevented by derivatisation of the selenium moiety by iodoacetic acid. The lability of the selenium compounds is exemplary of the precautions that have to be taken to prevent species conversion during electrophoresis: this point has to be checked by independent methods, *e.g.* chromatography or ultrafiltration, to be sure that no artifact is generated.

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5. Optimisation of the Detection Techniques and Applications

As already aluded to, one of the preferred detection methods in combination with gel electrophoresis is autoradiography in combination with radiotracers. Yet, in order to broaden the use of gel electrophoresis, detection methods that do not rely on radioactivity are necessary, especially for samples of human origin where the use of radiotracers is limited. Further, not all elements offer a radioisotope with a long enough half-life. The logical solution is to explore and apply the various methods for solid sampling analysis that are available and applicable to a sample such as a gel, namely electrothermal vaporisation – ICP-MS [Chéry, 2002] and laser ablation – ICP-MS [Chéry, 2003a].

5.1. Electrothermal vaporisation – ICP-MS

5.1.1. Introduction

In this chapter, the combination of SDS-PAGE and ETV-ICP-MS is studied. Advantages of both techniques are combined: fractionation with high resolution of the proteins by the former, overcoming the shortcomings of size-exclusion HPLC, and selective and sensitive detection of the selenium-containing proteins (possibly selenoproteins) by the latter, thus without the use of a radio-marker. Further, solid-sampling techniques minimize risks of contamination as well as analyte losses, as already mentioned for the determination of Se in biological samples [Pozebon, 1998]; it also prevents a time-consuming digestion of each band, as described by Chen *et al.* [Chen, 2002] for the analysis of electrophoresis gel by hydride generation-atomic fluorescence spectrometry.

5.1.2. Experimental

5.1.2.1. Sample preparation

The yeast protein fractions were obtained from a selenium-enriched yeast material (Pharma Nord), as described elsewhere [Chassaigne, 2002]. In brief, after extraction with an aqueous buffer (water-soluble proteins), the supernatant was injected onto a Superdex 75 column. The eluate corresponding to masses higher than 5 kDa (most probably proteins) was lyophilised.

Glutathione peroxidase (GSH-Px) was also obtained in lyophilised form. The lyophilisate contained 20 % GSH-Px, some protein impurities (14.5 and 18.7 kDa, communicated by the producer, Sigma-Aldrich), the rest of the lyophilisate being dithiothreitol, sucrose and sodium phosphate salts.

GSH-Px, as well as the yeast proteins, were first denatured with a non-reducing SDS-treatment (the proteins were dissolved in a 100 g L^{-1} SDS-solution, the sample was then left at 95°C for 5 min). The proteins were subsequently derivatised by treatment with iodoacetic acid as previously described in 4.2.2.1.4 [Chéry, 2001] in order to protect the side-chains containing selenium from oxidation. The latter solution, subjected to electrophoresis, contained 0.42 µg GSH-Px μL^{-1} or 1.54 ng Se μL^{-1}, since GSH-Px consists of four 22,612 Da monomers [Expasy, 2003] which each contain one selenocysteine amino-acid. The yeast proteins solution contained 4.4 ng Se μL^{-1}.

In order to repeat the study about the influence of the derivatisation step, a second GSH-Px solution was prepared following the same protocol, but omitting the derivatisation (see 4.2.2.1.4).

5.1.2.2. Instrumentation

5.1.2.2.1. Gel electrophoresis

SDS-PAGE was carried out with a Multiphor II electrophoresis unit (power unit: EPS 3500 XL; flatbed electrophoresis with water cooling) with precast gels (Homogeneous 15, *i.e.* in the separating gel a total acrylamide concentration equal to 15 %; a degree of crosslinking of 2 %; separation range 10-100 kDa) and SDS-buffers. Masses were estimated using a set of molecular-mass markers. The gels were silver stained (Silver Staining Kit, Protein), using a Hoefer autostainer, with the method of Heukeshoven and Dernick [Heukeshoven, 1985]. All products were from Amersham Pharmacia Biotech.

After staining, the gel was scanned using a densitometer (GS-690, Bio-Rad, Hercules), left to dry for a night and packed in a Mylar film to protect it from contamination until analysis. Each lane to be analysed was precisely cut in two in the direction of migration (see Figure 5.1). In this way, two strips of gel, identical the one to the other, were obtained from one lane, so that material was available for further experiments.

A lane is the length of the gel where the proteins have been separated and fixed after staining (the actual electropherogram). A band is the spot given by a single protein and is *ca.* 5 mm wide (see Figure 5.1).

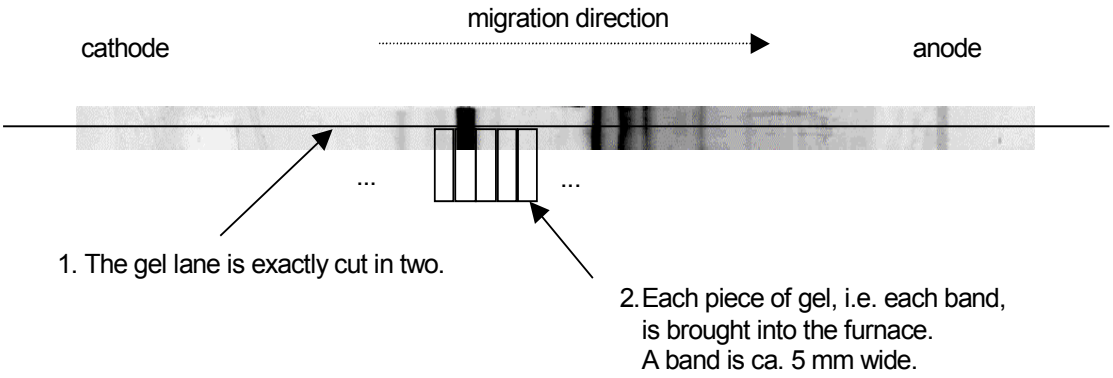


Figure 5.1: Preparation of a gel after SDS-PAGE for solid sampling ETV-ICP-MS.

5.1.2.2.2. ETV-ICP-MS

A PerkinElmer HGA-600MS electrothermal vaporiser (cup-in-tube technique for solid sampling), [Völlkopf, 1985] with modifications described in detail elsewhere [Resano, 2000], was coupled to a PerkinElmer SCIEX Elan 5000 quadrupole-based ICP-MS instrument. The samples were weighed with a microbalance (Sartorius M3P) with a precision of 1 µg.

5.1.3. Results

5.1.3.1. Optimisation of the solid sampling method

5.1.3.1.1. Description of the matrix

Three major difficulties have to be dealt with for the analysis of a gel after silver staining. First, the presence of silver itself. It was decided not to destain the gel, nor, as proposed by Sidenius *et al.* for electrothermal atomic absorption spectrometry, [Sidenius, 2000] to blot the proteins onto a polyvinylidene difluoride membrane (PVDF), nor to digest the bands, in order to obtain an easier matrix. In this way, we intend to keep the protocol straightforward and easy to use. Second, a gel after separation is, by definition, inhomogeneous: a band, revealed by silver staining, contains proteins and silver, although the background of the gel contains neither.

Third, in order to have a rapid analysis of a lane, only one ETV-ICP-MS signal was recorded, and the measurement was not repeated five times as is usually done. For all these reasons, the optimisation of the ETV parameters is of primordial importance.

For the optimisation of the multi-step furnace temperature program, measurements were carried out with solutions of selenium, tellurium and palladium added to a piece of stained gel. 10 µL of the various solutions were delivered by the autosampler onto the gel. The use of that gel was necessary to provide a correct concomitant matrix. It consisted of a normal polyacrylamide gel, soaked in an 0.16 g L^{-1} albumin solution and stained immediately afterwards. Therefore, it contained all chemicals present in a real gel: polyacrylamide, protein, silver, glycerol. It will be further referred to as 'blank gel'.

In order to correct for instabilities in the ETV signal, especially to avoid any correlation between sample mass and sensitivity for Se, the use of Te as an internal reference was investigated, as previously proposed [Fairman, 1997].

To get a better idea of the maximum amount of silver present on a stained gel, a piece of the blank gel was dissolved by means of microwave-assisted acid (HNO_3 / $HClO_4$) digestion. The solution was analysed for Ag using pneumatic nebulisation (PN) - ICP-MS and external calibration. 8.3 µg Ag cm^{-2} were found, or 3 µg Ag for a typical 0.3 cm^2 piece inserted into the furnace. In comparison, *ca.* 1 ng Se is expected in each band.

5.1.3.1.2. Temperature program

Since the boiling points of selenium compounds are relatively low (T_b(Se)=684°C, T_b(SeC)=125°C) [Grégoire, 2000], it was decided to use palladium as a chemical modifier. Although the mechanism of interaction of Pd with Se and Te is far from being totally understood, a physical or chemical interaction between Pd and these elements is assumed, [Volynski, 2001; Shiue, 2001] or an action as a carrier [Ediger, 1992; Pozebon, 1998] improving the transport efficiency of the analytes. However, too high an amount of Pd has to be avoided since this may lead to overloading of the plasma and pronounced matrix effects.

The pyrolysis curves of Se and Te obtained with 0.5 µg Pd are presented in Figure 5.2. Up to 1250°C, no noticeable loss of analyte occurred. If no Pd is used, loss of analyte is already observed above 200°C. Finally, a pyrolysis temperature of 800°C was chosen.

The efficiency of the pyrolysis of the polyacrylamide material was checked by weighing the boat before and after pyrolysis and was proved to be total.

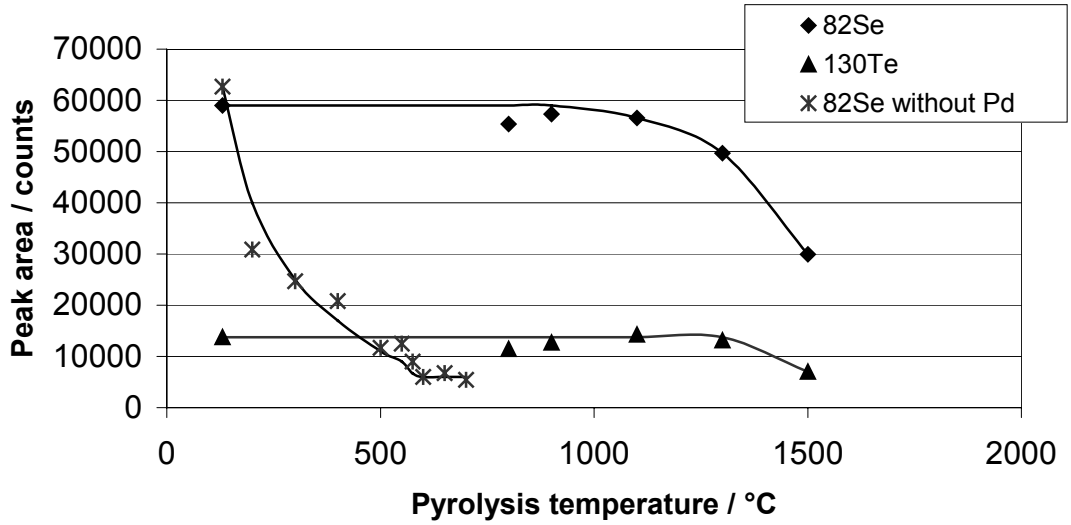


Figure 5.2: Pyrolisis curve of Se and Te spiked on a blank gel, in the presence or absence of $Pd(NO_3)_2$.

5.1.3.1.3. Sample mass: signal suppression

As illustrated in Figure 5.3, the Se sensitivity is dependent on the sample mass. This suppression effect is most probably due to Ag from the silver staining. In order to correct for this effect, an element was added to act as an internal reference. It has also the advantage of correcting for signal drift, instrument instabilities and variations in the vaporisation and transport efficiency. The use of Sb, as proposed for this purpose [Boonen, 1996], is impeded here by spectral interference from PdO^+ (*e.g.*, $^{105}Pd^{16}O^+$ and $^{121}Sb^+$). Te was preferred, as for the determination of Se in serum [Turner, 1999]. Measurements were carried out on ^{130}Te, because this signal is free from spectral overlap. After correction based on this internal reference, the relative signal obtained was independent from the mass of gel, for masses up to, at least, 1.5 mg (Figure 5.4). Such a mass corresponds to a piece of gel of *ca.* 0.4 cm^2, which is a very large surface for a protein band in half a lane. In all further work, 100 pg Te was added to the sample, an amount which gave a signal intensity comparable to the intensity of 1 ng Se (see Figure 5.5), the average Se amount in the bands.

To check whether significant signal suppression occurred, $^{38}Ar^+$ was monitored ($^{40}Ar^{40}Ar^+$, often used for this purpose [Vanhaecke, 1995], could not be

recorded since it overlaps with the $^{80}Se^{+}$ signal). This signal was indeed slightly suppressed at the maximum of the selenium signal (see Figure 5.5).

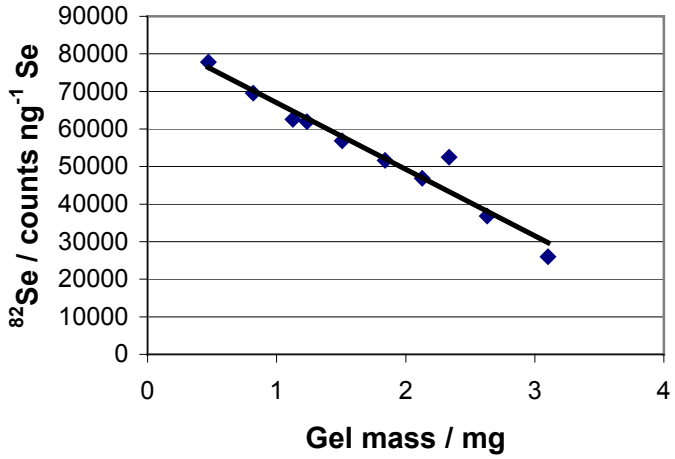


Figure 5.3: $^{82}Se^{+}$ sensitivity (1 ng Se) as a function of the sample mass.

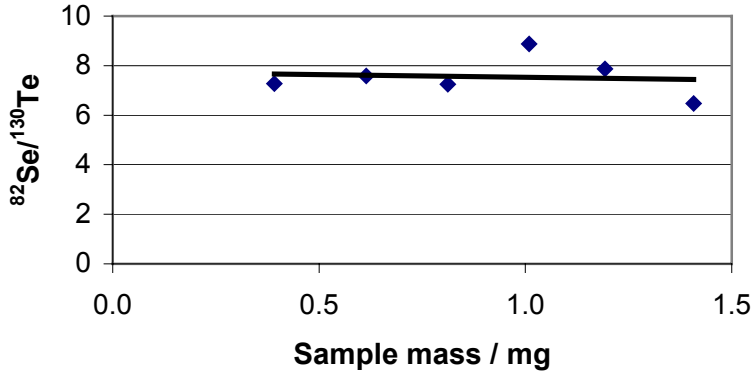


Figure 5.4: Signal ratio $^{82}Se^{+}$ / $^{130}Te^{+}$ (1 ng Se and 10 pg Te) as a function of the sample mass.

5.1.3.1.4. Optimisation of the ICP-MS instrumental conditions

It is well known that Se is a difficult element for ICP-MS, because of both spectral interference from $ArAr^{+}$, at mass-to-charge ratios of 76, 78 and 80, and from $ArCl^{+}$, at a mass-to-charge ratio of 77, and its high ionisation potential. For the latter reason it was studied whether the power setting of the ICP had a significant influence on the $^{82}Se^{+}$ signal. At 1250 W and an optimised carrier gas flow (1.20 L min^{-1}), a net signal of 230,000 counts was obtained, in comparison with 250,000 counts at 1000 W and 1.20 L min^{-1}. This latter set was preferred.

The optimised settings are summarised in Table 5.1. A typical signal profile is given in Figure 5.5.

Table 5.1: Optimised conditions for the ETV and ICP-MS parameters.
OmniRange: device of the mass spectrometer which can reduce the sensitivity by varying the transmission efficiency (P lens setting) at the exact time of the measurement of the mass-to-charge ratio selected.

