Isolation of Organic Selenium Compounds from Antarctic Krill after Enzymatic Hydrolysis and Bifunctional Chromatography

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1. Introduction

The essential trace element selenium plays a significant role in many biological functions such as regulation of thyroid hormone, antagonist to the heavy metals cadmium and mercury and is involved in the immune system activity [1, 2]. Low nutritional selenium status is correlated to high risks of cardiomyopathy, cardiovascular diseases and carcinogenesis. Selenium influence in reduced incidence of lung, colorectal and prostate cancer was studied and supporting evidence gathered from clinical trials encouraged further research on this topic [3, 4]. Biological samples contain selenium in bound form, incorporated in protein structures. Because of the reported low efficiencies for the aqueous and acid extraction, other more effective methods for the extraction of selenium amino acids involving proteolytic enzymes for sample digestion are of interest.

The combined treatment of natural samples with enzymes and ultrasound is a recently developed technique by which the sample processing time is significantly reduced and high recoveries for selenium species are obtained [5-7]. Selenomethionine (Se-Met), one of the organic forms of selenium presenting high bioavailability was isolated from grains, seafood and nuts [8-10]. The most common procedures applied for its quantitative and qualitative determination are ion exchange and reverse phase liquid chromatography with different detection systems such as atomic absorption spectrometry (AAS) or inductively coupled plasma-mass spectrometry (ICP-MS) [10-12]. Analyses of natural samples are generally difficult because of the encountered low concentrations of Se-Met and the interference with the other amino acids present in the sample.

Antarctic krill (*Euphausia superba*) is a small crustacean which is very attractive for a healthy human diet and represents an interesting resource for the future because of its high protein content [13, 14].

Solid phase extraction is a common procedure employed for sample preconcentration and purification before application of further analytical methods such as a chromatography. It comprises the sorption onto a disposable solid phase cartridge, followed by the elution of the analyte with a solvent appropriate

for instrumental analysis. Oasis MCX and Oasis MAX are mixed mode or bifunctional sorbents with reverse phase and ion exchange functionalities which are commonly applied in sample preconcentration and purification in clinical laboratory.

The scope of this research is the investigation of Se-Met extraction from Antarctic krill with proteolytic enzymes and of the applicability of mixed mode Oasis sorbents for the chromatographic separation of Se-Met from Antarctic krill. The extraction of selenomethionine from krill protein structure with various methods is also to be performed. The adsorption properties of the Oasis mixed mode sorbents MAX and MCX are to be experimentally determined and the adsorbent with best adsorption parameters to be employed as a stationary phase for the bifunctional chromatographic separation of Se-Met. The method validation with Se-Met standard solutions and with enzymatic krill extracts is to be performed. Analysis of the analyte recoveries and of the column employment for multiple separations under constant performance conditions are to examined.

2. Theoretical Background

2.1. Selenium

This part focuses on relevant information about selenium occurrence in nature, its chemical forms and its role to the human health.

2.1.1. Introduction

Selenium is a trace element essential for human health. After initial concerns regarding its toxicity and the later discovery of its nutritional importance, most of the studies concentrated on its metabolic function and on the importance for human and animal nutrition. Concentrations in soils and therefore in the food chain of animals and humans vary depending on the geographical areas. Certain regions in China, Venezuela, Columbia, Israel, Ireland and U.S.A. (western states) are extremely rich in selenium, while UK, Canada, Germany, France and Scandinavian countries are rather poor in selenium [2]. The daily intake of 30 to 70 μg, recommended by The German Nutrition Society (Deutsche Gesellschaft für Ernährung e.V.), might not be achieved by the population living in selenium poor areas. A common practice in countries with soils poor in selenium is the addition of selenite to the feed of animals or to the fertilizers. The soils in Finland are poor in Se and selenite was added to fertilizers since 1984 in order to increase concentrations in cereals and therefore foods such as meat, dairy products or eggs. Biomarkers of Se intake are the concentrations levels in serum, whole blood, toenails and liver tissue. Since time studies showed that selenium levels in humans are decreasing, special attention has to be given to concentration monitoring in population living in these areas [15].

Se content in foods

Even if the selenium content in foods depends on the geographical region, it is possible to identify certain nutrients which are rich in selenium. Good selenium sources are protein rich foods like meat, fish, grains and nuts (especially Brazil nuts). Selenium concentrations in marine animals are generally higher than in terrestrial ones [16]. Vegetables and fruits are poor in selenium and thus, vegetarians and vegans may be most at risk from low selenium intakes [17].

Representative values for US core-foods and for some nutrients with extremely high levels of selenium are shown in Table 2-1. The term “core foods” reflects the high frequency of consumption.

Table 2-1. Selenium content of U.S. core foods [18]

Food type (raw)	Se concentration (μg kg^{-1})*
fish/seafood	200 - 900
fruits and vegetables	1 - 13
beef	50 - 420
lamb	60 - 320
swordfish	2,540 - 3,440
pork kidney	1,900 - 3,220
beef kidney	1,450 - 2,320
lamb kidney	930 - 1,430
chicken liver	10 - 710

* values based on wet weight basis

Difficulties occur when comparing food concentrations published in different studies. Special care has to be taken not only because of alternating units of measurement but also because of wet/dry weight basis used for reporting Se concentrations. Fish and seafood contain high levels of selenium (approximately 250 - 450 μg kg^{-1} wet weight corresponding to 1,300 to 2,500 μg kg^{-1} dry weight) [19]. The analysis of other reported values [20, 21] showed that the concentrations in seafood/fish are relatively constant across the countries (with few exceptions), while the concentrations for cereals, meats or eggs significantly vary from country to country [18, 21]. Most foods contain Se in organic form such as amino acids Se-Met and selenocysteine (Se-Cys) as major species [22] but also inorganic forms such as selenate were detected [23].

Associated diseases of Se deficiency

Keshan's disease is the extreme form of selenium deficiency. This is a type of heart disease called cardiomyopathy (disease of the heart muscle) that affects population in some regions of China, where the soil concentrations are very low.

The relation between low nutritional selenium status and high risks of cardiomyopathy, cardiovasular diseases and carcinogenesis represents the target of many epidemiological studies and was discussed in several reviews [2, 24].

Physiological importance and roles

Se is part of the antioxidant system [25], is involved in the thyroid hormone metabolism (conversion of thyroxine hormone T4 into its biologically active form, triiodothyronine, known as T3) [1] and in the sperm function [26]. Selenium is also essential for the testicular function [27] and is regarded as an antagonist to heavy metals such as cadmium and mercury [2].

Cancer studies

The progress in basic research of selenium and cancer prevention during the past decade was reviewed in many journals [28-33]. Supporting evidences were gathered from epidemiological and experimental carcinogenesis studies and from clinical trials [34-36]. The long-term, placebo-controlled study (over 10 years) initiated by the US National Cancer Institute (NCI) and conducted by Clark et al. in 1996 was based on Se-yeast (200 μg Se daily dosis) and placebo tablets intake [3]. Reduced incidence of lung, colorectal and prostate cancer (54, 37, and 42%, respectively) was recorded and encouraged further research on this topic. The SELECT trial (Selenium and Vitamin E Cancer Prevention Trial) is another long term study using selenium (200 μg per day from Se-Met) and/or vitamin E supplementation for a minimum of 7 and maximum of 12 years, which was opened in June 2001 and was planned to study the role of the antioxidant agents Se and vitamin E in the prevention of prostate cancer in a population of 32,400 men. Se-Met was chosen by a panel of experts as the optimal form of selenium [37]. Significant reduction of cancer incidence (for example liver cancer) was also reported by Yu et al. in 1997 [38]. Therefore, organic selenium compounds such as Se-Met present highly protective action against toxicity induced by a variety of chemotherapeutic agents.

Bioavailability of selenium

The term bioavailability is related to the extent of ingested nutrient/drug which is available to the body and is relevant for physiological functions. Many studies showed that Se-Met is one of the most bioavailable forms of Se [39]. The enhanced retention of Se-Met, superior to retention of Se salts was confirmed in studies with selenium deficient population in China [40]. For nutritional supplements, naturally occurring forms are preferred. The amount and the chemical form(s) of selenium in yeast are varying according to the marketed product, because of the differences in preparation methods (yeast cultures) and further treatment [41, 42]. In addition, studies revealed the existence of unidentified Se species in yeast material [43]. The availability of selenium from fish and seafood was investigated and earlier results reported low selenium availability for rats [44, 45]. Recent comparative studies report significant differences in the retention of selenium from fish, selenate and yeast [46]. Superior retention was observed for fish and in this study the cooking did not affect selenium apparent absorption or retention from fish. Current evidence favors Se-Met over the other forms of selenium when considering nutritional supplements [22].

2.1.2. About organic selenium compounds

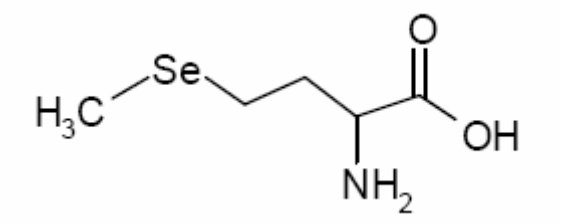
Se-proteins

Selenium is incorporated as Se-cysteine at the active site of many selenoenzymes and it may also be unspecifically incorporated into protein structure as Se-Met instead of methionine (Met). Depending on the Se precursor and the mechanism path of incorporation, one can distinguish four major classes of selenoproteins: Se-specific selenoproteins, Se-Met-specific proteins, Se-Cys-specific proteins and Se-binding proteins [47]. Selenium is a cofactor of glutathione peroxidase (GPX), which reduces hydrogen peroxide and organic hydroperoxides to water and organic alcohols, respectively [25]. The isolation, biochemical importance and molecular biology of GPX and other selenoproteins were reviewed elsewhere [48-50].

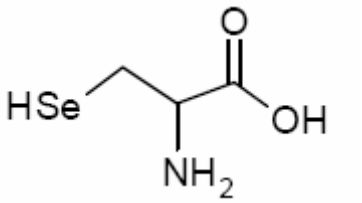
Se amino acids

The most common organic selenium amino acids are Se-Met and Se-Cys, which have similar structure to homologous sulfur amino acids methionine and cysteine, respectively). Se-Met can be the source of all selenoprotein types and can be converted into Se-Cysteine via the transsulfuration pathway. Inorganic selenium has to be firstly converted into Se-Met or Se-Cysteine in order to be metabolized. Se amino acids are the main dietary form, being adsorbed to a greater extent than inorganic species selenite and selenate [11]. The chemical structures of Se-Met and other organic selenium amino acids are presented below in Figure 2-1:

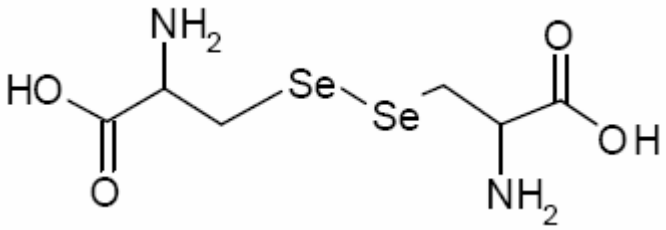
Se-Met



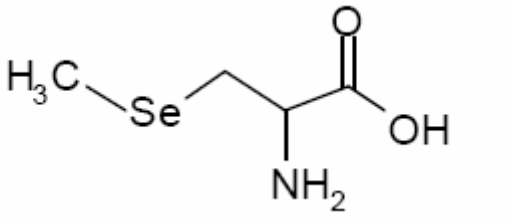
Se-Cysteine



Se-Cystine



Se-Methyl-Se-cysteine



Se-Methyl-Se-Met

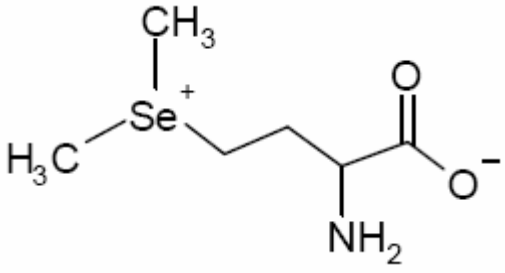


Figure 2-1. Chemical structure of common organic selenium amino acids

The chemical synthesis of Se-Cystine (Se-Cys_2), Se-Met and other monomethyl substituted methionines and selenomethionines were firstly developed about 70 years ago and later improved [51-54]. Reviews such as [55, 56] present their nutritional significance, metabolism, toxicology and biochemistry and the comparison to sulfur isologues.

2.1.3. Analysis of organic selenium compounds

Main difficulties encountered when analyzing and determining selenium and selenium compounds are related to the complexity of biological samples and to the low selenium concentrations in these samples. For the determination of selenium content, techniques such as gas chromatography (GC) [23], hydride generation - atomic absorption spectrometry (HG-AAS) [57, 58], hydride generation - electrothermal atomic absorption spectrometry (HG-ETAAS) [59], graphite furnace atomic absorption spectrometry (GF-AAS) [14, 60] have been intensively used. Especially the speciation of the amino acids Se-Met and Se-Cys or selenoproteins is at the present an intensively researched subject. Table 2-2 presents the most researched selenium compounds and the biological samples which have been investigated for this purpose.

Table 2-2. Selenium species in biological samples

Compound	Sample	Reference
Se-Met	yeast	[61-63]
	wheat, rice, corn	[8, 64]
	nuts	[9]
	oyster, dogfish, lobster	[60, 65]
	prawns	[57]
	Antarctic krill	[66]
Se-cystine	yeast	[63]
	prawns	[57]
	pig kidney CRM 186	[63]
	white clover CRM 402	[63]
Se-Methylselenocysteine	garlic, broccoli, onion	[67]
	phytoplankton	[11]
	ramp (*Allium tricoccum*)	[36]
Se-Methyl-Se-Met	*Brassica juncea* roots	[68]
Dimethylselenide	elephant garlic	[69]

Trimethylselenomium ion	urine	[58, 70]
	oyster	[65]
Se-cysteine	cockle (*Anadara trapezi*), mullet (*Mugil cephalus*)	[71]
	garlic, broccoli, onion	[67]
Selenocystamine	oyster, dogfish, lobster	[60]
Se-adenosyl-selenohomocysteine	Antarctic krill	[66]
Se-cystathione	ramp (*Allium tricoccum*)	[36]
Glutamyl-Se-methylselenocysteine	ramp (*Allium tricoccum*)	[36]
Se-Met-Asn-Ala-Gly-Arg	yeast	[72]

One factor of interest is the amount of organically bound selenium reported to the total selenium content of the natural samples. Research studies focus particularly on Se-Met content. As shown in Table 2-3, the concentration of Se-Met (as % of total Se) can strongly vary according to the sample. The highest Se-Met contingent was determined in swordfish, while in other marine products such as oysters or tuna less Se-Met was found.

Table 2-3. Se-Met as ratio of total Se in natural samples

Sample	Se-Met, % of total Se	Reference
Yeast	64 - 78	[61, 73, 74]
Oyster	46	[65]
Tuna	46	[19]
Swordfish	93	[19]

The extraction of selenium from biological matrices can involve solvent extraction, acid digestion or enzymatic hydrolysis. Table 2-4 summarizes the reported results for selenium extraction from yeast, marine and other samples in aqueous, acidic and enzymatic media. Quantitative extractions were rather unsatisfactory for the aqueous and acidic procedures. More than this, in case of acid hydrolysis additional problems occur because of amino acids instability during the hydrolysis. Since aqueous or solvent extractions release only weakly bound and soluble Se compounds, the hydrolysis with enzymes (which causes the cleavage of protein or peptide bonds) is the method with highest extraction yields. The original sample

matrix is therefore destroyed and the information regarding the native forms of selenium in peptide or protein form is therewith lost.

Table 2-4. Comparison of reported selenium extraction efficiencies

Sample	Extraction method	Efficiency, (%)	Reference
	Aqueous		
	Water		
yeast	H_2O	10 - 20	[12, 63, 75]
fish muscle	H_2O	55 - 70	[76]
oyster	H_2O	35	[65]
	Solvents		
cockle *Anadara trapezia*	$CH_3OH/CHCl_3$	6 - 30	[71]
yeast	Tris-HCl Puffer	15	[43]
	$H_2O/CH_3OH/CHCl_3$	11	[12, 63]
	Acid digestion		
brazil nuts	CH_3SO_3H	75	[10]
	Enzymatic digestion		
brazil nuts	proteinase K, protease XIV	25	[10]
oyster	subtilisin	100	[65]
prawns	protease VIII, lipase VII	60	[57]
tuna, mussels	protease XIV, subtilisin	92 - 100	[77]
yeast	proteinase K, protease XIV	46	[10]
	pepsin	82	[63]
	protease XIV	90 - 92	[12, 75]
	proteinase K	78	[61]
	pronase, lipase	92	[63]

Especially unspecific enzymes such as protease XIV (also known as pronase E) lead to the quantitative release of selenium compounds from samples such as yeast or fish/seafood.

2.2. Antarctic Krill

The research on krill started in the 1970s as a result of increasing demand of animal protein. Most of the papers deal with krill stock estimation, analysis of trace element content and krill utilization for various purposes. Antarctic krill (*Euphausia*

superba) is a small crustacean living in the ecosystem of the Antarctic region of the Southern Ocean and represents a vital food source for whales, penguins and seals. Antarctic krill typically occurs in aggregations that range from small, discrete swarms and schools through to layers and superswarms that extend horizontally for several kilometres [78]. Krill reserves are estimated to be ca. 100 to 2,500 million tons [13, 79-81] and krill catches are varying between 39,000 and 530,000 tons [79, 82-83]. According to the World Health Organization, Antarctic krill appears to be a food appropriate for healthy human diet, containing equal amounts of polyunsaturated, monounsaturated and saturated fatty acids, the last mentioned accounting for less than 6% of the total energy content. The cholesterol level of Antarctic krill is low (ca. 30 mg per 100 g), and the total level of polyunsaturated fatty acids is similar to that of tuna, salmon, anchovy and herring [13]. Krill represents an interesting resource for the future because of its high protein content. The protein content of fresh krill is 50 to 64% based on dry weight [13, 84] and 13 to 18% based on wet weight [85]. The literature reports moisture contents between 71 and 84% and very low fat concentrations of ca. 2% based on wet weight [84, 85]. As shown in Table 2-5, krill is richer in amino acids such as lysine and cystine when compared to beef, tuna or tiger shrimps [84, 86].

Table 2-5. Comparison of essential amino acids content in beef, marine products and krill [86]

Amino acid	Beef	Tuna	Tiger shrimp	Krill
isoleucine	0.30	0.32	0.25	0.35
leucine	0.55	0.57	0.43	0.52
lysine	0.57	0.58	0.41	0.72
methionine	0.14	0.16	0.14	0.17
cystine	0.07	0.08	0.07	0.09
phenylalanine	0.28	0.28	0.23	0.32
tyrosine	0.22	0.26	0.14	0.37
threonine	0.28	0.30	0.22	0.31
tryptophane	0.08	0.08	0.07	0.08
valine	0.34	0.37	0.25	0.33

Essential amino acids make up a significant percent of the total amino acid content in Antarctic krill. More detailed information about the acid content in krill and in other marine products is summarized in [79].

Krill can also be used as a bioindicator of environmental contamination because it can accumulate trace elements and other chemicals [87, 88]. The analysis of hazardous elements in *Euphausia superba* revealed almost the same as the lowest level of commercial fish [88]. Also the selenium content of Antarctic krill was investigated. The concentrations vary between 0.1 and 7.8 μg g^{-1} [13, 88-91]. The processing and storage of krill are strongly influenced by the high activity of its proteolytic enzymes. Since the storage time for the raw material should be as short as possible, the krill is peeled and frozen directly on the fishing boats. Additionally, soon after death occurs autolysis, accompanied by blackening and strong unpleasant odour. Krill is processed to fish bait, food for farmed fish and it is even suitable for direct human consumption (common in Chile, Japan, Poland, Russia). Recent publications about krill deal with the characterization of krill mineral content, chitin use in the industry or strategies for isolation and purification of digestive enzymes [14, 92, 93].

2.3. Chromatography

Chromatography is one of the most encountered methods in the separation and purification of one or more compounds from a mixture. The chromatographic methods of interest for this work are briefly presented below.

2.3.1. Liquid chromatography

Size exclusion chromatography

Size exclusion chromatography (SEC), also named gel permeation or gel filtration chromatography, separates the molecules according to their size. Therefore, this method is widely applied for the isolation, purification and characterization of biological macromolecules. As the sample passes through the column (filled with porous polymer beads or gel), the molecules larger than the largest pores in the gel are excluded from the column and are eluted in the void volume. The intermediate size molecules penetrate the gel according to their size; their flow

through the column is therefore retarded. Accordingly, small molecules such as salts will penetrate into the pores to the highest extent and will elute last from the column. The elution is performed isocratically, meaning only one buffer solution is enough for the separation. Symmetrical peaks are normally obtained with this method. For the determination of the molecular weight, the column must be calibrated with a series of appropriate standards. Typical detectors encountered in SEC analysis are refractive index (RI), ultraviolet (UV), infrared (IR), mass spectrometry (MS) or flame ionization (FI) detectors. Figure 2-2 presents the SEC separation process. SEC is commonly applied in the separation of monomers from dimers and higher aggregates, molecular weight estimation of proteins, molecular weight distribution of polymers, determination of equilibrium constants, salt removal or buffer exchange.

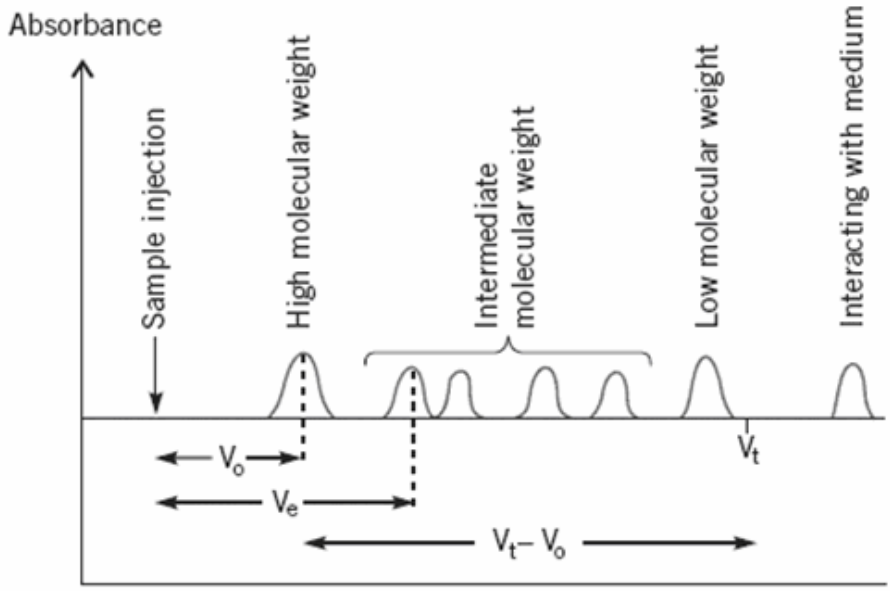


Figure 2-2. Separation process in SEC [94]
(V_t = total volume of packed bed, V_e = elution volume, V_0 = void volume)

Common commercially available stationary phases for SEC are Sephacryl, Superdex or Sepharose. Superdex is a composite medium based on highly cross-linked porous agarose particles to which dextran has been covalently bonded [94-96]. Separation of low and high molecular weight selenium compounds from yeast, animal, fish/seafood or plant materials was achieved using a wide separation range, as briefly summarized in Table 2-6.

Table 2-6. SEC applications for the determination of selenium compounds

Sample	SEC Column	Source
yeast	HiLoad 26/60 Superdex 30 Prep; Superdex peptide HR 10/30; G15 Sephadex; HiLoad 26/60 Superdex 30 Prep; Superdex 75 HiLoad 16/60; Superdex 200 HiLoad 16/60	[43, 72, 97-99]
nuts, yeast	Superdex peptide HR 10/30	[10]
Brazil nut	Superdex peptide HR 10/30	[9]
animal tissue	Superdex 200 10/30	[100]
shiitake mushroom	Asahipak GS-220 HQ; Asahipak GS-320 HQ; Asahipak GS-520 7G	[101]
indian mustard	Superdex 75 HR 10/30	[102]
green onion	Superdex peptide HR 10/30	[103]
krill	Superdex 75 HR 10/30	[66]
cod, plaice	Superdex 200 HR 10/30	[104]

The molecular weight range of employed columns vary from 10,000 - 600,000 Da for the Superdex 200 HR 10/30 column up to 100 - 7,000 Da for the Superdex Peptide HR 10/30 column. Tris-HCl in low concentrations (usually 10-20 mM) and ammonium acetate (AmAc) are commonly used mobile phases for the separation of selenium compounds.

