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ISOLATION AND CHARACTERIZATION OF ZINC SPECIES IN SELECTED COMPONENTS OF THE VEGETARIAN DIET

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MASTER'S THESIS

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IZOLACIJA IN KARAKTERIZACIJA CINKOVIH SPOJIN V IZBRANIH KOMPONENTAH VEGETARIJANSKE PREHRANE

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ABSTRACT

The main objectives of this thesis are to get information on Zn species, their differences regarding sample type (pumpkin seeds and iceberg lettuce) and their possible degradation in the human gastro-intestinal tract. A sequential analytical approach is applied focusing on total Zn, spatial Zn distribution, extractability of Zn, speciation of Zn and bioaccessibility of Zn. An array of procedures and techniques is used to aid in this research: microwave digestion, solid sampling by laser ablation, ultrasound-assisted variable volume extraction, ultrafiltration and size exclusion chromatography, where elemental detection is performed by FAAS or ICP-MS. Results show that pumpkin seeds and iceberg lettuce have different Zn species fingerprints (in water extracts) with a high (ca. 70%) low-MW fraction (ca. 500Da) in iceberg lettuce and a high (ca. 60%) intermediate/high-MW fraction (10 – 20kDa) in pumpkin seeds. When these Zn species are subjected to conditions simulating the human stomach (pH ~ 2) they break down completely, disproving conclusions of Zn speciation studies done in the past suggesting that low-MW Zn species may have nutritional value. However, these findings open up a wide range of further interesting research possibilities, especially in the case of pumpkin seeds, where results evidence that anti-nutrients (e.g. naturally present phytate) can reduce the uptake of Zn by complexing with Zn^{2+} in the intestines (pH ~ 7).

Key words: Zn, speciation, pumpkin seeds, iceberg lettuce, size exclusion chromatography, element-specific detection, physiologically based extraction test

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations:

AES	Atomic Emission Spectrometry
AAS	Atomic Absorption Spectrometry
CPS	Counts per second
DTT	Dithiothreitol
DAD	Diode Array Detector
CVAAS	Cold Vapor Atomic Absorption Spectrometry
ETAAS	Electrothermal Atomic Absorption Spectrometry
FAAS	Flame Atomic Absorption Spectrometry
GPC-AAS	Gel Permeation Chromatography – Atomic Absorption Spectrometry
HGAAS	Hydride Generation Atomic Absorption Spectrometry
HP	Hewett Packard
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductively Coupled Plasma – Mass Spectrometry
ICP-AES	Inductively Coupled Plasma – Atomic Emission Spectrometry
ICP-OES	Inductively Coupled Plasma – Optical Emission Spectrometry
ICP-TOF-MS	Inductively Coupled Plasma – Time-Of-Flight – Mass Spectrometry
IUPAC	International Union of Pure and Applied Chemistry
LA-ICP-MS	Laser Ablation – Inductively Coupled Plasma – Mass Spectrometry
LC	Liquid Chromatography
LMM	Low Molecular Mass
LMW	Low Molecular Weight
NMWL	Nominal Molecular Weight Limit
PC	Phytochelatin
PBET	Physiologically Based Extraction Test
PTFE	Polytetrafluoroethylene
RPM	Revolutions per minute (Frequency of rotation)
SEC	Size Exclusion Chromatography
SEC-ICP-MS	Size Exclusion Chromatography – Inductively Coupled Plasma – Mass Spectrometry
SRM	Standard reference material
TRIS	Tris(hydroxymethyl)aminomethane

TWEEN	Polyoxyethylenesorbitan monolaurate
UV	Ultraviolet
VIS	Visible
WHO	World Health Organization

Symbols:

Da	Dalton
kDa	Kilo Dalton
Hz	Herz
kHz	Kilo Herz
K	Kelvin
mA	Milli Ampere
t_0	Dead time in SEC
t_t	Total time in SEC
W	Watt

1 INTRODUCTION

Trace elements play an important role in the functioning of life on our planet. Some of them can be highly toxic, whereas others can be essential. These effects are very often related to a particular physico-chemical form in which the element is present. A number of definitions of essentiality have been proposed but the most frequently used definition refers to an essential element as one that is required for the maintenance of life and its deficiency causes an impairment of the function from optimal to suboptimal (Mertz, 1981). A biological requirement for Zn was first identified by Raulin (1869) when the common bread mould *(Aspergillus niger)* was found not to be able to grow in the absence of Zn. Zn deficiency under field conditions was first identified in 1932 (in Californian apple orchards and South Australian citrus trees) (Alloway, 2008). Attention was focused for years on Zn toxicity because of the general belief that Zn deficiency could not occur in humans because Zn was assumed to be ubiquitous and plentiful in our diets. About 40 years ago, Zn was recognized as an essential micronutrient for human health by Dr. Ananda Prasad, a nutrition chemist at Wayne State University in Detroit, Michigan (Prasad, 1991). Today, Zn deficiency is recognized as a nutritional problem worldwide, endemic in both developed and developing countries. The risk factors for a silent epidemic of Zn deficiency are primarily environmental in its origin. The causes of Zn deficiency fall in two main categories (i) nutritional causes such as consumption of food items with either low Zn contents or unavailable forms of Zn, and (ii) conditioned (secondary) deficiency related to diseases and genetic malfunctions that impair intestinal absorption and/or increase intestinal loss of Zn (Nriagu, 2007a).

1.1 Occurrence of Zn

Zn is the 25th most abundant element, accounting for approximately 0.02% by weight of the earth´s crust (Budavari, 1989). The average Zn content of rock in the earth´s crust is 78 mg kg^{-1} (Alloway, 2008). From Figure 1 it can be seen that Zn occurs in two valency states (0 and 2+) with the divalent zinc having the following potential forms: Zn^{2+}, $Zn(OH)_2$, $Zn(OH)_3^-$, $Zn(OH)_4^-$ (Beverskog & Puigdomenech, 1997). At mid-range pH values, Zn forms hydroxides that have low solubility in water, whereas at the pH extremes the solubility is increased, releasing Zn^{2+} at low and zincate ($Zn(OH)_3^-$ and $Zn(OH)_4^-$) at high pH (Sandstead & Au, 2007). In nature, Zn is a mixture of five stable isotopes: ^{64}Zn (49%), ^{66}Zn (28%), ^{68}Zn (19%), ^{67}Zn (4.1%), and ^{70}Zn (0.62%). A further 19 radioactive isotopes are known; ^{65}Zn is the most

stable with a half-life of 243.8 days (Simon-Hettich et al., 2001). Zn has a strong tendency to react with acidic, alkaline and inorganic compounds. Because of its amphoteric properties, Zn forms a variety of salts, which are all nonconducting, nonmagnetic and white or colorless, with the exception of those with a chromophore group, such as chromate.