ICP-MS instrument	*PerkinElmer Sciex Elan 5000*		
RF power	1000 W		
Ar gas flow rates			
Plasma	12 L min^{-1}		
Auxiliary	1.2 L min^{-1}		
Carrier	1.2 L min^{-1}		
ETV	*HGA-600MS*		
Sample mass	0.4 – 2 mg		
Chemical modifier	0.5 µg Pd		
Vaporisation programme			
	Temperature / °C	Ramp / s	Hold time / s
Drying step	130	10	20
Pyrolysis step	800	10	20
Vaporisation step	2500	0	10
Cleaning step	2700	1	3
Data acquisition			
Scanning mode	Peak hop transient		
Points per spectral peak	1		
Dwell time per acquisition point	30 ms		
Sweeps per readings	1		
Readings per replicate	95		
Signal monitored	$^{38}Ar^{+}$ (OmniRange 20), $^{82}Se^{+}$, $^{130}Te^{+}$		
Total measurement time	11 s		

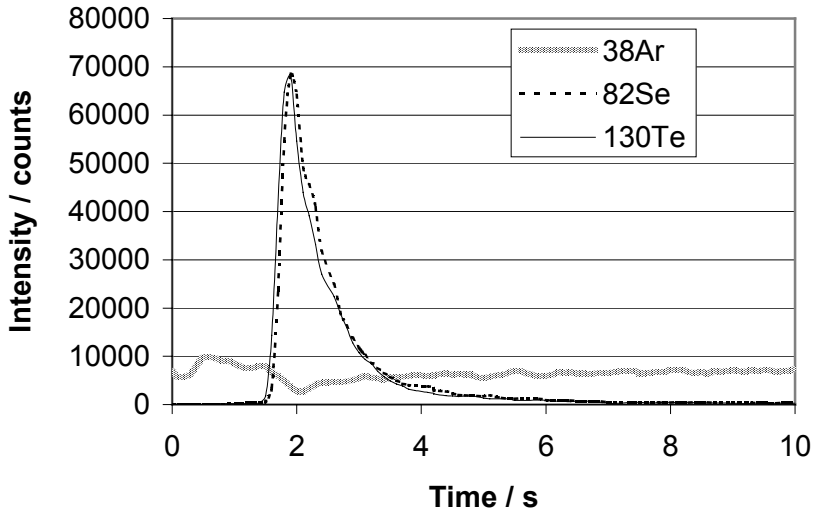


Figure 5.5: Signal profile of $^{38}Ar^{+}$, $^{82}Se^{+}$ and $^{130}Te^{+}$ recorded using the parameters described in Table 5.1. 1 ng Se and 100 pg Te spiked on 1.021 mg blank gel.

5.1.3.2. Determination of selenium

5.1.3.2.1. Calculations and limit of detection

The determination of Se in a band, with correction by Te, was carried out as follows:

- The mean background signal of Te was derived from 3 measurements of *ca.* 1 mg of blank gel (as defined in 5.1.3.1.1):

  Te_b = *background counts Te*

- The background signal of Se, corrected by Te, was derived from 3 measurements of *ca.* 1 mg of blank gel, spiked with Te:

  $$\left[\frac{Se}{Te}\right]_b = \frac{Se_b}{Te - Te_b} \qquad \text{(dimensionless)}$$

  where:

  Se_b =*(background) counts Se*

  Te = counts Te

- Each individual Se signal could be corrected:

  $$\left[\frac{Se}{Te}\right] = \frac{Se}{Te - Te_b} - \left[\frac{Se}{Te}\right]_b \qquad \text{(dimensionless)}$$

- The sensitivity for Se, corrected by Te, was derived from 3 measurements of *ca.* 1 mg of blank gel, spiked with Te and Se:

  $$s_{Se} = \frac{1}{m_{spiked}}\left(\frac{Se}{Te - Te_b} - \left[\frac{Se}{Te}\right]_b\right) \qquad (pg^{-1})$$

  with:

  Se = counts Se

  m_{spiked} = *spiked Se mass* (pg)

- The quantity of Se was determined by external standardisation:

  $$m = \left[\frac{Se}{Te}\right]\frac{1}{s_{Se}} \qquad \text{(pg)}$$

- Limit of detection: LOD

$$LOD = 3\frac{SD}{s_{Se}} \qquad \text{(pg)}$$

where *SD* is the standard deviation of the signal for the analysis of 10 blank (stained) gels. The blank was not simply given by a measurement with empty boats, as is usually done, but with exactly the same matrix. It is thus the most adequate blank measurement.

LOD = 40 pg, or 40 ng g^{-1}

It is worth noticing that, if a lower detection limit is necessary, a non-stained gel can be analysed. In this case, the LOD is equal to 5 pg or 5 ng g^{-1}. For such an analysis, the proteins in the gel can be fixed by an acidic treatment, the half of the lane of interest stained and the other half analysed with ETV-ICP-MS.

5.1.3.2.2. Differences between a stained and non-stained gel, repeatability

As already mentioned, the detection method for Se must have as little sensitivity to staining as possible. Otherwise, a faint protein band, where less Ag is present, would show a signal intensity other than a strongly coloured band, even if the same amount of Se were present.

It was experimentally shown that Te_b, $\left[\frac{Se}{Te}\right]_b$, $\left[\frac{Se}{Te}\right]$ and s_{Se} were not significantly different between stained and unstained gels (95 % confidence level, t-test on two series of at least 5 measurements). The repeatability of the method, 8.3 %, was assessed by the relative standard deviation on $\left[\frac{Se}{Te}\right]$ between ten measurements of pieces of gels spiked with 1 ng Se.

Yet, *SD* was higher in the case of a stained gel (F-test on two series of 10 measurements, 95 % confidence level). This explains the difference in LOD, but this difference does not affect the quantification. Therefore, this method is not sensitive to silver staining.

5.1.3.2.3. Validation of the method using GSH-Px

Glutathione peroxidase is, to our knowledge, the only selenoprotein commercially available and was therefore chosen as a model to check the applicability of the method.

Taking into account the sample preparation (1.54 ng Se μL^{-1} sample solution) and the amount of this solution brought onto a gel (4 µL), 6.2 ng Se from GSH-Px were present in a gel lane. Two types of experiments were conducted. First, an entire lane was analysed piece by piece, with results in very good agreement with the expected value (6.4 ng measured, *i.e.* 103 % recovery). Second, only the stained bands, expected to carry the selenoprotein, were analysed. The recovery was in good agreement with the expected value (two lanes measured, 6.0 ng were obtained twice, *i.e.* 97 % recovery).

The reconstituted Se profile of the first experiment, *i.e.* after analysis of the whole lane, is given in Figure 5.6. It is the real electropherogram of the Se-species. In order to take into account the difference in width of the band analysed, the Se amount was divided by the length of the band and is given in ng cm^{-1}; the area under the curve (ng cm^{-1} x cm) gives the amount of Se. The identity of the proteins was checked by comparing their migration distance with those of molecular mass markers.

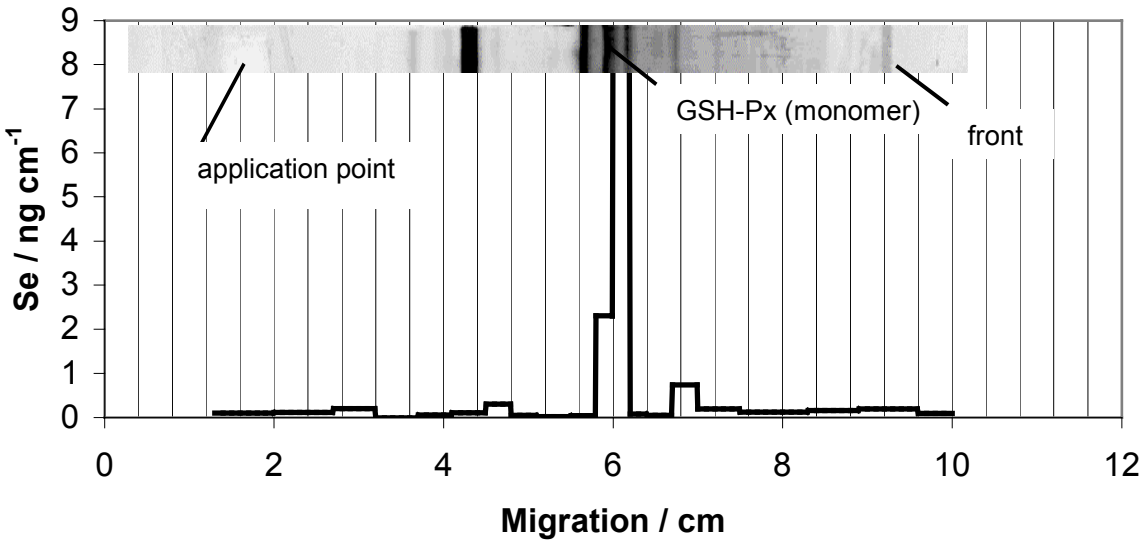


Figure 5.6: ^{82}Se electropherogram of GSH-Px. Analysis of the whole lane. The picture of the lane is given for comparison. Cathode and application point at the left.

This profile proves, first, that Se is found in the expected band, GSH-Px, and, second, that no Se is lost during the separation since no Se is detected at the front, where low molecular mass oxidation products, such as selenite, are expected.

Another experiment was done on a non-derivatised sample, and this time no Se was found in the GSH-Px band. This means that the selenoprotein had degraded, Se oxidised and removed from the protein during electrophoresis. Both sets of experiments prove that the derivatisation step was necessary and efficient.

5.1.3.3. Application: analysis of selenised yeast

The method was further applied to the analysis of the water-soluble proteins in a yeast extract using external calibration. The Se electropherogram is given in Figure 5.7. Two maximums can be discerned, first at a mass higher than 94 kDa and second at a mass lower than 14 kDa. Furthermore, proteins around 20 kDa also contain a fairly large amount of Se. This pattern is confirmed by size-exclusion preparative chromatography, but with a lower mass resolution [Chassaigne, 2002].

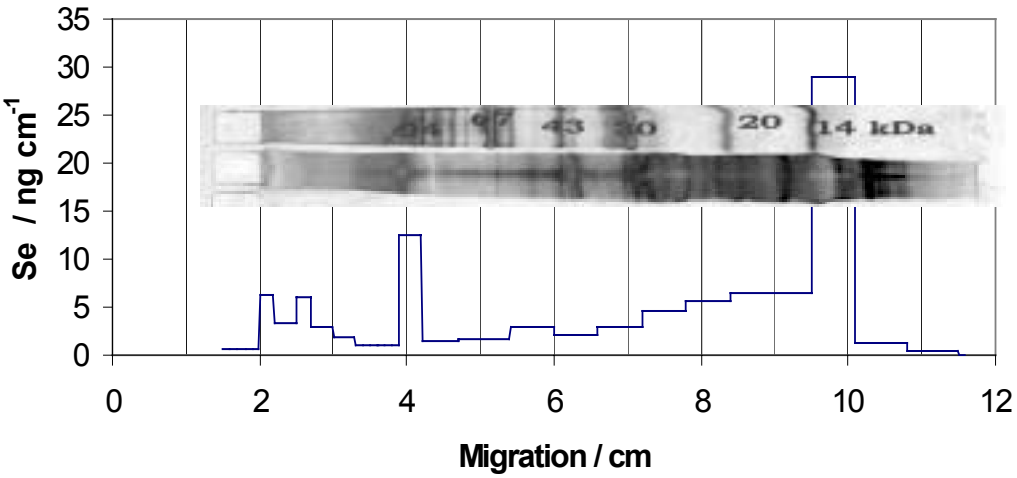


Figure 5.7: Se electropherogram of water-soluble proteins of selenium-yeast extract. Analysis of the whole lane. The picture of the lanes containing the yeast proteins is given for comparison. Cathode and application point at the left.

The amount of Se in the protein fraction of this sample has been determined by GF-AAS [Chassaigne, 2002]. Taking into account the Se amount in the protein fraction and the dilution due to the sample preparation, a 95 % recovery is obtained here.

It is normal that many yeast proteins contain Se, since this element is incorporated by yeast in a non-specific way. In other words, Se replaces S if the ratio Se/S is high, as is the case in the growth medium used for the production of this kind of selenium yeast. Instead of finding methionine or cysteine, selenomethionine and selenocysteine are encountered in these proteins.

It is worth noticing that the high resolution of the electrophoresis allows us to differentiate sharp protein bands. With the set of molecular markers used, 43 kDa is easily distinguished from 30 kDa. Still, the mass resolution can be higher if gels of different degrees of crosslinking or acrylamide concentrations are used.

5.1.4. Conclusions

The combined use of SDS-PAGE and ETV-ICP-MS allows the analysis of selenium in proteins with a high resolution. A gel lane with 10 bands can be entirely analysed in *ca.* 2 h. The use of an internal reference, Te, permits the quantification of Se. The LOD is 50 pg Se per piece of gel, or, assuming a dilution factor of 10 during the sample preparation preceding SDS-PAGE and a 10 µL sample brought onto the gel, 50 µg Se L^{-1} of original solution. This LOD is satisfactory for some biological samples. Yet, this method must be improved for samples such as serum: assuming a Se concentration of 100 µg L^{-1} serum and the same dilution factor and volume applied onto the gel, 100 pg Se are present in the sample. If Se is divided over 10 species, the LOD is insufficient. Since it is expected that ETV as an introduction system offers one of the best detection limits, the improvement had to come from the detection system, especially for alternative introduction systems as laser ablation. The use of the DRC technology was explored in the following in order to improve the LOD [Chéry, 2003b].

5.2. Improvement of the detection limit: dynamic reaction cell (DRC)

5.2.1. Vanadium

5.2.1.1. Introduction

An electrophoretic separation method has been optimised for the speciation of vanadium in biological fluids or tissues. The next step is to apply this method, but this time by using cold vanadium instead of a radiotracer. All separations take place in chlorine-rich media, because all buffers must be at physiological salinity. Otherwise, the species can be denatured [De Cremer, 1999]. Such large amounts of salts (0.15 M NaCl) are a major drawback for the detection of vanadium with ICP-MS, since the major and actually only measurable isotope of vanadium (^{51}V, 99.75 % isotopic abundance) is severely subject to interference from $^{35}Cl^{16}O^{+}$. In such a case, either the separation technique has to be adapted to enable accurate detection, thereby jeopardising the stability of the vanadium compounds, or the detection

technique has to be adapted to the separation. The latter strategy was preferred [Chéry, 2003b].

High-resolution ICP-MS was until recently the preferred way of non-radioactive detection, especially for on-line detection of vanadium after chromatography [Yang, 2002; Nagaoka, 2002].

In this work, the capabilities of the dynamic reaction cell (DRC) technology were studied. This technology has already been applied to the detection of vanadium in serum and urine [Nixon, 2002] and in seawater [Louie, 2002], but so far, no attention has been devoted to speciation. In these works, the $^{35}Cl^{16}O^{+}$ interference could be efficiently and selectively removed using ammonia as the reaction gas, without noticeable loss in sensitivity for vanadium.

The strategy of the following was to first prove the usefulness of the DRC technology on a transient signal as given by liquid chromatography, in our case size-exclusion chromatography. In a further step, this technology can be applied to LA-ICP-MS.

5.2.1.2. Experimental

5.2.1.2.1. Incubated human serum

Freshly sampled human blood was separated into serum and blood cells by centrifugation at 6000 x *g* for 20 min, the supernatant transferred to a second vial and centrifuged a second time following the same parameters. It should be noted that no particular precaution was taken to prevent contamination of this serum from external sources, like from stainless steel needles. Two aliquots of this serum were incubated with vanadate for at least 16 h, at a constant temperature of 37°C. This incubation brought the vanadium concentration to 50 and 300 µg V L^{-1} serum.

5.2.1.2.2. Instrumentation

5.2.1.2.2.1. Size-exclusion liquid chromatography

The fractionation was performed on an Åkta Purifier 10 (Amersham Pharmacia Biotech) equipped with, among others, a UV detector and two sets of pumps. First, it was checked whether the system could work contamination-free, contamination coming, *e.g.*, from the pump heads. The background signal on vanadium given by the chromatographic system was recorded and compared to the signal given by the

nebulisation of the same buffer at the same flow rate but this time introduced by a fully metal-free peristaltic pump. Identical background V-signals were recorded (chromatography: 41.5 cps, standard deviation on 75 replicates 5.1; peristaltic pump: 41.3 cps ± 4.8). Therefore, no contamination was to be feared from the HPLC system.

All samples were filtered through a 0.22-µm Millex-GV_{13} filter (Millipore) and manually injected onto a 100-µL loop without dilution. A Superose 12 HR 10/30 gel filtration column (Amersham Pharmacia Biotech) was used throughout this work. This type of column is made of crosslinked agarose (12%), has a volume of 24 mL with beads of 10-µm diameter and a separation range of 1-300 kDa. A single buffer made of 20 mM HEPES and 0.15 M NaCl adjusted to a pH of 7.5 with NaOH was used at a constant flow rate of 0.75 mL min^{-1}. All chromatograms were recorded in threefold.

5.2.1.2.2.2. Hyphenation

In the experiments without internal reference, the capillary at the column exit was inserted into a Meinhard concentric nebuliser, which was mounted into a cyclonic spray chamber (PerkinElmer). For the experiments with internal reference, the second set of pumps constantly added a 10 µg Y L^{-1} solution in a 0.14 M HNO_3 medium after the column to the eluent at 0.75 mL min^{-1} through a mix chamber (Valco). Immediately after the mix chamber, the flow was split in two by a T-piece (Amersham Pharmacia). In this way, the flow rate was brought back down to 0.75 mL min^{-1} before the nebuliser, *i.e.* the same flow rate as used without internal reference.