Reverse phase high performance liquid chromatography (RP-HPLC)

Reverse phase high performance liquid chromatography is intensively applied in biochemical separations and purifications. The term reverse phase is related to the use of a polar eluent and a non-polar stationary phase. Molecules such as proteins and nucleic acids can be separated by RP-HPLC based on their hydrophobic character. The non-polar stationary phase generally consists of hydrophobic C_4, C_8 and C_{18} alkyl chains, bonded to a solid matrix that is generally non-polar silica gel. Synthetic organic polymers, e.g. beaded polystyrene, are also available as reverse phase media. Gradient elution is generally employed for reverse phase chromatography of biomolecules [105, 106]. One of the most successfully applied highly sensitive detection method coupled to liquid chromatography is inductively

coupled plasma mass spectrometry (ICP-MS). Ion pairing reverse phase chromatography (IP-RP-HPLC) is a special mode of RP-HPLC applied in the separation of ionisable organic compounds such as carboxylic acids and organic bases. An ion pair is formed between the solute ion and an ion of the opposite charge in the mobile phase. The separation of the ion pair occurs on reverse phase column packed with neutral polystyrene divinylbenzene (PS-DVB) polymers or bonded silica materials [107]. Ion pairing reagents such as trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), pentafluoropropanoic acid (PFPA) or nonafluoropentanoic acid (NFPA) have been used for the speciation of selenium compounds [11, 108].

Ion Exchange Chromatography

Ion exchange chromatography (IEC) is applied for the separation of ionic compounds. The process is based on the different interactions between ionic species present in the mobile phase and an ion exchange matrix. This method can be employed for binding the compounds of interest and allowing the rest to leave the column (most common) or for binding of undesired substances and allowing the free passage of the desired ones through the column. The eluents generally consist of an aqueous salt or mixture of salts, sometimes with a small concentration of an organic solvent. The ion exchanger is an insoluble matrix (for example silica-based materials or synthetic resins) to which charged groups are covalently bound. The most common functional groups in ion exchange are presented in Table 2-7.

Table 2-7. Common anion and cation exchangers

Anion exchangers		Cation exchangers	
Type	Functional group	Type	Functional group
carboxylic acid	$-COO^-\ H^+$	primary amine	$-NH_3^+\ OH^-$
sulfonic acid	$-SO_3^-\ H^+$	secondary amine	$-NH_2(CH_3)^+\ OH^-$
phenolic	$-O^-\ H^+$	tertiary amine	$-NH(CH_3)_2^-\ OH^-$
selenonic acid	$-SeO_3^-\ H^+$	quaternary amine	$-N(CH_3)_3^-\ OH^-$

Sulfonic acid and quaternary amines are strong exchangers, the other functional groups present rather weak exchange properties [107, 109].

RP-HPLC, IP-RP-HPLC and IEC are common applications for the determination of inorganic selenium (IV and VI) [9, 11, 12, 103, 110], trimethylselenonium ion (TMSe) [65, 70, 77, 111], organic amino acids such as Se-Met [10, 73, 75, 112, 113], Se-Cystine [43, 108] or Se-Et [57] and higher molecular weight Se containing compounds [68, 75, 97, 99]. Most of the studies were performed with yeast extracts [73, 75, 99], mushrooms [110, 112], green onion [103], garlic [108] and fish/seafood such as oysters [65], mussels, tuna [77], praws and clams [57]. For samples containing high concentrations of lipids (such as Brazil nuts, walnuts, cashews or fish), the solvent extraction with a mixture of $CHCl_3$:CH_3OH (2:1 or 1:2) without Se losses was performed [9, 77]. Still, the extraction with water or the acid/enzymatic sample digestion followed by the separation on chromatographic column is an established method for the analysis of above mentioned compounds. Other reviews about the methods relevant for selenium speciation are presented in references [114, 115].

2.3.2. Gas chromatography

Gas chromatography (GC) is a method which can be applied only when the components of the mixture to be analyzed are volatile and stable enough to survive the oven temperature, which can be up to 350°C. Care must be taken to avoid thermal or catalytical decomposition of the sample. Some molecules such as amino acids must be chemically converted with derivatization agents prior to injection onto GC systems in order to increase their volatility and make them available to GC analysis. Adsorptive interaction between the components in the gas stream and the column coating leads to the separation of the components of the mixture, which are then swept in order through a detector flow cell. The most common detectors coupled to GC are flame ionization (FID), electron capture (ECD), photo-ionization (PID), flame photometric (FPD) [116]. Since many compounds possess the same retention time, the identification can not be achieved accordingly. Mass spectrometry (MS) is a well-established analytical technique used in conjunction to GC for the analysis of sample composition. The

mass spectrometer normally consists of an ion source, an analyzer and a detector. The injected material is ionized at high vacuum. The mass spectrometer propels and focuses the ions and their fragmentation products through a magnetic mass analyzer, and then collects and measures the amount of each selected ion in the detector. Mass spectrometry is thus a destructive method of analysis. The determination of the molecular structure of a compound based on its molecular weight and fragmentation spectra requires experience and spectral libraries are sometimes very helpful for the interpretation of fragmentation data [117]. The employment of gas chromatography in conjunction with selective and highly-sensitive detectors was reported for selenium compounds analysis [8, 67, 118, 119, 120-124]. As previously mentioned, amino acids have to be converted into volatile derivatives for GC analysis. The derivatization reaction implies the conversion of both the amine and the carboxylic acid functional groups. Various authors have taken different approaches to Se-Met derivatization, using methanol [8], propan-2-ol [8, 123], heptafluorobutyric-isobutanol (HFB-IBA) [125] as esterification agents and heptafluorobutyric anhydride (HFBA) [8, 125], TFA [8] or TFA anhydride [123] as acylation agents. The chemical reaction is often time consuming and occurs at high temperatures, for example at 135°C [125]. The derivatization with alkyl chloroformates is a fast one-step reaction, involving simultaneous reaction with both amino and carboxylic ends of the amino acid. The reagents are inexpensive and the reaction takes place at room temperature [126-128]. There are few studies reporting isobutyl [122], methyl and menthyl chloroformates [119] as derivatization agents for Se-Met. However, some authors reported the advantages of the derivatization with ethyl chloroformate (ECF) in ethanol [61, 67, 118, 120, 121, 123, 124].

2.4. Adsorption

Adsorption is one of the most effective gas and liquid separation and purification technique. Single components can be separated from a mixture based on their different adsorption properties. The most common industrial applications are metal removal, wastewater treatment and air pollution control.

2.4.1. Principles

Adsorption is described as the process by which the molecules from a gas or liquid phase bind onto a solid surface. The molecules that bind to the surface are called the adsorbate and the substance that holds the adsorbate is called the adsorbent, as illustrated in Figure 2-3. The term adsorptive describes the adsorbate species in the fluid phase before adsorption. Desorption is the removal of the molecules from the surface. The sorption stands for a general term and describes the attraction of adsorbate molecules to the solid surface.

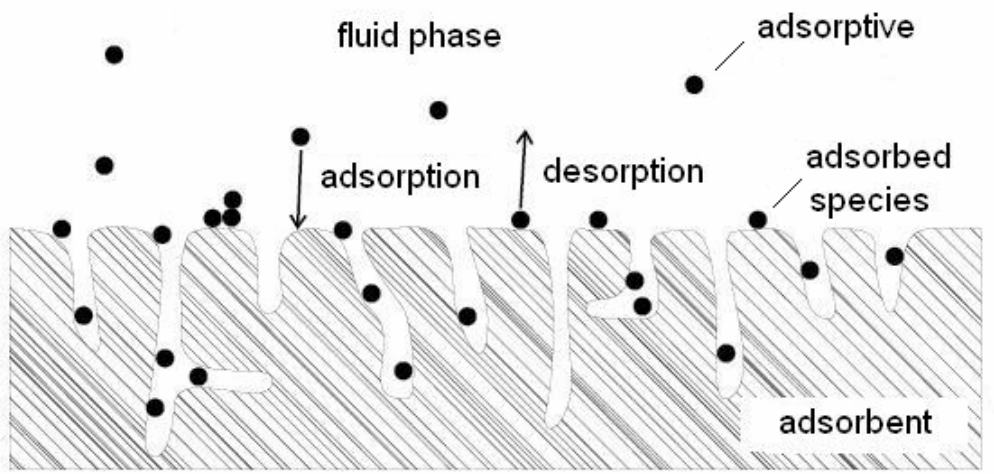


Figure 2-3. Adsorption on a solid adsorbent, adapted from [129]

Adsorption can occur through chemisorption (direct chemical bond between the surface and the adsorbate) or physisorption mechanism (no chemical bonds are formed and the binding is based on physical forces such as van der Waals or electrostatic forces). Chemisorption is characterized by higher energies than physisorption [130].

Most commercial applications are based on the following principle: the adsorbent material is packed in a separation column and the liquid sample, containing the adsorbate, is passed through the column bed. The adsorption process occurs through the continuous feed flow down through the column. As the solute moves through the column bed, target solute molecules from the fluid phase are being retained on the adsorbent surface and after a certain time a specific concentration profile is built. The column bed length in which mass transfer process takes place is called the mass transfer zone (MTZ) and illustrated in Figure 2-4.

The MTZ gradually moves through the column, as a function of the adsorption time. After a period of time, the MTZ reaches the end of the column bed. This moment is called the breakthrough and can be represented by plotting the effluent adsorbate concentration against volume or time in the breakthrough curve.

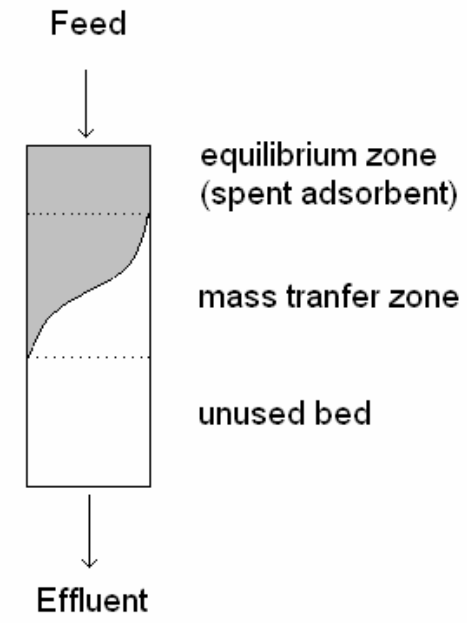


Figure 2-4. Adsorption profile in a fixed-bed adsorber

A typical plot of the outlet-to-inlet ratio solute concentration (C_{out}/C_{feed}) as a function of time from the flow start is showed in Figure 2-5. The S-shaped curve reveals information about the adsorption kinetics. Its steepness determines the extent to which the capacity of an adsorbent bed can be utilized.

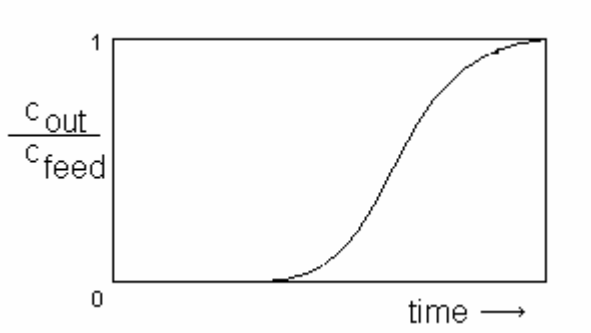


Figure 2-5. Typical breakthrough curve

A steep slope indicates good adsorption kinetics while a stretched curve with a low slope means a poor adsorption. Major factors influencing the adsorption capacity are flow rate, pH and flow rate. Before being employed in a further separation

process, the regeneration of adsorbent material must be carried out [131, 132]. Preliminary experiments must be performed for the characterization of a sorbent material before usage in large-scale applications. Studies under static and dynamic conditions are employed for the determination of kinetic and equilibrium adsorption parameters. A good sorbent should have following characteristics: favourable adsorption kinetic and transport properties resulting in a fast overall kinetic; high selectivity and capacity in order to minimize the amount needed; chemical, thermal, mechanical and biological stability, resistance against fouling and low cost.

2.4.2. Equilibrium considerations

The adsorption process can be quantitatively described by the adsorption isotherms. They describe the equilibrium between the bulk activity of adsorbate in the solution and the amount adsorbed on the surface under constant temperature conditions. The model parameters employed for the expression of adsorption isotherms are:

- c (the concentration of free A in the liquid phase at equilibrium, mg mL^{-1})
- q (the bound amount of adsorbate at equilibrium, mg g^{-1} adsorbent)
- q_{max} (the maximal bound amount of the target molecule onto the adsorbent's surface, mg g^{-1} adsorbent)
- K (the equilibrium constant, ratio of dissociation reaction rate and association reaction rate)

The most common adsorption isotherm types are Langmuir, BET (Brunauer-Emmet-Teller) and Freundlich (see Figure 2-6).

Langmuir isotherm [133] is derived from simple mass-action kinetics, assuming homogeneous pore surface of adsorbent and negligible forces of interaction between adsorbed molecules. At equilibrium, the rates of adsorption and desorption are equal. The Langmuir isotherm can be described by the equation:

$$q = \frac{q_{\max} \cdot c}{K + c} \qquad (2\text{-}1)$$

Systems that do not obey Langmuir type can be described by other adsorption isotherms such as BET or Freundlich. The BET isotherm is associated with

multimolecular adsorption on a homogeneous surface, assuming that the first layer of adsorbed molecules of an adsorbate A is available for the next layer.

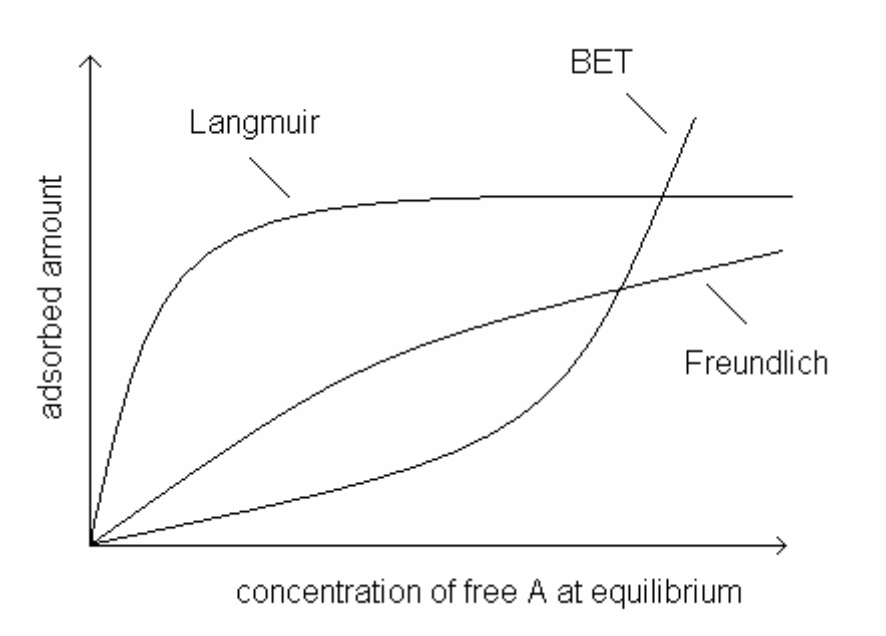


Figure 2-6. Graphical representation of common adsorption isotherms

The Freundlich model [134] is often applied for gas adsorption and enables the description of a multilayer sorption onto the adsorbent. The Freundlich isotherm can be described by the equation:

$$q = K_F \cdot c^{\frac{1}{n}} \qquad (2\text{-}2)$$

The linear isotherm (Henry's model) is the simplest method to describe the adsorption equilibrium and is valid for cases where the amount adsorbed is low [135]. Henry's isotherm can be described by the equation:

$$q = K_H \cdot c \qquad (2\text{-}3)$$

The Freundlich isotherm equation is empirical and assumes a heterogeneous surface with a non-uniform distribution of the heat of adsorption over the surface [131, 136]. The distribution coefficient K_F (also called Freundlich adsorption coefficient) and the linearity exponent $1/n$ are empirical constants. K_F is a measure of the sorbate affinity to the sorption sites and $1/n$ describes the isotherm curvature. If n is 1, the Freundlich isotherm reduces to the Henry's linear isotherm. The limiting factors for the maximal binding capacity of an adsorbent are the

accessible surface area per volume unit and the area of the ligand molecule bound to this surface. The binding constants (q_{max} and K) are obtained from the linearized Langmuir model (Scatchard plot analysis), as represented in Figure 2-7. This plot presents the specific binding divided by the free concentration in the liquid phase (q/c) on the Y axis versus specific binding q (X axis). This mathematical model gives information obtained about q_{max}/K (intercept of Y axis) and q_{max} (intercept of X axis). The slope of this function is -1/K.

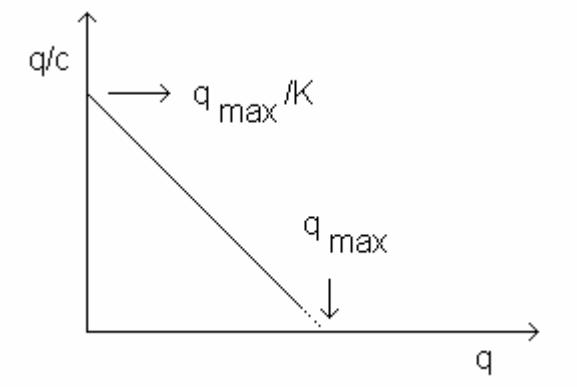


Figure 2-7. Graphical determination of maximal binding capacity (q_{max}) and equilibrium constant (K) from Scatchard-plots

These parameters are significant for the adsorptive separation process and allow the applicability of these models to chromatographic adsorbents for separation processes [137].

2.4.3. Kinetic and transport considerations

The adsorption of a solute onto the porous surface of an adsorbent consists of four major consecutive steps:

- interphase mass transfer of the solute from the bulk fluid through a boundary layer to the outer solid surface of the adsorbent (by convection)
- intraphase mass transfer of the solute to the inner solid surface of the adsorbent (by diffusion)
- diffusion of the solute through the adsorbent pores
- adsorption of the solute onto the porous adsorbent surface

These steps are occurring differently for chemisorption and physisorption. The last step may be rate determining for the chemisorption process and it is happening almost instantaneously for the physisorption. Heat transfer accompanies the

adsorption and desorption processes because of the exothermic heat of adsorption and endothermic heat of desorption. External heat transfer from the particle outer surface occurs by convection through the boundary layer surrounding each solid particle in the adsorbent bed and by conduction at points of contact by adjacent particles. Figure 2-8 presents the solute concentration profile for a given particle at a particular time for the adsorption and desorption.

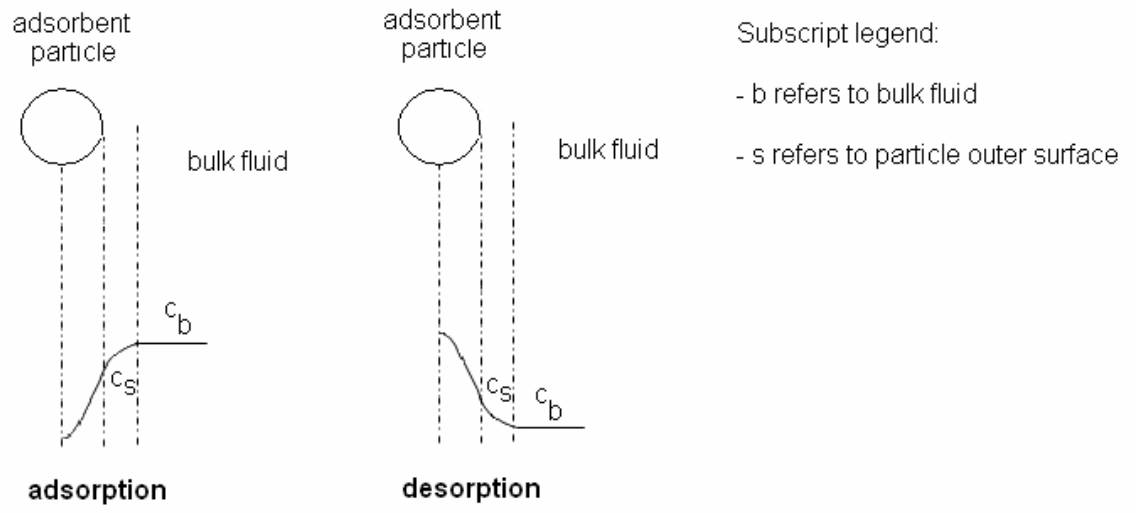


Figure 2-8. Solute concentration profile for a porous adsorbent particle surrounded by a fluid

The major resistance to heat transfer is usually external to the adsorbent particle while the major resistance to mass transfer is related to the inner of adsorbent particle [131].

2.5. Enzymes

Enzymes are very efficient agents in the synthesis or degradation of chemical compounds. In this work they are employed in the cleavage of krill protein structure and in the release of amino acids. Enzymes are biological catalysts which are essential to all living organisms. They consist (with very few exceptions) of large protein molecules and their mechanism of action is based the specific binding between the active site of the enzyme and the substrate. This temporary complex causes the reduction of the activation energy required for the chemical reaction and an increase of the reaction rate up to 10^{10} times [138]. The simplest enzymatic reaction can be represented by the unimolecular reaction developed by

Michaelis and Menten [139]. The equation 2-4 presents the enzyme-substrate interaction:

$$S + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} E - S \xrightarrow{k_3} P + E \qquad (2\text{-}4)$$

where E = free enzyme, S = substrate, E-S = enzyme-substrate complex, k_1, k_2 and k_3 = rate constants for the formation of E-S, release of S or release of P, respectively. Figure 2-9 presents a typical energy diagram for an enzyme-catalyzed reaction.

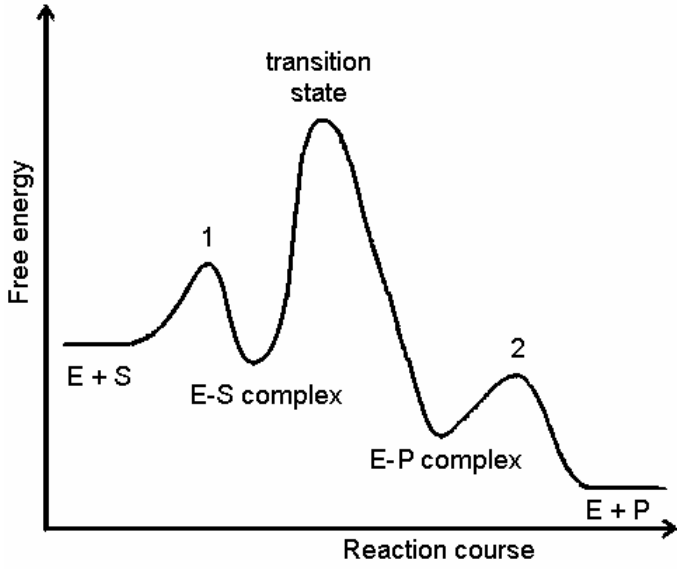


Figure 2-9. Energy diagram for an enzyme-catalyzed reaction, adapted from [140]. Small energy barriers exist at points 1 and 2.

Enzymatic reaction rate depends on factors such as ionic strength, pH and temperature conditions. Enzymes have specific temperature and pH ranges at which they work with optimal activity. Outside these optimal temperature and pH ranges, enzymes may undergo reversible or irreversible denaturation (unfolding) or alteration processes (the loss of the enzymatic activity caused by the changes in the functional structure due to bonds weakening and breakdown within the enzyme) [141]. Mild temperature and pH conditions are some of the advantages of enzymatic digestion of samples over conventional procedures such as acid or basic digestions. The enzyme concentration is also significant for the digestion process and the influence of the ratio enzyme to substrate must be considered for

optimal experimental results. Higher efficiencies were reported for enzymatic hydrolysis of chicken samples when the enzyme concentration increased [7].

Enzymes are divided into six major classes, each one divided into further subclasses, according to the nature of the catalyzed chemical reaction. The most encountered enzymes in the analytic procedures are hydrolases such as lipases, amylases and proteases. Hydrolases belong to the E.C. 3 class and catalyze the hydrolysis of chemical bonds. The enzymatic hydrolysis of peptide/protein bonds is associated to subclass E.C. 3.4 (proteases/peptidases). Proteases act on the amide bond and may have specific or unspecific action on the substrate. Trypsin (molecular weight 24,000 Daltons) and chymotrypsin (molecular weight 25,000 Daltons) are two of the most common enzymes applied for specific hydrolysis i.e. their mechanism of action depends on the amino acids near the peptidic bond (lysine or arginine for trypsin and aromatic amino acids such as phenylalanine, tryptophan or tyrosine for chymotrypsin). Figure 2-10 presents the substrate specificity of trypsin (A) and chymotrypsin (B).