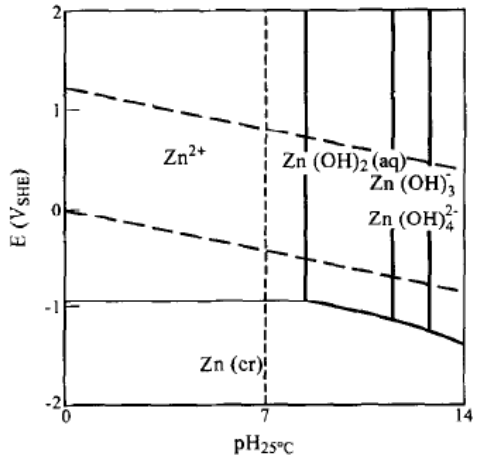


Figure 1: Pourbaix diagram for Zn at 25°C and $[Zn(aq)]_{tot} = 10^{-6}$ molal (Beverskog & Puigdomenech, 1997)

1.1.1 Zn in the environment

Zn is released to the environment from natural and anthropogenic sources. On a global scale, emissions from these sources are similar in magnitude. However, on a local scale anthropogenic sources may dominate (Simon-Hettich et al., 2001). Zn and its species are predominantly determined in the aquatic environment, soils and sediments (Günther & Kastenholz, 2005). There is a diversity of habitat types in both aquatic and terrestrial environments with different optimal concentration ranges. Zn is ubiquitous, except in some agricultural regions where Zn concentrations are very low or where antagonistic nutrient interactions occur (Simon-Hettich et al., 2001). Organisms may also develop tolerances on a local scale by acclimatization (physiological behavior) and adaptation (genetic changes) towards higher as well as lower concentrations. There is limited data available for performing a detailed assessment of the potential risk of Zn for each of the environmental media air, water, soil and sediment (Simon-Hettich et al., 2001). As the environment consists of different spheres or ecosystems, a comprehensive discussion would make his chapter too extensive. Therefore a partial environmental Zn cycle will be discussed, viz. the cycle dealing with the inherent relationship between soils and plants.

Zn in soil. Trace elements in soils are derived from the geochemical weathering of the rock fragments on which the soil has formed with inputs from atmospheric deposition and inputs from agricultural activities. Soils on river floodplains also receive trace elements from floodwaters and sediments. All of these sources and inputs can vary greatly in magnitude and result in soils having a wide range of total trace element concentrations (Alloway, 2008). Soils in some parts of the world are depleted in Zn and consumption of locally grown foods can result in endemic Zn deficiency in some communities (Nriagu, 2007a). Soils formed on limestone and sandstone tend to have low Zn concentrations, in contrast to soils developed on clay, shale, or igneous rock. The Zn content of soils and the bioavailability are critical for plant growth (Adriano, 1986). Soil factors that affect plant nutrition include total Zn, pH, organic matter, clay content, calcium carbonate content, redox conditions, microbes in the rhizosphere, soil moisture, amounts of other trace elements, and amounts of macronutrients, especially phosphorus (Sandstead & Au, 2007; Alloway, 2008). Zn is present in soil in five pools: a water-soluble pool; bound to soil particles by electrical charge; bound to organic ligands; nonexchangeably bound to clay and insoluble Zn complexes (Alloway, 2008). Low molecular weight organic acids that bind Zn^{2+} are an important source of soluble Zn. Adsorption mechanisms determine available Zn^{2+} for plants (Alloway, 2008). Low pH values increase cation exchange and high pH values increase chemisorption and complexation to organic ligands. In calcareous soils Zn^{2+} chemisorbs onto calcium carbonate and forms Zn hydroxycarbonate, which is very stable and forms a risk for Zn deficiency in plants (Sandstead & Au, 2007).

Zn in plants. Zn, in common with the other plant micronutrients, can limit growth when it is present both in low concentrations and also in excessive concentrations, due to deficiency and toxicity, respectively. Zn is essential for the normal healthy growth and reproduction of plants. If the supply of plant-available Zn is inadequate, crop yields are reduced and the quality of crop products is frequently impaired. In plants, Zn plays a key role as a structural constituent or regulatory co-factor of a wide range of different enzymes and proteins in many important biochemical pathways (Alloway, 2008):

- carbohydrate metabolism, both in photosynthesis and in the conversion of sugars to starch,
- protein metabolism,
- growth regulator metabolism,

- pollen formation,
- maintaining the integrity of biological membranes,
- resisting infection by certain pathogens.

Plants constitute important components of ecosystems as they provide the transport pathway from the abiotic to the biotic environment. Uptake of metals by plants reflects their bioavailability in soils and affects plant yield and crop quality to the extent that animal or human health may be jeopardized (Mingornace, 2002). Terrestrial plants assimilate Zn from soil. Zn is taken up mainly as Zn^{2+} although $ZnCl_2$ and Zn chelates may also be absorbed. The uptake of Zn^{2+} is active and metabolically controlled (Beneton Jones, 2002). Zn deficiency in plants is a worldwide problem that is not significantly mitigated by Zn entering the ecosystem from industry (Alloway, 2008). As a general rule, plants from environments poor in Zn are characterized by low Zn concentrations, those from Zn-enriched environments by high concentrations (Ernst, 1996). There is no convincing explanation as to why certain plant species have a higher uptake rate and accumulation pattern of Zn than others; although one possible reason may be to develop a defense against herbivores by accumulating high metal levels (Ernst, 1996). In plants, Zn does not undergo valency changes and its predominant forms are: low molecular weight complexes, storage metalloproteins, free ions and insoluble forms associated with the cell walls. Zn can become inactivated within cells by the formation of complexes with organic ligands or by complexation with phosphorus. Depending on the plant species, between 58% and 91% (Brown et al., 1993) of the Zn in a plant can be in a water-soluble form (low molecular weight complexes and free ions). This water-soluble fraction is widely considered to be the most physiologically active and is regarded as a better indicator of plant Zn status than total Zn contents. The low molecular weight complexes are normally the most abundant soluble form of Zn and are probably the most active form of the metal (Brown et al., 1993). These complexed forms of Zn are probably physiologically active because they can be degraded and may also be involved in homeostatic mechanisms where they may act as a buffer system to bind excess free Zn^{2+}. An example of this is the group of phytochelatins which have been identified in a wide range of species and are synthesized in response to exposure to excess concentrations of Cd, Zn and also Hg (Brown et al., 1993).

1.1.2 Zn related to nutrition

The primary source of most metals in food is the soil from which is food produced and there is an intimate connection between soil and the metals which are consumed and incorporated

in the human body (Conor, 2004). Sandstead & Au (2007) present one of the recent estimates from Food and Agriculture Organization based on food balance data from 176 countries, suggesting that approximately 20% of the world´s population is at risk of Zn deficiency. Major underlying determinants are economic status, food availability, and choice, but also diet quality (variety of foods and bioavailability of Zn) and diet quantity (amounts of food providing bioavailable Zn). The amounts in different kind of foods show a wide variation from less than 1 mg kg^{-1} in some fruits to more than 1000 mg kg^{-1} in certain shellfish (Conor, 2004). According to Scherz and Kirchhoff (2006), Zn concentrations and its variations characterize specific foods and food groups respectively, and do not depend on the origin of the food. In general, meat, eggs and dairy products contain more Zn than plants. But it is interesting that the recent movement to dietary habits that eschew red meat, which is high in Zn, in favor of fish, poultry and dairy products, may be a risk factor for Zn deficiency, similar to a purely vegetarian diet (Nriagu, 2007a). Regarding plants, pulses and cereals are the major source of dietary Zn for most people (Gibson, 1994). However, subsequently uptake of the element from different diets can vary depending on the selection of foods consumed. Indigestible plant ligands such as phytate, dietary fibers, lignin and products of nonenzymatic Maillard browning[1] formed during cooking inhibit intestinal Zn absorption (Sandstead and Smith, 1996), hence removal of Zn-binding ligands by milling or fermentation improves Zn bioavailability. Zn forms insoluble complexes with phytate at pH values usually found in foods (Shrimpton & Shankar, 2008). It is obvious that not only the total amount of Zn present determines how much will be taken up by the human body. More about Zn uptake will be discussed in chapter 1.2. Besides risk factors for Zn deficiency also the risk of an excess of Zn is present. Free access to uncontrolled amounts of Zn in nutritional supplements is the most common source of Zn excess (Sandstead & Au, 2007).