5.2.1.2.2.3. Detection: ICP-DRC-MS

An Elan DRC *plus* (PerkinElmer SCIEX) equipped with a dynamic reaction cell (DRC) and a dual gas manifold was used throughout this work, allowing the simultaneous use of two reaction gases. A complete description of this instrument is presented elsewhere [Tanner, 1999] and the principle of the DRC in 3.1.2.2.2.1. This instrument is also equipped with the *plus* option, *i.e.* an additional optional axial field in the reaction cell, which enhances the transmission of ions through the reaction cell [Bandura, 2002]. This earlier version of the axial field technology (AFT) has a constant potential difference of 150 V, a value that could not be further optimised.

5.2.1.3. Results

Since the fractionation method has already been optimised and presented previously [De Cremer, 2001], demonstrating the need for a high salinity, this work concentrated solely on the figure of merits of the hyphenated method and the compatibility of the ICP-DRC-MS with the aforementioned salinity.

5.2.1.3.1. Optimisation of the dynamic reaction cell DRC

5.2.1.3.1.1. Description of the system

In this case, the DRC is a system fully described by only two parameters: the flow rate of the reaction gas and the rejection parameter q (RPq) [Tanner, 2002], which are not correlated as a first approximation. It is worth mentioning that a step-by-step optimisation (in other words the optimisation of first the flow rate and then RPq) was therefore the most sensible procedure. This can be explained by the simple fact that the RPq setting does not noticeably affect the reaction efficiency, so that a full chemometrical optimisation, as a simplex optimisation, is not required.

As a first requisite, the reaction between reaction gas and interfering ion must take place. As a first approximation, the transmission of the interference as a function of the flow rate of the reaction gas is a step function: at a low reaction gas flow rate, the intensity of the signal of the interfering ion remains unchanged, but when a sufficiently high flow rate is used (in other words, when the partial pressure or the chemical potential of the reaction gas is high enough), the interference is removed. In some instances, a new interference originates from the reaction gas. Under these circumstances, low mass filtering must be used to destabilise the precursor of this interference. This is accomplished by optimising the RPq setting. The presence of a mass filter hardly influences the reaction efficiency of the gas; in other words, there is hardly any cross-effect of the RPq value on the reaction efficiency.

5.2.1.3.1.2. Gases and possible reaction mechanisms

The merits of five gasses were compared: CH_4, CO, NH_3, O_2 and a mixture of Ar and H_2. These gases can react with either the analyte or the interfering ion through various mechanisms. Theoretically, all can react through charge transfer; carbon monoxide and oxygen through O transfer; ammonia, methane and hydrogen could react through hydrogen transfer.

i. Charge transfer

In order to determine which charge transfer reactions are thermodynamically allowed, the ionisation energies (IE) of the molecules of interest have been displayed in Table 5.2. This set of data shows that only ammonia can react with ClO^+ [because IE(ClO) > IE(NH_3)] and that no gas is able to reduce V^+ [because IE(V) < IE(reaction gases)].

Table 5.2: Ionisation energies (IE) of the reaction gases, analyte and interfering ion.

	V	NH_3	ClO	O_2	CH_4	CO	H_2
IE (eV)	6.7	10.2	10.9	12.1	12.6	14.0	15.4
Reference	[Lide, 1991]	[Lide, 1991]	[Schwell, 1996; Thorn, 1996]	[Lide, 1991]	[Lide, 1991]	[Lide, 1991]	[Lide, 1991]

ii. O transfer

O transfer is the second possible class of reactions. The oxidation of vanadium is thermodynamically allowed, but the oxidation of ClO is endothermic, with the approximation of a negligible entropy factor (in the following, $\Delta_f H°$ is the standard formation enthalpy and $\Delta_r H°$ the standard reaction enthalpy). VO^+ can thus be measured without interference from ClO_2^+ originating from the DRC (data from [Bard, 1985], except $\Delta_f H°(ClO^+)$ = 1151 kJ mol^{-1} derived from [Schwell, 1996; Thorn, 1996] and $\Delta_f H°(ClO_2^+)$ = 1066 kJ mol^{-1} from [Mok, 2000]).

- $ClO^+ + O_2 \rightarrow ClO_2 + O^+$

  $\Delta_r H° = \Delta_f H°(ClO_2) + \Delta_f H°(O^+) - \Delta_f H°(O_2) - \Delta_f H°(ClO^+)$

  $\Delta_r H° = +\ 520$ kJ mol^{-1}

This calculation is given in more details in Figure 5.8.

- $ClO^+ + O_2 \rightarrow ClO_2^+ + O$

  $\Delta_r H° = \Delta_f H°(ClO_2^+) + \Delta_f H°(O) - \Delta_f H°(O_2) - \Delta_f H°(ClO^+)$

  $\Delta_r H° = +\ 164$ kJ mol^{-1}.

- The reduction of ClO^+ is highly endothermic and this reaction is thus not allowed:

  $CO + ClO^+ \rightarrow CO_2 + Cl^+$

  $\Delta_r H° = +\ 2695$ kJ mol^{-1}

iii. H transfer

Interestingly enough, when the same approach is followed to determine whether hydrogen transfer is a valid mechanism, we find that the reactions

$$H_2 + ClO^+ \rightarrow HClO + H^+ \text{ or } HClO^+ + H$$

are endothermic (with $\Delta_f H°(ClO^+)$ from [Thorn, 1999]).

The same is true for ammonia.

As a conclusion, for the elimination of the interfering ion only charge transfer with ammonia seems to be allowed. One has to address the question whether vanadium and ammonia can form clusters, $V(NH_3)^+$. However, no thermochemical data were found to confirm or infirm this hypothesis. An experimental approach was chosen to solve this problem (see 5.2.1.3.1.3).

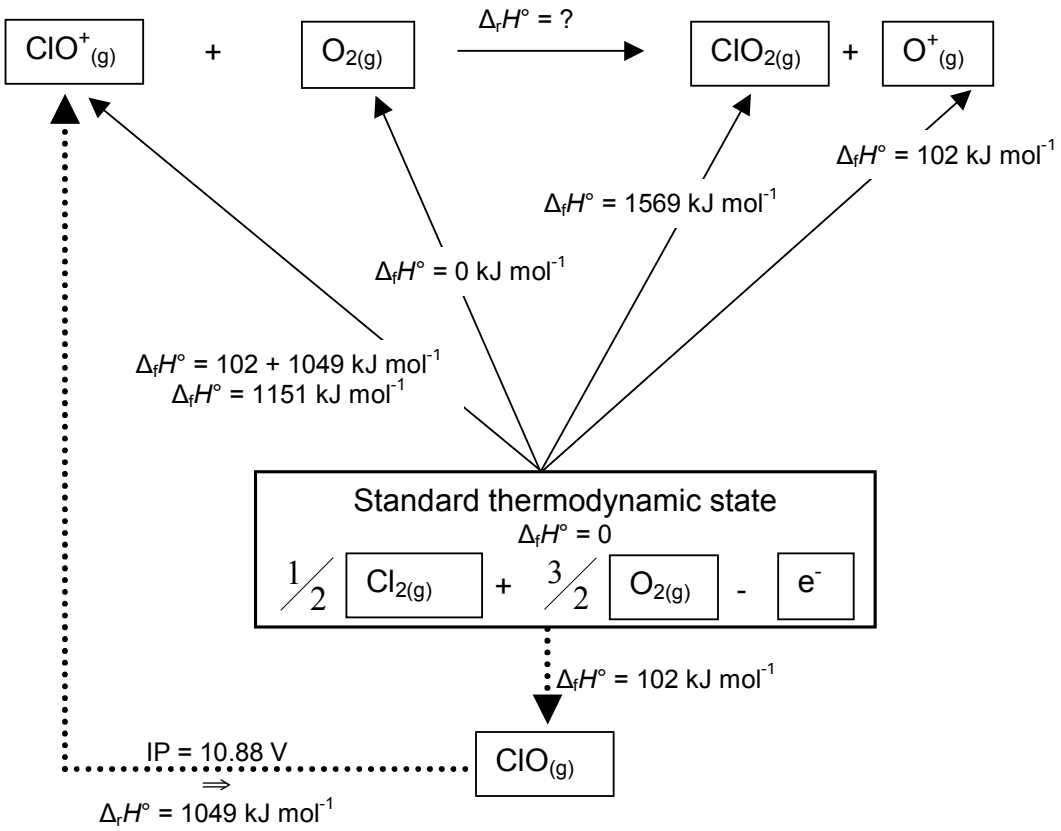


Figure 5.8: Diagram for the calculation of the reaction enthalpies of $ClO^+ + O_2 \rightarrow ClO_2 + O^+$. Note that $\Delta_f H°(ClO^+)$ is not tabulated as such but can be deduced from $IP(ClO^+)$.

5.2.1.3.1.3. Optimisation criterion

The signal-to-noise ratio was used as the criterion to optimise both reaction gas flow rate and rejection parameter. For RPq, the range was limited to [0, 0.8], as

above 0.8 the signal was observed to fluctuate intensely, although the signal-to-noise ratio is sometimes higher in this region. The signal given by a 50 µg V L^{-1} in 10 mL HCl L^{-1} solution was used as signal (S) and the signal given by a 10 mL HCl L^{-1} solution as noise (N). The signal-to-noise ratio (S / N) was calculated as [S-N]/N. Both solutions were delivered to the nebuliser with a peristaltic pump (0.75 mL min^{-1}). The same procedure was followed, but this time with all solutions in 10 mL HNO_3 L^{-1} in order to obtain the limits of detection (LOD) in a non-interfering matrix for comparison purposes.

The example of the optimisation of the flow rate for NH_3 as the reaction gas is given in Figure 5.9. An example of the signal-to-noise ratio as a function of the RPq setting obtained with Ar and H_2 is given in Figure 5.10. This figure illustrates the case where no major additional interference is caused by the reaction gas; changing the RPq does not drastically improve the signal-to-noise ratio. A very different behaviour is observed with NH_3 as already illustrated in Latino's work [Latino, 2001]. For the sake of clarity, a similar figure, obtained in our laboratory, is given in Figure 5.11. In this instance, ammonia is responsible for the formation of a new species that interferes with V^+ and setting the low-mass cut-off (RPq value) at a higher mass destabilises the precursor of this interfering ion.

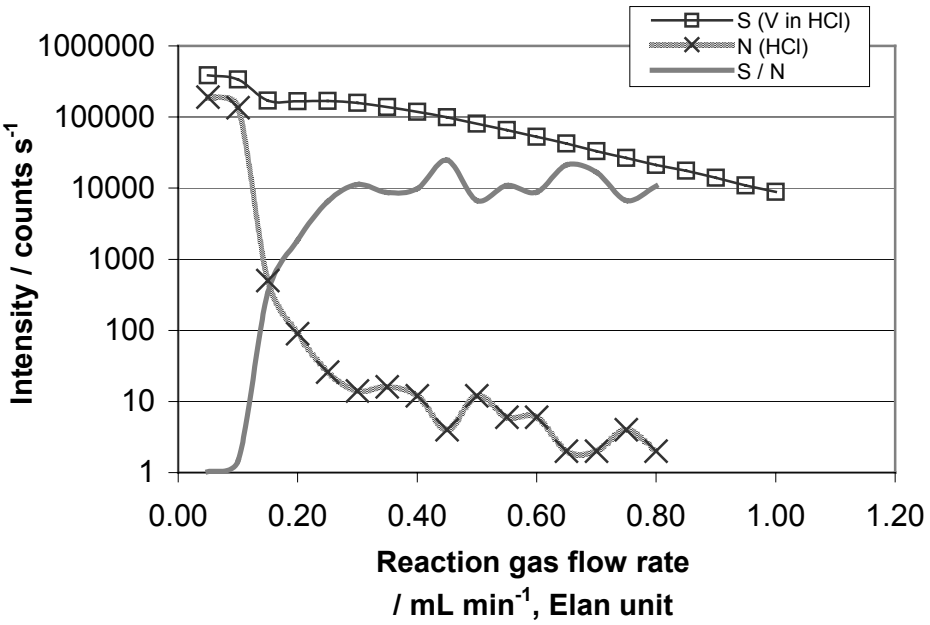


Figure 5.9: Optimisation of the reaction gas flow rate for NH_3 as reaction gas.
S: 50 µg V L^{-1} in 10 mL HCl L^{-1}; N: 10 mL HCl L^{-1}.
Elan units: the flow meter of the Elan instrument is calibrated on Ar, the unit of the flow rate read by the instrument, relative to Ar, is expressed Elan unit. A conversion is necessary to obtain the absolute flow rates.

A further interesting point about this figure is that it proves that hardly any clustering between vanadium and ammonia occurs. Otherwise, the vanadium signal, S, would be drastically affected and would drop as a function of the ammonia flow rate. A decrease is still present, but the slope is typical for collisional scattering. In other words, the analyte is brought out of a stable trajectory in the quadrupole because of collisions with the gas present in the cell. The higher the pressure, the higher the loss is.

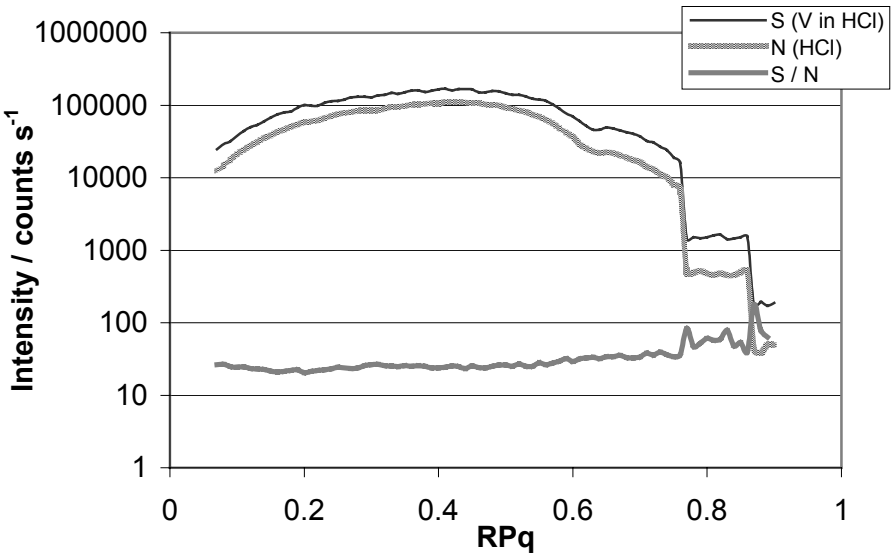


Figure 5.10: Optimisation of the RPq setting (low mass cut-off).
H_2 used as the reaction gas (0.40 mL min^{-1} Elan Units) and Ar as the non-reactive collision gas (0.20 mL min^{-1} Elan Units).
S: 50 µg V L-1 in 10 mL HCl L-1; N: 10 mL HCl L-1.

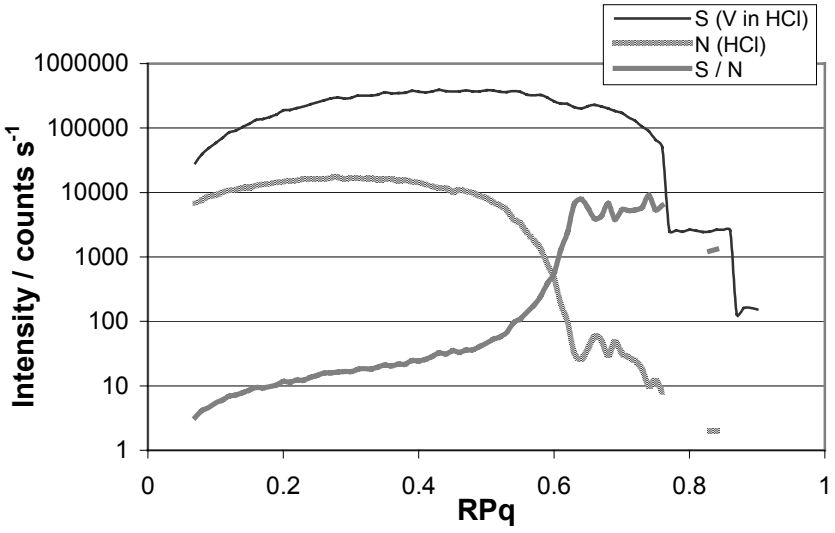


Figure 5.11: Optimisation of the RPq setting (low mass cut-off).
NH_3 used as the reaction gas, 0.30 mL min^{-1} Elan Units.
S: 50 µg V L^{-1} in 10 mL HCl L^{-1}; N: 10 mL HCl L^{-1}.

The detection limit was then determined at the optimised parameters and calculated as three times the standard deviation on 10 blank (either 10 mL L^{-1} HCl or HNO_3) replicates divided by the sensitivity of a standard in the same matrix. The optimised DRC settings and corresponding LODs are summarised in Table 5.3.

Table 5.3: Optimised DRC settings and limits of detection for vanadium (signals monitored: $^{51}V^+$ or $^{51}V^{16}O^+$).
Elan units: see Figure 5.9.