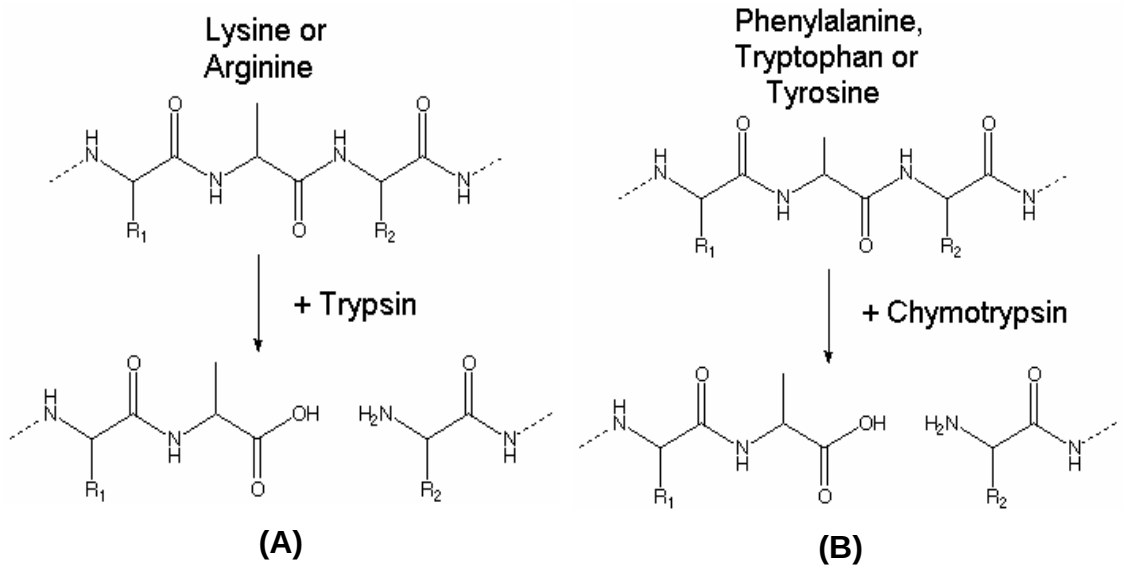


Figure 2-10. Mechanism of action for trypsin and chymotrypsin

The hydrolysis produces peptides suitable for analysis (15-20 amino acids). This property makes proteolytic enzymes suitable for peptide generation for sequencing or mapping. A high extent of enzymatic digestion (total hydrolysis, up to amino acids) can be achieved with unspecific enzymes such as subtilisin or protease type XIV.

The enzymes listed below are most commonly encountered in extraction of selenium species from natural samples.

Subtilisin is a bacterial proteinase obtained from *B. subtilis* (also known as Subtilisin Carlsberg) with a molecular weight of ca. 27,000 Daltons and presents largely non-specific activity. It cleaves most peptide linkages with a preference for aromatic amino acids and many esters [141]. Proteinase E is a chymotrypsin-like peptidase (molecular weight of ca. 27,000 Daltons) which preferentially cleaves after alanine, valine, serine and threonine in polypeptides and hydroxyl amino acid residues. Lipases (triacylglycerol hydrolases) are enzymes with excellent stereoselectivity which catalyze the hydrolysis and the re-esterification of triglycerides (i.e. natural fats and oils). They are active in a broad range of non-aqueous solvents and have also application in organic chemistry. Novo 0.6 MPX enzyme is an alkali-stable protease, with special applications in enzyme assisted chemical unhairing. The introduction of this enzyme in the tanning industry significantly reduced the demand of chemical substances and electricity for leather processing. Proteinase K is a subtilisin-like proteinase with a molecular weight of 27,000 to 29,000 Daltons and possesses a broad specificity spectrum. It cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. Pronase E (also known as proteinase type XIV) is a highly non-specific mixture of proteolytic enzymes obtained from *S. griseus*. This mixture contains at least 10 proteases (five serine-type proteases, two zinc endopeptidases, two zinc leucine aminopeptidases and one zinc carboxypeptidase). Digestion with Pronase is particularly useful since tryptophan, serine, threonine, asparagine and glutamine are easily destroyed by the usual acid hydrolysis procedures. It is used for extensive or complete degradation of proteins.

Proteases can also undergo self digestion (autolysis) - the partial or total hydrolysis of amide bonds in the same molecule. This reaction cannot be completely avoided, but it can be minimized by keeping the amounts of proteinase in lower ranges or by the addition of metal ions such as Ca^{2+} or Mg^{2+}. A good example of the function of these ions is the stabilization against autolysis through binding of Ca^{2+} to serine proteases such as trypsin or chymotrypsin [142]. Intramolecular autoproteolysis is a process in which a peptide bond hydrolysis

within a polypeptide chain is catalyzed by amino acid residues in the same polypeptidic chain, near this bond [143].

Applications of proteases are found in food and detergent production, in the manufacture of protein hydrolysates, leather industry, in processing of meat and fish residues and medicine [138, 141].

The digestion of natural samples such as marine products (oyster, prawns, tuna) and yeast with proteolytic enzymes is superior to other extraction methods including aqueous and acidic procedures. Higher recoveries for selenium species are reported for pronase, subtilisin and lipase, as summarized in Table 2-4.

2.6. Ultrasound

Common applications of ultrasound technique are cell disruption or treatment of the water and wastewater. The combination of enzymatic processes with ultrasound power extended the application area of this method.

2.6.1. Principles

Sound spreads through an elastic medium (gas, liquid or solid) as a longitudinal wave, i.e. as a series of compressions and rarefactions. Sound frequencies between 20 Hz and 20 kHz are the usual range for human hearing. Conventional technical ultrasound occupies the range between 20 kHz and 100 kHz on the sound frequency scale, as illustrated in Figure 2-11.

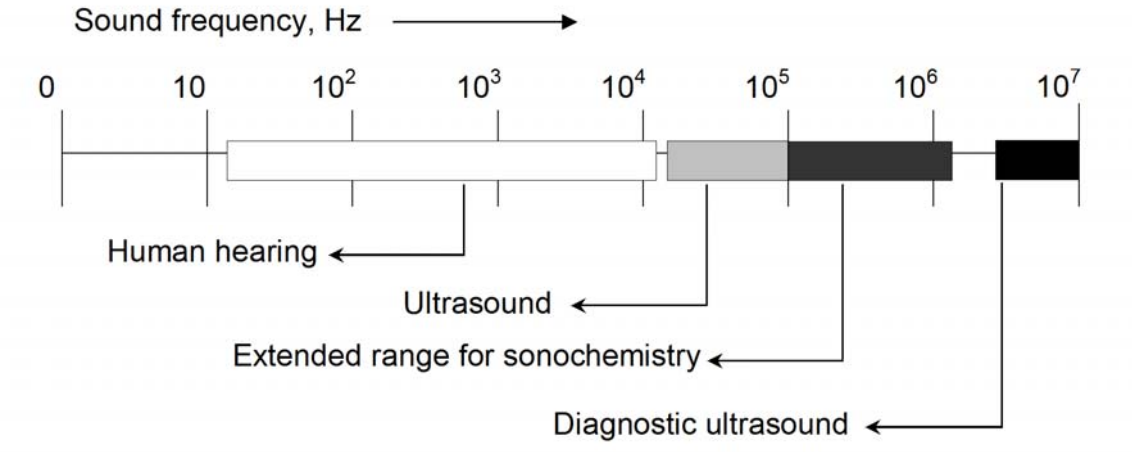


Figure 2-11. Sound frequency scale, adapted from [144]

Ultrasound is a form of energy which is introduced into media. It acts on molecular level as it modifies the average distance between molecules and causes an excitation in the form of enhanced molecular motion. In liquid systems, the liquid is heated up and, as a consequence, microbubbles are generated. When these cavitation bubbles collapse, a series of strong physical effects (shear forces, shock waves) are generated. Especially in heterogeneous systems this effect can be employed positively on chemical reactions due to increased contact area between phases [144, 145]. Enhanced uniformity in the reaction media can be achieved through the rapid reduction in particle size occurring as the most immediate effect in ultrasound assisted processes [145]. Additionally, the activation energy to start conversion can effectively be put into these systems resulting in a reduction of the overall energy consumption.

Reduction in processing time and enhanced uniformity in the reaction media are further benefits of ultrasound application. The reaction conditions and system parameters are directly influenced by the cavitation phenomenon and therefore also on the chemical effects of ultrasound treatment. The most important factors affecting cavitation are frequency and intensity of ultrasound, dissolved gases, solvent properties and temperature [146].

Ultrasound is generated by a transducer, which converts the applied alternating electrical energy into vibrations. The amplitude of vibration generated by the transducer is normally not high enough for practical applications. The attachment of a metal rod with a specially designed length to the transducer's end increases the amplitude of vibration. The "horn" system is one the most encountered methods for the direct introduction of ultrasound into a chemical system. Figure 2-12 illustrates the general scheme of an ultrasonic probe system.

During usage, heat may be generated by the immersed horn and the sample temperature has to be kept within the desired range via integrated cooling. Piezoelectric materials such as barium titanate or lead zirconium titanate are widely applied for this purpose. The ultrasound wavelength in a material depends on the material type and on the sound frequency.

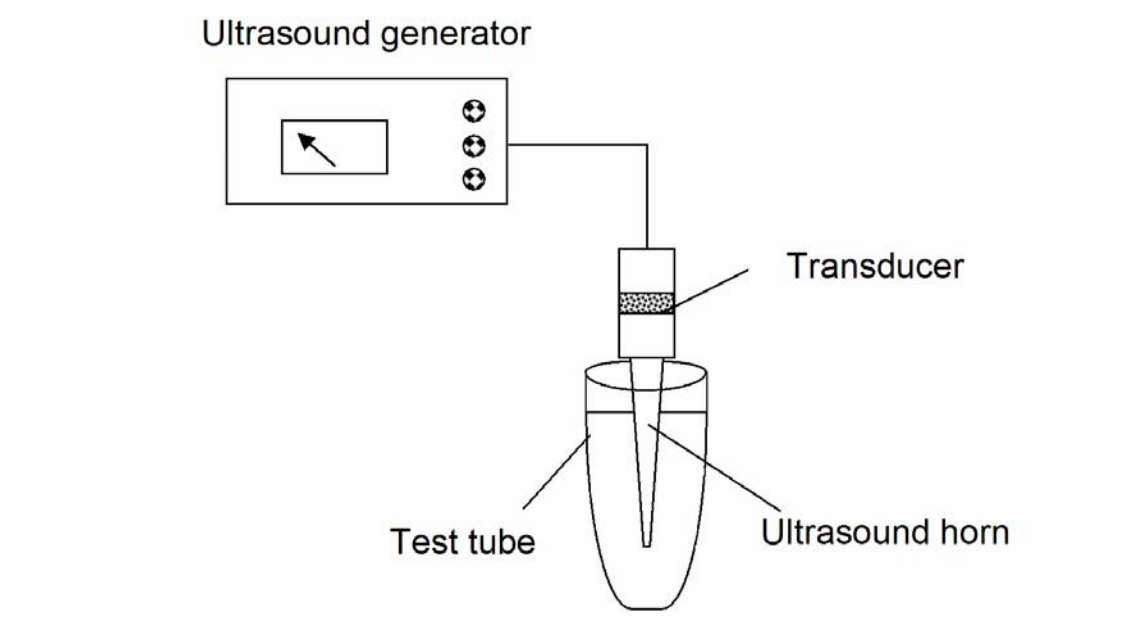


Figure 2-12: Ultrasonic horn system, adapted from [146]

The potential of ultrasound for the intensification of chemical and biological processes makes ultrasound attractive for many practical applications. Most common applications of ultrasound include homogenization and cell disruption, drilling and cleaning of teeth, cleaning and degassing of articles in ultrasonic baths, foetus observation and guidance of subcutaneous surgical implements [144, 145]. Recent studies deal with the application of the ultrasound sample treatment in environmental technologies such as water, wastewater and biosolids treatment [147, 148].

2.6.2. Enzymes and ultrasound

A frequent application of ultrasound in biotechnology is the disruption of the biological cells with the purpose of intracellular content release. It has been suggested that the enzymatic activity is positively influenced by ultrasound power [149-151]. Parameters such as temperature (and therefore molecular mobility and collision probability), concentration of reactants or presence of a catalyst may have a positive influence of the chemical reactivity between media components. The improvement of cellulase and pectinase performance in bio-processing of cotton textiles and the successful application of ultrasound in hazardous water treatment were reported [152-156]. The successful proteolytic digestion of proteins in gel and

in solution and its advantages compared to conventional enzymatic digestion were also achieved [157]. Important parameters in combined enzymatic and ultrasound procedures are enzyme ratio, sample volume, type of solvent, pH, power, duty cycle, time and temperature. These parameters should be optimized specifically for each enzyme, since negative side-effects such as enzyme deactivation due to local sample overheating during sonication might occur. Sample cooling on ice helps keeping the temperature changes under control [158]. Sample processing involving enzymatic digestion is time-consuming. During incubation time, the contact between enzyme and substrate is usually improved by mechanical agitation in a shaker, under temperature and pH control. Enzymatic processes normally take 12 up to 24 h. Time reduction up to minutes level was reported for sonicated samples such as mussel tissue, yeast or chicken muscle [5-7].

There are only few papers describing the combined treatment with enzymes and sonication for the extraction and analysis of selenium and selenium compounds. Subtilisin and pronase E are two of the most encountered enzymes in conventional enzymatic digestion procedures including the monitoring of organic compounds such as Se-Met or TMSe [7, 65] and they are also encountered in procedures involving combined treatment with ultrasound. The ultrasound assisted enzymatic digestion was performed, beside pronase E and subtilisin, with other enzymes such as pepsin, trypsin and pancreatin [5, 6, 65]. Samples such as yeast, chicken, seafood including krill were analyzed for this purpose and the quantitative recovery for selenium together with the preservation of selenium species (Se-Met) was achieved within seconds/minutes [6, 7, 159].

3. Materials and Methods

3.1. Materials

The chemical substances and the instrumentation employed for the analyses are specified in the Appendix (chapters 7.3 and 7.4).
The biological samples, tested sorbent materials and buffer solutions employed in this work are presented below.

3.1.1. Krill samples

Deep-frozen krill (*Euphausia superba*) was granted by Schoppenhauer GmbH (Bremerhaven, Germany) and stored at -20°C. The delivered frozen blocks weighted between 1 and 1.5 kg.

3.1.2. Adsorbents

The concept of mixed mode or bifunctional extraction implies the combined mode use of adsorbent-adsorbate interactions, usually hydrophobic and ion exchange [160]. Mixed phase sorbents consist of a reverse phase (C_4-, C_8- or C_{18}- alkyl chains) and an ionic phase (either anionic or cationic). Most of the mixed mode products are actually a mixture of two different materials (one reverse phase and one with ion exchange groups). The only products available on the market which possess both functions on the same backbone are the Oasis® MCX and Oasis® MAX from Waters (Waters GmbH (Eschborn, Germany). These sorbents are employed mainly in solid phase extraction process.

Oasis MCX (mixed mode cation exchanger)

This adsorbent is obtained by precisely controlled sulfonation of a patented copolymer synthesized with a composition that is hydrophilic-lipophilic-balanced for both strong reverse phase retention and water-wettability (m-Divinylbenzene & *N*-vinylpyrrolidone copolymer, with sulfonic acid substituents [-SO_3H, 1.0 meq g^{-1}]). Figure 3-1 presents the chemical structure of the Oasis MCX sorbent. It is stable over a pH range from 1 to 14 and it is used for the adsorption of polar and non-polar, neutral and cationic compounds. The mixed mode cation exchange and reverse phase mechanisms are acting simultaneously. Typical applications include the separation of basic drugs and their metabolites from biological fluids,

environmental pollutants from water, drugs of abuse and their metabolites from urine [161]. The nominal pore size is 80 Å and the particle size 30, and 60 µm, respectively.

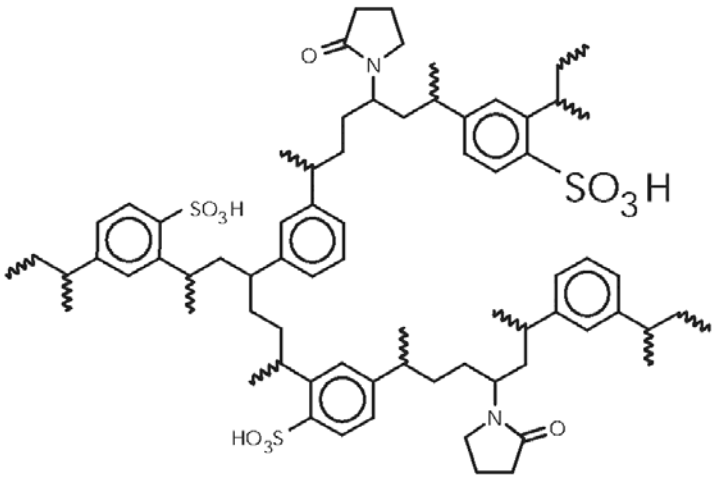


Figure 3-1. Chemical structure of Oasis MCX sorbent

The dual mode of retention of the Oasis MCX adsorbent is based on the reverse phase mechanism and on the interaction between the charged species of the adsorbent and the sample (as shown in Figure 3-2 for the sulfonic group of MCX and the positively charged amine group of a basic drug such as propanolol).

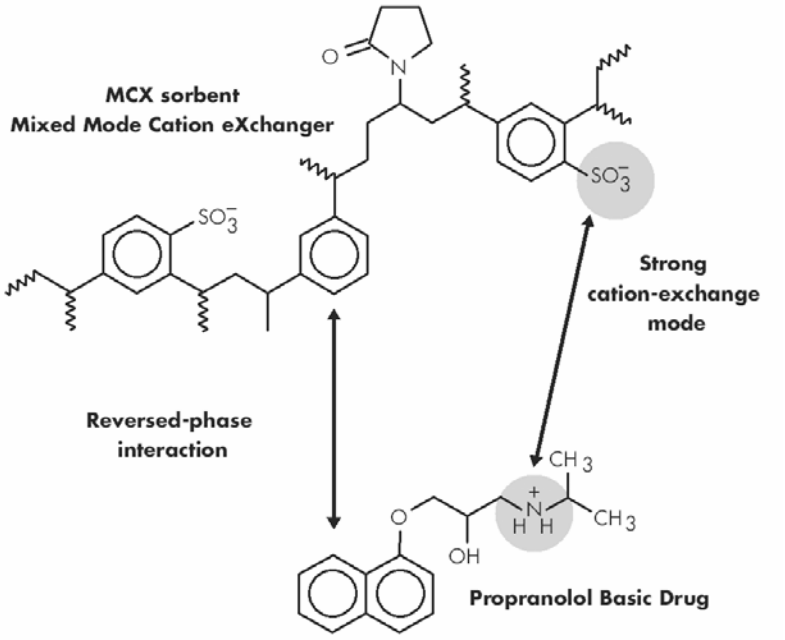


Figure 3-2. Example of a drug-sorbent interaction on Oasis MCX sorbent [161]

Oasis MAX (mixed mode anion exchanger)

This adsorbent is obtained by precisely controlled functionalization of a patented copolymer (m-Divinylbenzene & *N*-vinylpyrrolidone copolymer, with quaternary amine substituents, 0.3 meq g^{-1}) synthesized with a composition that is hydrophilic-lipophilic-balanced for both strong reverse phase retention and water-wettability. Figure 3-3 presents the chemical structure of the Oasis MAX sorbent. Like MCX, it is stable over a pH range from 1 to 14 and it is used to adsorb polar and non-polar, neutral and anionic compounds from aqueous media. The mixed mode anion exchange and reverse phase mechanisms are also acting simultaneously.

Typical applications include the separation of acidic drugs and their metabolites from biological fluids, environmental pollutants from water, drugs of abuse and their metabolites from urine. The nominal pore size is 80 Å and the particle size 30, and 60 µm, respectively [161].

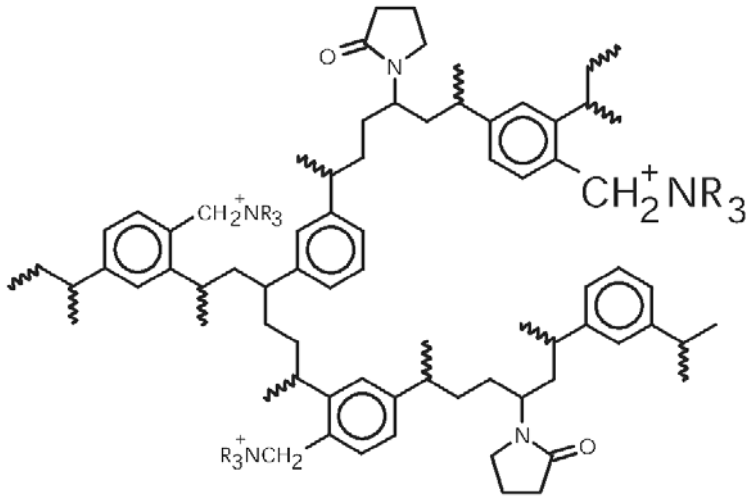


Figure 3-3. Chemical structure of Oasis MAX sorbent

The dual mode of retention of the Oasis MAX adsorbent is based on the reverse phase mechanism and on the interaction between the charged species of the adsorbent and the sample. Figure 3-4 illustrates an example for the quaternary amine of Oasis MAX and the carboxyl group of an acidic compound such as suprofen.

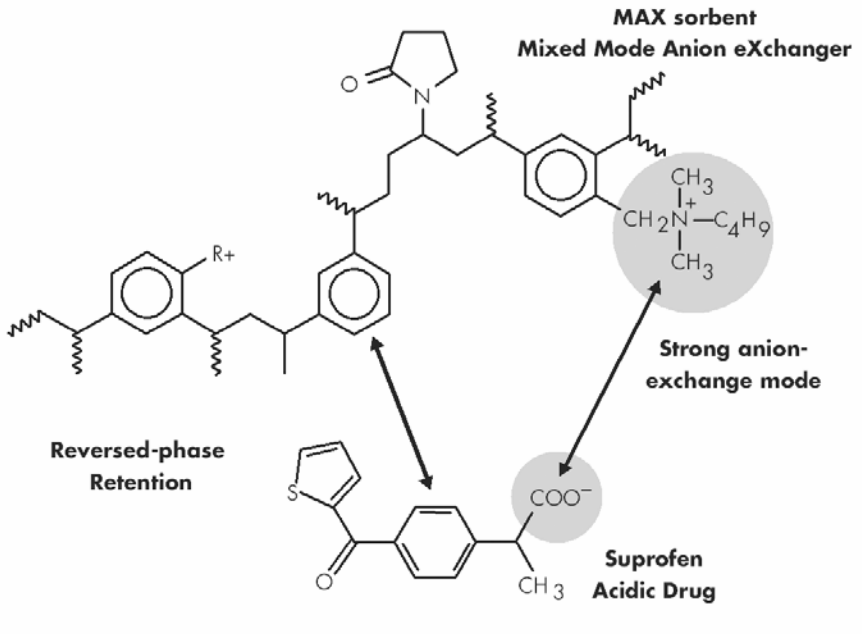


Figure 3-4. Example of a drug-sorbent interaction on Oasis MAX sorbent [161]

The few papers published until now report the successful application of Oasis sorbents in preconcentration and purification procedures. Table 3-1 presents some of the reported Oasis practical applications.

Table 3-1. Common applications of the Oasis MCX and MAX sorbents

Sorbent	Application	Reference
MCX	General screening method for acidic, neutral and basic drugs in whole blood	[162]
MAX, MCX	Acrylamide determination in foods	[163]
MCX	Anticoagulants determination in human plasma	[164]
MAX	Rutin determination in human plasma	[165]
MCX	Cytokinins analysis in coconut water	[166]
MCX	Basic drug determination in rat plasma and human urine	[167]

Amberlite XAD adsorbents

The Amberlite XAD polymeric adsorbents (Rohm and Haas, Philadelphia, USA) have numerous applications such as the removal and recovery of phenols, antibiotics and pesticides. Amberlite XAD-2 is a nonionic polystyrene divinylbenzene resin, normally applied for removal of hydrophobic compounds up

to 20,000 Da. XAD-4 is a hydrophobic polyaromatic resin used for the removal of small hydrophobic compounds, and it is widely encountered in pharmaceutical manufacturing and in the removal of chlorinated organics and pesticides. XAD-9 is a polar resin with a sulfoxide functional group. XAD-16 is hydrophobic polyaromatic, used to remove hydrophobic compounds up to 40,000 Da, commonly for the separation of large organic molecules, especially proteins. The Amberlite XADs are available with a nominal particle size of 20-60 mesh (equivalent to 0.25-0.84 mm) and a pore size of 40-90 Å.

3.1.3. Buffers and solutions

Buffer solutions were applied in procedures such as krill homogenization, enzymatic hydrolysis, ultrasound treatment and separation via chromatography. The solutions were prepared in house with ultra pure water (Millipore Milli-Q water, 18 MΩ cm). The most used buffers were 20 mM Tris-HCl pH 7.5 and 20 mM Tris-HCl pH 7.4 containing 0.15 M ammonium acetate. The pH of the buffer solutions was adjusted with 1 M HCl solution. The buffer solutions were filtered at 0.45 µm and stored in a cool room at 4°C. Stock solutions were kept for maximum 2 days after preparation. The GC-MS solvents (pyridine, chloroform and ethyl chloroformate) were also stored at 4°C and the experimental work with these substances was executed under hood conditions.