1.1.3 Zn in the human organism

Zn abundance in humans is approximately half that of Fe. For example, a 70 kg adult male contains approximately 2-3g of Zn. Approximately 60% is in muscles, 30% in bones, 8% in skin and hair, 5% in liver and 3% in the gastrointestinal tract and pancreas (Wastney et al., 1986). Simon-Hettich and co-workers (2001) present several studies which give evidence that the highest concentrations of Zn in humans are found in the liver, kidney, pancreas, prostate

[1] The Maillard reaction is a chemical reaction between an amino acid and a reducing sugar

and eye. Besides that, Zn is also present in plasma, erythrocytes and leukocytes. In healthy subjects, the normal plasma Zn concentration is ca. 1 mg l^{-1}. Zn is mostly bound to albumin (60-80%) and to a lesser extent to α-2-macroglobulin and transferrin (Wastney et al., 1986). Zn is also known to bind to ca. 1000 different proteins in humans and there are more than 300 enzymes involved in key metabolic processes in humans which contain Zn (Graham, 2008).

1.1.4 Zn interactions with other metals

A chemical balance in living organisms is a basic condition for their proper growth and development. Interactions between chemical elements may be antagonistic or synergistic and their imbalanced reactions may cause chemical stress to the biological system. Kabata-Pendias & Pendias (2001) define these two terms as follows: antagonism occurs when the combined physiological effect of two or more elements is less than the sum of their independent effects and synergism occurs when the combined effect of these elements is greater.

Zn interactions in plants. Higher concentrations of Cu in the soil pore solution, relative to Zn, can reduce the availability of Zn to a plant and *vice versa* due to competition for the same sites for absorption into the plant root. Also Zn-Cd interactions occur but seem to be somehow controversial. Kabata-Pendias & Pendias (2001) quote several studies done in the past which either observe synergism in rice plants in terms of Zn competition for Cd sites (resulting in an increased Cd solubility and translocation) or antagonism of these cations in the uptake and transport. It may be stated that the ratio of Cd-to-Zn ratio in plant media controls the occurrence of synergism and antagonism (Kabata-Pendias & Pendias, 2001). High soil phosphate levels are one of the most common causes of Zn deficiency in plants encountered around the world. However, although this interaction with phosphate has been recognized for many years, the actual mechanisms responsible are still not completely understood (Alloway, 2008). Nitrogen appears to affect the Zn status of crops by both promoting plant growth and by changing the pH of the root environment (Alloway, 2008). Excessive Zn depresses Fe uptake and can result in the development of Fe deficiency symptoms (Beneton Jones, 2002). Zn is also known to interact with Mn, B, As and C (Kabata-Pendias & Pendias, 2001).

Zn interactions in the human body. Uptake in the human body is inhibited by divalent cations (Ca, Fe, Cu, Ni and Mg). Hettich and co-workers (2001) present several studies which give evidence that Zn can behave antagonistically in combination with Cu and synergistically with Pb or Fe alone or in combination and with Hg or Ni. High Zn intakes (50 mg or more per day) inhibit the absorption of Cu by competing directly for serosal transport in the gut or by inducing metallothionein in the intestinal cells (UNEP et al., 2002). High affinity of Cu for Zn-induced metallothionein can be negative (excessive ingestion of Zn promotes Cu deficiency) or positive (use of high Zn levels for the treatment of Wilson´s disease (UNEP et al., 2002). Daily intake of Fe such as those found in some supplements could decrease Zn absorption (O´Brien et al., 2000). Supplementation with Zn has been shown to prevent the teratogenic and carcinogenic effects of Cd. However, it is important to stress that metal interactions can vary depending on the physiological conditions of the human body.

1.2 Zn related to human health

Regarding the human body, Zn is an essential trace element that can cause symptoms of deficiency and can be also toxic when exposures exceed physiological needs. Health disorders as a result of Zn deficiency have been well documented in humans and animals (Simon-Hettich et al., 2001). The relationship between essentiality and toxicity is described by a model that takes the form of a U-shaped curve (Fig. 2) and depends on the pool of Zn in the body and the amount of Zn ingested.

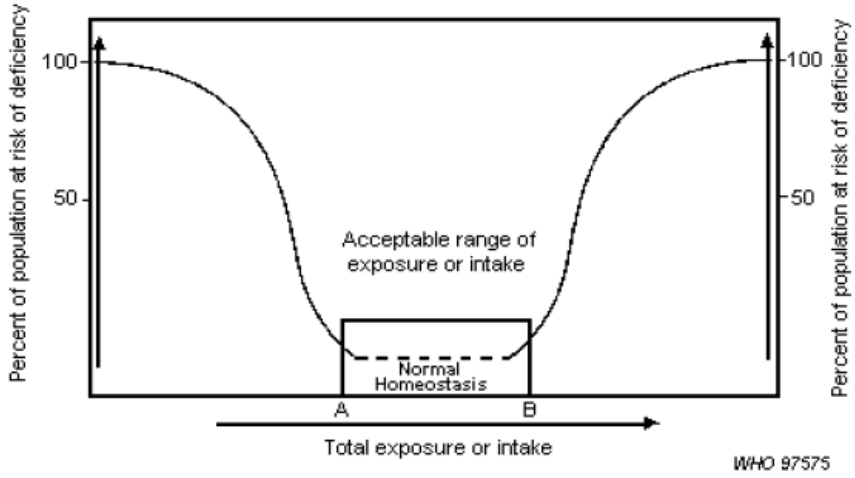


Figure 2: Percent of population subjected to deficiency and toxicity effects according to exposure/intake. As intake drops below A the risk for deficiency increases; at extremely low exposures or intakes all subjects will manifest deficiency. As exposure or intake increases beyond B a progressively larger proportion of subjects will exhibit effects of toxicity. (Simon-Hettich et al., 2001)

The relationship between intake and health is affected by physiological factors (homeostasis) and by extrinsic factors discussed in previous chapters that affect the availability of Zn for absorption and utilization (Simon-Hettich et al., 2001). Environmental levels of Zn providing exposures or intakes within the acceptable range do not produce adverse effects among the general human population. However, there are individuals or groups with imbalances in relation to other trace elements, or with disorders in homeostatic mechanisms that experience effects, of deficiency or toxicity, from exposures within the acceptable range. These disorders may be acquired or are of genetic origin (Simon-Hettich et al., 2001). Zn deficiency is one of the most prevalent risk factors for nutrient-related diseases, and is a leading contributor to the global burden of anemia (as a direct proximate cause or by potentiating the role of iron in anemia). Although severe Zn deficiency is rare, the incidence of mild to moderate deficiency is common throughout the world (Nriagu, 2007a). Besides inadequate Zn absorption also increased losses or increased requirements lead to several health disorders (Scherz & Kirchhoff, 2006). Although metabolic changes underlying human Zn deficiencies are still not completely understood it is known that Zn has a fundamental role in the structure and function of numerous proteins, including metalloenzymes, transcription factors and hormone receptors (Simon-Hettich et al., 2001). The widespread role of Zn in metabolism is underscored by the occurrence of Zn in all tissues, organs and fluids of the human body. However, large numbers of people are believed to ingest insufficient bioavailable Zn. The effects of Zn deficiency include impaired neuropsychological functions, oligospermia[2], growth retardation, impaired reproduction, immune disorders, dermatitis[3] and impaired wound healing (Simon-Hettich et al., 2001). Most of these effects are treatable with adequate amounts of Zn. Some physiological conditions may lead to increased requirement for Zn, including pregnancy, lactation, dilutional effects of rapid growth (such as the catch-up growth of premature infants), stress, obesity, trauma and rehabilitation after starvation (Nriagu, 2007a). Toxic effects in humans are most obvious from accidental or occupational inhalation exposure to high concentrations of Zn compounds. Although intentional or accidental ingestion of large amounts of Zn leads to gastrointestinal effects, such as abdominal pain, vomiting and diarrhea (Simon-Hettich et al., 2001). High levels of Zn may also disrupt the homeostasis of other essential elements. There is no convincing evidence that excess Zn plays an etiological role in

[2] Defined as semen with a low concentration of sperm (medical dictionary).
[3] Inflammation of the skin (medical dictionary)

human carcinogenesis. The weight of evidence supports the fact that Zn is not genotoxic or teratogenic but it can be cytotoxic at high concentrations (Simon-Hettich et al., 2001).