Reaction gas	Flow rate		RPq	LOD in 10 mL HCl L^{-1}		LOD in 10 mL HNO_3 L^{-1}	
	mL min^{-1}			ng L^{-1}		ng L^{-1}	
	Elan units	Absolute		$^{51}V^+$	$^{51}V^{16}O^+$	$^{51}V^+$	$^{51}V^{16}O^+$
CH_4	0.40	0.23	0.45	/		$2\ 10^4$	
CO	0.40	0.28	0.63	$1.5\ 10^3$		$6\ 10^1$	
O_2	0.40	0.28	0.45	$2\ 10^3$	$3\ 10^1$	$1\ 10^1$	$3\ 10^1$
Ar + H_2	0.20 + 0.40	0.20 + 0.29	0.65	$7\ 10^1$		$1\ 10^1$	
NH_3	0.40	0.22	0.65	***1 10¹***		***3***	
Standard	0		0.25	/		$1\ 10^2$	
Data acquisition							
Sweeps per reading		10					
Readings per replicate		1					
Replicates		10					
Other ICP-DRC-MS settings as summarised in Table 5.4							

Two gases, CH_4 and CO, seem to be unsuitable for the elimination of the interfering ion. This was expected for CO, since both mechanisms through which this gas might react (reduction and charge transfer) are thermodynamically forbidden. Oxygen cannot remove the interfering ion but is efficient to convert V^+ into VO^+. The argon - hydrogen mixture is also efficient at removing the ClO^+ interfering ion, but we must recognize that we do not fully understand the reaction process in question, which may involve traces of water and thus facilitate other types of reaction [Tanner, 2002].

The lowest LOD is obtained when using NH_3 as a reaction gas. This gas has also been chosen by Latino et al. [Latino, 2001] for the detection of V with the same RPq. They proposed that the new polyatomic ion species, created within the DRC, is a Cl-NH_x adduct. Another theoretical explanation for this interference could be $(NH_3)_3^+$. Yet, the analysis of ultra-pure water using the DRC with ammonia but with the low-mass filter 'switched off' (RPq = 0.45), which allows the formation of the latter species, did not reveal any noticeable interference on the mass-to-charge ratio of 51, excluding this hypothesis.

These DRC settings were also tested with the Plus option. Nevertheless, the axial field technology did not further improve the detection limit. A second advantage of the Plus option is the much faster transmission of the ions through the reaction cell, decreasing the risk of signal mix between two sweeps, which can occur because of the long settling time of the DRC. Since we have to do with relatively long transient signals from the HPLC (typically 30 s peak width at half-height), the advantage offered by the Plus option is much less pronounced. That is why this option was not further used in this work.

5.2.1.3.2. Choice of an internal reference for liquid chromatography – ICP-MS hyphenation

In ICP-MS, an internal reference corrects for both (i) plasma instabilities, during the time-span of a chromatographic run, which means that a corrected chromatogram should be free from ghost peaks or signal suppression, and (ii) for differences in sensitivity among acquisitions, and hence it improves the repeatability.

Co is normally a good choice as an internal reference for V. Under the conditions used, however, $^{59}Co^{+}$ suffers from a DRC-based interference, as V does. The identity of this interference could not be elucidated. Still, the DRC can overcome this problem by using an appropriate RPq setting (RPq = 0.6 gives the highest signal-to-noise ratio). Nevertheless, we preferred to explore other elements as potential internal references, since Co is present in serum, among others, in the form of cyanocobalamin, also known as vitamin B12. The total concentration of Co in serum is *ca.* 100 ng L^{-1}, so that a high concentration of this element would have to be added post-column to mask the cyanocobalamin chromatographic peak. For the same reason, candidate elements like Al, Cr, Mn, Ni, Cu, Mo and Sr had to be rejected (concentration between 0.1 and 1000 µg L^{-1} serum; all concentrations in serum were taken from the reference book by Versieck and Cornelis [Versieck, 1988] and the value for Sr from [Krachler, 1999]).

Solubility is another selection criterion. Nb and Ta, although from the same chemical group as V, are hardly soluble. Sc, Ti, Zr and Ga are no good options either, since they readily form insoluble hydroxides at a pH of *ca.* 5. One must bear in mind that the internal reference is added to a pH buffer round neutrality.

The choice is then between Ge and Y. Since Y has an ionisation potential close to that of V (6.38 and 6.74 eV respectively), this element was preferred.

5.2.1.3.3. Need for an internal reference

The correction with ^{89}Y was accomplished as follows:

$$\left[\frac{V}{Y}\right] = \frac{^{51}V}{^{89}Y - Y_b}$$

where ^{89}Y and ^{51}V are the signal intensities recorded on mass-to-charge ratios of 89 and 51 respectively during chromatography and Y_b the signal intensity recorded on a mass-to-charge ratio of 89 without chromatography.

However, it should be realised that in this case, the use of an internal reference also leads to a reduction in sensitivity, since the eluent is diluted after the column by a factor of two. In order to assess if an internal reference is strictly necessary, the background signal observed after the injection of a serum sample not incubated with vanadium was recorded. The resulting chromatogram is given in Figure 5.12.

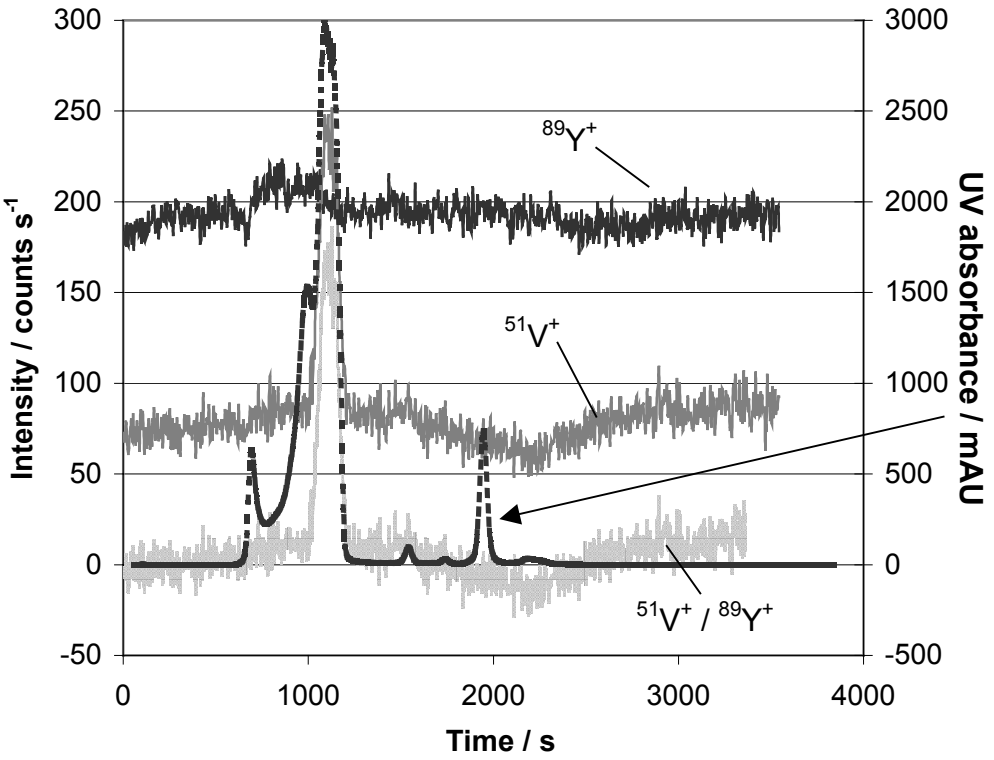


Figure 5.12: Chromatogram for serum with Y used as internal reference.
10 µg Y L^{-1} added post-column (SEC buffer : internal reference = 1 : 1).
The values for $^{89}Y^+$ (signal divided by 5) and $^{51}V^+$ / $^{89}Y^+$ were brought to the scale of the $^{51}V^+$ signal.

It can be noted that a signal is recorded at the position of transferrin. This transferrin-bound vanadium was also observed by other research groups at these low

concentrations (first mentioned in Sabbioni's work [Sabbioni, 1978]) and is probably vanadate that stems from both 'natural' origins (already present in serum) and from the sampling needle (stainless steel needle).

Since the stability of the Y-corrected signal (8.7 % RSD on the section of a chromatogram without V) is similar to the stability of the $^{51}V^{+}$ signal without correction (8.9 % RSD), while no signal suppression was observed during the recording of an entire chromatogram for serum (matrix background) no internal reference was further used. Instead, it was preferred to use external standardisation in order to correct for long-term drift of the instrument.

5.2.1.3.4. Example: speciation of V in serum at therapeutical level

A chromatogram recorded using the parameters summarised in Table 5.4 is given in Figure 5.13.

Table 5.4: Optimised settings for the HPLC-ICP-DRC-MS system.

ICP-MS instrument	*Elan DRC plus*
RF power	1200 W
Ar flow rates	
Plasma	17.0 L min^{-1}
Auxiliary	1.2 L min^{-1}
Nebuliser	1.0 L min^{-1}
DRC	
NH_3 (reaction gas) flow rate	0.4 mL min^{-1} Elan unit
RPq	0.65
Data acquisition	
Scanning mode	Peak hopping
Acquisition points per spectral peak	1
Dwell time per acquisition point	50 ms
Sweeps per reading	70
Readings per replicate	1000
Signal monitored	$^{51}V^{+}$
Total measurement time	1 h
HPLC	*Åkta Purifier 10*
Injection loop	100 µL
Column	Superose 12 HR 10 / 30
Flow rate	0.75 mL min^{-1}
Buffer	20 mM HEPES, 0.15 M NaCl, pH = 7.5
UV wavelength	280 nm
Running time	64 min

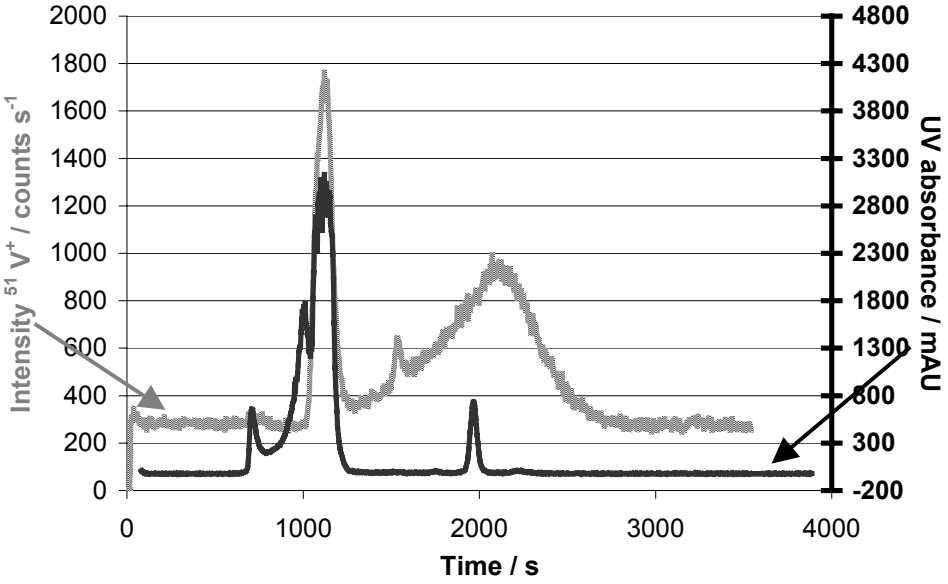


Figure 5.13: Chromatogram for serum incubated with vanadate at 300 µg V L^{-1}. Experimental conditions as in Table 5.3.

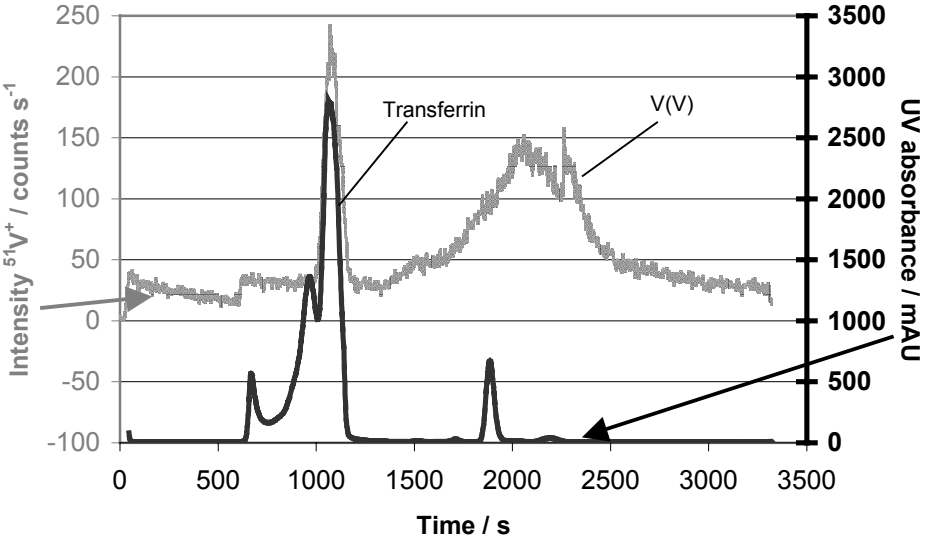


Figure 5.14: Chromatogram for serum incubated with vanadate at 50 µg V L^{-1}. Experimental conditions as in Table 5.3.

In contrast with the chromatogram shown in Figure 5.12, vanadium was added here to serum at a concentration much higher than the basal concentration, estimated to be *ca.* 0.03 µg L^{-1} serum in healthy patients. It is worth noticing that this concentration, 300 µg L^{-1}, is in the range observed in serum after administration of vanadium-complexes in the treatment of diabetes [Willsky, 2001]. Once again, the peak of transferrin is to be seen at a retention time of 1120 s. An unknown low molecular mass species is present at 1545 s. Further, at such a high concentration, a

non-negligible part is not bound and present as ready-exchangeable vanadate (peak at 2133 s) or bound to low-molecular species. This experiment was repeated with a concentration of 50 µg L^{-1}. The chromatogram is shown in Figure 5.14. The peak of transferrin represents 10 µg V L^{-1} serum. This amount was calculated by external standardisation by injections of vanadate. A series of unknown low-molecular mass species is present from 1530 s to *ca.* 2200 s. The sum of these low-molecular mass vanadium compounds amounts to 34 µg V L^{-1} serum. The recovery for this chromatogram, typical of these experiments, is thus 88 % on this single assay. The missing 12 % are assumed to be due to signal instability and not to a loss of vanadium in the chromatographic process. If a better accuracy is required, the use of an internal reference is mandatory.

5.2.1.3.5. Figures of merit of the hyphenated method

Various figures of merit for this method were estimated.

Repeatability of both retention time and peak area was calculated on the basis of three injections of serum incubated with vanadate.

The RSD of the area of the V(V)-transferrin complex was 4.5 % and of free V(V) 7.0 %. The RSD on the retention time of V(V)-transferrin complex was 0.9 %.

The limit of detection for vanadate is theoretically the poorest of the eluted compounds since it has the longest retention time and hence it shows the broadest peak width at half height according to the theory of size-exclusion. The LOD was calculated using the three SD method and replicate injections of vanadate to determine the sensitivity.

- SD is the standard deviation on the background signal during chromatography at a mass-to-charge ratio of 51, thus without injection of vanadium, on a length at least three times the half width of the vanadate peak.
- The sensitivity for vanadate is the sum of counts recorded for the vanadate peak divided by the concentration (or quantity) of vanadium in the sample.
- $LOD = 3\frac{SD}{sensitivity_V}$.

The LOD calculated in this way is equal to 40 ng V L^{-1} serum or, in absolute terms, 4 pg V.

The long-term performance of the instrument was also checked. Indeed, the high salt concentration causes a decrease in the sensitivity of the instrument after a couple of weeks operation. Yet, a simple monthly cleansing procedure of the components of the introduction system (nebuliser, nebulisation chamber, injection tube and skimmer cone), which only lasts a couple of hours, suffices to restore the sensitivity of the instrument. No damage has ever been noted to any of these components, even after months of use with this buffer.

5.2.1.4. Conclusions

The use of size-exclusion chromatography coupled on-line to ICP-DRC-MS allows a rapid screening of biological samples such as serum for the speciation analysis of vanadium. This method offers huge advantages in comparison with the use of a vanadium radiotracer, namely its hyphenated character and its applicability to samples of human origin. The retention time alone is not sufficient to prove the identity of a compound, but it is already a first step that can be followed by submitting the fraction of interest to, *e.g.*, MS-MS [Templeton, 2000].

Further, the LODs obtained in this work are comparable to those presented for HR-ICP-MS [Yang, 2002]. These LODs are lower than the quantity of vanadium expected in serum after treatment of diabetes with vanadium-complexes.

The conclusion of this chapter in the frame of this work is twofold. First, the DRC technology is an efficient solution to spectral interferences and it is applicable to transient signals as those given by LA-ICP-MS. Second, the results obtained by gel electrophoresis about the identity of the vanadium-protein complex are confirmed here, namely a vanadium-transferrin complex.