3.2. Methods

3.2.1. Krill lyophilization

Homogeneous samples are required for reliable and reproducible experiments. The quality of the different frozen samples delivered is not constant and therefore the desired homogeneity cannot be guaranteed. In addition to this, the handling of frozen samples represents a time-consuming procedure. Therefore, lyophilization (freeze-drying) of krill was performed. Frozen krill samples were allowed to thaw over night at 4°C and afterwards homogenized with 20 mM Tris-HCl buffer (1:1 v/v) pH 7.5 in a Braun domestic mixer for 10 minutes at maximal stirring level. Representative homogenized krill samples (ca. 20-25 g) were lyophilized in a

Christ Alpha 1-2 freeze-dryer and stored in a desiccator under protection of direct sunlight.

3.2.2. Determination of protein concentration

Protein concentration was measured in fresh and freeze-dried krill samples. The fresh krill samples were prepared by thawing the frozen krill over night at 4°C and further homogenization in a Braun domestic mixer for 10 minutes at maximal stirring level. Water was added up to a krill concentration of 30 mg mL^{-1} and the suspension was sonicated for 30 s on ice. The protein determination was performed by the Biuret method (cuvette assays). Bovine serum albumin in the concentration range 0.4 - 4 mg mL^{-1} was used for calibration. Sample dilution was performed in order to fit to the linear absorption range of the calibration curve. For protein determination in lyophilized krill, the krill samples were mixed with water up to a concentration of about 5 mg mL^{-1} and after appropriate dilutions, the same procedure was applied. Absorbance measurements were carried out with an Uvikon XL spectrophotometer at λ = 540 nm.

3.2.3. Selenium extraction from krill samples

Procedure in aqueous media

Lyophilized krill (0.4 g) was suspended in 10 mL H_2O or 10 mL 20 mM Tris-HCl pH 7.5 solution. The samples were incubated at 37°C in a shaker (200 rpm) for different times, and afterwards centrifuged at 48,000 × g for 30 min at 4°C (Beckman centrifuge, JA-20 rotor). Selenium concentrations in the supernatant were determined by inductively coupled plasma mass spectrometry (ICP-MS) coupled to MS analysis. Experimental conditions are presented in Table 3.2.

Enzymatic Digestion

0.4 g lyophilized krill and 0.02 g proteolytic enzyme were suspended in 10 mL 20 mM Tris-HCl pH 7.5 (pH 10 for Novo 0.6 MPX enzyme). The samples were incubated at 37°C in a shaker (200 rpm) for 24 h. The hydrolysates were centrifuged for 30 min at 48,000×g and 4°C. Selenium concentration in the supernatant was determined by ICP-MS. In parallel, control samples consisting only of enzyme and buffer were incubated under the same conditions.

The protein content in the supernatant and in the control samples was determined by the Biuret method in order to estimate the extent of the enzymatic hydrolysis [168].

Ultrasonic treatment

The influence of ultrasonic power on selenium extraction was studied without and with proteolytic enzymes, following the same conditions as for the enzymatic digestion. Ultrasonic irradiation was applied with a Branson Sonifier 450 equipped with a microtip of 3 mm diameter. The output power was set to 20 W, the frequency at 20 kHz and the vibrational amplitude at 50%. The sample was cooled on ice and the temperature profile during the sonication was recorded. For a better understanding of the influence of ultrasound on the enzymatic hydrolysis, similar tests were performed with the cell free protein bovine serum albumin (BSA) and pronase E. The sample sonication was performed for 15 minutes on ice. The same temperature profile was applied as for the ultrasound assisted enzymatic krill digestion.

3.2.4. Determination of total selenium content

In krill

The selenium content in lyophilized krill was analyzed in the Central Laboratory of the Hamburg University of Technology. GF-AAS using a Perkin–Elmer SIMAA 6000 GF-AAS with Zeeman background correction was used as analytical method.

In enzymatic/ultrasonic extracts

The total selenium content in the soluble fractions after enzymatic or ultrasound assisted enzymatic digestion of krill samples and in the fractions obtained after separation by SEC was determined by ICP-MS. The operating conditions are given in Table 3-2.

The instrument employed was an Agilent 7500s inductively coupled plasma mass spectrometer. The isotopes 77Se and 82Se were monitored. Rhodium and Yttrium were added as internal standards. The quantification of the total Se was realized by a standard addition calibration (at µg L^{-1} Se levels) of the ICP-MS signal. Individual calibrations were performed according to sample origin (aqueous or buffer media).

Table 3-2. Operating parameters for the ICP-MS measurements

ICP-MS Parameters	
forward power	1450 W
reflected power	< 0.5 W
plasma gas (Ar) flow rate	15 L min^{-1}
auxiliary gas (Ar) flow rate	1.0 L min^{-1}
carrier gas (Ar) flow rate	0.82 L min^{-1}
nebulizer type	PFA Microflow 100
monitored isotopes	^{77}Se and ^{82}Se

Before the analysis, each sample was diluted with 5% HNO_3 (1:3 v/v) and filtered with a 0.45 μm syringe-driven filter.

3.2.5. Determination of Se-Met

Two methods were applied for the analysis of Se-Met. Liquid chromatography was employed for the experiments where the total selenium contents in the enzymatic extracts and the sonicated samples were analyzed as well, while gas chromatography (after derivatization with ECF) was chosen for the analysis of samples and standard solutions obtained after bifunctional chromatography separation onto the MCX-packed column.

RP-HPLC-ICP-MS method

This specific method was investigated for the identification and quantification of Se-Met in the SEC fractions of krill enzymatic extracts obtained after conventional and ultrasound assisted enzymatic hydrolysis. The chromatographic separation was performed with an Agilent 1100 system, consisting of an on-line vacuum degasser, a binary pump and an autosampler, with a Synergi Hydro reverse phase column (4 μm, 150 mm × 1.0 mm) from Phenomenex) with an injection loop of 5 μL and ICP-MS detection. Calibration was performed with Se-Met solutions in the concentration range from 5 to 200 ng mL^{-1}. The separation conditions are presented in Table 3-3. Before being injected onto the chromatographic system, each sample was diluted and filtered as described above for the ICP-MS analysis. An Agilent 7500s ICP-MS device was used as selenium-specific detector.

Table 3-3. Operating conditions for RP-HPLC analysis of Se-Met

HPLC Parameters	
analytical column	C18 Synergi Hydro, 4 μm, 80 Å, 1.0 mm × 150 mm
mobile phase	0.1% TFA/CH_3OH (98:2 v/v)
eluent flow rate	0.150 mL min^{-1}
sample injection volume	5 μL
elution program	Isocratic
operation temperature	30°C

GC-MS method

Se-Met derivatization was performed according to [118]. Prior to GC measurements, a derivatization reaction with ECF was performed in order to convert Se-Met to a volatile compound, suitable to gas chromatography. The derivatization reaction with ECF was selected because ECF reacts with both the amino and the carboxylic groups of the Se-Met and because of its easiness and fast processing time. The chemical reaction for the derivatization of Se-Met in the presence of a water, ethanol and pyridine mixture is shown in Figure 3-5. Se-Met (4.0 mg) was dissolved in 3.0 mL 0.1 M HCl. A volume of 0.3 mL from this solution was mixed with 0.15 mL ethanol, 0.05 mL pyridine and 0.05 mL ECF in a 1.5 mL vial. The vial was gently shaken and, after CO_2 evolution has ceased, a volume of 0.5 mL $CHCl_3$ containing 1% (v/v) ECF was added to the mixture. Derivatized analytes were stable in tightly closed vials for at least 1 week at 4°C when the organic phase was separated from the aqueous phase. Aliquots (0.2 mL) from the chloroform phase were further analyzed by GC-MS. Pure $CHCl_3$ was employed for the appropriate dilution of the samples.

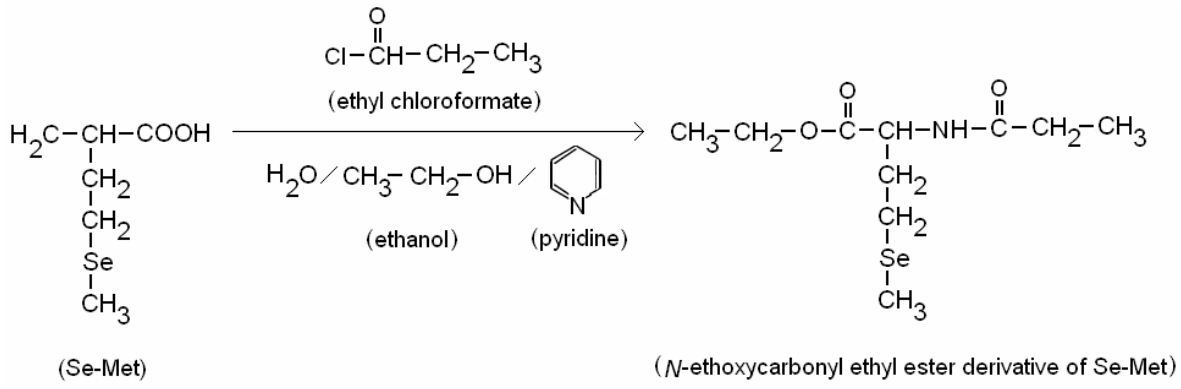


Figure 3-5. Derivatization reaction of Se-Met with ECF for GC-MS analysis

The same procedures were applied for the analysis of Se-Met in enzymatically hydrolysed krill samples and in the fractions collected after chromatographic separation. The 1.5 mL fractions were evaporated to dryness and afterwards dissolved in 0.2 mL 0.1 M HCl and reacted as described above.

The GC-MS experiments were performed with a Hewlett-Packard HP G1800A GCD system. An Agilent HP-5MS capillary column (30 m × 250 µm ID, 0.25 µm phase thickness, cross-linked 5% phenylpolydimethylsiloxane) provided analysis of the sample which was injected in a splitless mode at 250°C. The column was operated with helium as a carrier gas (inlet pressure 40 kPa) at 0.6 mL min^{-1} constant flow rate. The GC-MS analysis was performed with an oven temperature program starting at 120°C and increasing with 20°C min^{-1} up to a final temperature of 290°C (with 5 min stand-by time for each ramp level). The total time for the analysis lasted for 13.5 min. The mass-selective detector was operated in the electron impact ionization mode. A scan time of 1.0 s was applied in the scanning range *m/z* 45 - 425. The selected-ion mode was applied for quantitation with a solvent delay time of 2.0 min, by injecting standard solutions of Se-Met.

The *m/z* values of the ions were 297 (molecular ion), 251, 224, 202, 175, 128 and 109, respectively. The analysis with standard Met solution (expected to be present in a higher concentration than Se-Met in the samples) was also performed at similar derivatization conditions as for Se-Met. In the reference GC-MS analysis for Met, the *m/z* values of the respective methionine derivative were 249 (molecular ion), 188, 175, 129 and 101, respectively.

3.2.6. Separation of selenium rich fractions with SEC

This analytical method allows the separation of compounds from a mixture according to their molecular weight. Preparative Size Exclusion Chromatography (SEC) was employed for the isolation of low molecular weight compounds from krill samples.

SEC was performed with a Pharmacia LKB 2249 solvent delivery system, LKB Bromma 2151 variable wavelength detector and Superdex Peptide HR 10/30 column with a fractionation range of 100 - 7,000 Da and a total volume of 24 mL.

The column calibration was performed with homologous standards (Dextran Blue, cytochrome C, aprotinin, vitamin B12, H-Phe-Leu-OH and tyrosin). The injection

loop was 230 µL and the detector was set to λ = 280 nm. Elution was performed with 20 mM Tris-HCl buffer, pH 7.4 containing 0.15 M ammonium acetate. The eluted fractions were collected with a Pharmacia LKB RediFrac autosampler. At a flow rate of 0.5 mL min^{-1}, 2 mL fractions were collected during 60 min and analyzed off-line for their selenium content by ICP-MS.

3.2.7. Microscopy

The difference in the structure of the krill samples before and after enzymatic treatment and ultrasound assisted enzymatic treatment was observed by microscopy with a Zeiss Axioskop and a magnification of 100. Images were captured using a JVC TK-C1381 colour video camera and further processed using Microsoft Paint version 5.1.

3.2.8. Adsorption behaviour of Se-Met onto XAD and Oasis adsorbents

Solid phase extraction (SPE) is an established sample preparation method, widely employed for sample preconcentration and purification procedures, especially in environmental analysis, food chemistry and medicine. This procedure was employed for the analysis of Se-Met retention from standard solutions and krill samples. The advantages of SPE over other separation techniques are high recoveries, automation easiness, higher degree of sample purity and low costs. It comprises the concentration and the purification of analytes from solution by sorption onto a disposable solid phase cartridge, followed by the elution of the analyte with a solvent appropriate for instrumental analysis. This technology can be applied for the retention of product and elution of interferences and for the retention of interferences and elution of desired product as well. SPE procedures can involve ion exchangers, size exclusion, normal and reverse phase sorbents [160]. The normal phase procedures involve a polar stationary phase and a polar analyte. The retention is due to interactions between polar groups of analyte and sorbent surface (hydrogen bonding, pi-pi or dipole-dipole interactions, etc.). For the elution, a solvent that disturbs the binding mechanism (e.g. more polar than the sample's original matrix) is chosed [169]. A very important role in SPE procedures plays the pH factor. Silica-based packings are stable in a pH range of 2 - 7.5. Polymeric based materials such as Oasis HLB, MAX, MCX, ENVI-Chrom P

or ENVI-Carb are stable over the pH range of 1-14. One of the SPE related methods is SPME (solid phase microextraction), which involves the use of a microfiber immersed into a sample for sorption, followed by direct desorption in the inlet of the gas chromatograph [160].

Since selenium concentrations in natural samples are low, sample preparation methods such as SPE and SPME are often applied in preconcentration procedures and therefore represent an alternative of improving detectability. Another advantage of the SPE and SPME is the preservation of selenium species over the preconcentration procedure. Most of the reported SPE technical works deal with the inorganic species Se (IV) and Se (VI) [170] and only few concentrate on seleno amino acids [122, 171]. Sorbents such as Cellex T, Dowex 50Wx4, Amberlite IRA-400, AG MP 1 or Chelex 100 were involved in Se-Met studies. Selenomethionine was chosen as the organic form of selenium because it is a good model for naturally incorporated selenium in the food chain. Very low detection limit for Se-Met (32 ng L^{-1} as selenium) and the successful separation of Se-Met from inorganic Se (as Se IV and Se VI) with a dual-column system implying the Cu-Chelex resin (a chelating resin with iminodiacetic acid exchange groups) for Se-Met retention and the Cellex T material (highly purified cellulose powder with quaternary amine exchange groups) for inorganic Se retention were reported [171].

Batch experiments of Se-Met adsorption onto tested adsorbents

Batch experiments are the first step in the adsorbent characterization and deliver information about Se-Met adsorption parameters. The XAD bulk material was prepared before usage as follows: 30 mg were washed with 1 mL ultra-pure water in a 2 mL Eppendorf cup, centrifuged at 13,000 rpm for 2 min and after water removal, gently dried at 35°C overnight before use. Se-Met samples (0.35 mg in 1 mL of 20 mM Tris-HCl buffer) were added to the sorbent and the samples were incubated for 90 min at room temperature under gentle rotation (6 rpm) at various pH values. After incubation, samples were filtered and Se-Met concentrations in the supernatants were determined by GC-MS, as described above. The adsorption was quantified by subtracting the amount measured in the supernatant solution from the initial amount present in the sample. Adsorption of Se-Met on Oasis MCX

and Oasis MAX mixed mode adsorbents was performed according to the manufacturer instructions, as shown in Table 3-4.

The contact time, pH and temperature were optimized. Since bulk material was not commercially available, the syringe barrels were cut and the frits removed in order to release the packed adsorbent. Adsorption experiments were carried out in a discontinuous operation by incubation of 30 mg adsorbent (content of one cartridge) and 1 mL 5 mM Se-Met solution in 0.1 M HCl and in sodium carbonate buffer pH 11, I = 0.1 mol m^{-3} respectively for 30 min at gentle rotation (6 rpm).

Table 3-4. Standard protocol for Oasis adsorbents (30 mg cartridge)

Step	MAX	MCX
Conditioning	1 mL CH_3OH	1 mL CH_3OH
Equilibration	1 mL H_2O	1 mL H_2O
Loading	1mL sample	1 mL sample
Wash I	1mL H_2O + 2% NH_4OH (1 min)	1mL 0.1 M HCl (1 min)
Wash II	1 mL CH_3OH (1 min)	1 mL CH_3OH (1 min)
Elution	1mL CH_3OH + 5% HCOOH	1mL CH_3OH + 5% NH_4OH

Se-Met solutions without adsorbent were also incubated under similar conditions as reference samples. The adsorption protocol for the Oasis sorbents is given in Table 3-5. The experiments showed that a second washing step with methanol, as recommended by the manufacturer, was not necessary. Therefore only one washing step was employed in our experiments. After incubation, the samples were centrifuged at 13,000 rpm (Heraeus Minifuge) for 3 min. The supernatant was removed with a pipette. Se-Met concentrations in the eluted samples were determined by GC-MS, as described above. Prior to GC-MS analysis, the samples were evaporated to dryness in a Speed Vac Concentrator at room temperature and afterwards dissolved in 0.1 M HCl.

Table 3-5. Adsorption protocol for Se-Met on Oasis adsorbents

Step	MAX	MCX
Conditioning	1 mL CH_3OH	1 mL CH_3OH
Equilibration	1 mL H_2O	1 mL H_2O
Loading	1mL sample (pH 11)	1 mL sample (pH 1)
Washing	1mL H_2O + 2% NH_4OH	1mL 0.1 M HCl
Elution	1mL CH_3OH + 5% HCOOH	1mL CH_3OH + 5% NH_4OH

The Speed Vac Concentrator step was necessary because the presence of methanol in the eluted fractions would influence the derivatization reaction with ECF (formation of by-products).

Adsorption isotherms

Experiments were performed with Oasis sorbents (MCX and MAX) and with XAD bulk materials (XAD-2, XAD-4, XAD-9 and XAD-12). The XAD bulk materials were investigated and, for the adsorbent showing best performance in individual experiments, the adsorption isotherm was determined. Samples with different concentrations of Se-Met (0.15 to 18.5 mg mL^{-1} in 0.1 M HCl, sample volume 1.0 mL) were incubated with 30 mg adsorbent for 30 min at room temperature and gentle rotation (6 rpm). Experiments with the XAD material showing optimal adsorption properties were performed as described above (for 30 min incubation time as well). After centrifugation at 13,000 rpm (2 min), the supernatant was separated and its Se-Met concentration was determined by GC-MS. The amount of bound Se-Met per mass unit of adsorbent was calculated by subtracting the amount of unbound amino acid from the total added (initial concentration in the solution). The equilibrium solid phase concentration was plotted against the equilibrium liquid phase concentration of the adsorbate. The maximal binding capacity (q_{max}) and the equilibrium constant (K) were determined by linearizing of binding data and representing them in a Scatchard plot, as described in the chapter 2.4.2.

3.2.9. Bifunctional chromatography of Se-Met on MCX column

Bifunctional chromatography was performed in order to obtain information about Se-Met adsorption under dynamic conditions. A chromatographic column (4.6 mm

× 250 mm) packed with Oasis MCX adsorbent (a research sample prepared by Waters GmbH, Eschborn, Germany) was coupled to a Kontron HPLC-system with UV detector. The column specifications are presented in Table 3-6. The MCX column was conditioned with 100% CH_3OH for 4 min, washed with pure H_2O for 4 min and equilibrated with 0.2 M HCl-KCl buffer solution (pH = 2, I = 0.1) for 4 min at a flow rate of 1.5 mL min^{-1} and room temperature. 20 µL sample with 2 mg mL^{-1} Se-Met in 0.1 M HCl was injected at a flow rate of 1 mL min^{-1}. Four minutes after the sample injection a step gradient 0-50% CH_3OH containing 10% NH_4OH was applied at a flow rate of 1.5 mL min^{-1}. The elution profile was monitored at a wavelength λ = 214 nm. The eluted fractions (1.5 mL) were collected with a Pharmacia LKB RediFrac autosampler, transferred with an Eppendorf pipette into 2 mL Eppendorf cups and immediately subjected to evaporation until dryness in a Speed Vac Concentrator at room temperature and analyzed off-line for their Se-Met content by GC-MS.

Table 3-6. Oasis MCX column specifications, manufacturer parameters

Parameter	Average Value
Specific surface area ($m^2 g^{-1}$)	782
Average pore diameter (Å)	83
Total pore diameter ($cm^3 g^{-1}$)	1.25
Average particle diameter (µm)	33.5
Sulfonic acid content (meq g^{-1})	0.84

Experiments with standard solutions of Met and Se-Met

For the determination of the column dynamic capacity, 5 mg mL^{-1} Se-Met solution was loaded onto the column in 1 mL consecutive injection steps. The procedure was repeated until no more adsorption occurred and this was recorded as dynamic column capacity.

Experiments with krill samples

After separation of selenium rich fractions from krill enzymatic hydrolysates with SEC on the Superdex peptide HR 10/30 column, the collected fractions (2 mL) were freeze-dried and suspended in 0.1 M HCl (dried sample to acid ratio 1:32 v/v)

prior to the bifunctional chromatography on the MCX column. This step was necessary because of the low selenomethionine concentrations in the fractions collected after size exclusion chromatography of krill hydrolysate samples. The volume of HCl solution added was kept to minimum needed for solubilizing the dry SEC fractions, in order to reduce the sample volume which will be further on injected onto the MCX packed column. A sample with a final volume of 5 mL was loaded onto the chromatographic column packed with the MCX adsorbent in 5 consecutive steps, each step with an injection loop of 1 mL. The splitting of the sample in the 1 mL fractions was performed as a safety measure because the column overload was suspected.

Chromatographic samples with a volume of 1.5 mL were collected over the whole range with a RediFrac autosampler device. The collected fractions were analyzed by GC-MS. Se-Met was detected only in the eluted peak after the application of the gradient. The spiking with Se-Met solution was performed as an additional identification method of Se-Met in krill samples (chromatographic fractions collected after bifunctional chromatography on MCX column). For this purpose, 50 µL of the collected fraction containing Se-Met were added to 100 µL acidic standard solution of Se-Met (1.33 mg mL^{-1} Se-Met in 0.1 M HCl)

The ion of the *N*-ethoxycarbonyl ethyl ester derivative of Se-Met (obtained after the derivatization with ethyl chloroformate) was recorded at m/z = 297 in the mass spectrum. The result is identical to Se-Met standards analyzed before and coincides with previously reported data [118, 123].

4. Results and Discussion

4.1. Krill lyophilization

Fresh krill samples are difficult to handle because they are not homogeneous and special care has to be taken for storage conditions. Especially the fat distribution in the fresh krill samples may alter the experiments. Increased homogeneity was achieved by sample lyophilization. The sample mass loss versus lyophilization time is represented in Figure 4-1.

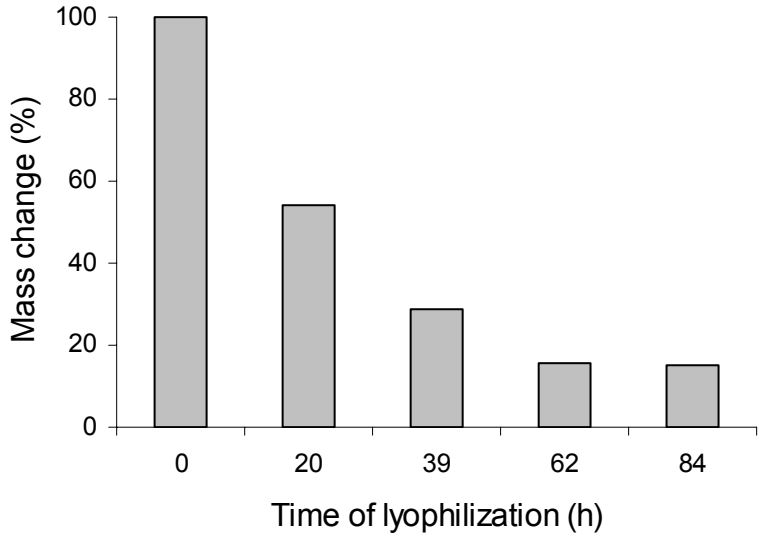


Figure 4-1. Mass loss of krill samples during lyophilization process

A mass reduction to approximately 16% was recorded at the end of the freeze-drying process. After ca. 84 h, the sample mass remained constant and the procedure was stopped. The freeze-dried krill samples were stored as described in the chapter 3.2.1.

4.2. Analysis of protein content in krill samples

Protein content of the krill samples was determined in fresh and freeze-dried samples. The krill homogenates obtained from fresh samples contained an average of 17±3% protein. This value corresponds to other reported values (17-22%) for fish species [172]. During the freeze-drying procedure, the water is removed from fresh krill samples and the mass remaining afterwards consists

mainly of protein mass. This explains the high protein content measured in the lyophilized krill samples (ca. 95%, reported to freeze-dried mass).
The Biuret method was chosen after preliminary comparison of different protein assays. The Bradford technique yielded values up to tenfold lower, which has also been encountered by other authors [173].

4.3. Selenium extraction from krill

Total selenium content of Antarctic krill determined by GF-AAS method was 2.4 μg g^{-1} (dry weight). This value is in the range 1-8 μg g^{-1}, previously reported in the literature for krill samples [91, 174].