1.3 Zn uptake, retention and excretion

Zn can be absorbed in the human body via three major pathways (inhalation, ingestion and dermal uptake). In this chapter only the uptake via ingestion will be presented. In humans the absorption of Zn in the diet ranges widely. Bioavailability can be affected by *i*) abnormalities in the gastrointestinal tract, *ii*) the concentration of transport ligands and/or *iii*) the presence of substances that interfere with Zn absorption. A decreased absorption was noted for elderly subjects (Simon-Hettich et al., 2001). Also morphological alterations of the intestine as an intervention strategy for obesity by young healthy persons can lead to loss of absorption (Simon-Hettich et al., 2001). Several naturally occurring components of the diet can have considerable effect on Zn absorption. Animal proteins and low molecular weight organic substances like sulphur-containing amino acids (methionine, cysteine, citric and lactic acid) have a positive effect and enhance the uptake (Conor, 2004) but there are also several inhibitors already mentioned in sections 1.1.2 and 1.1.3. Persons with adequate nutritional levels of Zn absorb approximately 20–30% of all ingested Zn (Hunt et al., 1995), whereas greater proportions of dietary Zn are absorbed in Zn-deficient subjects if presented in a bioavailable form. In the small intestine both a passive and an active, carrier-mediated process are involved (Simon-Hettich et al., 2001; Lee et al, 1989). An intestinal protein which binds Zn during transmucosal transport, not apparently influenced by Zn status, is a 6–8 kDa protein with seven cystein residues (Tapiero & Tew, 2003). It has also been suggested that the pancreas secretes a ligand (probably metallothionein) that enhances jejunal zinc absorption (Oberlas, 1996). During periods of active growth and physiological states like infancy, pregnancy and lactation, when Zn requirements are high, there is a corresponding increase in the absorption of Zn (Conor, 2004). After absorption in the small intestine Zn is initially transported to the liver and distributed through the body. As already mentioned Zn is found in all tissues and body fluids but it is primarily an intracellular ion. Therefore, unlike some other elements, there is no particular body store or storage organ for Zn. That is why the metabolic Zn requirement must be met by intake of food or food supplements (Nriagu, 2007a). The gastrointestinal tract is the primary route of endogenous Zn excretion. Endogenous fecal zinc excretion is directly related to the total amount of zinc absorbed. If it is low, endogenous fecal

zinc excretion is also low (Tapiero & Tew, 2003). Some secreted Zn is reabsorbed by the jejunum, ileum and colon while the remainder is lost in the feces. When dietary Zn is restricted, fecal losses decrease and absorption increases (Krebs & Hambidge, 2001). Urinary and sweat Zn are affected by the Zn status. In humans, most ingested Zn is eliminated in the feces (5−10 mg day^{-1}) (Simon-Hettich et al., 2001) and comprises unabsorbed Zn and endogenous Zn from bile, pancreatic fluid and intestinal mucosa cells. Increased urinary excretion of Zn has been reported in a number of conditions including liver diseases, kidney diseases, alcoholism and during treatment with some chelating agents such as ethylenediamine tetraacetate (EDTA) and penicillamine (Nriagu, 2007a).

1.4 Zn species

1.4.1 Speciation

IUPAC[4] defines speciation as "the process yielding evidence of the atomic and molecular form of an analyte". According to Lobinski (1998) speciation is an excellent term to describe the state of distribution of an element among different chemical species in a sample. The definition of species is based on different atomic and molecular structures where chemical forms of the same element are manifested as: (1) isotopic composition, (2) electronic or oxidation state, (3) inorganic and organic compounds and their complexes, (4) organometallic species, and (5) macromolecular compounds and complexes (Templeton et al., 2000). Elemental speciation at the macromolecular level is important in physiology, biochemistry and nutrition. Differences in valence state, complexation by inorganic and organic ligands and the occurrence of covalent organometallic species determine the availability and toxicity of metals, thus having high importance in food chemistry and also in clinical and biological fields (Gonzalves et al., 2009). The identification of the elemental species involved is a critical step toward a complete understanding of the nutritional aspects or toxicity of elements of interest and also the key to the understanding of many metal-involving biochemical processes in the cell (Lobinski et al., 2006). Since the purpose of this work is speciation of Zn in vegetal samples, only Zn species and speciation methodologies in such matrices will be elaborated.

[4] International Union of Pure and Applied Chemistry

1.4.2 Zn and its species in vegetables

While some of the biological functions of essential elements are quite well understood, the chemical form in which elements naturally occur in plants is mostly unknown (Weber & Konieczynski, 2003). In contrast to elements like As, Hg, Se or Pb, only little information has been published on the physico-chemical forms of Zn, especially in the field of vegetables. In the scientific literature only a few cases of Zn speciation in plant samples are mentioned. Walker and Welch (1987) quote different authors which mention chemical compounds such as anionic compounds, phytochelatins and ellagic acid as potential ligands to form complexes with metals. As possible ligands which bind Zn in leaves of green salad they mention reducing sugars, amino acids and compounds which contain sulfur. A low molecular mass (LMM) fraction which contains Zn was partly purified; the major Zn-binding fraction had a molecular mass of about 1250 Da. The isolated LMM fractions contained 73% of the total soluble Zn and may be of nutritional importance (Walker & Welch, 1987). Waldner and Günther (1996) established characteristics of low molecular Zn complexes. They found that Zn complexes in various vegetables (kohlrabi, Chinese cabbage, chard, leek, spinach, Jerusalem artichokes) are similar and contain low molecular weight species and are anionic at pH 8. They show similar elution behavior in gel permeation and anion exchange chromatography. They suppose that Zn in kohlrabi and Chinese cabbage is bound to an unknown glutamic acid derivate, possibly a malic acid ester. Szpunar et al. (1999) gave evidence that Zn, Cu and Mg in aqueous leachates of apple and carrot samples elute as LMM complexes with non-carbohydrate compounds in the water-soluble fraction. Weber and Messerschmidt (2000) mention isolation of carbohydrates with low molecular mass as metal-binding ligands for Zn, Mg and Pt in plant roots extract but they do not give details. Günther and Kastenholz (2005) give evidence that by conducting GPC-AAS the Zn in several vegetal samples is predominantly present as species with a molecular mass lower than 5000 Da. Weber and Messerschmidt (2000) warn that low molecular weight metal species are often relatively labile and therefore warn for unwanted changes during the chromatographic separation, viz. dissociation, ligand exchange and/or irreversible adsorption to the resin. Conor (2004) claims that a large fraction of the Zn in plants and animals is incorporated into organic compounds like metalloenzymes and other Zn-containing proteins. Some of the Zn may also be present in an inorganic form, especially in plant foodstuffs.

1.5 Measurement of Zn and its species

Speciation analysis of trace elements in foodstuffs is mostly based on hyphenation of liquid chromatography or electrophoresis with atomic absorption spectrometry (AAS), inductively coupled plasma - optical emission spectrometry (ICP-OES) or inductively coupled plasma - mass spectrometry (ICP-MS) (Szpunar, 2000). Speciation analysis is often complicated by the fact that the trace or ultra trace amount of the element is distributed over species with a whole array of physico-chemical properties (e.g., charge, protonation, etc.). The often unknown molecular identity of the ligand and the sometimes low thermodynamic stability of the complexes make the analytical task even more challenging (Szpunar, 2000). A clear trend exists toward using the techniques that combine highly selective separation with very sensitive elemental detection in an online system. In such a system the selectivity is achieved by application of powerful separation modules, while the use of atomic or mass spectrometric techniques assures high sensitivity (Wrobel et al., 2005). The choice of a hyphenated technique depends primarily on the research objective. More about choosing an appropriate technique is discussed below.