5.2.2. Selenium

The DRC was optimised using a pneumatic nebuliser as the method of sample introduction (thus with solutions) since optimisation is easier when using a stable and continuous signal. The strategy of optimisation of the DRC is similar to the one followed by Hattendorf and Günther [Hattendorf, 2003] for Se: the parameters RPq (low mass cut-off) and the reaction gas flow rate were set at the values giving the highest signal-to-noise ratio. The merits of several reaction gases were compared: methane, carbon monoxide, ammonia, oxygen and the combination of argon (collision gas) and hydrogen (reaction gas). The LODs obtained with these reactions

gases, at the optimised DRC parameters, are given in Table 5.5. The order of the LODs is the same as those reported by Hattendorf, but CO, which was not used by this group, proved to give a lower LOD than methane. Logically, the most abundant Se isotope, ^{80}Se, offered the lowest LOD.

The reaction mechanism is a charge transfer reaction:
$CO + ArAr^+ \rightarrow CO^+ + ArAr$
which is thermodynamically allowed, since:
IE(CO) < IE(ArAr)
where IE stands for ionisation energy.
(and goes further with $ArAr \rightarrow 2\ Ar$)

Se cannot react with CO, since:
IE(Se) < IE(CO)

These experiments were repeated in a chlorine-rich matrix, to evaluate the utility of this approach in the case a non-denaturing electrophoresis with a chlorine-based buffer. The lowest LOD was still obtained with CO, but it was increased fivefold due to signal suppression.

Table 5.5: Optimised DRC settings and limits of detection for selenium.

ICP-MS instrument	*Elan DRC plus*
Data acquisition	
Sweeps per reading	10
Readings per replicate	1
Replicates	10
Ar flow rates	
Plasma	17.0 L min^{-1}
Auxiliary	1.2 L min^{-1}
Nebuliser	1.0 L min^{-1}
RF Power	1200 W

Gas	Flow rate		RPq	LOD in 10 mL HNO_3 L^{-1}					
	mL min^{-1}			ng L^{-1}					
	Elan units	absolute		$^{77}Se^+$	$^{80}Se^+$	$^{82}Se^+$	$^{77}Se^{16}O^+$	$^{80}Se^{16}O^+$	$^{82}Se^{16}O^+$
CO	0.40	0.28	0.45	$2.5\ 10^2$	**$1\ 10^1$**	$1\ 10^2$			
CH_4	0.40	0.23	0.45	$1\ 10^3$	$2.5\ 10^1$	$2\ 10^2$			
$Ar + H_2$	0.20 + 0.40	0.20 + 0.29	0.65	$4\ 10^2$	$1\ 10^2$	$3\ 10^2$			
O_2	0.40	0.28	0.45	$2\ 10^2$	$3\ 10^2$	$3\ 10^2$	$1\ 10^4$	$2\ 10^2$	$9\ 10^2$
NH_3	0.40	0.22	0.45	$2\ 10^2$	$7\ 10^2$	$1\ 10^2$			
Standard	0		0.25	$2\ 10^2$	/	$9\ 10^1$			

5.3. Laser ablation – ICP-MS

5.3.1. Introduction

Laser ablation is the most recent method for the detection of trace elements in gels after electrophoresis. This method is a solution to extend the application range of this fractionation approach to 'cold' isotopes, *i.e.*, non-radioactive isotopes.

An alternative had to be found to electrothermal vaporisation – ICP-MS, which shows limitations: a rapid screening of a two-dimensional gel, with hundreds of spots, is impossible because the analysis of a single spot already lasts 4 min. Laser ablation is able to overcome this shortcoming.

This chapter is divided in two parts: first, a study about the figures of merit of this combined method; second, the application of the method to real samples, in the frame of the speciation of selenium among proteins, using the dynamic reaction cell technology in order to improve the detection limit of this typically difficult element for ICP-MS detection.

5.3.2. Experimental

5.3.2.1. Sample preparation for 1DE and 2DE

The glutathione peroxidase extract, a lyophilisate extracted from red blood cells and partially purified, and the selenised yeast extract were first denatured with a non-reducing SDS-treatment (thus without DTT, see 2.1.5.2.1). The proteins were subsequently derivatised by treatment with iodoacetic acid, in order to prevent the degradation of the side-chain of the Se-amino acids, especially Se-cysteine (see 4.2.2.1.4).

5.3.2.2. Instruments

5.3.2.2.1. Electrophoresis material and apparatus

The IPGphor unit for isoelectric focusing with immobilised pH gradient, the horizontal electrophoresis unit (Multiphor II electrophoresis system) for SDS-PAGE, the SDS-buffers, all polyacrylamide gels:

- for matrix matching, blank and standards: CleanGel 25S, *T* = 10 % and *C* = 2 %; this type of gel is in a dry form and can be hydrated in the solution of choice;
- for the separations: Homogeneous 15, *T* = 15 % and *C* = 3 % in the separation zone,

as well as the gel staining unit (Hoefer automated gel stainer) were from Amersham Pharmacia Biotech.

All separations were carried out following standard procedures as described by the manufacturer (see 2.1.5.2.2 for SDS-PAGE and 2.1.5.2 for 2DE). After separation, the gels were stained, left to dry overnight and packed in a Mylar film to protect them against contamination until analysis.

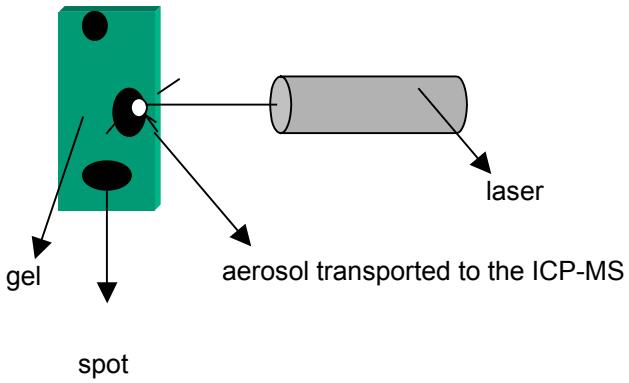
5.3.2.2.2. Laser Ablation

Two 193-nm ArF laser instruments were used throughout this work: a MicroLas C System at the ETH Zurich and a GeoLas System at Ghent University. It is worth noticing that the ablation cell is mounted on a stage that allows translation, monitored by a computer, in all three spatial directions and that the cell is directly monitored by a camera connected to the same computer, making it easy to change the ablation site on a gel. Further, a translation speed can be programmed, allowing the ablation of the sample in its length, in our case the ablation of the gel in the direction of migration. Figure 5.15 illustrates the two ablation procedures used in this work.

5.3.2.2.3. Detection of the trace elements: ICP-MS

The laser ablation system was coupled to an Elan DRC *plus* (PerkinElmer-SCIEX) quadrupole-based ICP-MS. This instrument is equipped with a dynamic reaction cell. Selective ion-molecule chemistry in this cell allows the drastic reduction of interferences that the analyte might suffer from. In our application, this technology permits the elimination of the $^{40}Ar^{40}Ar^{+}$ ion that interferes with the determination of Se via the signal of $^{80}Se^{+}$. The principle of the DRC is given 3.1.2.2.2.1.

a. Ablation on one spot, single hole drilling. Most relevant for 2DE.



b. Ablation with translation. Most relevant for 1DE.

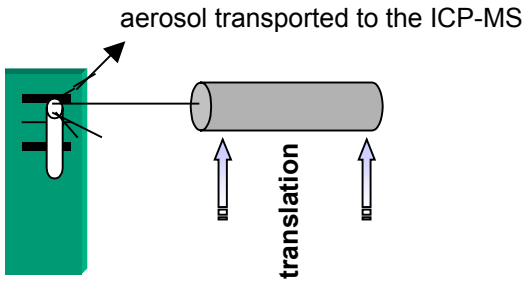


Figure 5.15: Schematic of laser ablation

5.3.3. Results

5.3.3.1. Applicability of the method

In the first part of this work, the applicability of LA-ICP-MS to the detection of trace elements in gels was systematically assessed for the elements Li, V, Cr, Mn, Ni, Cu, Zn, As, Se, Mo, Pd, Ag, Cd, Pt, Tl, Pb. These elements were chosen because of their importance in the field of speciation in biological samples. For completion, some data have also been acquired for Al, Fe, Co, Ga, Sr and Ba and will be shown here. In order to determine the figures of merit of the detection technique, LA-ICP-MS, two sets of samples were necessary, namely a blank and a standard. First, the blank is readily available in the form of a piece of CleanGel, a pristine polyacrylamide gel without buffer, soaked in a 10 mL HNO_3 L^{-1} solution and, second, the standard, in the form of a piece of CleanGel, soaked in a 10 mL HNO_3 L^{-1} containing the target analyte at the desired concentration.

5.3.3.1.1. Contamination of the gels with trace elements

In order to assess the presence of contaminants in the gels and buffers necessary for electrophoresis, a 'blank' separation was performed. A standard SDS-PAGE procedure was followed, but without bringing any sample on the gel. After separation, an aliquot of this latter gel, as well as a part of a pristine gel, were mineralised using a microwave-assisted digestion in acid (50 % HNO_3, 33 % H_2O, 17 % H_2O_2). The sample solution obtained was analysed with ICP-MS with a pneumatic nebuliser as the introduction system. No element of interest was found to be above the detection limit of the instrument. Such a gel is thus readily useable for analysis with LA-ICP-MS without significant contribution from contamination.

5.3.3.1.2. Hydration of the gels with standard solutions

Once the blank was checked, the reliability of the standard had to be estimated. It had to be proved that no partitioning occurred between the element in solution and in the gel, in other words that the gel contained the analyte at the desired concentration after it was hydrated. To do so, a gel was hydrated in the standard solution and after this hydration, it was mineralised. To the same amount of gel, the expected quantity of the target analyte was added, without hydration this time, and this mixture was directly mineralised. The two solutions were measured using PN-ICP-MS. No significant difference in ICP-MS signals was observed between both solutions, (except for Zn, with a recovery in the hydrated gels of 120 %), indicating that no partition occurred during hydration. The problem with Zn can either be due to contamination during the sample preparation, which seems unlikely on the basis of the experiments reported on earlier, or the consequence of a higher affinity of Zn for the polyacrylamide matrix.

5.3.3.1.3. Figures of merit: detection of metals in gels with LA-ICP-MS

Three characteristics of this combined method were quantified, namely the limit of detection, the linearity and repeatability. The parameters used for the measurements are summarised in Table 5.6. All these experiments were performed at the ETH Zurich.

Table 5.6: Measurements conditions for the determination of the figures of merit of the method. All measurements were done at the ETH Zurich.

ICP-MS instrument	*Elan DRCplus*	
RF Power	1470 W	
Ar flow rates		
Plasma	15 L min^{-1}	
Auxiliary	0.75 L min^{-1}	
Ar make-up gas	0.82 L min^{-1}	
Data acquisition		
Scanning mode	Peak hop	
Dwell time per acquisition point	10 ms	
Sweeps per reading	1	
Readings per replicate	1	
Laser ablation system	*193-nm ArF laser, MicroLas C*	
	Without translation	Translation, 20 µm s^{-1}
Crater diameter	120 µm	120 µm
Repetition rate	5 shots s^{-1}	10 shots s^{-1}
Energy	100 mJ	120 mJ
Fluence	15 J cm^{-2}	17 J cm^{-2}
He (transport gas) flow rate	1.3 L min^{-1}	1.3 L min^{-1}

5.3.3.1.3.1. Limits of detection (LOD)

The methodology followed to determine the LOD was as follows. Blank gels were ablated and the standard deviation calculated on ten signals. The sensitivity was determined by the ablation of gels hydrated with a known amount of the analyte.

The LOD was then calculated with the 3 x SD criterion (SD: standard deviation on the ten blanks). In the case of ablation of one spot, an estimation of the absolute LOD is also given. It was calculated by assuming a removal by ablation of the whole thickness of the gel, with a constant crater diameter of 120 µm. This absolute LOD, in pg, is thus simply the LOD in µg analyte g^{-1} gel multiplied by the amount of gel and solution in a cylinder of 500 µm height and 120 µm diameter. All LODs are in the fmol range, from 5 10^{-2} fmol for ^{95}Mo to 90 fmol for ^{77}Se.

In order to check the relevance of the LODs determined in this way, gels were ablated with a concentration ten times as high as the LOD without translation. With 95 % confidence, the signal given by the ablation for all elements was significantly higher than the signal given by the ablation of blank gels. The LODs are presented in Table 5.7.

The limits of detection determined here are typical of an ICP-MS detection. Elements that are not interfered and / or have a low ionization energy, like lead or

thalium, offer the lowest LODs. Elements that are interfered and / or have a high ionization energy, like selenium, have poorer LODs.

Table 5.7: LODs of trace elements detected in gels by LA-ICP-MS.
LOD determined using the 3 x SD criterion (SD: standard deviation on ten blanks).

Isotope	LOD No translation		LOD Translation
	µg analyte g^{-1} gel	pg	µg analyte g^{-1} gel
^{7}Li	$4\ 10^{-2}$	$2\ 10^{-1}$	$3\ 10^{-3}$
^{51}V	$1\ 10^{-2}$	$5\ 10^{-2}$	$3\ 10^{-3}$
^{53}Cr	$2\ 10^{-1}$	$9\ 10^{-1}$	$6\ 10^{-2}$
^{55}Mn	$7\ 10^{-3}$	$4\ 10^{-2}$	$2\ 10^{-1}$
^{60}Ni	$8\ 10^{-2}$	$4\ 10^{-1}$	$4\ 10^{-3}$
^{62}Ni	$4\ 10^{-2}$	$2\ 10^{-1}$	$9\ 10^{-3}$
^{63}Cu	$5\ 10^{-2}$	$2\ 10^{-1}$	$3\ 10^{-3}$
^{65}Cu	$2\ 10^{-2}$	$9\ 10^{-2}$	$4\ 10^{-3}$
^{66}Zn	$4\ 10^{-1}$	2	1
^{67}Zn	$4\ 10^{-1}$	2	$7\ 10^{-1}$
^{75}As	$2\ 10^{-2}$	$9\ 10^{-2}$	$4\ 10^{-2}$
^{77}Se	1	7	$1\ 10^{-1}$
^{82}Se	$1\ 10^{-1}$	$6\ 10^{-1}$	$7\ 10^{-2}$
^{95}Mo	$1\ 10^{-3}$	$5\ 10^{-3}$	$1\ 10^{-3}$
^{105}Pd	1	5	$7\ 10^{-3}$
^{107}Ag	$4\ 10^{-2}$	$2\ 10^{-1}$	$9\ 10^{-3}$
^{109}Ag	$3\ 10^{-2}$	$2\ 10^{-1}$	$9\ 10^{-3}$
^{114}Cd	$9\ 10^{-2}$	$4\ 10^{-1}$	$4\ 10^{-3}$
^{195}Pt	$8\ 10^{-4}$	$4\ 10^{-3}$	$1\ 10^{-3}$
^{205}Tl	$2\ 10^{-3}$	$1\ 10^{-2}$	$4\ 10^{-4}$
^{208}Pb	$2\ 10^{-2}$	$1\ 10^{-1}$	$3\ 10^{-3}$

5.3.3.1.3.2. Repeatability

Repeatability over 10 ablations was also assessed, using the $^{13}C^{+}$ signal as an internal reference to correct for difference in ablation efficiencies, plasma instabilities, etc. All RSDs were between 12 and 19 % (see Table 5.8), which is not excellent, but the use of another internal reference is difficult, if not impossible in practice. Yttrium, an element which is hardly encountered in biological samples, could normally fulfill this role. However, this internal reference should be homogeneously distributed in the gel. If it is not ^{13}C, it has to be added to the gel, but it is most difficult to add an element homogeneously into a gel, before or after electrophoresis. For example, should Y be added before electrophoresis, the risk is high that it would migrate and its concentration build up in certain areas of the gel. After electrophoresis, if an

element is added by soaking the gel in a solution, the proteins could show a higher affinity for Y than the gel background, increasing once again the Y concentration in certain areas of the gel.

5.3.3.1.3.3. Linearity and quantification

The linearity expressed as R^2 was calculated using 0.5, 1, 2, 5 and 10 µg g^{-1} solutions, with ten ablations at each concentration. As can be seen in Table 5.8, the linearity is once again linked to the ability of the instrument to efficiently detect the element and shows the same trend as for the LOD. Since R^2 is close to unity for all elements investigated here, except for an interfered element like Se (R^2 = 0.95), quantification seems possible. A reference material, a NIST 610 glass containing all the target analyte at known concentrations, was used for external calibration: the glass and the hydrated gel were placed in the ablation cell, the signals given by the ablation of the gel and the reference material recorded and compared. Even with such a non-matrix-matched calibration, a satisfactory recovery of 90 to 110 % was calculated for all those elements.

Table 5.8: Correlation and RSD.
Correlation calculated between signals given by 0.5, 1, 2, 5, 10 µg analyte g^{-1} gel.
RSD calculated for 10 µg analyte g^{-1} gel on 6 signals. Note that the higher the concentration, the lower the RSD (at 50 µg analyte g^{-1} gel, RSD (Pb) = 11 %).