4.3.1. Aqueous method

After aqueous extraction of krill samples, about 24% of total Se was detected in the soluble fraction. This result is in good agreement with recent studies (23-34%) [172]. The remaining 76% of the total Se were found in the pellet, bound in high molecular weight insoluble proteins.

4.3.2. Enzymatic method

Several enzymes were tested to improve the total Se extraction from the krill protein and the release of low molecular selenium organic compounds. The enzymatic extraction procedure with pronase E, subtilisin Carlsberg, trypsin, α-chymotrypsin, proteinase and proteinase N from *B. subtilis* and Novo 0.6 MPX protease was performed for an incubation time of 24 h. The lowest residual protein content, indicating a higher extent of proteolysis, was determined in the samples treated with pronase E, followed by subtilisin Carlsberg, whereas the other enzymes did not hydrolyse more than 25-40% of the initial protein content (Figure 4-2).
These results can be explained by the broader specificity of pronase E and subtilisin compared to the other used enzymes. Trypsin, for example, preferably cleaves at lysine and arginine residues, whereas α-chymotrypsin acts on hydrophobic residues such as tyrosine, phenylalanine and tryptophan.

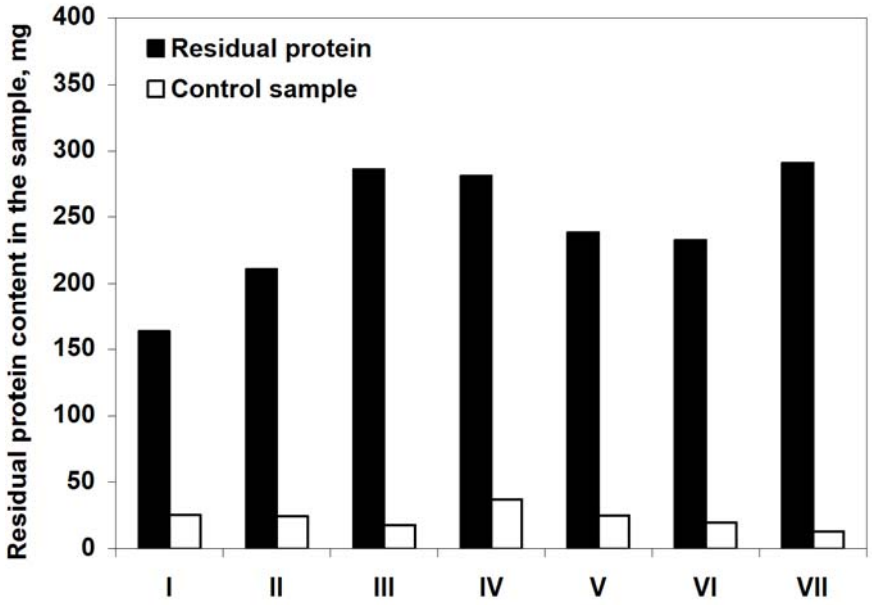


Figure 4-2. Protein digestion of Antarctic krill with proteolytic enzymes: pronase E (I), subtilisin Carlsberg (II), trypsin (III), trypsin/α-chymotrypsin mixture (1:1 w/w) (IV), *B. subtilis* proteinase (V) and proteinase N (VI), and Novo 0.6 MPX enzyme (VII). Initial protein content was 380 mg (consisting of 360 mg krill and 20 mg enzyme). The samples were incubated for 24 h at 37°C and 200 rpm. Control samples were incubated under similar conditions and consisted of enzyme and buffer solutions only.

The enzymatic digestions were initially performed for 24 h. Lower incubation periods were tested in order to check the possibility of time shortage for the experiments. The results of enzymatic digestion in water and in 20 mM Tris-HCl buffer pH 7.5 are presented in Figure 4-3. The experiments showed that best extractions yields are achieved when samples are incubated for 24 h and Tris-HCl buffer is used as incubation media. This can be explained by the more favourable conditions (controlled pH level in the sample) in Tris-HCl media, which ensure enzyme activity during the hydrolysis. The appropriate enzyme/sample ratio was chosen by testing different amounts of enzyme (1:10 to 1:40 w/w). Optimal digestion results (based on the amount of hydrolysed substrate and experiment reproducibility) were obtained with an enzyme:substrate ratio of 1:20 w/w and this was used in further digestion experiments. Successive analysis of digested samples confirmed the quantitative Se extraction with this enzyme:substrate ratio. Higher substrate to enzyme ratios were often reported by other authors for experiments related to protein structure cleavage and quantitative selenium

recovery [11, 77] but the experiments in this study showed that optimal results could be achieved also with a smaller amount of enzyme [175].

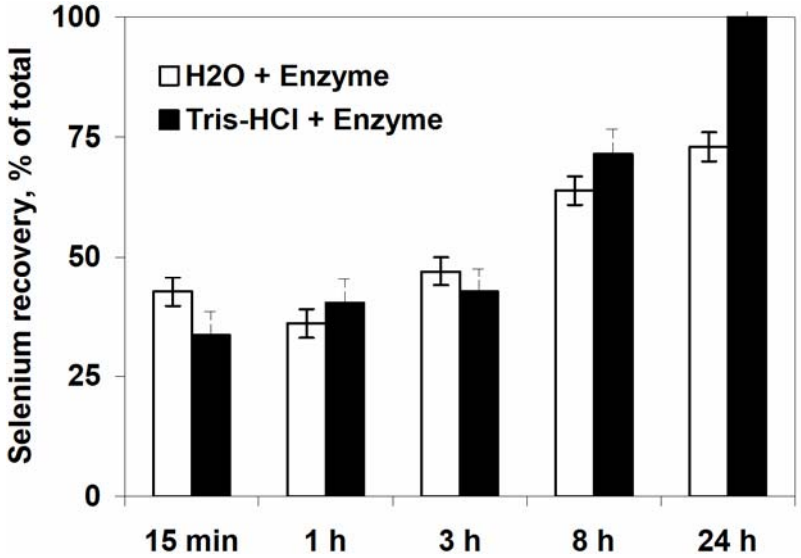


Figure 4-3. Enzymatic digestion of Antarctic krill samples in water and buffer (20 mM Tris-HCl pH 7.5) media with pronase E (1:20 w/w) at 37°C and 200 rpm.

The highest enzyme efficiency (expressed as the ratio between selenium content in digested liquid extracts and the initial selenium content in the samples) was achieved with pronase E (approximately 96%), as shown in Figure 4-4.

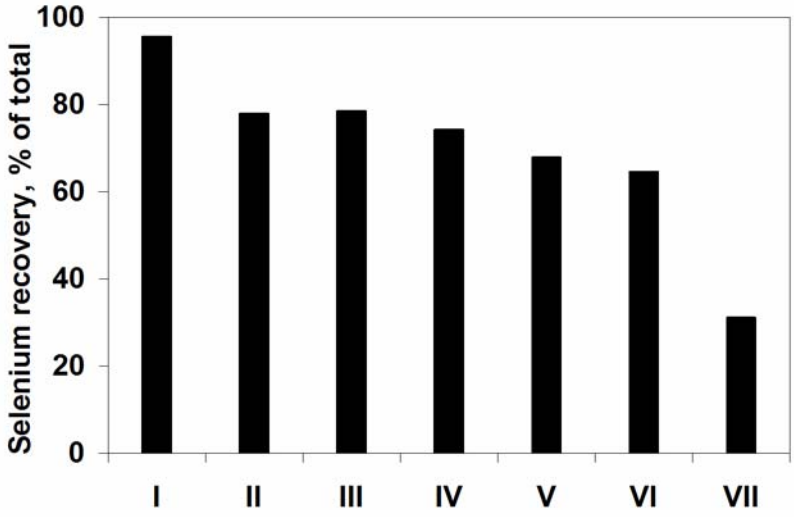


Figure 4-4. Selenium recovery from Antarctic krill samples after treatment with proteolytic enzymes: pronase E (I), subtilisin Carlsberg (II), trypsin (III), trypsin/ α-chymotrypsin mixture (1:1 w/w) (IV), *B. subtilis* proteinase (V) and proteinase N (VI), and Novo 0.6 MPX enzyme (VII). The samples were incubated for 24 h at 37°C and 200 rpm. Control samples were incubated under similar conditions and consisted of enzyme and buffer solutions only.

The enzymatic extracts obtained after digestion with pronase E were used for the further experiments.

4.4. SEC of selenium rich fractions after enzymatic digestion

The SEC on a Superdex peptide HR 10/30 column of krill hydrolysate, obtained after digestion with pronase E, is presented in Fig. 4-5 A.

4.4.1. Selenium content in SEC fractions

In order to analyze selenium distribution in the hydrolysate, SEC fractions were collected according to the calibrated molecular weight ranges and their selenium content was determined off-line by GF-AAS (Fig. 4-5 B). 80% of the total Se was found in the fractions corresponding to compounds with molecular weight 600-150 Da; the remaining 20% were still bound to proteins and larger peptides. The sum of the selenium content in the collected fractions was equal to the initial total selenium amount. This quantitative recovery implies that the amount of selenium in each fraction was correctly determined and that the detection limit of the GF-AAS method (4 µg L^{-1}) was precise enough for this purpose. The chromatograms of standard solutions of inorganic selenium compounds such as selenate and selenite showed that these compounds did not elute within the total column volume. The fact that all selenium compounds in the krill hydrolysate eluted before the total column volume, indicates that the recovered selenium species were organically bound.

The selenium distribution profile shows also that the enzymatic hydrolysis is an appropriate approach for releasing selenium compounds from the krill proteins.

The HPLC-ICP-MS analysis of the fraction 600-150 Da showed only two intense peaks for Se-containing organic compounds (Figure 4-6).

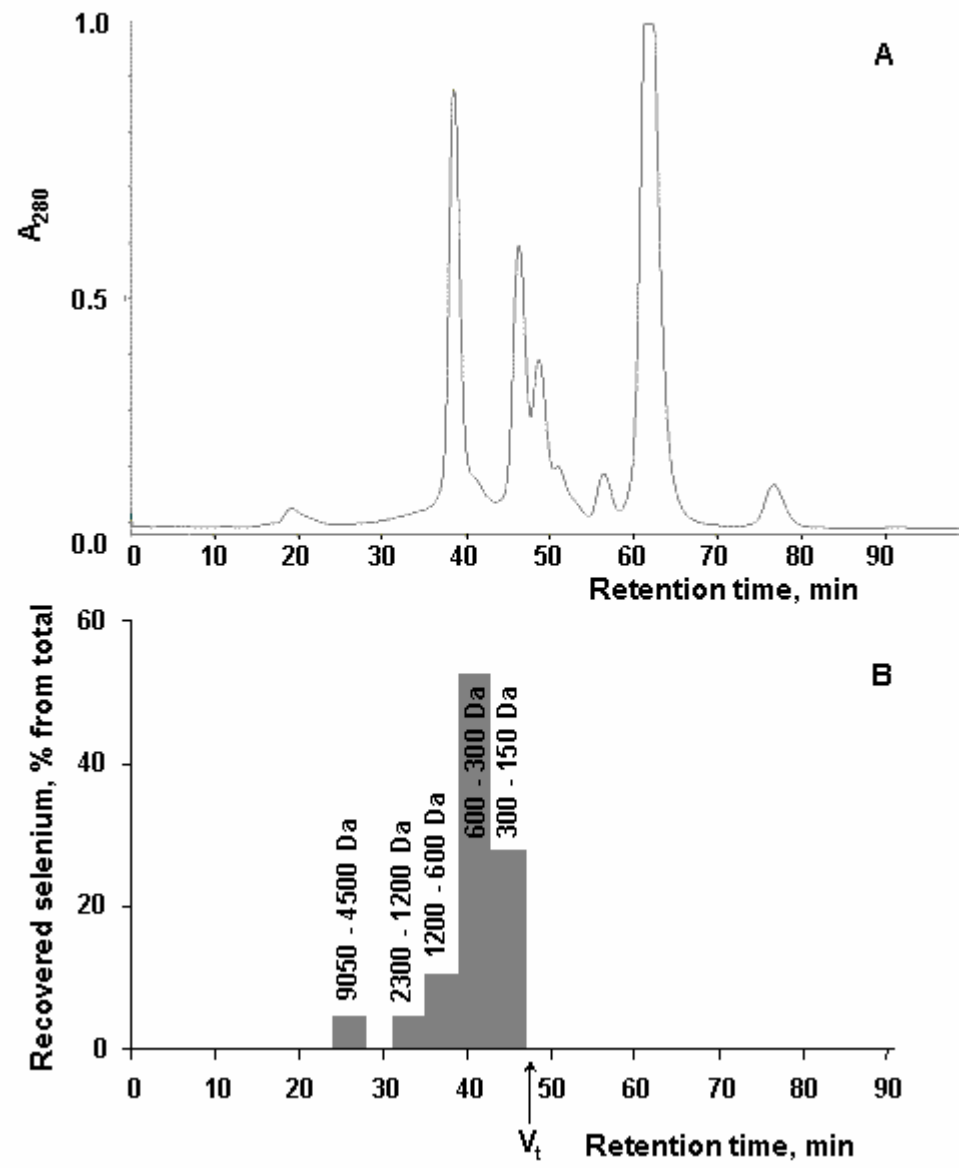


Figure 4-5. (A) Size exclusion chromatogram on Superdex peptide HR 10/30 column of krill homogenate obtained after digestion with pronase E as described in chapter 3.2.3. Elution conditions: 20 mM Tris-HCl pH 7.4, containing 150 mM ammonium acetate at flow rate 0.5 mL min^{-1} and 25°C. (B) Selenium distribution in fractions collected during the SEC of enzymatically digested krill as presented in A. The arrow indicates the total column volume.

The peak with the retention time of approximately 2.5 min matched with standard Se-Met and was identified by the method of standard addition. The peak with the retention time of approximately 1 min matched to Se-Cys_2 standard solution, and its intensity increased by standard addition to the sample [175].

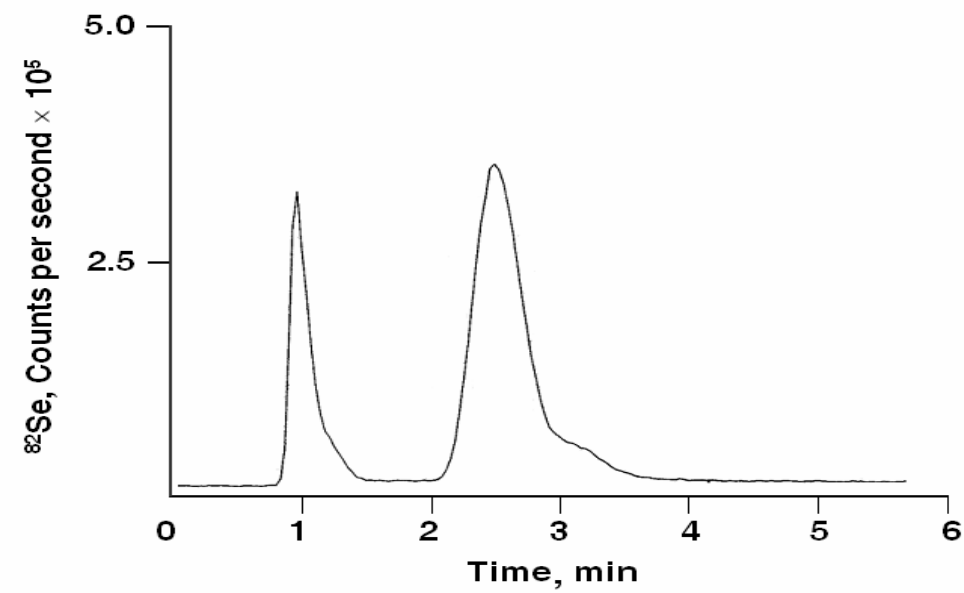


Figure 4-6. HPLC-ICP-MS chromatogram of the 600-150 Da fraction obtained from Antartic krill after digestion with pronase E.

Since Se-Cys$_2$ eluted near the void volume, one could not exclude a presence of other Se species within the molecular weight range of the fraction, most probably Se-Cys$_2$/Se-Cys$_2$ derivatives [108].

4.4.2. Se-Met content in SEC fractions

The selenomethionine content was determined in the enzymatic extracts obtained after digestion of krill samples with the proteolytic enzyme pronase E and the in fractions collected after size exclusion chromatography of these enzymatic digests. The Se-Met content after enzymatic digestion summarized about 40% of the total initial amount of selenium present in the original krill samples. The recovery of Se-Met over the chromatographic column was quantitative. The analysis of chromatographic fractions showed that Se-Met was present only in the fractions with molecular weights lower than 600 Da (amino acids range). The results for the SEC experiments are presented in Table 4-1. The analysis of chromatographic fractions showed that Se-Met within the detection limit was present only in the fractions with molecular weights lower than 600 Da. The recovery of Se-Met by the SEC procedure was quantitative as well and about 97% of the initial amount was found in the fractions with molecular weights corresponding to the respective amino acids. In these fractions the recovery of

total Se was about 80% (related to the whole amount measured in all SEC fractions).

Table 4-1. Recovery of Se and Se-Met after SEC of hydrolysed krill samples

Sample		Concentration, ng mL^{-1}	Recovery, %
Pronase E hydrolysate of krill [a]	Se	98±2	98±2
(24 h incubation time)	Se-Met	101±3	- [c]
SEC fractions of pronase E	Se	4.6±0.5	80±2
hydrolysate of krill (600-150 Da) [b]	Se-Met	5±1	40±2 [d]

[a] The initial concentration of lyophilized krill in the sample was 40 mg mL^{-1}

[b] 0.23 mL from the krill extract after enzymatic digestion with pronase E were subjected to SEC. The fractions corresponding to a molecular weight range of 600 to 150 Da were pooled and analyzed together (4 mL)

[c] Initial concentration of Se-Met in lyophilized krill not detected.

[d] Further recovery values for Se-Met are based on concentrations measured in the krill hydrolysate obtained by digestion with pronase E.

4.5. Ultrasound assisted digestion

The ultrasound method was applied to samples obtained after aqueous extraction with water and buffer solutions and to enzymatic extracts obtained after digestion with pronase E.

The total selenium content in the lyophilized krill (2.5±0.2 µg g^{-1}) and in the solubilized fractions was used to compare the efficiencies of the different procedures for the extraction of Se and Se organic compounds. The maximal selenium recovery did not differ much for aqueous extraction with pure water (24%) and with 20 mM Tris-HCl pH 7.5 (27%). Thus, the reference experiments for aqueous extraction with and without ultrasonication were carried out in pure water. The enzymatic digestion of krill with and without ultrasonication and the successive extraction of selenium organic compounds were performed in both pure water and buffer. The results are presented in Figure 4-7.

Ultrasonication in aqueous media without enzyme for 15 min resulted in a total selenium extraction efficiency of about 38%. Similar selenium recoveries have

been reported for yeast [6] and chicken muscle [7]. This value is not much higher than in the case of aqueous extraction without ultrasound treatment (Fig. 4-7, experiment I). In addition, the Se extraction yield without ultrasound application did not substantially differ for the three procedures compared. This implies that the selenium species dissolved within 15 min are probably not included in high molecular weight protein structures which would require longer action of proteolytic enzymes.

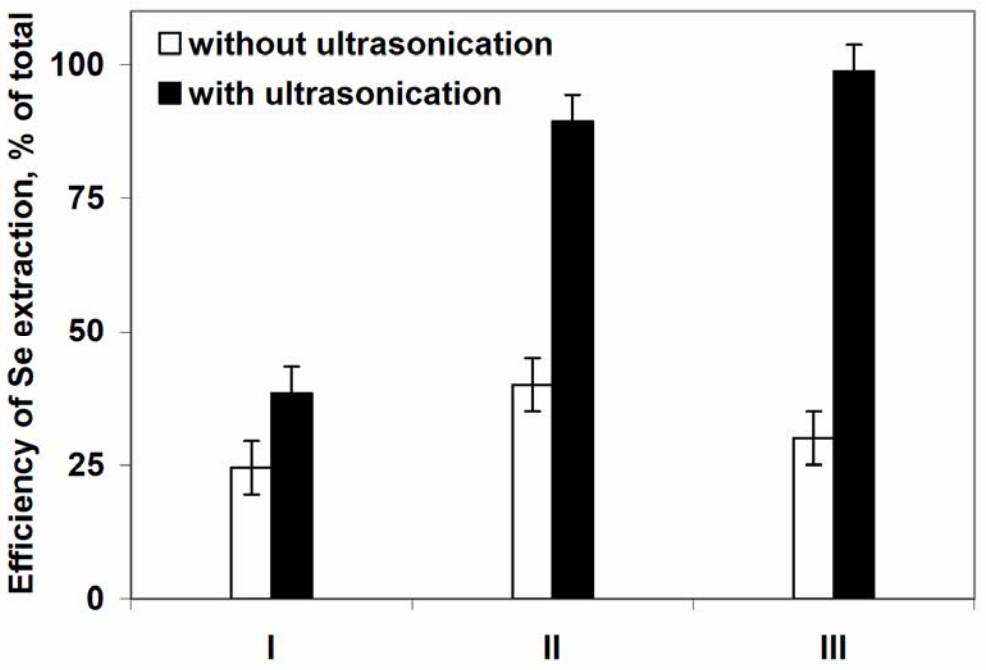


Figure 4-7. Selenium extraction from Antarctic krill with and without simultaneous ultrasound treatment: (I) extraction with pure water; (II) digestion with pronase E in aqueous medium; (III) digestion with pronase E in 20 mM Tris-HCl pH 7.5. All experiments were performed at 37°C and 200 rpm for 15 min and with a substrate concentration of 40 mg mL^{-1}. The enzyme concentration in samples II and III was 2 mg mL^{-1}. Each extraction procedure was performed three times and then the Se concentration in the hydrolysate was determined by ICP-MS. The calculated standard deviation intervals were based on three measurements.

Compared to the aqueous extraction, the enzymatic hydrolysis with pronase E was much more strongly influenced by ultrasound application. Whereas the use of proteolytic enzymes in extraction of selenium from various natural products leads to quantitative recoveries in 24 to 48 h incubation periods [12], by the combined use of enzymes and ultrasonication, the processing time was reduced by 2 orders

of magnitude and quantitative Se recovery was achieved within 15 min (Figure 4-8).

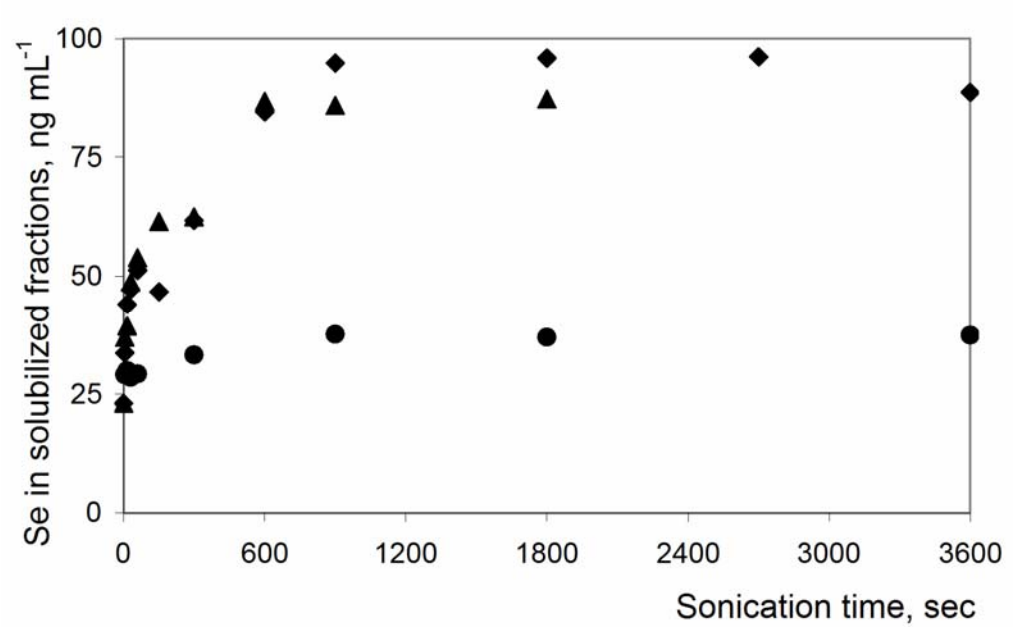


Figure 4-8. Influence of sonication time on the extraction yield of total selenium from Antarctic krill: •: sonication without enzyme in water; ▲: ultrasound assisted digestion with pronase E in water; ♦: ultrasound assisted digestion with pronase E in 20 mM Tris-HCl pH 7.5. In all cases the concentration of the lyophilized krill was 40 mg mL^{-1}, and the ratio of krill to pronase E was 20:1 (w/w).

Highest Se recovery was achieved for the experiments performed in the Tris-HCl medium (96±2%). The ultrasound sample treatments in aqueous media lead to a lower recovery (ca. 10% less than for Tris-HCl media). The pH control in the buffer media offered optimal reaction conditions for the enzyme, prevented from enzyme inactivation during treatment and may be the reason for the relatively higher efficiency of Se extraction [176]. Better efficiency for ultrasound assisted enzymatic hydrolysis was also observed by other authors [7] in relatively similar experiments with chicken samples (28% higher values for Se recovery in Tris-HCl media). The shorter sonication time reported in the literature for the extraction of selenium from yeast or chicken samples can be explained by the higher enzyme concentration used in these cases [6, 7].