1.5.1 Sample preparation

Usually the most critical step in speciation analysis is the sample preparation because mistakes made in this stage can influence the results further on and lead to misinterpretation of the results. Because Zn is ubiquitous in the environment special care is required during sampling, sample preparation and analysis to avoid sample contamination. In general laboratory operations care should be taken to avoid galvanized laboratory fittings, rubber materials and powdered gloves, all of which contain Zn (Kebbekus, 2003). Therefore the sample preparation for trace-level samples should be done in a clean area, with samples protected from atmospheric contamination as much as possible. Samples being analyzed for low trace metallic elements often require the use of clean-room technologies to allow satisfactory blanks to be obtained (Kebbekus, 2003). Plant sample tissues chosen for analysis usually include whole shoots, young leaves and, in some cases, grain or fruit. Potential problems may arise when sampling whole shoots because elemental concentrations are a function of the age of the plant. The leaves need to be washed in ultra-pure water to remove any contaminating particles on the surface, mopped dry and then oven dried, ground in a mill, taking care to avoid any possible contamination (Alloway, 2008). Plant tissues can also be cut in pieces and extracted as fresh tissues, frozen in liquid nitrogen and ground or freeze dried

and ground (Szpunar et al., 2003). For non-homogeneous solid samples, the gross sample may weigh several hundred grams and so reduction to a finely ground and homogeneous laboratory sample is necessary (Skoog et al., 2004). In the field of metal analysis we need to be careful by choosing the tools for sample preparation. A serious error can arise during grinding and crushing as a consequence of sample contamination resulting from the mechanical wear and abrasion of the grinding surfaces (Skoog et al., 2004). Further steps of sample preparation for determination of their metal content serves several purposes, which vary with the type of sample and the demands of the particular analysis. According to Kebbekus (2003) the major functions of sample preparation are:

- Degradation of the matrix to release metals for analysis;
- Extraction of metals from the sample matrix into solvent to make it more suited to the analytical method used;
- Concentration of metals present in very low levels to bring them into a concentration range suitable for analysis;
- Dilution of the matrix so that the effect of the matrix on the analysis is negligible or constant and measurable;
- Separation of a single analyte or group of analytes from other species that might interfere in the analysis.

Günther and Kastenholz (2005) quote several studies which describe sample pretreatment for further analysis. For cell fractionation (freeze-dried) vegetables were either crushed in the presence of fine-grained quartz and extracted with a buffer solution or subjected to liquid shearing by treatment with an electrical dispersant (ultra turrax) in a buffer. Supernants or cytosol (soluble fraction) and pellets or cell membranes and cell organelles (particulate fraction) are then usually separated with centrifugation. Only supernant is usually analyzed because it can be used directly for the analysis. The most practical difficulty present in speciation analysis is to preserve the integrity of the sample and consequently the species of interest during sampling, storage and pretreatment. Because of that procedures which alter equilibria or result in a transformation of species must be avoided (Gomez-Ariza et al., 2001). Species preservation during analysis requires “unaggressive” extraction and “gentle” separation procedures, in contrast to procedures for total acid digestion (Gonzalves et al., 2009).

Sample preparation for total metal analysis. Total elemental analysis requires digestion of the sample and is generally performed by classical hot plate digestion or open vessel digestion

and microwave-assisted digestion (Zhang, 2007). Digestion of the sample must assure the sufficient destruction of the matrix to permit the total analysis of the element of interest. However, sometimes the concentrations of different elements differ considerably depending on the digestion method used (Juranovic et al., 2003). Acid digestion requires a combination of acids (HNO_3, HCl and/or H_2SO_4), in the presence of a strong oxidizing agent (mostly H_2O_2 or $HClO_4$), and an external heat source to decompose the matrix and liberate metals in analyzable form. In hot plate digestion methods usually glass (Juranovic et al., 2003) or Teflon vessels are used. The major disadvantage of this method is that it is quite time consuming in comparison to other methods and presents more possibilities for contamination. Minogorance (2002) summarizes the results of several studies using microwave-assisted digestion as an alternative to classical digestion, offering faster and more reproducible results (Zhang, 2007). Besides wet digestion also dry ashing can be applied, especially for samples which contain significant amounts of organic matter and are being analyzed for nonvolatile metals (Kebbekus, 2003).

Ultrasound assisted extraction for speciation. Ultrasound-assisted leaching is an effective way of extracting a number of analytes from different types of samples. It is also an effective method for extracting heavy metals from environmental samples. Metals may be encapsulated within cell walls which must be broken to release the metals into the liquid extractant. The mechanism of ultrasound activity is well described by Luque-Garcia & Luque de Castro (2003) and Filgueiras et al. (2000), but in brief the most important property of ultrasonic waves passing through a liquid medium comes from a physical phenomenon called cavitation. Cavitation refers to a process by which numerous micro-gas bubbles are formed as the ultrasonic waves compress and decompress the liquid media as they pass through it. These bubbles can grow, oscillate, split and implode. Nowadays there are many types of ultrasonic devices; ultrasonic probes, ultrasonic baths, sonoreactors, and cup horns are ultrasonic systems that can be used for sample treatment in analytical chemistry (Vale et al., 2008). Generally, ultrasonic baths and probe units are used. Although ultrasonic baths are more widely used, they have two main disadvantages that substantially decrease experimental repeatability and reproducibility: (i) lack of uniformity in the distribution of ultrasound energy and, (ii) decline of power with time so that the energy supplied to baths is wasted. Ultrasonic probes have an advantage over ultrasonic baths in that they focus their energy on a localized sample zone, thereby providing more efficient cavitations in the liquid (Luque-Garcia &

Luque de Castro, 2003). However, the number of samples that can be treated at once is higher for the ultrasonic bath than for the sonoreactor (the ultrasonic probe allows only one sample treatment at the time).

Variable volume extraction approach. Every time extraction is used as a sample pretreatment step in speciation analysis, it is essential that we know the maximal amount of analytes extractable from the sample. The extraction efficiency may be limited, mostly through the interactions between target analytes and sample matrix. It is impossible to give defined species distribution concentrations when the species are not completely extractable. Therefore a so called variable volume extraction approach may be applied to give insight in the extraction efficiency and a possibility to extrapolate to maximal extractability (under the extraction conditions applied). The background (theory) of this approach is well discussed by Van Elteren et al. (2007) but in brief, when an element is present in a solid material, its total concentration may be subdivided into sub concentrations of species containing the element of interest. It is not necessary that all species have the same extractability (completely extractable, moderately extractable, non extractable) as with the variable volume extraction approach we can then get information about the concentration of species in solid material using their maximal extraction yield. There are also other possibilities of sample pretreatment as enzymatic extraction, controlled enzymatic degradation and sequential enzymatic extraction which are well described by Szpunar et al. (2003) and Schwedt et al. (1998).

1.5.2 Separation techniques

The separation component of the coupled system becomes of particular concern when the species of interest have close physico-chemical properties (Szpunar, 2000), and any additional sample manipulation increases the opportunity for sample contamination. For separation a simple separation technique as e.g. ultrafiltration through membranes with a defined molecular weight cut off or more sophisticated chromatographic techniques can be used. In this chapter only those techniques which were used in the framework of this study will be discussed in detail.