Isotope	R^2	RSD (%)	Isotope	R^2	RSD (%)
^{7}Li	0.990	15	^{71}Ga	0.9992	
^{27}Al	0.995	14	^{75}As		19
^{51}V		15	^{77}Se	0.97	
^{53}Cr	0.9999		^{82}Se	0.97	15
^{55}Mn	0.99993	15	^{88}Sr	0.9997	17
^{57}Fe	0.9998		^{107}Ag	0.9998	
^{59}Co	0.9998	14	^{109}Ag	0.99990	
^{62}Ni	0.999	16	^{114}Cd	0.999	
^{65}Cu	0.997	15	^{138}Ba	0.9998	18
^{66}Zn	0.996		^{205}Tl	0.9998	
^{69}Ga	0.9993		^{208}Pb	0.99999	17

5.3.3.1.4. Conclusion

From those preliminary experiments, it can be concluded that LA-ICP-MS is an attractive method for the determination of various trace elements like Li, V, Cr, Mn, Ni, Cu, Zn, As, Se, Mo, Pd, Ag, Cd, Pt, Tl, Pb. The LODs are low enough for the

detection of these elements in a variety of biological samples. The semi-quantitative aspect of the method is established but quantification does not lie far away since an ideal matrix matching is available in this case. This matrix matching is simply the gel, polyacrylamide hydrated with the target analyte, as the standards used in our study.

5.3.3.2. Application: Se in red blood cells and Se in yeast

As mentioned above, the detection of Se with ICP-MS suffers from argon-based interferences. This reason, plus the fact that its ionisation potential is relatively high, explains why its LOD is relatively poor. In order to solve the former problem, the DRC technology had to be applied (see 5.2).

5.3.3.2.1. Ablation parameters and LOD with PAGE-LA-ICP-DRC-MS

The optimised DRC settings with CO as determined in 5.2.2 were applied to the detection of Se in gels. Since Se is covalently bound to the proteins to be analysed, silver staining is allowed in order to detect the proteins before ablation. Hence, for realistic determination of the LOD, a different blank had to be used: a polyacrylamide gel soaked in an albumin solution and silver stained, containing thus all chemicals as in a real gel after separation. This blank is thus perfectly matrix-matched. The standard used in the following is this type of gel, an originally blank gel, hydrated in a Se solution. The various ablation parameters, summarised in Table 5.9, were chosen so that the highest Se signal could be obtained (for ablation energy and translation speed) or the lowest LOD obtained (for the ablation repetition rate and transport gas). Helium and argon were compared as transport gases since it was already reported that helium offered a better transport efficiency than argon [Günther, 1999].

Our observations can be summarised as follows:

- The ablation energy does not seem to influence the signal. This is probably due to the ease with which such a soft material is ablated. The ablation threshold is very low and increasing the energy has no further effect on the ablation efficiency.
- The higher the ablation repetition rate, the lower the LOD (the higher the sensitivity). This dependence is grossly linear.
- The use of He as the transport gas (still with Ar as plasma gas) improves the LOD by a factor of at least 2 in comparison to Ar.

- The LODs obtained with translation are systematically poorer than those with the ablation on a single spot. The nature of the sample seems to play a determining role in this case. Since silver from the staining is present mainly at the surface, the ablation of the surface causes a more pronounced signal instability than the ablation deeper in the gel. This instability directly increases the standard deviation between ablations on the surface, with translation, and the consequence is a poorer LOD.

Table 5.9: Optimised ablation parameters for Se.

ICP-MS instrument	*Elan DRC plus*
RF power	1200 W
Ar flow rates	
Plasma	17.0 L min^{-1}
Auxiliary	1.2 L min^{-1}
Ar make-up gas	1.0 L min^{-1}
DRC	
CO (reaction gas) flow rate (Elan units)	0.4 mL min^{-1}
Rpa	0.00
RPq	0.45
Data acquisition	
Scanning mode	Peak hopping
Dwell time per acquisition point	50 ms
Sweeps per reading	10
Acquisition points per spectral peak	1
Signals monitored	$^{13}C^+$, $^{23}Na^+$, $^{77}Se^+$, $^{80}Se^+$, $^{82}Se^+$, $^{107}Ag^+$
Laser Ablation	*193-nm ArF laser, GeoLas*
He (transport gas) flow rate	0.75 L min^{-1}
Repetition rate	20 shots s^{-1}
Energy	80 mJ
Translation speed	30 µm s^{-1}

With translation, an LOD of 0.15 µg Se g^{-1} gel was obtained, while for the ablation on one spot, the LOD was equal to 0.07 µg Se g^{-1} gel (3 x SD criterion, SD standard deviation on a blank signal at least 150 s long). Without DRC, in other words by measuring $^{82}Se^+$ in the standard mode, the LODs using the same definition, with the same matrix, were respectively 0.6 and 0.7 µg Se g^{-1} gel. The improvement offered by the DRC is thus of an order of magnitude. It is related to the isotopic abundance of the measured isotopes, ^{80}Se (49.7 % relative abundance in nature) instead of ^{82}Se (9.2 %).

Note that $^{13}C^+$, $^{23}Na^+$, and $^{107}Ag^+$ were monitored in order to follow the ablation process. A high $^{107}Ag^+$ signal proves that the ablation occurs at the site of the protein, $^{13}C^+$ and $^{23}Na^+$ are good indicators of the ablation depth.

5.3.3.2.2. 1DE: glutathione peroxidase in red blood cells

The first application of the method was done in combination with 1DE. To our knowledge, glutathione peroxidase (GSH-Px) is the only selenoprotein commercially available. It is obtained by partial purification from red blood cells. Figure 5.16 shows the ablated gel as well as the Se signal as given by LA.

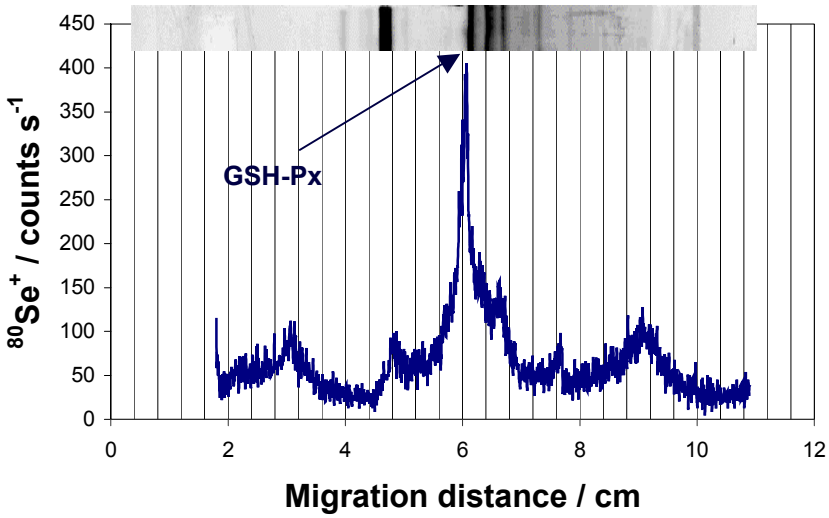


Figure 5.16: Detection of Se after 1DE (SDS-PAGE, anode at the right).
Sample: GSH-Px extract from red blood cells, represents in total 6.75 pg Se.

On this gel 6.75 pg Se were present in the fractionated GSH-Px sample. The gel, with a migration length of 11 cm, had to be cut in three to be inserted into the ablation cell, which has a diameter of 4 cm. GSH-Px was identified on the gel by comparing its migration distance with molecular mass markers. The Se signal is thus in fact the Se electropherogram. At a migration distance of 6 cm, the Se peak from GSH-Px is clearly seen. The peak at 9 cm is probably due to the partial decomposition of selenocysteine into low molecular weight selenium compounds and the peak at 3 cm comes from high-molecular-mass compounds that were too big to go from the stacking gel to the separation gel. The peaks at 4.9 and 7.7 cm are artifacts, due to the opening of the cell to change the gel portions; a correction with ^{13}C as internal reference was not successful in solving this problem.

5.3.3.2.3. 2DE: Se in yeast

This detection method was also applied to 2DE. As can be seen from Figure 5.17, the acquisition for one spot only lasts a couple of seconds, the restricting parameter being the displacement of the sample to another ablation site and subsequent focusing, which takes a time typically of the order of 30 s. The picture taken in Figure 5.16 is the actual gel after ablation, with the interesting feature that the ablation craters are hardly to be seen. In fact, the formation of a crater of 120-µm diameter is hardly noticeable in the protein spots, which show a diameter of a couple of mm. This method leaves thus the majority of the material available for further analysis, *e.g.*, for the determination of the protein of interest by MS.

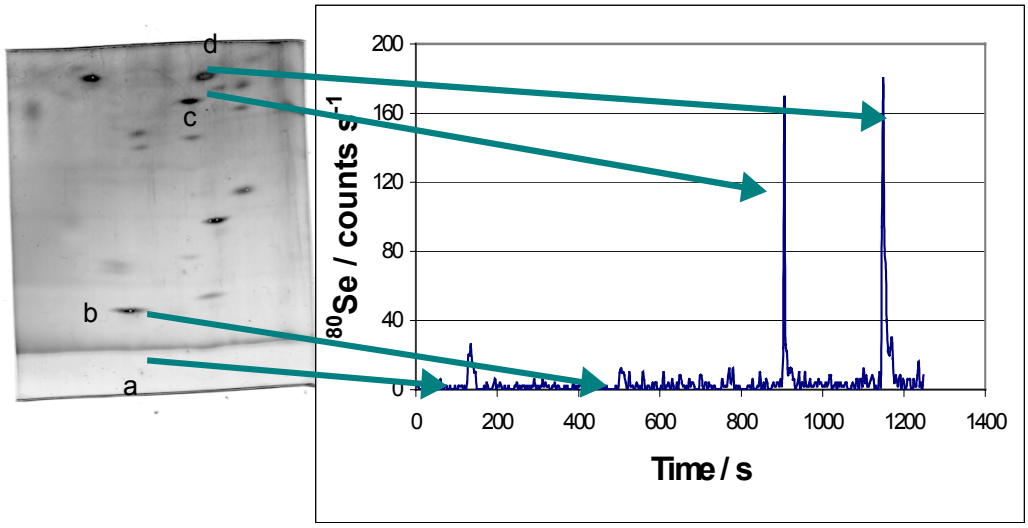


Figure 5.17: 2DE of selenised yeast. The actual part of the gel which was analysed is shown (3 cm x 3.5 cm; pI *ca*. 5-7; M_r < 30 kDa; (+,+) bottom right).
a: beyond the front, no protein present
b: protein containing no Se
c and d: two Se-containing proteins

5.3.4. Conclusions

This work has demonstrated that the off-line detection of trace elements present in gel after electrophoresis is possible with laser ablation – ICP-MS. Some elements may still be difficult to detect, due to spectral interferences, but the dynamic reaction cell technology is able to improve the limit of detection for these elements.

The quantitative aspect of the method is still a problem. Although it would seem that this method is at least semi-quantitative from the study with hydrated gels (see 5.3.3.1.3), the experiments with selenium could not deliver any quantitative results. This is due to the actual design of the ablation cell which does not allow the presence

of a whole gel, the size of the cell allowing the ablation of a piece of gel of maximum 3 cm x 3 cm. Each time the cell must be opened, a signal drift is recorded. In other words, even if the repeatability is good, the reproducibility seems poorer. The design of a new ablation cell must be considered if quantitative results are expected from the analysis with this method. Automation of the system is also possible, allowing a rapid screening of a given sample.

Nonetheless, this off-line detection is a powerful tool for the study of Se-proteins, especially when applied to 2DE. This method will now be applied to the research towards Se-proteins in samples of human or other mammalian origin, where the type of information sought after is qualitative: does the protein contain selenium or not.

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6. Summary and Conclusions

This work has shown that gel electrophoresis is a relevant fractionation method for trace elements bound to or complexed by macromolecules. Furthermore, detection methods of the trace elements that do not rely on the use of radiotracers were applied, making this combination relevant for materials of human origin, where the use of radioactive material is restricted for reasons of safety.

6.1. Slab gel electrophoresis as fractionation method for elemental speciation

The first part of this work was dedicated to the applicability of gel electrophoresis as a fractionation method for proteins with bound or complexed trace elements. Two distinct cases were identified: first, the fractionation of trace element – protein complexes (or weak associations between the macromolecule and the trace element), exemplified by the case of vanadium bound to proteins; second the fractionation of proteins where the trace element is covalently bound, exemplified by selenium.

This distinction dictates the fractionation mechanism that has to be used, more precisely whether denaturation can be applied. Denaturation, which is achieved with detergents and chaotropes, is normally used for a better solubilisation and more reproducible separation of the proteins. Because such a step has as consequence the loss of the tertiary structure of the proteins, and thus the loss of the active centre responsible for the complexation of the element, it is incompatible with the analysis of complexes.

At this stage of the method development, radiotracers were used for the detection of the trace elements, in combination with autoradiography, more specifically the phosphor-screen technology.

6.1.1. Native fractionation

Vanadium was chosen as the trace element in order to optimise the fractionation method, since it is known that the complexes it forms with proteins, especially transferrin, are among the weakest in comparison with other metal-transferrin complexes. The use of Coomassie blue, already described for non-denaturing separations of enzymes, where it is important to preserve the tertiary

structure of the enzyme, was explored. It could be proved that this chemical allowed the efficient separation of the proteins in a complex sample such as serum, without drastic loss of resolution in comparison with the reference fractionation method, sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE).

This method using Coomassie blue is an adaptation of the blue native – polyacrylamide gel electrophoresis separation, an adaptation necessary to ensure the stability of the vanadium-protein complex. Coomassie blue was used to mask the charges of the proteins for a separation towards the anode and to stabilise the vanadium complex. The detection of the vanadium species was made possible by the use of the ^{48}V radiotracer and the phosphor-screen technology.

The method was first developed using transferrin, incubated with ^{48}V, as a model. After it was proved that the vanadium-transferrin complex was stable during separation, the method was used for the fractionation of serum incubated with ^{48}V.

The efficiency of the separation was assessed according to two parameters: resolution and conservation of the species. First, the resolution of the separation was as expected from a native separation. Second, the release of free vanadium from the transferrin complex, which was the main vanadium species expected, was negligible, which proves that the species are kept intact during separation.

In accordance with the literature, it was found that vanadium binds to transferrin in incubated serum at these low concentrations.

6.1.2. Denaturing fractionation

Selenium as a trace element can be covalently bound to proteins, in the form of the amino-acids selenomethionine or selenocysteine. The nature of the chemical bound between selenium and the protein allows the denaturation of the protein prior to gel electrophoresis. Not only is SDS-PAGE allowed, but also two-dimensional high-resolution gel electrophoresis (2DE).

2DE was applied to the fractionation of ^{75}Se-containing proteins in yeast, grown in ^{75}Se-containing medium, and autoradiography was used for the detection of the ^{75}Se-containing proteins. It was checked by means of gel filtration and ultrafiltration whether the selenium side-chains were stable in the presence of the chemicals used for lysis and 2DE. Since it was not the case, a carboxylation of the side-chains had to be implemented in order to protect them from oxidation. The mass distribution of the selenium-containing proteins was estimated by applying gel filtration and the results were compared with the distribution obtained by 2DE. A 2DE

map of selenium-containing proteins in yeast is presented in comparison with a total protein map of yeast.

6.2. Detection of the trace elements after slab gel electrophoresis without radiotracers

Although radiotracers offer definite advantages, among other things low detection limits and contamination-free analysis, other detection methods were explored in order to be able to analyse samples of human origin. The most logical choice was thus solid sampling techniques. For a gel, a polymer, the two applicable methods are electrothermal vaporisation and laser ablation in combination with ICP-MS (inductively coupled plasma – mass spectrometry) for the most sensitive detection. Both were used in this work for the detection of selenium after gel electrophoresis.

6.2.1. Electrothermal vaporisation – ICP-MS

This technique requires a careful optimisation of the various vaporisation parameters, among other things the temperature and the possible use of a modifier, but allows the precise and sensitive detection of the trace element in the gel.

After separation, the protein bands are excised from the gel and brought one by one to the oven of the ETV (electrothermal vaporisation). External standardisation with the use of an internal reference (Te) was applied. A detection limit of *ca*. 40 pg Se per band and a complete recovery were obtained. A single measurement, *i.e.* the measurement of the amount of selenium in a protein band, is accomplished in less than 4 min and thus a gel lane, after a separation of *ca*. 1.5 h, can be entirely analysed with ETV-ICP-MS in 3.5 h. If limited to 10 bands, a gel lane is analysed in 2 h (calibration included). The analysis is directly carried out on the stained gel, without blotting, which makes the analysis even more practical.

This method was optimised using the selenoprotein glutathione peroxidase as model. Then, it was applied to the fractionation of proteins from a selenium-yeast candidate reference material. It was then possible to reconstruct the Se-electropherograms and to compare these with the stained gels. The major

advantages of this method are the high resolution of the protein fractionation and the straightforward quantification of selenium.