The more pronounced influence of ultrasound on the enzymatic extraction raises the question about the mechanism of enhancement of the biocatalytic reaction. Similarly to the influence of the sonication time on the total Se recovery, the

maximal Se-Met extraction yield was also achieved within 15 min. Further extension of the sonication time up to 60 min showed that the HPLC-ICP-MS signal for Se-Met in the hydrolysate did not change, i.e. its concentration remains constant. No changes in the chromatogram pattern were observed. The extended sonication time did not cause the interconversion of Se species, as reported in the literature for other food samples as well (where Se-Met in standard solutions and in enzymatic extracts remained stable for at least 24 h after sonication at room temperature [7].

4.6. SEC of selenium rich fractions after sonication

The chromatogram of krill extracts after 15 min ultrasound assisted enzymatic hydrolysis presented a profile similar to the chromatogram obtained for enzymatically digested krill fractions (pronase E, Figure 4-5 A).

4.6.1. Se content in SEC fractions

The total selenium content was determined in the collected SEC fractions (Figure 4-9). The sum of the selenium content in the fractions with molecular weights lower than 1200 Da was more than 90% of the total selenium content in the extracts [176]. This suggests that most of the selenium was released from the protein structures and was present in the hydrolysate as amino acids or was incorporated in small peptides and only 10% remained in the structure of proteins and larger peptides. The sum of the selenium content in the collected fractions was equal to the initial total selenium amount. The chromatograms of standard solutions of inorganic selenium compounds such as selenate and selenite showed that these compounds did not elute within the total column volume. For the krill hydrolysate obtained by combined enzymatic and ultrasound treatment, no Se was found in the SEC fractions eluted after the total column volume. The fact that all selenium compounds in the krill hydrolysate eluted before the total column volume, indicates that the recovered selenium species were organically bound.

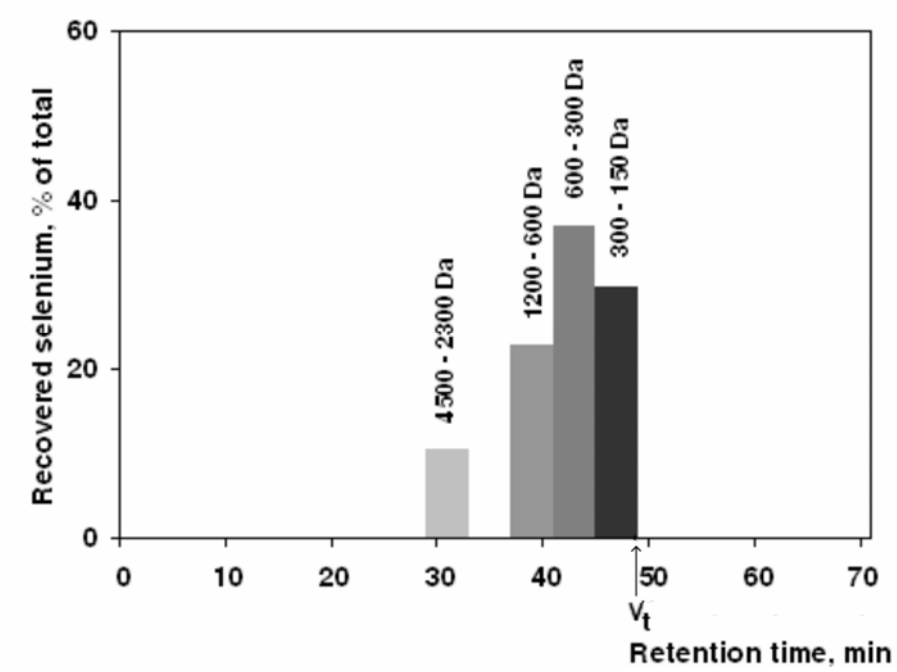


Figure 4-9. Selenium content in the fractions collected during SEC on Superdex peptide HR 10/30 column of krill hydrolysate obtained by ultrasound assisted enzymatic hydrolysis after 15 min. Elution with 20 mM Tris-HCl pH 7.4 containing 150 mM ammonium acetate at a flow rate of 0.5 mL min^{-1} and 25°C. The arrow indicates the total column volume.

4.6.2. Se-Met content in SEC fractions

The Se-Met content in the krill extract after enzymatic hydrolysis, in the krill extract after enzymatic hydrolysis combined with sonication and in the 600-150 Da fractions from SEC shown in Figure 4-8 determined by HPLC-ICP-MS is given in Table 4-2. Similar Se-Met recoveries resulted from the two enzymatic procedures. The SEC step for Se-Met isolation from the sample after 15 min ultrasound assisted hydrolysis with pronase E was quantitative (the Se-Met amount determined in the hydrolysate obtained after ultrasound assisted hydrolysis was completely recovered in the 600-150 Da SEC fractions, summarizing ca. 35% of the total Se content in the enzymatic extract).

The results show a significant increase in the released Se-Met when subjecting enzymatic samples to ultrasound treatment. The treatment of krill samples in the absence of enzyme released a minimal amount of Se-Met from the krill matrix. A Se-Met concentration of only 3 ng mL^{-1} was measured in the samples subjected without enzymes to ultrasound treatment. Thus, the ultrasound assisted enzymatic

hydrolysis of krill followed by SEC offers a fast and efficient procedure for extraction of this amino acid.

Table 4-2. Recovery of Se and Se-Met after SEC of sonicated krill samples

Sample		Concentration ng mL^{-1}	Recovery %
Krill extract after ultrasound assisted enzymatic hydrolysis for 15 min [a]	Se	97±2	97±2
	Se-Met	86±2	35±2 [c]
600-150 Da fractions of krill hydrolysate obtained by ultrasound assisted enzymatic hydrolysis for 15 min [b]	Se	4±1	70±2
	Se-Met	5±1	35±2 [c]

[a] The initial concentration of lyophilized krill in the sample was 40 mg mL^{-1}

[b] 0.23 mL from the krill extract after ultrasound assisted enzymatic digestion were subjected to SEC and the 600-300 Da and 300-150 Da fractions were pooled (total 4 mL)

[c] referred to Se-Met concentration of pronase E krill hydrolysate

The results in Table 4-2 show that half of the Se identified in the SEC fractions with molecular weights corresponding to amino acids is present as Se-Met. The part of Se present as Se-Met (35% of total Se in krill) is lower than the values reported for swordfish, where Se-Met makes up ca. 93% of the total Se [19] and it is comparable to measured levels in oysters or tuna fish [19, 65], as presented in Table 2-3. The percent of Se-Met detected in samples such as yeast (64-78% in [61, 73, 74]) or different types of nuts (19-25% in [9], 42% in [12], or 75% in [10]) varies considerably and therefore the comparison with these values is not significant for data interpretation.

Remaining Se may be present in other organic forms such as trimethylselenonium ion, Se-cystine or methyl-Se-cysteine, compounds which were previously detected beside Se-Met in samples such as yeast [12, 75] or oyster tissue [65].

The mechanism of enhancement of the enzymatic digestion of biological samples and the successive extraction of selenium organic compounds has not yet been

investigated. In the few published papers on enzymatic reactions combined with ultrasonication, only general considerations on this have been noted [6, 7, 153].
In order to gain more information on whether the enhancement of the biocatalytic reaction is a result of reduction of the mass transfer limitations or wether the ultrasound modulates the enzyme/substrate interactions, reference experiments in a homogeneous system with cell-free protein were performed. Bovine serum albumin is a commonly encountered standard protein in ultrasound studies [157, 177]. Enzymatic hydrolysis of BSA with pronase E with and without simultaneous sonication was performed and the results are presented in Figure 4-10.

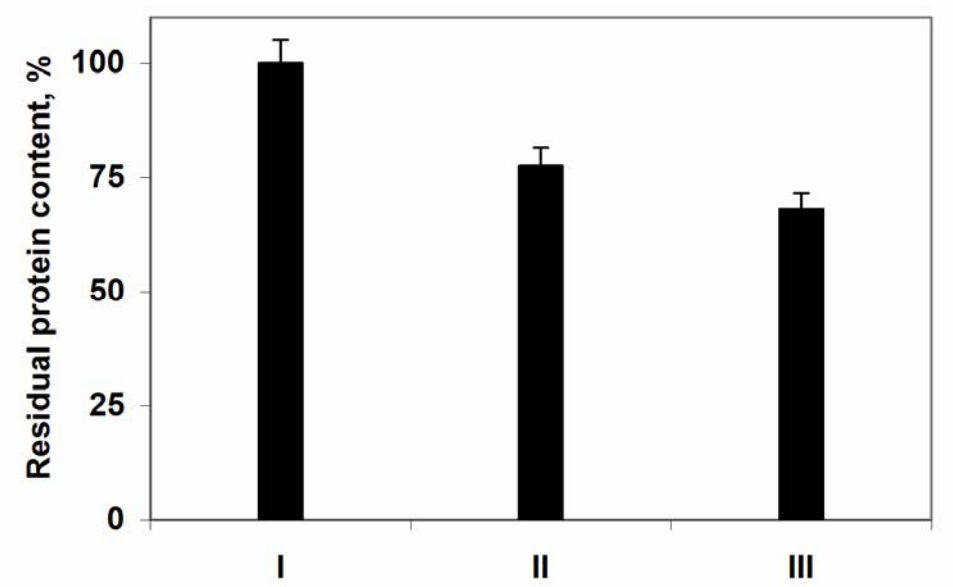


Figure 4-10. Influence of ultrasound on the enzymatic hydrolysis of BSA: I: initial protein content; II: residual protein content after treatment with pronase E; III: residual protein content after ultrasound assisted treatment with pronase E. The reference experiments were performed for 15 min under the same reaction conditions and the same temperature profile as for the krill hydrolysis given in the legends to Figures 4-7 and 4-8. The calculated standard deviation intervals were based on triplicate determination.

Whereas the efficiencies of Se extraction from Antarctic krill for 15 min in the absence and the presence of ultrasound differ by up to 70% (Fig. 4-7), the extent of bovine serum albumin hydrolysis catalyzed by pronase E without (Fig. 4-10, experiment II) and with (Fig. 4-10, experiment III) ultrasonication differ by only 10%. Thus, the reference experiment does not allow definitive conclusions on whether the ultrasound directly influences the rate of the enzymatic reaction. In

order to monitor the transformations occurring during the sonication process, krill samples were analyzed by microscopy (Figure 4-11).

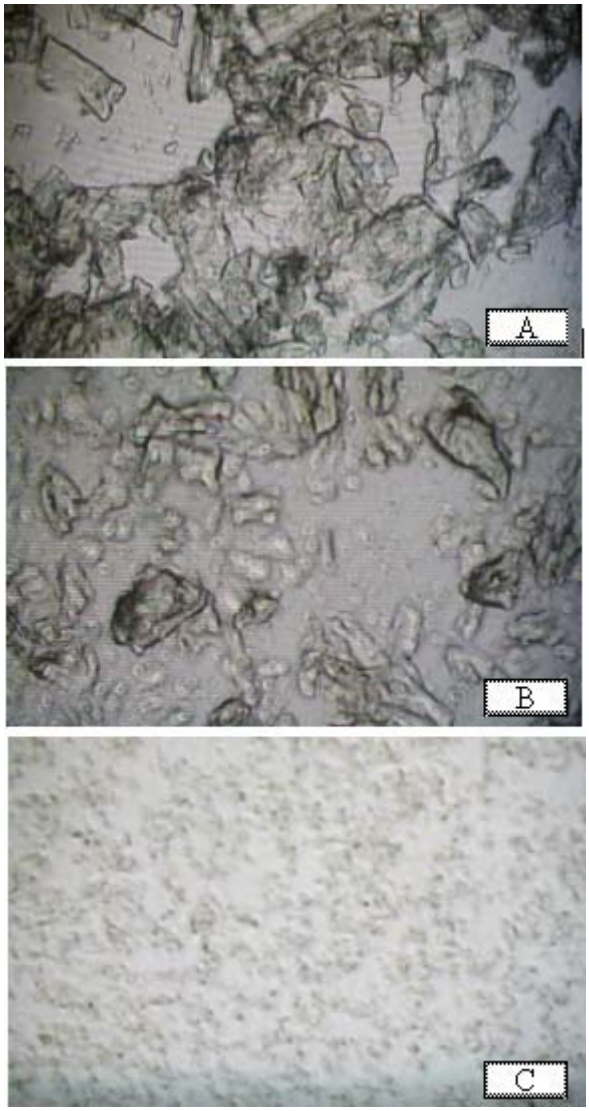


Figure 4-11. Microscopy of lyophilized krill samples before and after enzymatic treatment. A: Suspension of freeze-dried krill in 20 mM Tris-HCl pH 7.5; B: suspension of freeze-dried krill in 20 mM Tris-HCl pH 7.5 after 15-min treatment with pronase E; C: suspension of freeze-dried krill in 20 mM Tris-HCl pH 7.5 after 15 min ultrasound assisted digestion with pronase E. In all cases the krill concentration was 40 mg mL^{-1}. The enzyme concentration in samples B and C was 2 mg mL^{-1}.

The images show a clear particle size reduction by the action of pronase E (Fig. 4-11 B), and the reduction becomes more obvious when a combined treatment with pronase E and ultrasound (Fig. 4-11 C) is applied to the suspended freeze-

dried krill samples. This implies an increased contact area between enzyme and sample and respective substantial reduction of the mass-transfer limitations in the heterogeneous system [178]. The reference experiment for pronase E catalyzed hydrolysis of cell-free protein in the homogeneous system (Fig. 4-10) showed a slightly higher degree of protein hydrolysis under ultrasonication. This could be partly due to the efficiency of mixing, but does not definitely exclude direct influence of ultrasound on the enzyme–substrate interaction and on the intrinsic rate constants of the biocatalytic reaction. This phenomenon requires advanced investigation.

4.7. Bifunctional sorbents for chromatographic isolation of Se-Met

The scope of this experimental part was to develop an analytical procedure for the novel separation of Se-Met from krill samples. In the first part, adsorption of Se-Met (as standard solution) on different sorbents was analyzed and the sorbent presenting optimal binding capacity was selected for bifunctional chromatography. Afterwards, the adsorption of Se-Met as standard solution and the selective adsorption/desorption of Se-Met from enzymatic krill extracts with bifunctional chromatography was performed. The third part concentrates on the identification of Se-Met in the collected chromatographic fractions obtained after bifunctional chromatography of enzymatic krill samples.

4.7.1. Sorbent selection

Various Amberlite XAD bulk materials (XAD-2, XAD-4, XAD-9 and XAD-12) were tested for their ability to bind Se-Met. Figure 4-12 presents the results of adsorption experiments with Se-Met onto Amberlite bulk materials for pH values between 7 and 8.5. Lowest adsorption values were recorded for XAD-4 and XAD-12. Only 23 and 24%, respectively, of the initial amount of Se-Met was adsorbed by XAD-4 and XAD-9. A higher amount of Se-Met was retained by XAD-2 (45%), but the adsorption on Amberlite XAD-9 was superior (50%). The adsorbed Se-Met onto XAD-2, XAD-4 and XAD-12 adsorbents remained relatively constant in the considered pH range. A certain pH influence on the adsorption efficiency was observed only for the XAD-9 material.

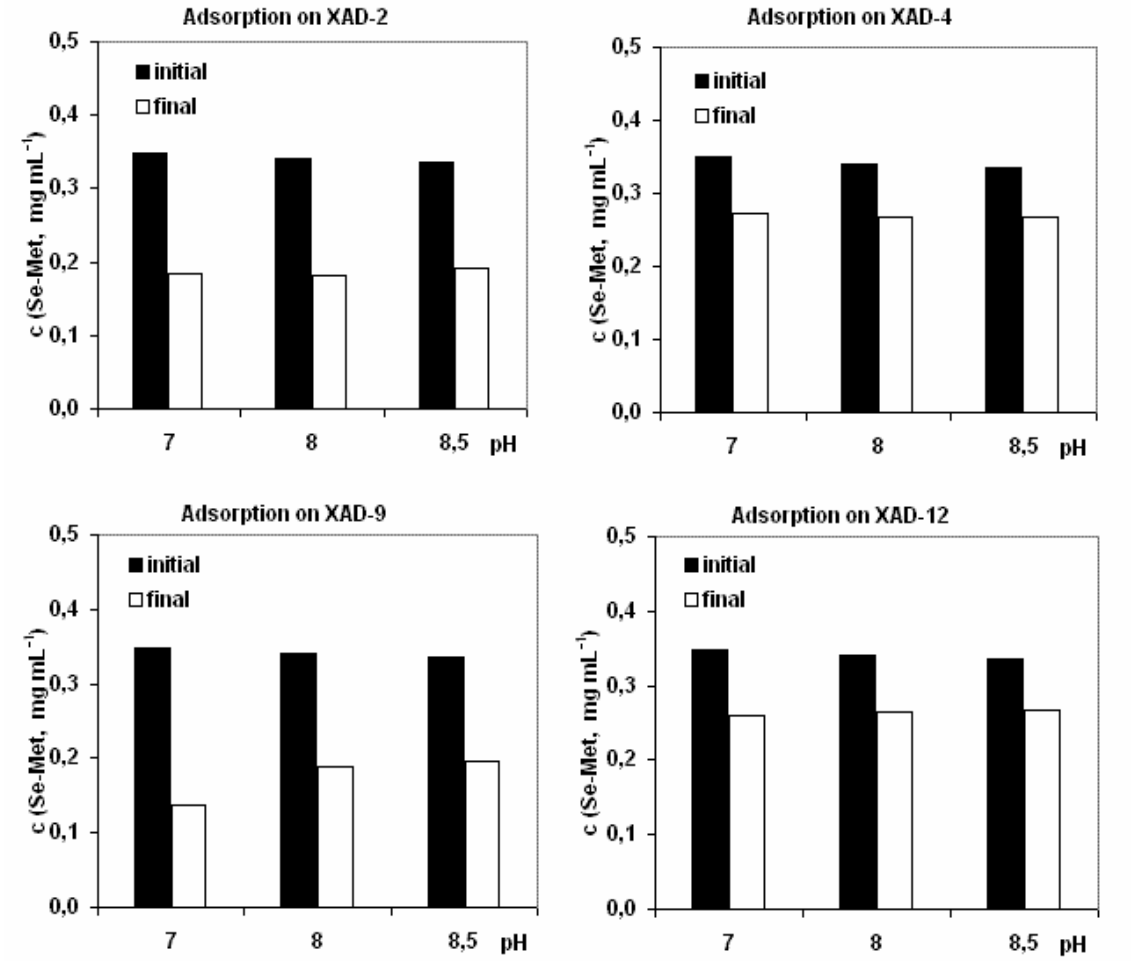


Figure 4-12. Adsorption of Se-Met on various XAD sorbents. 30 mg XAD bulk material was incubated for 90 min. at T = 25°C and 6 rpm with 1 mL Se-Met solution (0.35 mg Se-Met in 1 mL 20 mM Tris-HCl buffer).

At pH = 7, the highest adsorption was recorded for XAD-9 (approximately 60%). This adsorbent material was selected for further experiments (determination of adsorption isotherm).

Further adsorption experiments for Se-Met adsorption were performed with two mixed mode Oasis adsorbents, MAX and MCX. The manufacturer recommends the adsorption at two pH units above a pK_a of an analyte for MAX and at 2 pH units below this pK_a value for MCX [161]. The pK_a values of the two ionisable groups of Se-Met are 2.19 and 9.05 [179].

Thus, the standard Se-Met solutions were prepared in sodium carbonate buffer solution pH = 11, I = 0.1 and in 0.1 M HCl (pH = 1), respectively. The adsorption was optimized by varying the incubation time, pH and temperature.

Influence of the incubation time for the Oasis sorbents

Figure 4-13 presents the influence of the incubation time on adsorption of Se-Met on MAX and MCX. For both adsorbents the adsorbed Se-Met reached saturation after 30 min incubation time and longer incubations did not improve the adsorbed amount. Therefore, the incubation time was set to 30 min.

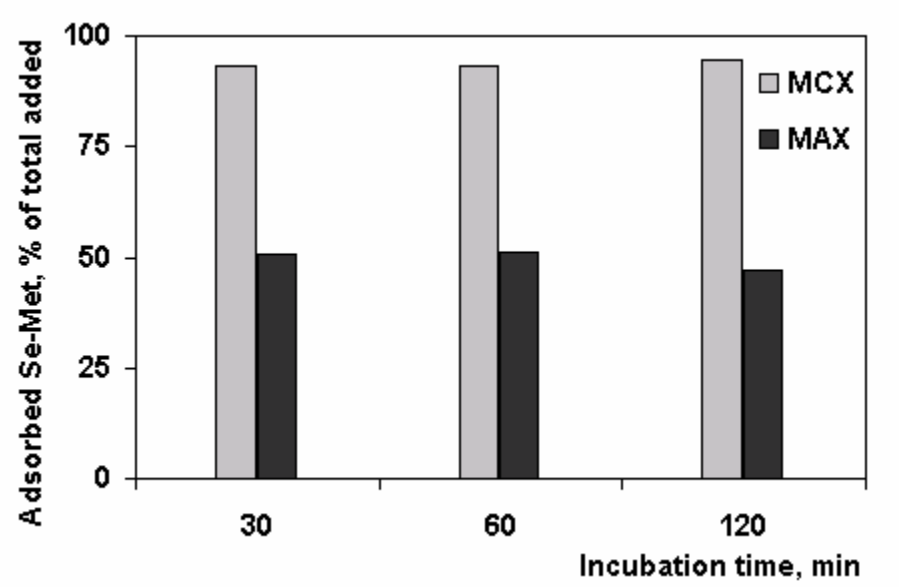


Figure 4-13. Influence of the incubation time on the adsorption of Se-Met on Oasis MAX and MCX adsorbents. 30 mg of each adsorbent were incubated with 1 mL 5 mM Se-Met in sodium carbonate buffer pH = 11, I = 0.1 and in 0.1 M HCl (pH = 1), respectively, at room temperature and at gentle rotation (6 rpm).

The adsorption of Se-Met on MCX was better than on MAX material. The incubation of standard Se-Met solutions with the Oasis MCX sorbents showed a quantitative adsorption of 93 to 95%, while less Se-Met was adsorbed during similar experiments with Oasis MAX (47 to 51%). Because of its higher adsorption capacity, MCX was chosen for further optimization experiments and finally for the column chromatography.

Influence of the temperature for the Oasis sorbents

The temperature dependence on the adsorption of Se-Met on MCX was analyzed at three different temperatures. The results (see Fig. 4-14) proved that the adsorption does not depend much on temperature in the studied range. Thus, 25°C (room temperature) was chosen for further adsorption experiments.

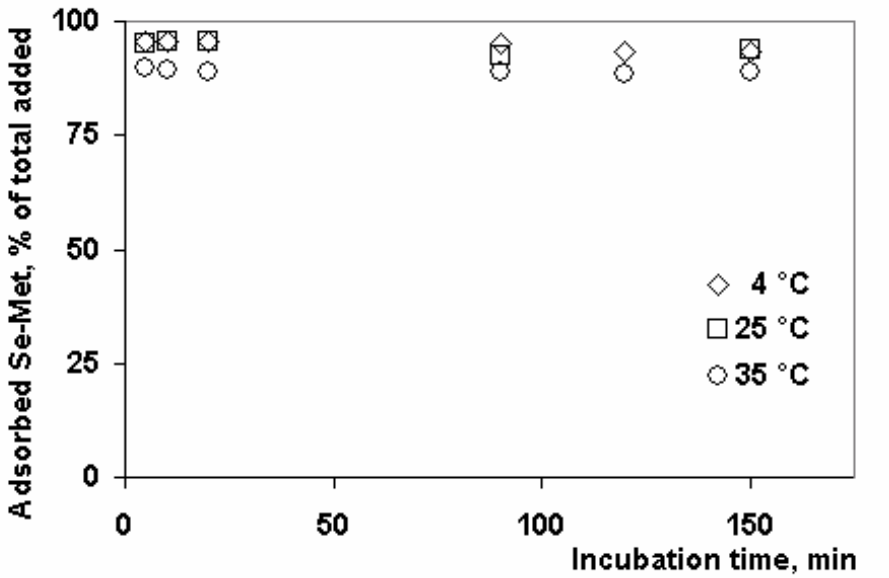


Figure 4-14. Influence of the incubation temperature on the adsorption of Se-Met on Oasis MCX. 30 mg adsorbent were incubated with 1 mL 5 mM Se-Met in 0.1 M HCl (pH = 1) at different temperatures and rotation at 6 rpm.

Influence of the pH for the Oasis sorbents

Another important parameter determining the ionization state of the amino acids and controlling the adsorption is the pH value of the sample. The experiments performed for the optimization of adsorption performance with respect to pH conditions are shown in Figure 4-15.