Ultrafiltration. Ultrafiltration is a membrane separation technique used to fractionate substances according to molecular weight. It is based on a pressure differential across a semipermeable membrane. Particles with a molecular weight less than the membrane

molecular cut-off pass through the membrane while solutes with greater molecular weight are retained on the membrane. Ultrafiltration offers fast separation and minimal denaturation of molecules compared to precipitation methods. However, it is important to indicate that some smaller molecules such as metallothioneins and phytochelatins do not pass the expected cut-off filters because their apparent molecular masses are higher than the theoretical ones (Szpunar et al., 2003).

Size exclusion chromatography (SEC). The theoretical principles of SEC are well presented and discussed by Mayer (2004), but in brief the separation mechanism of SEC is not based on chemical interactions as in other types of liquid chromatography (LC) but rather on the ability of analyte to penetrate the pores of the stationary phase. The mobile phase does not play a large role in the separation and is usually selected on its ability to solubilize the analytes of interest. The use of an aqueous mobile phase prevents structural changes, denaturation of proteins and destruction of protein-metal complexes. In practice, various aqueous mobile phases of fairly high ionic strength have been used to avoid interactions with the packing material (Szpunar & Lobinski, 2003). The SEC system must be calibrated with molecules of known molecular weight that have similar properties to those of the analyte of interest but one of the main problems in the analysis of real world samples is the unavailability of standards. Only a very restricted number of standards can be used for peak identification (Szpunar & Lobinski, 2003). The average time a substance spends on the column (in the pores) can usually be directly related to its molecular weight. As the interactions between the sample components and the gel support are limited on SEC, the formation of artefacts during chromatography is less probable then in the case of ion exchange or reversed phase chromatography (Mestek et al., 2002), but there can be some difficulties especially in case of ions with a high charge-to-mass ratio when secondary absorption and ion-exchange effects can occur (Szpunar, 2000). SEC has an advantage over other LC techniques because of its high tolerance to matrix components and the possibility to avoid a buffer. SEC is a gentle separation method and does normally not result in loss of element species or on-column alterations. Consequently, SEC has a predominant role in the separation of labile and weak metal-complexing biopolymers (Michalke, 2002). The optimum separation conditions may not always be used in speciation studies. Compromises are sometimes necessary to avoid possible changes in natural species distribution due to their interaction with stationary and mobile phases (Wrobel et al., 2005). Kannamkumarath et al. (2005) quote several studies which give evidence that SEC has proved to be a convenient technique for protein

fractionation. In many applications SEC has been used as an initial fractionation step for determination of metalloproteins or metals bound to biomolecules (Willoud et al., 2004; Kannamkumarath et al., 2004; Kannamkumarath et al., 2005).

1.5.3 Detection techniques

The choice of the detector becomes crucial when the concentration of analyte species in the sample is very low and low limits of detection are required. An important problem is usually the interface between the chromatograph and the detector as the separation conditions may not be compatible with the detector requirements in terms of flow rate and mobile phase composition (Szpunar, 2000). In this chapter only the methods which were used in the framework of this study will be presented more in detail.

AAS. AAS is predominantly a single element technique but with some instruments, up to four elements can be measured simultaneously, which is sufficient for a number of practical applications of AAS. The major applications include the detection of metals that give the most intense response in AAS (Cd, Zn, Cu) (Szpunar, 2000). There are several AAS techniques depending on the mode of sample introduction and atomization (FAAS, ETAAS, HGAAS, CVAAS) although in this chapter only FAAS will be discussed. The mechanistic operation of FAAS is well described by Ebdon et al. (1998) and Broekaert (2005) but in brief in FAAS the liquid sample is pneumatically nebulized in a spray chamber where the aerosol is produced. The aerosol is usually mixed with air and acetylene and introduced in the flame atomizer. This allows the temperature to reach 2300K which is sufficient for atomization of most elements and is low enough to circumvent significant ionization interferences. The selectivity is provided by the radiation source (hollow cathode lamp) delivering the exact radiation required since the lamp emits the atomic spectrum of the element of interest. The amount of light absorbed in the flame by the atoms of the element under study is directly proportional to the amount of analyte initially present. FAAS is applicable for quantitative analysis of nearly 70 elements (Ortega, 2002) but poor detection limits for trace elements and poor sensitivity for some of them remain the culprits although some improvement can be achieved with selective preconcentration techniques (Zhang & Zhang, 2003).

ICP-MS. Inductively coupled plasma mass spectrometry has initially been designed for solution nebulization analysis and although this remains the major application (Tanner & Günther, 2009) other sampling techniques have been pioneered. In comparison with AAS it offers the advantage of multielement operation. The mechanistic operation of ICP-MS is well described by Ortega (2002), Szpunar & Lobinski, (2003) and Nelms (2005) but in brief this technique converts the sample into an aerosol by pneumatic nebulization. An argon plasma is used as an ion source which ensures almost complete decomposition of the sample into its constituent atoms and the ionization conditions result almost exclusively in singly charged ions. Mass analysis is achieved by ion separation and depends on mass-to-charge (m/z) ratios. An electron multiplier usually serves as an extremely sensitive detector (Vanhaecke & Köllensperger, 2003). Among element-specific detectors, ICP-MS has become the primary tool in speciation analysis because of its unique features such as (i) monitoring effluents for their elemental composition with high sensitivity, (ii) determination of the target element with a high selectivity over co-eluting elements, (iii) isotope ratio, and isotope dilution capabilities, and (iv) detection of a number of elements virtually simultaneously (Wrobel et al., 2005). ICP-MS offers not only extremely good sensitivity and selectivity for the majority of elements but is also a robust detector that can be hyphenated with many chromatographic separation techniques. The major problem is presented by keeping blank levels low enough to exploit the extreme sensitivity. In general, ICP-MS requires more dilute buffers and tolerates lower concentrations of organic solvents than ICP-AES (Szpunar, 2000).

1.5.4 Hyphenated techniques in speciation analysis

According to the scientific literature numerous combinations of separation and detection techniques are possible; however, in this chapter only those combinations which were used in this work will be presented and discussed further.

LA-ICP-MS. Laser ablation coupled with ICP-MS is a powerful solid sampling technique for spatial distribution analysis of elements, both for lateral and depth profiling purposes. Besides these advantages LA-ICP-MS is a microanalytical techniques that provides high sensitivity, good accuracy and precision for most elements of the perodic table (Wu et al., 2009a). The ablated material is ionized and transported into the ICP using argon as a carrier gas. Next to analysis of geological, ceramic and metallurgical samples, LA-ICP-MS can be applied to the imaging of biological tissues such as cryostat-cut slices of brain tissues and plant leaves with

relatively high special resolution and good sensitivity (Wu et al., 2009b). The limited application of LA-ICP-MS in biological and medical research can be explained by quantification difficulties. Because of a lack of suitable matrix-matched standard reference materials calibration strategies need to be developed (Lobinski et al., 2006) which requires the laborious in-house preparation of matrix-matched standards or solution-based calibration for specific quantification problems (Becker et al., 2005). The LA-ICP-MS approach was reported for the study of essential element accumulation in leaves of a Cu-tolerant plant (Wu et al., 2009a, Wu et al., 2009b) and for studying the distribution of nutrient and toxic elements in cross-sections of tobacco tissues (Becker et al., 2008). A nanometer scale spatial resolution of elements on sample surfaces by LA-ICP-MS was reported for element imaging in a leaf sample using near-field LA-ICP-MS (Becker et al., 2006). Another possibility is the use of LA-ICP-MS for speciation purposes by lateral scanning of gels after gel electrophoresis (Szpunar & Lobinski, 2003).