Application to fractionation as given by 2DE is, however, difficult. Thousands of protein spots are present on a gel and the analysis would thus be too time-consuming. That is why LA-ICP-MS (laser ablation – ICP-MS) was explored as the detection method.

6.2.2. Laser ablation – ICP-MS

6.2.2.1. Capability of the method

The capabilities of laser ablation – ICP-MS for the detection of trace elements in a gel after gel electrophoresis were systematically studied. Figures of merit such as limit of detection, linearity and repeatability were evaluated for various elements (Li, V, Cr, Mn, Ni, Cu, Zn, As, Se, Mo, Pd, Ag, Cd, Pt, Tl, Pb). Two ablation strategies were followed: ablation on a single spot, relevant for ablation of spots after two-dimensional separations, and ablation with translation, following a line, relevant for one-dimensional separations, along a whole gel strip.

6.2.2.2. Improvement of the limit of detection

Since only a minute amount of material is effectively ablated and brought to the plasma, the detection procedure had to be further improved by using the dynamic reaction cell technology (DRC). It was first optimised for the detection of vanadium and then for the detection of selenium.

6.2.2.2.1. Vanadium

The dynamic reaction cell (DRC) technology was applied in the context of the speciation analysis of vanadium in serum, with size-exclusion as the fractionation method as a first step. The goal is then to move away from size-exclusion to apply gel electrophoresis in the near future. The DRC technology was necessary in order to detect vanadium on-line after size-exclusion chromatography with a buffer at physiological salinity (0.15 M NaCl). This salinity was compulsory to assure the stability of the vanadium compounds during chromatography, in other words to prevent inter-species conversion. In fact, the DRC allowed the detection of vanadium without adapting the conditions of the chromatographic separation to ICP-MS.

Neither will an adaptation be necessary with native gel electrophoresis, using chlorine based chemicals.

First, the merits of various reaction gases were compared: methane, carbon monoxide, ammonia, oxygen and the combination of argon (collision gas) and hydrogen (reaction gas). In each instance, the reaction cell parameters were optimised in order to obtain the lowest detection limit for ^{51}V (as $^{51}V^{+}$ or as $^{51}V^{16}O^{+}$ with O_2 as the reaction gas) in chlorine-rich solution, Cl being the parent element of the $^{35}Cl^{16}O^{+}$ interference. Ammonia was found to offer the best detection limit (3 x SD criterion, 10 ng L^{-1} with pneumatic nebulisation as the sample introduction system).

The detection limit with size-exclusion chromatography ICP-DRC-MS for vanadate, expected to be the worst among all vanadium chemical species, was found to be 4 pg V and the repeatability 7 %.

This on-line separation method was used in order to speciate vanadium in serum after incubation with vanadate, at a concentration level that is representative of the pharmacological concentration range of vanadium when used as an insulin-like agent. The results on the speciation of vanadium in serum obtained by size-exclusion – ICP-DRC-MS also confirmed the results obtained by gel electrophoresis with a vanadium radiotracer, namely the presence of a major vanadium-transferrin complex.

6.2.2.2.2. Selenium

The DRC technology allowed the removal of the Ar_2^{+} interference and hence the use of the most abundant Se isotope, ^{80}Se. The same reaction gases as for vanadium were compared [methane, carbon monoxide, ammonia, oxygen and the combination of argon (collision gas) and hydrogen (reaction gas)]. In each instance, the reaction cell parameters were optimised in order to obtain the lowest detection limit for Se (as $^{80}Se^{+}$, $^{82}Se^{+}$ or $^{77}Se^{+}$; and as $^{80}Se^{16}O^{+}$, $^{82}Se^{16}O^{+}$ or $^{77}Se^{16}O^{+}$ with O_2 as the reaction gas). Carbon monoxide was found to offer the best performance.

6.2.2.3. Application to selenium

This technique was applied to the detection of selenoproteins in red blood cells extracts after a one-dimensional separation (SDS-PAGE) and the detection of selenium-containing proteins in yeast after two-dimensional electrophoresis (2DE).

The detection limit with the use of DRC and He as transport gas was found to be 70 ng Se g^{-1} gel for ablation on one spot (relevant for 2DE) and 150 ng Se g^{-1} gel for ablation with translation (relevant for 1DE).

6.3. Perspective

The figures of merit for gel electrophoresis qualify this method as an integral part of the fractionation techniques for elemental speciation analysis, its main assets being its high resolution and the various separation mechanisms it offers. A gel can be stored for years after separation, which allows further analysis with methods such as tandem mass spectrometry. It was demonstrated that gel electrophoresis could be combined with methods such as laser ablation – ICP-MS for the detection of the trace elements, allowing the analysis of samples of human origin.

Various samples can now be analysed using the methods optimised in this work. Gel electrophoresis will be further applied, as well as other separation techniques such as size-exclusion chromatography as control, for the analysis of:

- protein extracts to determine the presence of selenium-containing proteins;
- samples of human origin, such as serum, analysed for the speciation of vanadium; an interesting question is to know whether vanadium, present in one of its reduced forms, vanadyl, is transported by transferrin or albumin; the prerequisite for this type of work is to perform the separation under a non-oxidising atmosphere;
- samples from patients to whom vanadium compounds were administrated; the goal is to determine the metabolic fate of these compounds.

These studies, however, will have to rely on other methods, such as tandem mass spectrometry, to investigate the identity of the compounds of interest. The electrophoretic migration distance, although an information about the identity of a compound, is surely not enough to fully characterise a compound.

7. Samenvatting en Besluit

Dit werk heeft aangetoond dat gelelektroforese een geschikte fractioneringsmethode is voor spoorelementen gebonden aan of gecomplexeerd door macromoleculen. Verder werden detectiemethodes voor spoorelementen toegepast die geen gebruik maken van radiotracers. Zulke combinatie maakt het mogelijk om stalen van menselijke oorsprong te analyseren aangezien het gebruik van radioactiviteit beperkt is omwille van veiligheidsredenen.

7.1. Dunne-laaggelelektroforese als fractioneringsmethode voor speciatie van elementen

In het eerste deel van dit werk werd de toepasbaarheid van gelelektroforese als fractioneringsmethode voor eiwitten met erop gebonden of gecomplexeerde spoorelementen nagegaan. Er werd een onderscheid gemaakt tussen twee verschillende gevallen: ten eerste, fractionering van spoorelement-eiwit complexen of zwakke verbindingen tussen de macromolecule en het spoorelement, in het kader van dit werk vanadium gebonden op eiwitten; ten tweede, fractionering van eiwitten waarop het spoorelement covalent gebonden is, in het kader van dit werk seleen.

Dit onderscheid bepaalt de fractioneringstechniek die kan worden aangewend en meer bepaald of denaturatie kan worden toegepast. Denaturatie wordt bekomen door het toevoegen van detergenten en chaotrope chemicaliën. Dit wordt toegepast om de eiwitten beter in oplossing te krijgen en een meer reproduceerbare scheiding te garanderen. Omdat deze stap het verlies van de tertiaire structuur van de eiwitten impliceert, en dus ook het verlies van de actieve kooi verantwoordelijk voor de complexering van het element, is het ongeschikt voor de analyse van complexen.

Tijdens deze methodeontwikkeling werden radiotracers gebruikt voor de detectie van de spoorelementen in combinatie met autoradiografie, en meer bepaald de fosforschermtechnologie.

7.1.1. Niet-denaturerende fractionering

In dit geval werd vanadium gekozen als spoorelement voor het optimaliseren van deze fractioneringsmethode. Het is namelijk bekend dat vanadium-eiwit complexen heel zwak zijn, en meer bepaald het vanadium-transferrine complex, in

vergelijking met andere metaal-transferrine complexen. Het gebruik van Coomassie blue werd onderzocht, dat reeds beschreven werd voor het scheiden van enzymen onder niet-denaturerende omstandigheden, waar het belangrijk is om de tertiaire structuur van de actieve kooi van het enzym te behouden. Er kon worden aangetoond dat dit product een efficiënte scheiding van eiwitten toeliet, in een complex monster zoals serum, zonder een drastisch verlies aan resolutie in vergelijking met de referentie fractioneringsmethode, SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis, natrium dodecylsulfaat – polyacrylamide gelelektroforese).

Het gebruik van Coomassie blue in deze methode is een aanpassing van de 'blue native'-polyacrylamidegel-elektroforese scheiding, een aanpassing die nodig is om de stabiliteit van het vanadium-eiwit complex te garanderen. Coomassie blue werd gebruikt om de lading van de eiwitten integraal negatief te maken voor een scheiding in de richting van de anode en om het vanadium-complex te stabiliseren. De detectie van de vanadium verbindingen werd mogelijk gemaakt door het gebruik van ^{48}V radiotracer en de fosforschermtechnologie.

De methode werd eerst en vooral ontwikkeld aan de hand van transferrine, geïncubeerd met ^{48}V. Nadat kon bewezen worden dat het vanadium-transferrine complex stabiel bleef tijdens de scheiding, werd de methode toegepast op serum geïncubeerd met ^{48}V.

De efficiëntie van de scheiding werd beoordeeld op basis van twee parameters: resolutie en behoud van de species. Ten eerste, de resolutie van de scheiding is zoals verwacht onder niet-denaturerende omstandigheden. Ten tweede, het vrijkomen van vanadium uit het transferrine complex, het belangrijkste vanadium complex, was verwaarloosbaar. Dit bewijst dat de complexen intact bleven gedurende de scheiding.

In overeenstemming met de literatuur werd gevonden dat vanadium vooral bindt op transferrine in geïncubeerd serum bij deze lage concentraties.

7.1.2. Denaturerende fractionering

Seleen kan als spoorelement covalent gebonden zijn op eiwitten, in de vorm van de aminozuren selenomethionine en selenocysteïne. De aard van de chemische binding tussen seleen en het eiwit laat een voorafgaande denaturatie toe van het eiwit vóór gelelektroforese. Hierdoor kan niet alleen SDS-PAGE uitgevoerd worden, maar ook tweedimensionale hoge resolutie gelelektroforese (2DE).

2DE werd gebruikt voor de fractionering van ^{75}Se-bevattende eiwitten in gist, gekweekt in een ^{75}Se-bevattend medium, en autoradiografie werd gebruikt als detectie van ^{75}Se-bevattende eiwitten. Door middel van gelfiltratie en ultrafiltratie werd nagegaan of de seleenbevattende zijketens stabiel bleven in de aanwezigheid van producten gebruikt voor lysis en 2DE. Aangezien dit niet het geval was, werden de zijketens gederivatiseerd om hen te beschermen tegen oxidatie. De massadistributie van de seleenbevattende eiwitten werd geschat door het toepassen van gelfiltratie en de resultaten ervan werden vergeleken met de distributie bekomen na 2DE. Een 2DE kaart van de seleenbevattende eiwitten in gist wordt voorgesteld in vergelijking met een kaart waarop alle in gist aanwezige eiwitten weergegeven zijn.

7.2. Detectie van de spoorelementen na gelektroforese zonder radiotracer

Alhoewel radiotracers zekere voordelen bieden, waaronder lage detectielimieten en niet-gecontamineerde analyses, werden andere detectiemethodes onderzocht om zo ook monsters van menselijke oorsprong te kunnen analyseren. De meest logische keuze waren vast-monsteranalysetechnieken (solid sampling techniques). In het geval van een gel, een polymeer, waren de twee toepasbare methoden elektrothermische vervluchtiging en laserablatie in combinatie met ICP-MS (inductief gekoppeld plasma massaspectrometrie), voor de meest gevoelige detectie. Beide technieken werden in dit werk gebruikt voor de detectie van seleen na gelelektroforese.

7.2.1. Elektrothermische vervluchtiging – ICP-MS

Deze techniek vereist een nauwgezette optimalisatie van de verschillende vervluchtigingsparameters, waaronder de temperatuur en het mogelijk gebruik van een modifier, maar laat een precieze en gevoelige detectie van het spoorelementen in de gel toe.

Na scheiding, worden de banden met eiwitten uit de gel geknipt en één voor één in de oven van de ETV (elektrothermische vervluchtiging) gebracht. Externe standaardisatie met telluur als interne referentie werd toegepast. Een detectielimiet van ongeveer 40 pg Se per band en een volledige opbrengst werden behaald. Een

enkelvoudige meting, d.i. de meting van de hoeveelheid seleen in een eiwitband, wordt uitgevoerd in minder dan 4 min en een volledige gelstrip, na een scheiding van ongeveer 1,5 u, kan geanalyseerd worden met ETV-ICP-MS in 3,5 u. In het geval dat niet meer dan 10 eiwitbanden aanwezig zijn in een gelstrip, kan dit gedaan worden in 2 u, kalibratie inbegrepen. De analyse wordt onmiddellijk uitgevoerd op de gekleurde gel, zonder blotting, hetgeen de analyse nog praktischer maakt.

Deze methode werd geoptimaliseerd door gebruik te maken van het seleeneiwit glutathione peroxidase als model. Vervolgens, werd het toegepast op de fractionering van eiwitten in een kandidaat referentiemateriaal (seleenbevattende gist). Het was dan mogelijk om de Se-elektroferograms te reconstrueren en deze te vergelijken met gekleurde gels. De belangrijkste voordelen van deze methode zijn de hoge resolutie van de eiwitfractionering en de directe kwantificatie van het seleen.

Toch is deze techniek moeilijk toepasbaar bij 2DE scheidingen, omdat duizenden eiwitten aanwezig zijn op de gel en dat een analyse daardoor enorm tijdrovend zou zijn. Daarom werd LA-ICP-MS (laserablatie – ICP-MS) ook getest als detectiemethode.

7.2.2. Laserablatie – ICP-MS

7.2.2.1. Mogelijkheden van de methode

De mogelijkheden van laserablatie – ICP-MS voor de detectie van spoorelementen in een gel na gelelektroforese werden systematisch onderzocht. De eigenschappen zoals detectielimiet, lineariteit en herhaalbaarheid, werden geëvalueerd voor verschillende elementen (Li, V, Cr, Mn, Ni, Cu, Zn, As, Se, Mo, Pd, Ag, Cd, Pt, Tl, Pb). Twee ablatiestrategieën werden gevolgd: puntablatie op eenzelfde plaats, relevant voor de ablatie van spots na 2DE scheidingen, en lijnablatie, relevant voor de ablatie van een volledige gelstrip.

7.2.2.2. Verbetering van de detectielimiet

Aangezien slechts een heel kleine hoeveelheid materiaal effectief geableerd wordt en naar het plasma gebracht wordt, werd de detectieprocedure verder verbeterd door het gebruik van de dynamische reactiecel technologie (DRC). Deze werd eerst geoptimaliseerd voor de detectie van vanadium en nadien voor de detectie van seleen.

7.2.2.2.1. Vanadium

De dynamische reactiecel technologie werd in een eerste stap toegepast in het kader van de speciatieanalyse van vanadium in serum, met gelfiltratie als fractioneringsmethode. Het uiteindelijke doel is gelfiltratie te vervangen door gelelektroforese in de nabije toekomst. De DRC technologie was noodzakelijk om vanadium on-line te detecteren na gelfiltratie chromatografie, met een buffer met fysiologische samenstelling (0,15 M NaCl). Dit zoutgehalte was nodig om de stabiliteit van de vanadiumverbindingen tijdens chromatografie te verzekeren, met andere woorden om interspecies omzetting te voorkomen. De DRC liet de detectie van vanadium toe zonder dat een aanpassing van de chromatografische condities nodig was. Een aanpassing zal evenmin bij niet-denaturerende gelelektroforese nodig zijn, die gebruik maakt van chloorhoudende chemicaliën.

Ten eerste, werd de invloed van verschillende reactiegassen vergeleken: methaan, koolstofmonoxide, ammoniak, zuurstof en de combinatie van argon (als botsingsgas) en waterstof (als reactiegas). Voor alle gevallen werden de reactiecelparameters geoptimaliseerd om zo een laag mogelijke detectielimiet voor ^{51}V (als $^{51}V^+$ of met O_2 als reactiegas) in een chloorrijke oplossing te bekomen. Chloor is immers het precursorelement van de $^{35}Cl^{16}O^+$ interferentie. Ammoniak bood de beste detectielimiet (3 x SD criterium, 10 ng L^{-1} met pneumatische verstuiving als monsterintroductiesysteem).

De detectielimiet met gelfiltratiechromatografie ICP-DRC-MS voor vanadaat, vooraf als de minst gunstige van alle vanadiumspecies verwacht, bedroeg 4 pg V en de herhaalbaarheid was 7 %.

Deze on-line scheidingsmethode werd gebruikt voor de speciatie van vanadium in serum na incubatie met vanadaat, op een concentratieniveau dat vergelijkbaar is met de farmacologische concentratie aan vanadium bij gebruik als een insulineachtige component. De resultaten van vanadium in serum behaald met gelfiltratie – ICP-DRC-MS, bevestigden ook de resultaten bekomen met gelelektroforese, namelijk de aanwezigheid van het belangrijke vanadium-transferrine complex.