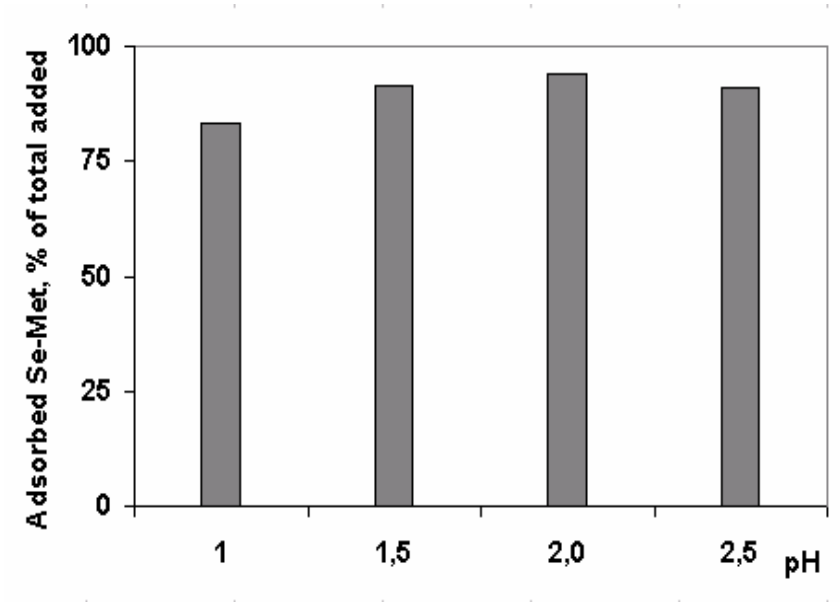


Figure 4-15. Influence of the pH on the adsorption of Se-Met on Oasis MCX. 30 mg adsorbent were incubated with 1 mL of 5 mM Se-Met in 0.1 M HCl (pH range 1 to 2.5) at room temperature and gentle rotation (6 rpm).

Significant adsorption of Se-Met on MCX was obtained for the entire analyzed pH range (87 to 97%). The lowest adsorption was measured at pH = 1. One possible explanation for the poorer adsorption (compared to measured values for pH between 1.5 and 2.5) is the loss of adsorbent performance under strong acidic conditions. The other values were relatively similar (above 94%), with the highest Se-Met amount adsorbed (about 97% of the total added) at pH = 2. Thus, this pH value was used for further experiments.

4.7.2. Adsorption isotherms for Se-Met

The adsorption isotherms for Amberlite XAD-9, Oasis MCX and Oasis MAX are presented below. The isotherms fitted to the Langmuir type and the adsorption parameters q_{max} and K were determined in each case from the linearized Scatchard plots, as described in the chapters 2.4.2. and 3.2.8. These parameters are important for the selection of the sorbent appropriate for bifunctional chromatography of standard Se-Met solutions and of enzymatic krill digests.

The adsorption isotherm for XAD-9 is presented in Figure 4-16.

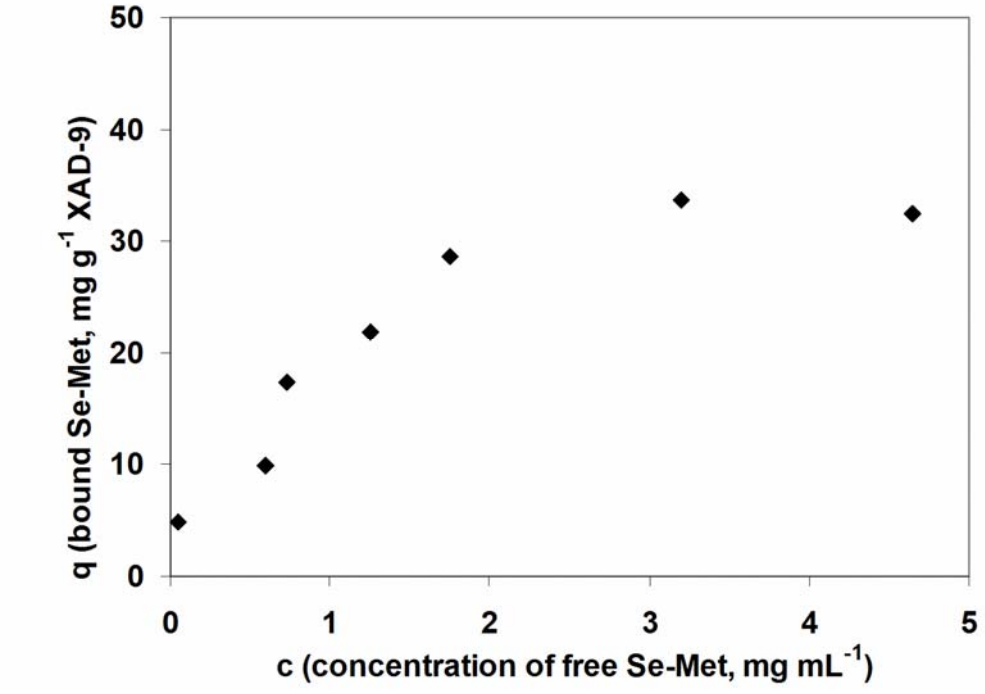


Figure 4-16. Adsorption isotherm of Se-Met on XAD-9 sorbent

According to the adsorption isotherm, the maximum binding capacity of XAD-9 (q_{max}) was found to be ca. 35 mg g^{-1} adsorbent and the dissociation constant *K* was evaluated to be $2.6 \cdot 10^{-3}$ M. The binding should be strong enough in order to achieve quantitative adsorption. The lower the *K* value, the stronger the binding; if *K* value is too low, then the elution takes too much time and even sample denaturation may occur. The upper limit for a quantitative adsorption is 10^{-3} M and the lower limit for the dissociation constant is 10^{-11} or 10^{-8} M for low molecular weight molecules or biopolymers, respectively [137]. The *K* value for XAD-9 is within the applicability range for chromatographic separation.

Figure 4-17 shows the results of the adsorption equilibrium measurements for Se-Met solutions on Oasis MCX.

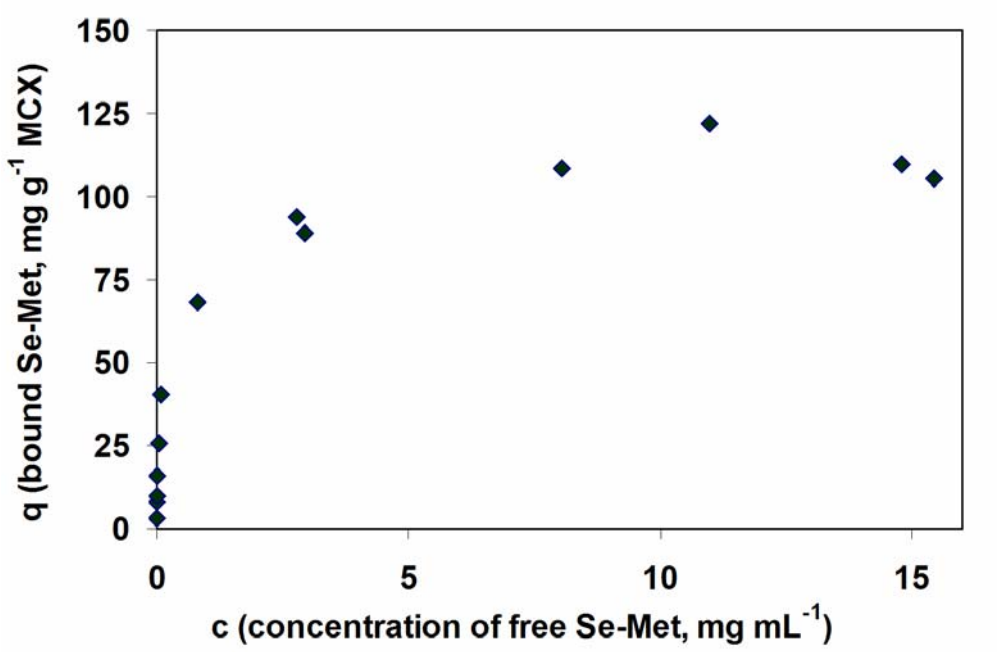


Figure 4-17. Adsorption isotherm for Se-Met on Oasis MCX

According to the adsorption isotherm, the maximum adsorption capacity of MCX (q_{max}) was found to be 115 mg g^{-1} adsorbent and the dissociation constant *K* was evaluated to be ca. $5 \cdot 10^{-4}$ M. This value lies also within the range of 10^{-3} to 10^{-11} M, appropriate for chromatographic separations of low molecular weights molecules [137]. Expressed in mol L^{-1}, the maximal static binding capacity q_{max} for Oasis MCX is 0.017 mol L^{-1} (considering the Oasis MCX cartridge volume of 1 mL, for 30 mg contained adsorbent). The dissociation constants for proteins are usually between the 10^{-5} to 10^{-11} M [180], but higher values in milimolar range

were also determined for lower molecular weight compounds such as glutamine or asparagine [181]. The fact that the *K* value for Oasis MCX is lower than the *K* value obtained for XAD-9 (Fig. 4-16) stands for the better suitability of MCX for further chromatographic separations of Se-Met [182].

The adsorption isotherm for the second Oasis sorbent (MAX) is presented in Figure 4-18. According to the adsorption isotherm, the maximum binding capacity of MAX (q_{max}) was found to be ca. 8 mg g^{-1} adsorbent and the dissociation constant *K* was evaluated to be $3.5 \cdot 10^{-3}$ M.

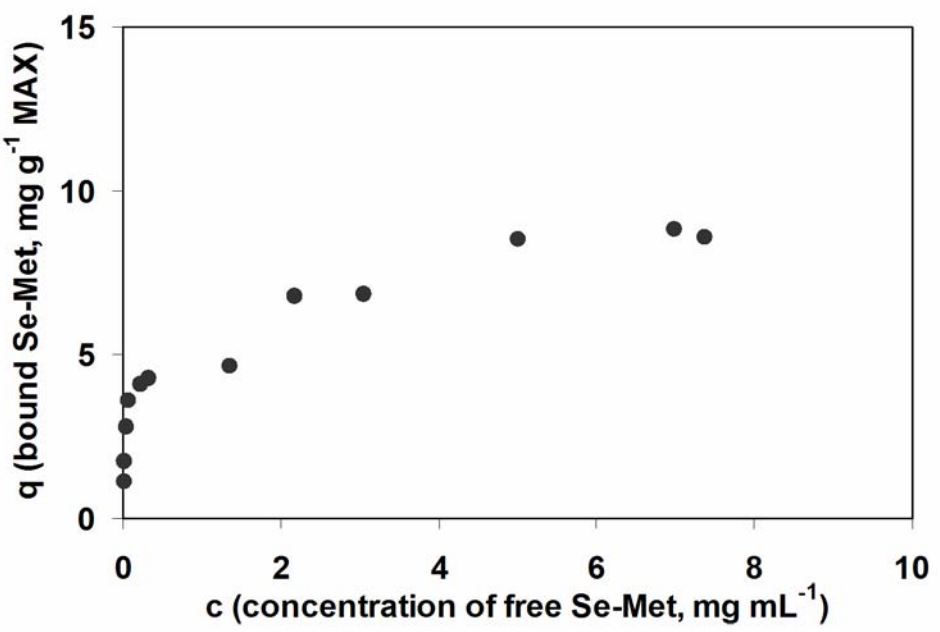


Figure 4-18. Adsorption isotherm for Se-Met on Oasis MAX

Expressed in mol L^{-1}, the maximal static binding capacity q_{max} for Oasis MAX is 0.0012 mol L^{-1} (considering the Oasis MAX cartridge volume of 1 mL, for 30 mg contained adsorbent). The binding strengths (*K* values) of Se-Met on MAX and XAD-9 sorbents are similar and indicate a weaker binding to Se-Met compared to Oasis MCX.

Table 4-3 presents the experimental binding capacities of Se-Met on Oasis materials and of other compounds with higher molecular weights such as trypsin, chymotrypsin and *E. coli* penicillin amidase on Sepharose material with biospecific ligands. The maximal binding capacities for the Oasis adsorbents presented in Table 4-3 are higher than the reported values for trypsin, α-chymotrypsin and penicillin amidase on Sepharose. This could be explained by the lower molecule

size of the amino acid Se-Met, which can penetrate the adsorbent pores to a higher extent and has therefore access to a higher active area of the adsorbent.

Table 4-3. Binding capacities for Oasis and Sepharose with biospecific ligands

Compound bound	Molecular weight	Adsorbent	q_{max} (mol L^{-1})
trypsin *	~ 25,000 Da	Sepharose	0.0001
α-chymotrypsin *	~ 25,000 Da	Sepharose	0.00027
E. coli penicillin amidase *	~ 88,000 Da	Sepharose	0.00001
Se-Met	~ 196 Da	Oasis MAX	0.0012
Se-Met	~ 196 Da	Oasis MCX	0.017

* values according to [137]

Summarizing the results for the adsorption experiments, one can conclude that best Se-Met adsorption was achieved on Oasis MCX. The dissociation constants for XAD-9 and Oasis MAX were similar. The lowest dissociated constant and therefore the stronger binding was determined for Oasis MCX. The maximum adsorption capacity was also determined for Oasis MCX (115 mg g^{-1} adsorbent), followed by the XAD-9 and the Oasis MAX (35 and 8 mg g^{-1} adsorbent, respectively). As a consequence, the Oasis MCX adsorbent was selected for bifunctional chromatography of Se-Met.

4.7.3. Bifunctional chromatography on MCX column

Bifunctional chromatography was performed on a novel chromatographic column packed with Oasis MCX material. The adsorption of Se-Met from standard solutions was analyzed. After setting up of the analytical procedure with standard Se-Met solutions, the krill samples obtained after enzymatic digestion with pronase E and separation with SEC were subjected to bifunctional chromatography. The chromatographic fractions were collected and the Se-Met presence in these fractions was determined. The recovery of Se-Met by this novel separation procedure was also analyzed.

Experiments with Se-Met standard solutions

The application of MCX as a stationary phase for bifunctional column chromatography is shown on Fig. 4-19. From all collected fractions only the peak

with retention time 42 min (indicated by an arrow) contained Se-Met as determined by GC-MS. For the determination of the dynamic capacity of the column, 5 mg mL^{-1} Se-Met was loaded onto the column in 1 mL injection steps. The procedure was repeated until no more adsorption occurred. An amount of 20 mg Se-Met was retained on the column up to the moment Se-Met signal was recorded in the column effluent. Considering ca. 1.1 g as the amount of the stationary phase packed into the column (evaluated from the column volume and MCX density), the dynamic column capacity was calculated to be 18.2 mg Se-Met g^{-1} adsorbent.

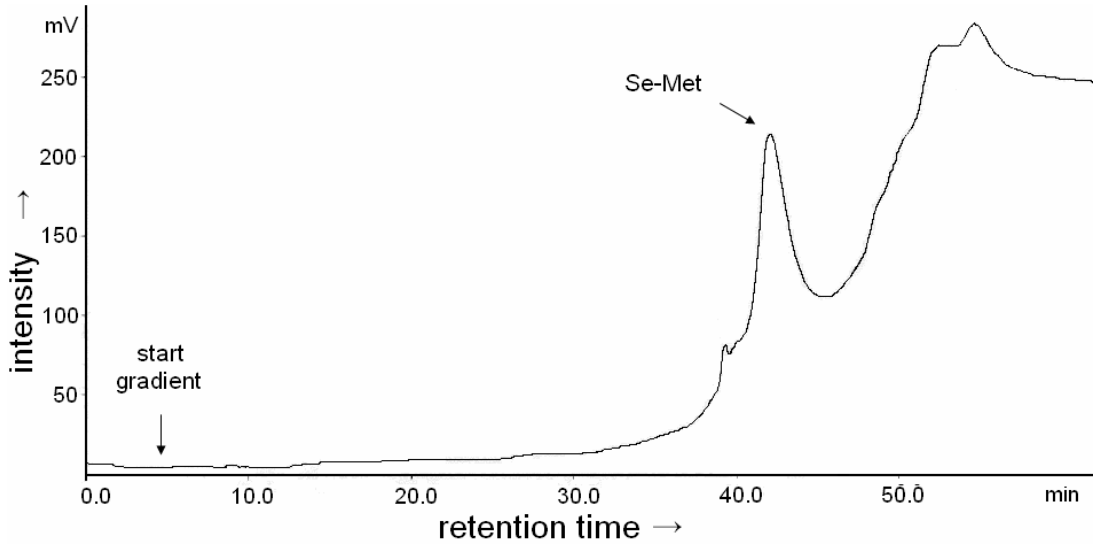


Figure 4-19. Bifunctional chromatographic separation of Se-Met on a column packed with Oasis MCX adsorbent. The column was conditioned for 4 min with 100% CH_3OH, washed for 4 min with 100% H_2O and equilibrated for 4 min with 0.2 M HCl-KCl buffer (pH = 2, I = 0.1) at room temperature and a flow rate of 1 mL min^{-1}. 20 µL Se-Met solution (2 mg mL^{-1} Se-Met in 0.1 M HCl) were injected onto the column at a flow rate of 1 mL min^{-1}. Four min after sample injection a step gradient 0-50% CH_3OH containing 10% NH_4OH was applied at a flow rate 1.5 mL min^{-1}. The detector was set at λ = 214 nm.

Compared to the stationary capacity (ca. 115 mg Se-Met g^{-1} MCX), the dynamic capacity was reduced to about 16%. The lower dynamic capacity observed in our experiments can be explained by the lack of more precise data from the manufacturer about the real amount of adsorbent packed in the column. It is assumable that the column length has direct influence on the dynamic column capacity. The difference between the experimentally determined static binding capacity and the dynamic capacity could be positively influenced by a longer chromatographic column.

Experiments with krill samples

Chromatographic experiments were performed also with samples obtained after enzymatic hydrolysis of Antarctic krill. Selenium rich fractions with molecular weights in the range of amino acids were isolated by size exclusion chromatography. These fractions were freeze-dried and suspended in 0.1 M HCl (with a ratio of freeze-dried sample to HCl solution 1:32 v/v) prior to the bifunctional chromatography on the MCX column. A sample with a final volume of 5 mL was loaded onto the chromatographic column in 5 steps using an injection loop of 1 mL. A breakthrough was observed after each loading, corresponding to non-adsorbed compounds eluting from the column. After starting the gradient, a peak was eluted as shown in Fig. 4-20.

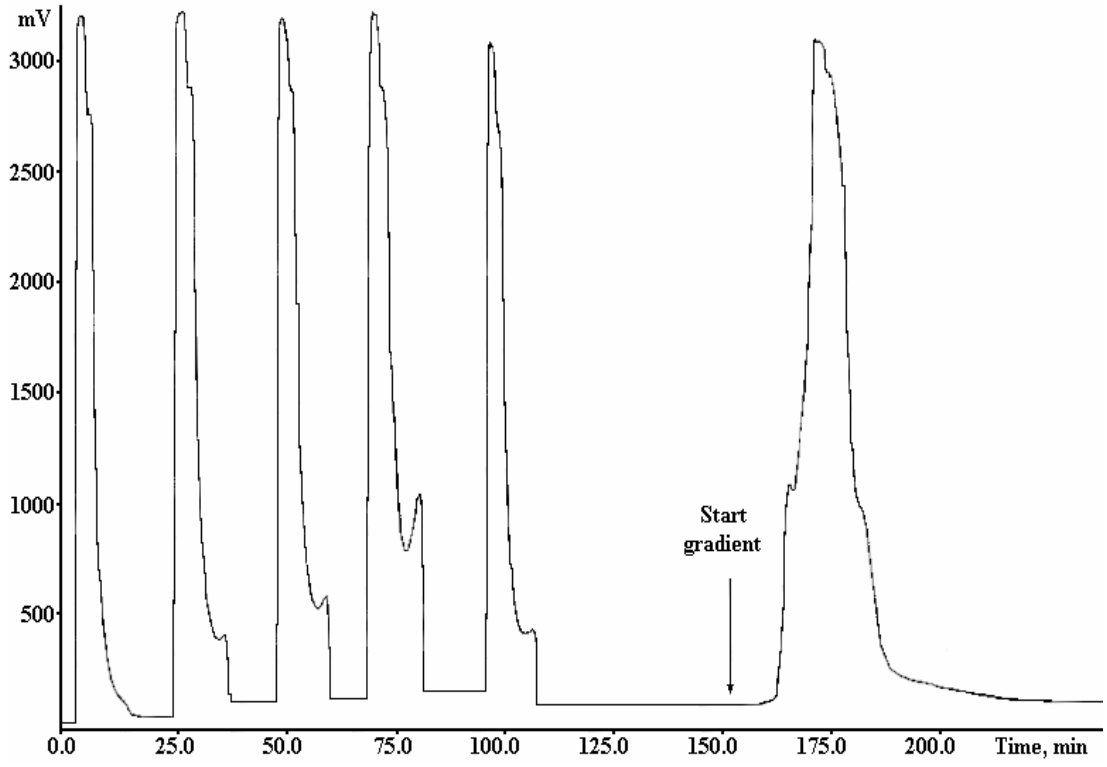


Figure 4-20. Dynamic separation of Se-Met from SEC fractions of krill homogenate obtained after enzymatic digestion. The MCX column was conditioned for 4 min with 100% CH_3OH, washed for 4 min with 100% H_2O and equilibrated for 4 min with 0.2 M HCl-KCl buffer (pH = 2, I = 0.1) at room temperature and a flow rate of 1 mL min^{-1}. 5 mL sample was injected onto the column in 5 × 1 mL steps at a flow rate of 1 mL min^{-1}. After base line stabilization a step gradient 0-50% CH_3OH containing 10% NH_4OH was applied at a flow rate 1.5 mL min^{-1}. The detector was set at λ = 214 nm.

The sample was injected in consecutive steps because column overload was suspected (krill hydrolysates contain a multitude of digestion products) [182]. Peak tailing and peak broadening were observed in the chromatogram from Figure 4-20. Peak tailing is particularly encountered in the separation of basic compounds. Possible explanations are the sample mass overload and the injection of samples in a solvent which is stronger than the mobile phase. The difference between the sample pH and the mobile phase is too low in order to cause such disturbances, but, on the other hand, the mobile phase pH is relatively close to pK_a value of 2.19 of Se-Met [179]. Since the amount of Se-Met present in the injected samples was expected to be very low, the volume of the injected sample was not changed.

The peak irregularities (peak symmetry) can be also explained by the variety of compounds present in the enzymatic krill hydrolysate, which may introduce disturbances into the chromatographic system. The peak broadening recorded at retention time of 165 min can be caused by the simultaneous desorption of interfering compounds which elute at the same time with Se-Met from the column.

The collected fractions were analyzed by GC-MS. Gas chromatograms are illustrated in Figure 4-21. Se-Met was detected only in the eluted peak after the application of the gradient. The gas chromatogram of the sample is shown in Figure 4-21 A. The peak containing Se-Met was recorded at 4.7 min (Se-Met derivative is indicated by the peak 1 in the figure 4-21 A). Sample spiking with Se-Met was performed for confirming the Se-Met determination in the digested krill samples.

Figure 4-21 B represents the gas chromatogram of spiked sample obtained as described in the chapter 3.2.5. (Se-Met derivative is indicated by peak 2 in the figure 4-21 B). The ion of the *N*-ethoxycarbonyl ethyl ester derivative of Se-Met was recorded at *m/z* = 297 in the mass spectrum of this peak at 4.7 min (Fig. 4-22) and it is identical to Se-Met standard and fits to previously reported data [118, 123]. The other *m/z* values of the ions were 251 (caused by the loss of an ethanol molecule), 224 (caused by the loss of a CH_3-CH_2-COO group), 202 (caused by the loss of CH_3-Se), 175 (caused by the loss of CH_2-CH_2-Se-CH_3), 128 (caused by the loss of CH_3-CH_2-COO and CH_3-Se groups) and 109 (caused by the loss of CH_2-Se-CH_3), respectively.