SEC-ICP-MS. Among the HPLC techniques, SEC is considered to be the most suitable for coupling with ICP-MS regarding compatibility with the mobile phases used (Wei et al., 2003). The coupling of HPLC to ICP-MS is technically very simple and consists of connecting the exit of chromatographic column to the nebulizer. The optimization of the interface is limited to assuring the stability of the plasma in the presence of mobile phase (Szpunar & Lobinski, 2003). Although the application of SEC–ICP–MS coupling for metal speciation has some limitations (Szpunar & Lobinski, 2003), it has been accepted as a very useful hyphenated technique in speciation studies to estimate the association of elements to the compounds present in the sample. The main advantage of SEC-ICP-MS can be seen in its simplicity (Mestek et al., 2002) and the low detection limits achievable. On-line coupling, where the element-selective detector is connected directly to the separation system, give faster results compared to off-line systems. Furthermore, the risk for contamination or losses are reduced and the sample throughput is much higher than for off-line methods where chromatographic fractions are collected prior to analysis. Coupling of HPLC to ICP-MS has been widely applied to elemental speciation analysis in foodstuffs of animal and plant origin. (Szpunar et al., 1999; Weber & Konieczynski, 2003; Wuilloud et al., 2004b). Using this hyphenated technique the Caruso group has extensively studied different trace metals, including Zn, in a variety of nuts (Wuilloud et al., 2004b; Kannamkumarath et al., 2004; Kannamkumarath et al., 2005).

1.5.5 Estimation of Zn bioaccessability/bioavailability

It is important to note that there is an essential difference between bioaccessibility and bioavailability. The concept of bioavailability is interpreted in the literature in various ways. According to the general interpretation in pharmacology, oral bioavailability is mainly defined as the fraction of an orally administered dose that reaches the systemic circulation (Oomen et al., 2003). According to Oomen et al. (2003) the bioavailability is a function of several factors (see Figure 3) which determine the final internal exposure. The fraction of the element that is mobilized from the matrix into the digestive juice is defined as the bioaccessible fraction (Ruby et al., 1999).

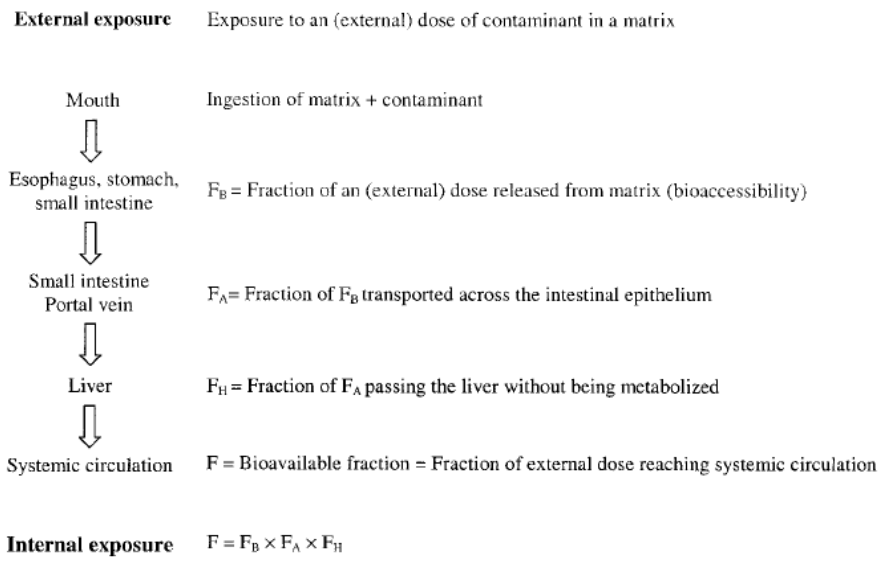


Figure 3: ***Various steps of oral bioavailability (Oomen et al., 2003)***

According to Ruby et al. (1999) the oral bioavailability is defined as the fraction of an administered dose that reaches the central (blood) compartment from the gastrointestinal tract, also named "absolute bioavailability", while the bioaccessible fraction represents the fraction that is soluble in the gastrointestinal environment and is available for absorption. This bioaccessible fraction is considered to represent the maximum amount of an element available for transport across the intestinal epithelium. To get insight into the release of metals from ingested foodstuff and otherwise, a so-called Physiologically Based Extraction Test (P.B.E.T) is used to simulate the processes occurring in the gastro-intestinal tract, representing the bioaccessibility. In a paper by Oomen et al. (2002) we can a find description and comparison of five in vitro digestion models which can be used for PBET modeling. Although these

models were primarily developed for human health risk assessment after ingestion of soil, they were also applied in the field of bioavailability regarding foodstuffs (Glahn et al. 1996; Jovani et al., 2001 Versantvoort et al., 2004; Versantvoort et al., 2005).

2 JUSTIFICATION OF ZN SPECIATION IN PUMPKIN SEEDS AND ICEBERG LETTUCE

In the scientific literature there is limited information available on Zn species in iceberg lettuce (*Lactuca sativa*) and pumpkin seeds (*Cucurbita pepo spp.*). Furthermore, insight into their fate upon digestion in the gastro-intestinal tract is largely lacking although this is of prime importance from a bioaccessibility and bioavailability point of view. Hence, the main objectives of this thesis are to get information on i) Zn species in the mentioned dietary products and ii) degradation of these species in the gastro-intestinal tract. To this end a "sequential" analytical approach was applied as schematically shown in Figure 4. By gradually focusing on more elaborate procedures, insight is obtained in total Zn, distribution of Zn, extractability of Zn, speciation of Zn and bioaccessibility of Zn, respectively. Bioaccessibility is studied by physiologically-based extraction tests (PBET) which simulate the digestion conditions in the gastro-intestinal tract. An array of procedures and techniques is used to aid in this research: microwave digestion, solid sampling by laser ablation, ultrasound-assisted variable volume extraction, ultrafiltration and size exclusion chromatography; elemental detection is performed by FAAS or ICP-MS, either off-line or hyphenated as in the case of Zn speciation by SEC-ICP-MS. Since two different dietary products are used it is expected that the Zn species encountered are different whereas their absolute and relative amounts will undoubtly vary as well, as will be their behavior in the gastro-intestinal tract. Results expected will potentially fill up gaps in this field of research and will provide nutritionists with novel information to improve dietary recommendations. To some extent these results could be also interesting to those who are dealing with plant breeding.

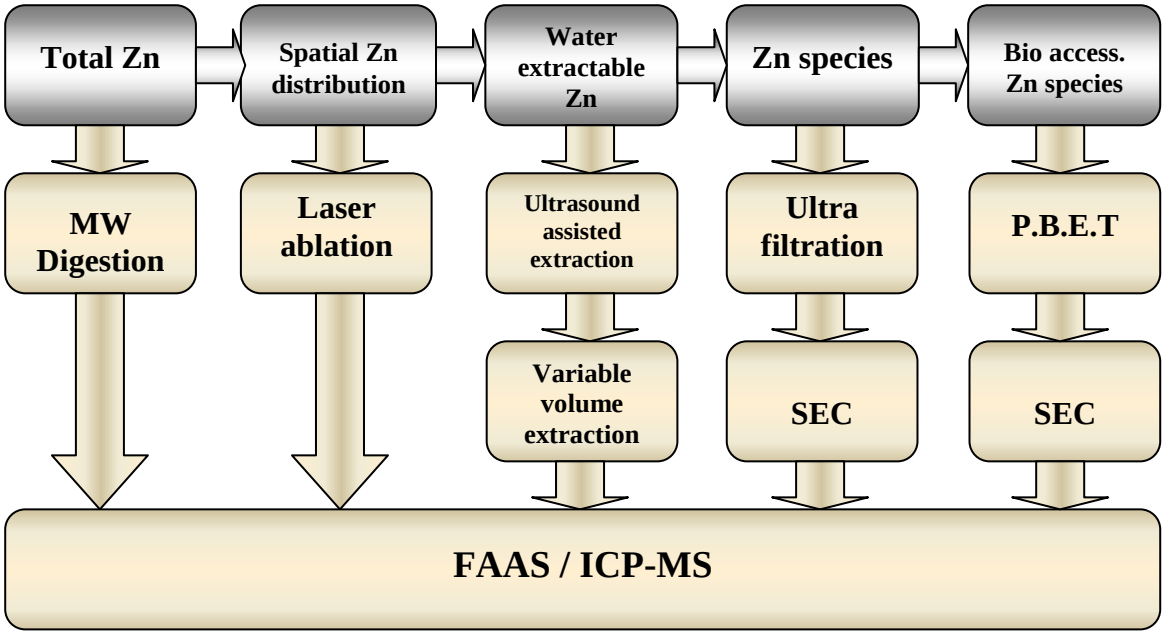


Figure 4: Analytical approach for determination of Zn and its species in pumpkin seeds and iceberg lettuce