7.2.2.2.2. Seleen

De DRC technologie liet de verwijdering toe van de Ar_2^+ interferentie en liet dus ook het gebruik toe van de meest voorkomende seleenisotoop, ^{80}Se. Dezelfde reactiegassen als voor vanadium werden vergeleken [methaan, koolstofmonoxide,

ammoniak, zuurstof en de combinatie van argon (botsingsgas) en waterstof (reactiegas)]. Voor elk geval werden de reactiecelparameters geoptimaliseerd om een zo laag mogelijke detectielimiet voor Se te bekomen (als $^{80}Se^{+}$, $^{82}Se^{+}$ of $^{77}Se^{+}$; en als $^{80}Se^{16}O^{+}$, $^{82}Se^{16}O^{+}$ of $^{77}Se^{16}O^{+}$ met O_2 als reactiegas). Koolstofmonoxide gaf de beste resultaten.

7.2.2.3. Toepassing voor seleen

Deze techniek werd toegepast voor de detectie van seleeneiwitten in het extract van rode bloedcellen na eendimensionale scheiding (SDS-PAGE) en voor de detectie van seleenbevattende eiwitten in gist na tweedimensionale gelelektroforese (2DE).

De detectielimiet bij het gebruik van de DRC en helium als transportgas bedroeg 70 ng Se g^{-1} gel voor de puntablatie (relevant voor 2DE) en 150 ng Se g^{-1} gel voor lijnablatie (relevant voor 1DE).

7.3. Perspectief

De eigenschappen van gelelektroforese bezorgen deze methode zijn plaats tussen de fractioneringstechnieken voor de speciatie van spoorelementen. De belangrijkste voordelen zijn de hoge resolutie en de verschillende scheidingsmechanismen. Deze gels kunnen na scheiding jarenlang bewaard worden en laten een verdere analyse toe met methoden zoals tandemmassaspectrometrie. In dit werk werd aangetoond dat gelelektroforese gecombineerd kan worden met methoden zoals laserablatie – ICP-MS voor de detectie van spoorelementen, hetgeen de analyse van menselijke monsters toelaat.

Verschillende monsters kunnen nu geanalyseerd worden, gebruik makend van de methoden die in dit werk geoptimaliseerd werden. Gelelektroforese zal toegepast worden, in combinatie met andere scheidingstechnieken zoals gelfiltratie als controle, voor de analyse van:

- Eiwitextracten, om de aanwezigheid van seleenbevattende eiwitten na te gaan;
- Monsters van menselijke oorsprong, zoals serum, geanalyseerd voor de speciatie van vanadium; interessant om te weten is of vanadium,

aanwezig in een van zijn gereduceerde vormen, vanadyl, getransporteerd wordt door transferrine of albumine; de voorwaarde voor dit type van experiment is de scheiding uit te voeren onder niet-oxiderende omstandigheden;

- Monsters van patiënten die vanadiumsupplementen toegediend kregen; het doel hiervan is het nagaan van de metabolische activiteit van deze verbindingen.

Deze studies zullen echter moeten steunen op andere methoden, zoals tandemmassaspectrometrie, om de identiteit van deze verbindingen na te gaan. De elektroforetische migratieafstand, nochtans nuttige informatie over de identiteit van een verbinding, is zeker en vast niet voldoende om een verbinding volledig te identificeren.

8. Résumé et Conclusions

Ce travail a démontré que l'électrophorèse sur gel est une méthode de fractionnement applicable aux oligoéléments liés à ou complexés par des macromolécules. De plus, des méthodes de détection de ces mêmes oligoéléments qui ne reposent pas sur l'usage de radiotraceurs (traceurs radioactifs) ont été appliquées. Ces couplages entre l'électrophorèse sur gel et une détection 'froide' permettent d'analyser des échantillons d'origine humaine, où l'usage de composés radioactifs est limité.

8.1. L'électrophorèse sur gel comme méthode de fractionnement pour la spéciation élémentaire

La première partie de ce travail a été consacrée à démontrer l'applicabilité de l'électrophorèse sur gel au fractionnement de protéines auxquelles sont liés ou complexés des oligoéléments. Deux cas distincts ont été identifiés : premièrement, le fractionnement de complexes entre protéines et oligoéléments (ou des associations faibles entre des macromolécules et des oligoéléments), où le cas de complexes protéines – vanadium a été pris en exemple ; deuxièmement, le fractionnement d'oligoéléments avec une liaison covalente avec la protéine, comme dans le cas du sélénium.

Cette distinction impose le mécanisme de fractionnement, plus précisément autorise ou non la dénaturation des macromolécules. La dénaturation, qui est obtenue avec des détergents ou des chaotropes, est utilisée normalement pour obtenir une meilleure solubilisation des protéines et une séparation plus reproductible. Puisqu'une telle opération entraîne la perte de la structure tertiaire de la protéine, et donc la perte du centre complexant l'élément, elle est incompatible avec l'analyse de complexes.

A cette étape du développement de la méthode, des radiotraceurs ont été utilisés pour la détection des oligoéléments, en combinaison avec l'autoradiographie, plus précisément la technologie des écrans à phosphore.

8.1.1. Fractionnement natif

Le vanadium a été sélectionné comme oligoélément afin d'optimiser la méthode de fractionnement. En effet, il est connu que les complexes vanadium-

protéines sont relativement faibles, et plus particulièrement le complexe entre le vanadium et la transferrine en comparaison avec d'autres métallocomplexes de la transferrine. L'utilisation du 'Coomassie blue', déjà décrite pour les séparations non-dénaturantes d'enzymes, où il est important de préserver la structure tertiaire des enzymes, a été étudiée. Il a été possible de prouver que cette molécule permet une séparation efficace des protéines d'un échantillon aussi complexe que le sérum, sans perte notable de résolution en comparaison avec la méthode de fractionnement de référence, l'éléctrophorèse sur gel polyacrylamide avec dodécylsulfate de sodium (SDS-PAGE).

Cette méthode faisant appel au Coomassie blue est une adaptation de la méthode native d'électrophorèse sur gel dite 'blue', adaptation nécessaire pour s'assurer de la stabilité des complexes vanadium-protéine. Le Coomassie blue a été utilisé pour, d'une part, masquer la charge des protéines pour une séparation vers l'anode et, d'autre part, pour stabiliser le complexe avec le vanadium. La détection des espèces du vanadium a été rendue possible par le radiotraceur ^{48}V et la technologie de l'écran à phosphore.

Cette méthode a tout d'abord été mise au point en utilisant la transferrine, incubée avec ^{48}V comme modèle. Après qu'il fut prouvé que le complexe vanadium-transferrine était stable pendant la séparation, la méthode a été employée au fractionnement de sérum incubé avec ^{48}V.

L'efficacité de la séparation a été estimée suivant deux paramètres : sa résolution et la préservation des espèces. Premièrement, la résolution de la séparation correspondait à une séparation native. Deuxièmement, le complexe avec la transferrine n'était que faiblement déplacé vers la production de vanadium non-complexé, ce qui prouve que les espèces restent intactes pendant la séparation.

Tel que mentionné dans la littérature, le vanadium, à ces faibles concentrations, s'est rencontré complexé par la transferrine dans du sérum incubé.

8.1.2. Fractionnement dénaturant

Le sélénium est un oligoélément qui peut être lié de manière covalente aux protéines, sous la forme des acides aminés sélénométhionine et sélénocystéine. La nature de la liaison entre le sélénium et la protéine autorise la dénaturation de cette dernière avant l'électrophorèse. La SDS-PAGE est autorisée mais aussi l'électrophorèse sur gel bidimensionnelle à haute résolution (2DE).

La 2DE a été appliquée au fractionnement de protéines séléniées de levure, levure élevée dans une solution contenant du ^{75}Se et l'autoradiographie a été utilisée pour la détection des protéines contenant ^{75}Se. La filtration sur gel et l'ultrafiltration ont permis de tester la stabilité des chaînes séléniées des protéines en présence des composés nécessaires à la lyse et à la 2DE. Puisque ces chaînes n'étaient pas stables, elles ont dû être carboxylées afin de les protéger de l'oxydation. La distribution des masses des protéines séléniées a été estimée par filtration sur gel et les résultats comparés à la distribution obtenue par 2DE. Une carte 2DE des protéines séléniées de la levure est présentée et comparée à la carte de toutes les protéines de la levure.

8.2. Détection des oligoéléments après l'électrophorèse sur gel sans radiomarqueurs

Bien que les radiotraceurs offrent des avantages certains tels qu'une limite de détection basse et une analyse sans risque de contamination, d'autres méthodes de détection ont été étudiées afin de rendre possible l'analyse d'échantillons d'origine humaine. La solution la plus logique était donc les techniques d'analyse de solides. Pour un gel, un polymère, les deux méthodes utilisables étaient la vaporisation électrothermique et l'ablation laser combinées à la spectrométrie de masse à plasma à couplage inductif (ICP-MS) pour la détection la plus sensible. Les deux techniques furent utilisées lors de ce travail pour la détection du sélénium après l'électrophorèse sur gel.

8.2.1. Vaporisation électrothermique – ICP-MS

Cette technique exige une optimisation minutieuse des divers paramètres de vaporisation, entre autres les températures et l'utilisation éventuelle d'un modificateur, mais permet la détection précise et sensible des oligoéléments dans un gel.

Après la séparation, les bandes de protéines sont prélevées du gel et amenées une par une dans le four de la vaporisation électrothermique (ETV). Il a été fait appel à l'étalonnage externe avec application d'une référence interne, le tellure. Une limite de détection par bande d'environ 40 pg a été obtenue ainsi qu'une

récupération totale. Une mesure, c'est-à-dire la détermination de la quantité de sélénium dans une seule bande de protéine, prend moins de 4 min et donc une languette entière de gel, après une séparation d'environ 1,5 h, peut être entièrement analysée en 3.5 h par ETV-ICP-MS. Si l'analyse est limitée à une dizaine de bandes, une languette est analysée en 2 h, étalonnage inclus. L'analyse est menée directement sur le gel révélé, sans blotting, ce qui rend cette analyse encore plus pratique.

Cette méthode a été optimisée avec la sélénoprotéine glutathione peroxydase comme modèle. Puis elle a été appliquée au fractionnement de protéines d'un matériau de référence candidat, une levure séléniée. Il a été possible de reconstruire les électrophérogrammes du sélénium et de les comparer avec les gels révélés. L'avantage majeur de cette méthode est la haute résolution du fractionnement des protéines et la quantification directe du sélénium.

Elle est cependant difficilement applicable à un fractionnement obtenu après 2DE. Des milliers de spots sont présents sur un gel et l'analyse serait alors trop longue. C'est pourquoi l'ablation laser – ICP-MS a été étudiée en tant que méthode de détection.

8.2.2. Ablation laser – ICP-MS

8.2.2.1. Applicabilité de la méthode

L'applicabilité de l'ablation laser – ICP-MS (LA-ICP-MS) pour la détection d'éléments trace dans un gel après électrophorèse a été systématiquement étudiée. Les caractéristiques telles que la limite de détection, la linéarité et la répétabilité ont été déterminées pour différents éléments (Li, V, Cr, Mn, Ni, Cu, Zn, As, Se, Mo, Pd, Ag, Cd, Pt, Tl, Pb). Deux stratégies d'ablation ont été suivies: l'ablation d'un spot, sur une même position, après une séparation bidimensionnelle, et l'ablation avec translation, sur une ligne, utilisable pour un gel monodimensionnel, le long d'une languette.

8.2.2.2. Amélioration de la limite de détection

Puisque seulement une infime quantité de matière est effectivement ablatée et amenée au plasma, la procédure de détection a dû être améliorée en utilisant la

technologie de la chambre dynamique de réaction (DRC). Elle a tout d'abord été optimisée pour la détection du vanadium puis pour celle du sélénium.

8.2.2.2.1. Vanadium

La technologie de la chambre dynamique de réaction (DRC) a été appliquée à l'analyse de spéciation du vanadium dans le sérum, avec la chromatographie d'exclusion stérique comme méthode de fractionnement. Le but est de passer de la chromatographie d'exclusion stérique à l'électrophorèse sur gel dans un avenir proche. La technologie de la DRC était nécessaire pour détecter le vanadium en ligne après la chromatographie d'exclusion stérique utilisant une solution tampon à salinité physiologique (0,15 M NaCl). Cette salinité était obligatoire pour s'assurer de la stabilité des composés vanadiés lors de la chromatographie, en d'autres termes pour empêcher une interconversion des espèces. En fait, la technologie de la DRC a permis la détection du vanadium sans adapter la séparation chromatographique à l'ICP-MS. Une adaptation ne sera pas non plus nécessaire pour l'électrophorèse sur gel native, qui fait appel à des composés chlorés.

Tout d'abord, les performances de différents gaz de réaction ont été comparées : méthane, monoxyde de carbone, ammoniac, oxygène et le mélange d'argon (gaz de collision) avec de l'hydrogène (gaz de réaction). Pour chaque gaz, les paramètres de la chambre de réaction ont été optimisés afin d'obtenir la plus basse limite de détection pour ^{51}V (en tant que $^{51}V^{+}$ ou en tant que $^{51}V^{16}O^{+}$ avec O_2 en gaz de réaction) dans des solutions riches en chlore, le chlore étant le précurseur de l'interférence $^{35}Cl^{16}O^{+}$. L'ammoniac présentait la meilleure limite de détection (critère des 3 écarts-types, 10 ng L^{-1} utilisant la nébulisation pneumatique comme méthode d'introduction de l'échantillon).

La limite de détection du vanadate avec la chromatographie d'exclusion stérique couplée à l'ICP-DRC-MS se montait à 4 pg V et la répétabilité à 7 %. Cette limite de détection devrait être la plus haute pour les composés vanadiés.

Cette séparation en ligne a été utilisée pour la spéciation du vanadium dans le sérum après incubation avec du vanadate, à une concentration qui est représentative de concentrations thérapeutiques quand le vanadium est utilisé comme substitut de l'insuline.

8.2.2.2.2. Sélénium

La technologie de la DRC a aussi permis d'éliminer l'interférence Ar_2^+ et donc de faire appel à l'isotope du sélénium le plus abondant, ^{80}Se. Les mêmes gaz de réaction que ceux utilisés avec le vanadium ont été comparés [méthane, monoxyde de carbone, ammoniac, oxygène et le mélange d'argon (gaz de collision) avec de l'hydrogène (gaz de réaction)]. Pour chaque gaz, les paramètres de la chambre de réaction ont été optimalisés afin d'obtenir la plus basse limite de détection pour Se (en tant que $^{80}Se^+$, $^{82}Se^+$ ou $^{77}Se^+$; et en tant que $^{80}Se^{16}O^+$, $^{82}Se^{16}O^+$ ou $^{77}Se^{16}O^+$ avec O_2). Le monoxyde de carbone présentait la meilleure performance.

8.2.2.3. Application au sélénium

Cette technique a été appliquée à la détection de sélénoprotéines dans des extraits de globules rouges après une séparation monodimensionnelle (SDS-PAGE) et la détection de protéines séléniées de levure après séparation bidimensionnelle (2DE).

La limite de détection en utilisant la DRC et en utilisant He comme gaz de transport se montait à 70 ng Se g^{-1} gel pour l'ablation sur un spot (applicable à la 2DE) et se montait à 150 ng Se g^{-1} gel pour l'ablation avec translation (applicable à la 1DE).

8.3. Perspectives

Les capacités de l'électrophorèse sur gel en font une partie intégrale des techniques de fractionnement pour l'analyse de spéciation élémentaire, ses avantages principaux étant sa haute résolution et les différents mécanismes qu'elle offre. Un gel peut être stocké pendant des années après la séparation, ce qui permet des analyses complémentaires telles que la spectrométrie de masse en tandem. Il a pu être montré que l'électrophorèse sur gel pouvait être combinée à des méthodes comme l'ablation laser – ICP-MS pour la détection des oligoéléments, ce qui permet l'analyse d'échantillon d'origine humaine.

Différents échantillons peuvent maintenant être analysés en faisant usage des méthodes optimalisées lors de ce travail. L'électrophorèse sur gel sera utilisée, en

combinaison avec d'autres techniques de séparation comme la chromatographie d'exclusion stérique, pour l'analyse :

- d'extraits de protéines pour déterminer la présence de protéines séléniées ;
- d'échantillons d'origine humaine, tels que le sérum, analysés pour la spéciation du vanadium ; une question intéressante est de savoir si le vanadium, présent sous une de ses formes réduites, le vanadyle, est transporté par la transferrine ou l'albumine ; la condition *sine qua non* pour ce type d'analyse est de réaliser la séparation sous une atmosphère non-oxydante ;
- d'échantillons de patients à qui des composés vanadiés ont été administrés ; le but est de déterminer la voie métabolique de ces composés.

Ces expériences devront cependant faire appel à d'autres méthodes, telles que la spéctrométrie de masse en tandem, pour rechercher l'identité des composés d'intérêt. La distance électrophorétique de migration, quoiqu'une information sur l'identité d'un composé, n'est certainement pas suffisante pour caractériser complètement un composé.