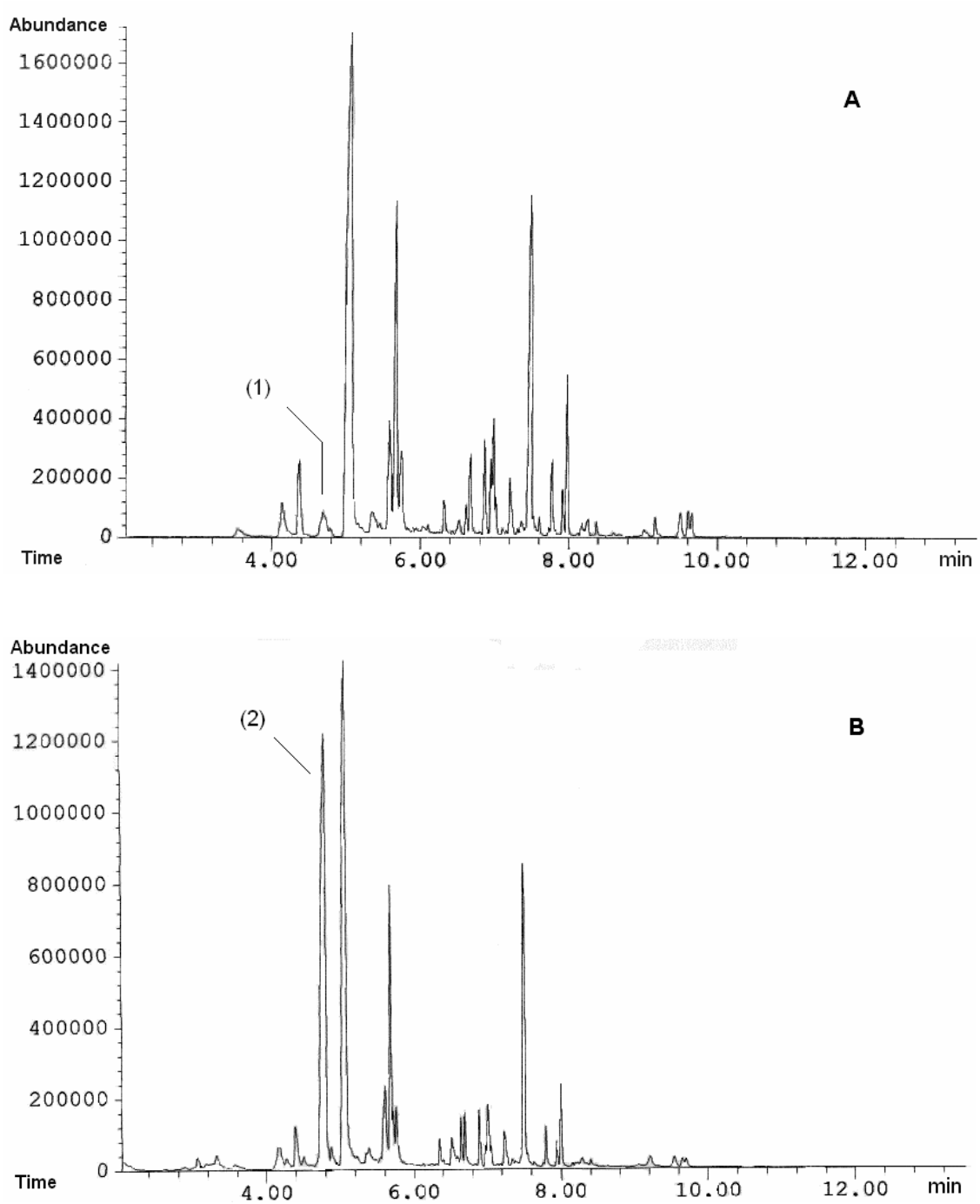


Figure 4-21. Gas chromatograms of digested krill samples obtained after bifunctional chromatography on MCX column (A) and after spiking of these samples with Se-Met (B). Se-Met peaks are indicated (1 for the original sample and 2 for the spiked sample).

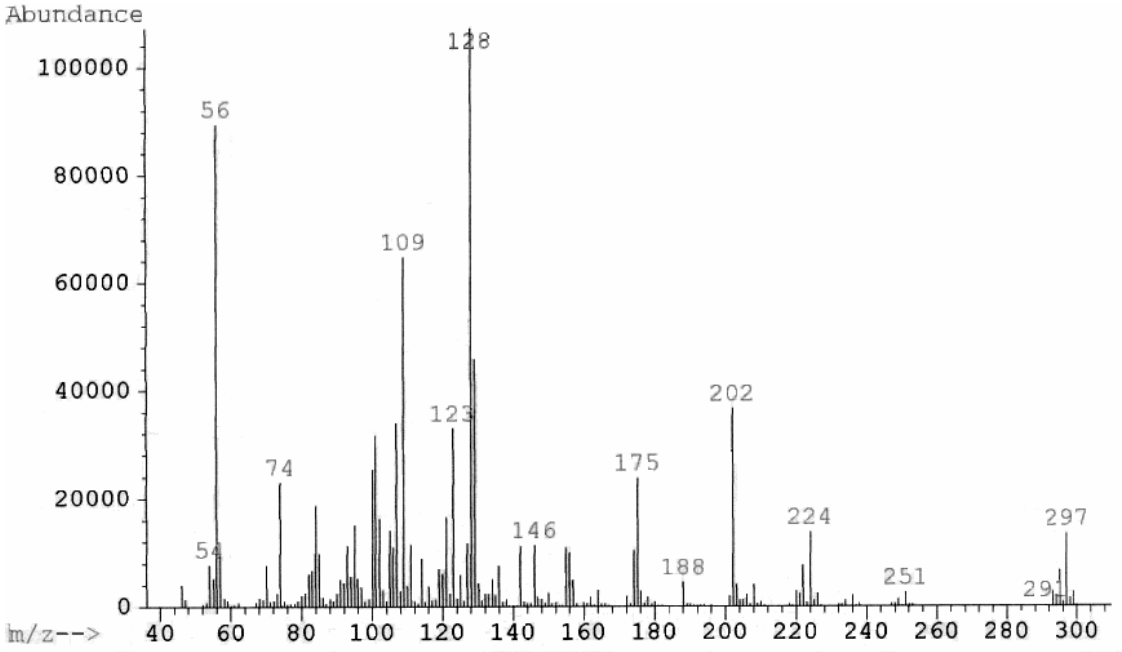


Figure 4-22. Mass spectrum of ECF derivative of Se-Met present in the fractions obtained after bifunctional chromatography of hydrolysed krill samples on MCX column.

Krill hydrolysates contain a diversity of low molecular weight compounds, the consequence of the digestion with proteolytic enzyme pronase E. Among these compounds, amino acids such as methionine, could also be present. The derivatization reaction represented in chapter 3.2.5., Figure 3-5, could also act on the methionine present in the krill digests. Due to similar chemical structures of Met and Se-Met amino acids, interferences in the GC-MS analysis because of similar fragmentation patterns of the *N*-ethoxycarbonyl ethyl ester derivatives of Se-Met and Met are theoretically possible. Thus, the same derivatization reaction was performed for Met as well in order to have more evidence for the detected Se-Met peak. The GC-MS analysis with Met and Se-Met (single or mixed) standard solutions did not show any interference as clear peak separation was observed (Figure 4-23). This agrees with the results obtained by other authors [119, 124, 125].

The ion of the *N*-ethoxycarbonyl ethyl ester derivative of Met was recorded at *m/z* = 249 in the mass spectrum at 4.46 min, as presented in Figure 4-24. The other *m/z* values of the ions were 203 (caused by the loss of an ethanol molecule), 188 (caused by the loss of a CH_2-S-CH_3), 88 (probably caused by the

CH-CH_2-CH_2-S-CH_3 radical), 61 (caused by the CH_2-S-CH_3 group) and 47 (caused by the S-CH_3 group), respectively.

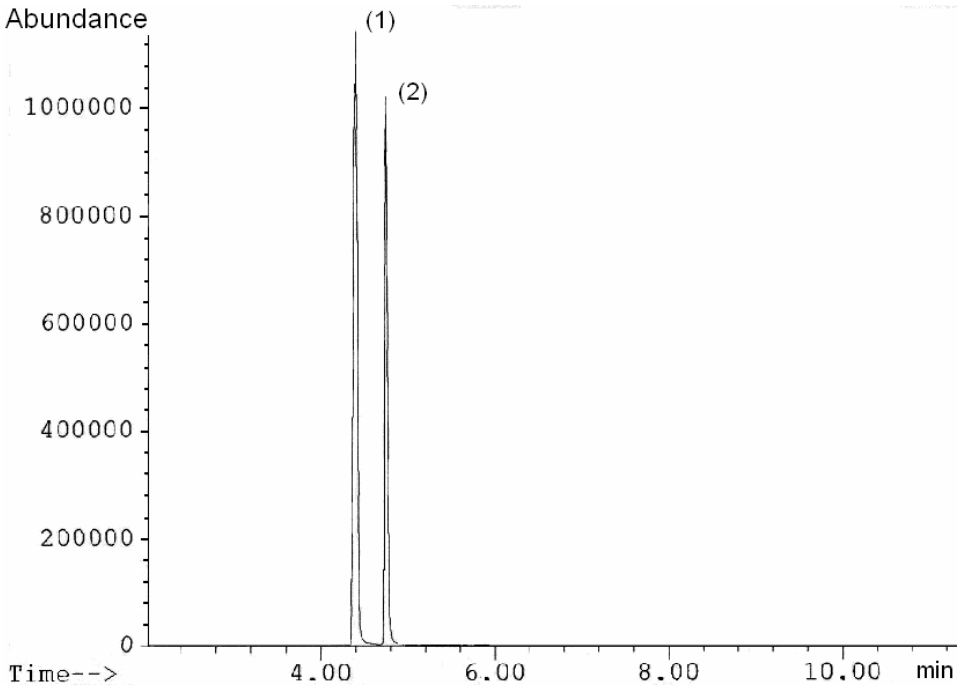


Figure 4-23. Gas chromatogram of a Met (peak 1) and Se-Met (peak 2) mixture (100 µg mL^{-1} each), after derivatization with ECF.

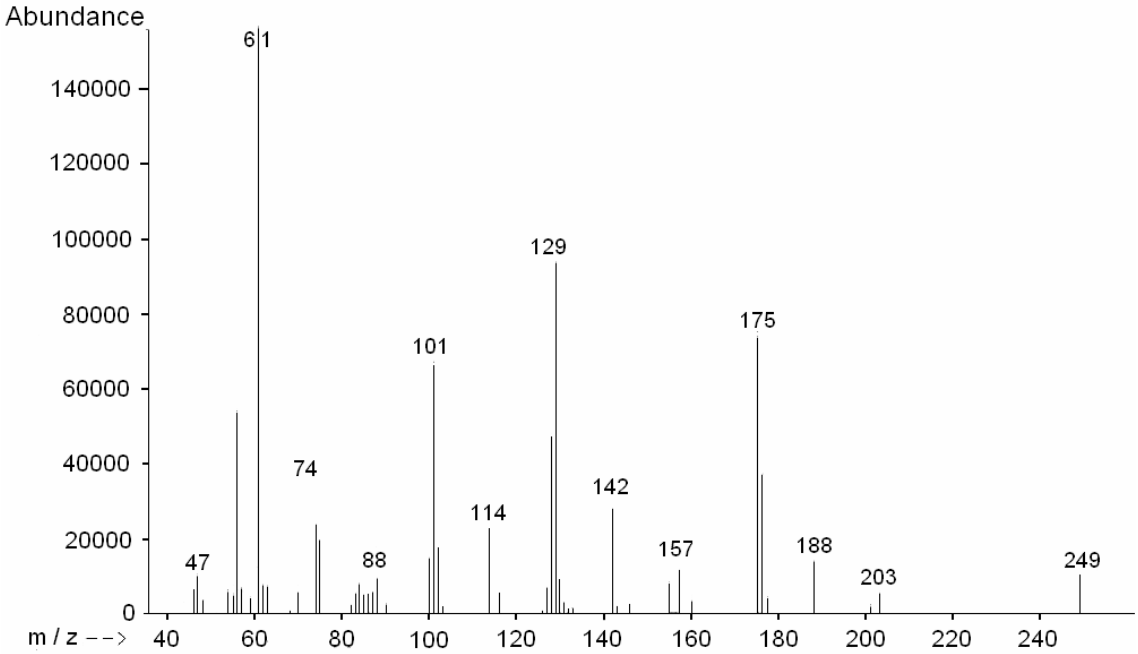


Figure 4-24. Mass spectrum of ECF derivative of Met in a Se-Met and Met mixture (100 µg mL^{-1} each)

The Se-Met amount detected in the selenium rich fractions with molecular weights under 600 Da collected after size exclusion chromatography was compared to the Se-Met amount detected in the fractions collected after bifunctional chromatography on Oasis MCX packed column. A recovery of 83±2% was achieved. The employment of the developed method for samples with higher Se-Met contents could increase this recovery. The GC-MS procedure was simple, fast and suitable especially for processing samples with low volumes.

More than 60 runs were performed with this column, without any reduction in performance. As far as this adsorbent has been designed and applied for single-use solid phase extractions, our results open new possibilities for its utilization for bifunctional chromatography.

5. Conclusion

Selenium is a trace element with recognized nutritional essentiality for human health. Main dietary selenium sources are grains, nuts, meat and fish. High bioavailability for the selenium species encountered in marine products was reported [8-10] and makes the quantitative determination and isolation of organic selenium compounds from biological samples a challenging subject. Antarctic krill (*Euphausia superba*) is a small crustacean living in the ecosystem of the Antarctic region of the Southern Ocean and is regarded as one of the most important protein sources of the future, because of its low cholesterol level and the high levels of essential amino acids and of polyunsaturated fatty acids [13]. Se-Met is the main isolated organic form of selenium in the food chain. Only sparse number of papers deals with the isolation of Se-Met fractions from Antarctic krill. Solid phase extraction is a common analytical procedure for sample cleanup or preconcentration before chromatographic separations and is based on the retention of onto a disposable solid phase cartridge, followed by the elution of the analyte with a solvent appropriate for instrumental analysis.

This work focuses on the extraction of Se-Met from Antarctic Krill and on the consecutive adsorption studies with the mixed mode Oasis MCX and MAX adsorbents. For this purpose, various extraction methods such as enzymatic digestion and ultrasound sample treatment were analyzed. A novel chromatographic procedure for the Se-Met determination in Antarctic krill samples after enzymatic digestion on an Oasis MCX packed column was developed.

In the first part, the total selenium content in the Antarctic krill samples was determined. An average total selenium content of 2.4 µg g^{-1} dry weight was measured in the freeze-dried krill samples. Freeze-drying of krill samples ensured homogeneous quality and easy storage of the krill samples.

The second part is dedicated to the extraction of organic selenium compounds from krill samples. Since biological samples contain selenium mostly in bound form such as proteins or peptides, the release of its organic compounds from the protein matrix was required. The most common extraction methods for the

cleavage of proteins or peptides to lower molecular weight compounds are based on the hydrolysis with solvents, acids or enzymes. Digestion experiments with proteinases such as pronase E, subtilisin Carlsberg, trypsin, chymotrypsin, proteinase and proteinase N from *Bacillus subtilis* and Novo 0.6 MPX enzyme were performed. Among these, the enzymatic treatment with pronase E led to best selenium recovery. 98±2% of the total Se initially measured in freeze-dried krill samples was detected in the enzymatic hydrolysate. This hydrolysate was further subjected to size exclusion chromatography. The separation on a Superdex peptide HR 10/30 column (100 - 7,000 Da separation range) and the analysis of collected chromatographic fractions showed that ca. 80% of the selenium species have a molecular weight under 600 Da (Table 4-1). The release of low molecular weight Se species was achieved also by combining ultrasound technique and enzymatic hydrolysis. The simultaneous treatment led to a significant reduction of digestion time and an increase in sample handling easiness. The conventional 24 h needed for the enzymatic extraction with pronase E were reduced to approximately 15 min. About 70% of the total Se (reported to initial Se concentration in freeze-dried krill samples) was detected in the chromatographic fractions with the molecular weight under 600 Da (Table 4-2). The percent of Se-Met from the total Se content in the krill samples obtained after digestion varied between 35 and 41%, depending to the chosen digestion method (Tables 4-1 and 4-2). The fractions collected after separation with size exclusion chromatography were further subjected to bifunctional chromatography.

The third part describes the selection of an appropriate sorbent material and its employment for the adsorption of Se-Met from standard solutions and from enzymatic hydrolysates. Several adsorbent materials were investigated on their ability to adsorb Se-Met. Parameters such as adsorption time, pH and temperature conditions were considered. Best results were obtained with Oasis MCX, a mixed mode cation exchanger from Waters GmbH. The adsorption isotherm and the linearization of adsorption data showed a maximal binding capacity of ca. 115 mg g^{-1} adsorbent and a dissociation constant K of ca. $5 \cdot 10^{-4}$ M. A chromatographic column packed with the MCX material was employed for the dynamic capacity determination and for the subsequent processing of Se-Met standards and krill

samples. The dynamic capacity was ca. 16% of the stationary capacity. After the validation of the chromatographic procedure with Se-Met standards, krill samples (obtained after enzymatic digestion and size exclusion chromatography) were subjected to bifunctional chromatography onto the MCX column. Good recovery (83±2%) and simple GC-MS determination of Se-Met after derivatization with ethyl chloroformate was achieved (Figures 4-21 and 4-22).

The repeated employment of the Oasis MCX packed column did not show any performance diminution after multiple experiments. Hence, we proved that this adsorbent is suitable also for chromatography procedures, beyond its traditional application for sample preconcentrations in disposable columns and cartridges.

6. Literature

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7. APPENDIX

7.1. Notations

Symbol	Measure unit	Significance
c	mg mL^{-1}	free concentration in liquid phase at equilibrium
	mM, M	molar concentration
	N	normal concentration
c_{out}/c_{feed}	-	outlet to inlet solute concentration ratio (column bed)
f	Hz	sound frequency
I	mol m^{-3}	ionic strength
IEC	meq g^{-1}	ion exchange capacity
K	M	equilibrium constant
K_F	-	constant in Freundlich equation
k_1	s^{-1}	rate constant for the formation of E·S
k_2	M^{-1} s^{-1}	rate constant for the release of S
k_3	s^{-1}	rate constant for release of P
m	ng, µg, mg, g, kg	mass
M	g mol^{-1}	molecular weight
m/z	-	mass to charge ratio
MW	Da	mass, molecular weight
n	-	linearity exponent
P	kPa	pressure
q	mg g^{-1}	bound amount of adsorbate
q_{max}	mg g^{-1}	maximal binding capacity in Langmuir equation
r	rpm	rotation speed
R	mΩ cm	water resistivity
T	s, min, h	time
t	°C	temperature
V	µL, mL, L, m^3	volume
v/v	-	volume to volume ratio
w/w	-	weight to weight ratio
λ	nm	wavelength

7.2. Abbreviations

AAS	atomic absorption spectrometry
Ala	alanine
AmAc	ammonium acetate
Asn	asparagine
Arg	arginine
BSA	Bovine serum albumin
ECD	electron capture detection
ECF	ethyl chloroformate
FI	flame ionization
FID	flame ionization detection
FPD	flame photometric detection
GC	gas chromatography
GF-AAS	graphite furnace atomic absorption spectrometry
Gly	glycine
GPX	gluthatione peroxidase
HFBA	heptafluorobutyric acid
HFB-IBA	heptafluorobutyric-isobutanol
ICP-MS	inductively coupled plasma-mass spectrometry
ID	Internal diameter (here for chromatographic column)
IR	infrared
IEC	ion exchange chromatography, ion exchange capacity
IP-RP-HPLC	ion pairing reverse phase high performance liquid chromatography
Met	methionine
MS	mass spectrometry
MTZ	mass transfer zone
NFPA	nonafluoropentanoic acid
PFPA	pentafluoropropanoic acid
PID	photo-ionization detection
PS-DVB	polystyrene divinylbenzene
RI	refractive index
RP-HPLC	reverse phase high performance liquid chromatography
SEC	size exclusion chromatography
Se-Cys	selenocysteine
$Se\text{-}Cys_2$	selenocystine
Se-Et	selenoethionine
Se-Met	selenomethionine
SPE	solid phase extraction

SPME	solid phase microextraction
TFA	trifluoroacetic acid
TMSe	trimethylselenonium ion
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UV	ultraviolet

7.3. Chemicals

Chemical substances

Ammonium acetate (AmAc)	Carl Roth GmbH (Karlsruhe, Germany)
Biuret, Bradford reagents	Merck (Darmstadt, Germany)
Chloroform ($CHCl_3$)	Carl Roth GmbH (Karlsruhe, Germany)
Ethanol (CH_3CH_2OH)	Carl Roth GmbH (Karlsruhe, Germany)
Ethyl chloroformate (ECF)	Sigma-Aldrich Chemie (Steinheim, Germany)
HCl (37%), KCl	Merck (Darmstadt, Germany)
Methanol (CH_3OH)	Carl Roth GmbH (Karlsruhe, Germany)
Na_2SeO_4, Na_2SeO_3	Sigma-Aldrich Chemie (Steinheim, Germany)
Sodium hydroxide (NaOH)	Carl Roth GmbH (Karlsruhe, Germany)
Pyridine	Sigma-Aldrich Chemie (Steinheim, Germany)
Selenocystine (Se-Cys_2)	Sigma-Aldrich Chemie (Steinheim, Germany)
Selenomethionine (Se-Met)	Sigma-Aldrich Chemie (Steinheim, Germany)

Proteins and Enzymes

bovine serum albumin	Biomol Feinchemikalien GmbH (Hamburg, Germany)
α-chymotrypsin from bovine pancreas	Serva Feinbiochemica (Heidelberg, Germany)
Novo 0.6 MPX protease	Novo Nordisk (Bagsvaerd, Denmark)
pronase E (*S. griseus*)	Serva Feinbiochemica (Heidelberg, Germany)
proteinase (*B. subtilis*)	Fluka (Buchs, Switzerland)
proteinase N (*B. subtilis*)	Fluka (Buchs, Switzerland)
subtilisin Carlsberg	Sigma-Aldrich Chemie (Steinheim, Germany)
trypsin	Difco Laboratories (Detroit, MI, USA)

All other reagents were of analytical grade.

Disposal of toxic materials

Toxic substances were avoided when possible. Toxic compounds were collected according to their chemical classifications and disposed according to the regulations imposed by Hamburg University of Technology.

7.4. Technical Equipment

Equipment	Type and Manufacturer
Freeze-drier	Christ Alpha 1-2, Martin Christ (Osterode, Germany)
Centrifuge	J2-21 M/E with JA-20 rotor, Beckman (Munich, Germany) Biofuge 13, Minifuge T (2250 rotor), Heraeus (Osterode,Germany)
Ultrasound	Model 450, 3 mm microtip, Branson Inc. (Danbury, USA)
Photometer	Uvikon XL, Bio-Tek Instruments (Bad Friedrichshall, Germany)
Filter	Rotilabo, 0.2, 0.45 µm, Carl Roth GmbH (Karlsruhe, Germany)
SEC	HPLC Pumps Type LKB 2249; UV Detector LKB Bromma 2151; Superdex peptide HR 10/30; Injector Rheodyne 7725; Autosampler RediFrac, Amersham Pharmacia Biotech (Freiburg, Germany)
HPLC-ICP-MS	Agilent 1100 system; detector Agilent 7500s , Agilent Technologies (Waldbronn, Germany) C18 Synergi Hydro, 4 µm, 80 Å, 1.0 mm × 150 mm Phenomenex (Aschaffenburg, Germany)
GC-MS	Hewlett-Packard HP G1800A GCD series system, Auto-sampler HP 7673B, Hewlett-Packard (Waldbronn, Germany) Capillary column Agilent HP-5MS (30 m × 250 µm ID, 0.25 µm phase thickness, cross-linked 5% phenylpolydimethyl-siloxane, Agilent Technologies (Waldbronn, Germany)
GF-AAS	Perkin-Elmer SIMAA 6000 with Zeeman background correction, PerkinElmer (Überlingen, Germany)
pH-meter	pH 535 Multical, WTW (Weilheim, Germany) pH-meter 761 Calimatic, Knick (Mannheim, Germany)
Microscopy	Zeiss Axioskop, Otto Steiner GmbH & Co KG (Hamburg, Germany) JVC TK-C1381 Colour video camera (JVC, Japan)

Vacuum Concentrator	SAVANT Speed Vac Concentrator SVC100, Savant Instrument Inc. (Farmingdale, N.J., USA)
Shaker	Environmental Incubator Shaker, New Brunswick Scientific (Edison, N.J., USA)
Water supply	Milli-Q Water Purification System, Millipore (Eschborn, Germany)

7.5. List of Tables

7.6. List of Figures

Curriculum Vitae

Mariana Siwek born Dorovei
born on 14.04.1975 in Bucharest

Education

12/2001 - 02/2005	Research assistant studying for doctoral degree at the Hamburg University of Technology and Institute of Coastal Research, GKSS Research Center, Geesthacht. Cooperation with the Helmut-Schmidt-University / University of the Federal Armed Forces Hamburg.
09/1999 - 11/2001	International Master Course in Process Engineering Hamburg University of Technology
09/1998 - 07/1999	Master Course in Chemical Engineering, English Stream University „Politehnica“ of Bucharest
09/1993 - 07/1998	Diploma in Chemical Engineering University „Politehnica“ of Bucharest
09/1989 - 07/1993	„Traian Vuia“ Metrology High School, Bucharest
09/1981 - 07/1989	Primary school no. 97, Bucharest

Work Experience

since 03/2006	Process Engineer Aker Kvaerner Germany, Langenfeld (Rheinland)
12/2004 - 02/2005	Research Assistant Helmut-Schmidt-University / University of the Federal Armed Forces Hamburg, Hamburg
12/2001 - 11/2004	Research Assistant Hamburg University of Technology
08/2001 - 11/2001	Chemical Engineer Silica Verfahrenstechnik GmbH, Berlin
10/1997 - 01/2000	Research Assistant University „Politehnica“ of Bucharest
10/1998 - 02/1999	Teaching Assistant „Titu Maiorescu“ University, Medical Faculty, Bucharest