A detailed overview of the procedures and techniques used will be given to rationalize the above "sequential" analytical approach. Determination of the total Zn is necessary as a reference point for further steps of the analytical approach. For this purpose a microwave digestion technique was chosen since the destruction of organic material is in general more complete than with e.g. classical hot plate techniques, resulting in better reproducibility. The study of the spatial Zn distribution by LA-ICP-MS can give insight in the Zn behavior in relation to other elements, which may contain valuable information about similarities or discrepancies between elements which can act either synergistically or antagonistically in plants or in the gastro-intestinal tract. In the case of pumpkin seeds it can be an important tool to examine the Zn distribution in the husk and the kernel individually, especially with regard to the fact that also seeds without husk can be purchased. For speciation purposes the mildest possible extraction is with water to minimize the risk of unintentionally altering the Zn speciation (as a result of structural changes, denaturation of proteins and damage of metal-protein complexes). However, the extraction of Zn species in water is a function of the extractant volume-to-sample mass (V/m) ratio. To counteract for extraction discrepancies related to V/m ratios a so-called variable volume extraction was applied which extrapolates the V/m ratio to infinity thereby yielding the maximum extractable amount in water. Ultrasound-assisted water extraction was chosen as an effective technique for efficient release of Zn species incorporated in the sample matrix. Speciation of Zn in the extracts was

performed by low (ultrafiltration) and high (size exclusion chromatography) resolution separation techniques with the goal to unravel the MW distribution of Zn species. Although physiologically-based extraction tests are generally used for human health risk assessment after ingestion of soil, only a few papers describe the actual use of these tests for nutritional purposes; their involvement in speciation studies has not been reported as yet. In this thesis physiologically-based extraction test will be used for i) simulation of the release of Zn in the gastro-intestinal tract upon ingestion of the mentioned dietary products and ii) investigation of the degradation of the Zn species during the digestion process. Element-specific detectors used were ICP-MS and FAAS; although FAAS is less susceptible to interferences, ICP-MS is the detector of choice for sensitivity allowing the successful hyphenation with SEC.

3 EXPERIMENTAL

3.1 Determination of total Zn by ICP-MS

Sample preparation for total Zn determination

Pumpkin seeds and iceberg lettuce were purchased from nearby markets in Ljubljana and originated from Slovenia and Italy, respectively. Prior to analytical treatment both products were washed with deionized water, followed by drying in an oven at 40°C for 48 h. Subsequently, iceberg lettuce leaves were ground in an agate ball mill, whereas seeds were ground in a domestic grinder. All the equipment for sample preparation was washed with 10% *v/v* HNO_3 and rinsed with ultrapure water prior to preparation. Iceberg lettuce samples were acquired from different batches while pumpkin seeds were from one batch only. Thus, the natural variation in Zn concentration may lead to results which are not directly comparable for iceberg lettuce.

For pumpkin seeds, besides determination of the total Zn amount in whole seeds, also determination of the total amount in the husk and kernel was performed individually (husk was carefully removed with a scalpel). For all samples (seeds and lettuce) dried and ground sub-samples were weighed (0.5 g) in PTFE[5] vessels in triplicate. For digestion, 7 ml of HNO_3 (65%) and 1 ml of H_2O_2 (30%), both p.a. quality, were added to the PTFE vessels, followed by microwave digestion in a Milestone Ethos 1 Advanced Microwave Digestion Labstation. Both seeds and lettuce were digested according to the following standard digestion protocol: in 15 min the temperature in the vessels reached 200°C and was kept constant for the next 15 min; after cooling of the vessels the digests were transferred to 50 mL polyethylene centrifuge tubes and the solutions made up with deionized water; blank solutions were prepared in the same way.

Determination of total Zn in digests

Two ICP-MS instruments were used and the typical instrumental conditions applied are given in Table 1. For calibration purposes standard solutions were prepared from a 1000 mg l^{-1} multielement solution (ICP Multielement Standard IV, Merck) in the concentration range 0 – 500 μg l^{-1} with a matrix resembling the sample solution as closely as possible. To correct for

[5] Polytetrafluoroethylene

potential instrumental drift internal standardization was used by spiking both the standard and sample solutions with Y, Sc, Ge and Gd (typically 50 μg l^{-1}).

Table 1: ICP-MS instrumental parameters

INSTRUMENTAL PARAMETER	ICP-MS INSTRUMENT	
	Agilent 7500ce	HP 4500
RFpower (*W*)	1500	1300
Plasma gas flow rate (l min^{-1})	15.0	15.0
Auxiliary gas flow rate (l min^{-1})	1.0	1.0
Carrier gas flow rate (l min^{-1})	0.8	0.98
Sampling depth (mm)	8.0	6.0
Acquisition time (s)	0.2	0.2

3.2 Determination of the spatial Zn distribution by LA-ICP-MS

A quadrupole ICP-MS (Agilent 7500ce, Palo Alto, USA) interfaced with a LA system (NewWave Research UP 213, Fremont, USA) was used. The laser ablation device contained a frequency-quintupled Nd:YAG laser (wavelength, 213 nm; pulse width, 4 ns) with a motorized stage and a laser ablation chamber, a so-called SuperCell (New Wave Research) optimized for monitoring of fast transient signals as encountered in solid sampling of heterogeneous samples by laser ablation. Elemental distribution in samples was investigated by rastering and depth profiling. Typical operational parameters are given in Table 2. In the case of pumpkin seeds rastering (longitudinal and cross-sectional) was done on the freshly cut surface of a pumpkin seed which was cut in half with a scalpel. In the case of iceberg lettuce one part of a dried leaf (approx. 14 cm^2) was used for analysis. The time resolved signals (in cps) were used for elemental distribution purposes.

Table 2: Operational LA-ICP-MS conditions for solid sampling

Laser ablation system	NewWave Research UP 213
Wavelength of Nd:YAG laser (nm)	213
Ablation mode	raster or crater
Repetition frequency (Hz)	20
Energy output (J cm^{-2})	0.50 – 0.70
Scan speed (μm s^{-1}) or dwell time (s)	50 (raster mode) or 130 (crater mode)
Spot diameter (μm)	100
He purge gas flow rate (l min^{-1})	0.95
ICP-MS	Agilent 7500ce
RF Power (*W*)	1500
Carrier gas flow rate (l min^{-1})	1.0
Auxiliary gas flow rate (l min^{-1})	1.0
Carrier gas flow rate (l min^{-1})	0.8
Sampling depth (mm)	8.0
Acquisition time (s)	0.25

3.3 Extraction of Zn and its species

Ultrasound assisted extraction

For ultrasound assisted extraction a Sartorius Labsonic®M device equipped with a titanium probe (diameter: 3 mm and length: 80mm) with 30 kHz operating frequency under maximum energy density and amplitude (460 W/cm^2 and 180 μm, respectively) was applied. Samples were weighed into 50 ml polyethylene centrifuge tubes and 25 ml of deionized water was added. Between extractions the probe was cleaned with 1% *v/v* HNO_3 (65%) and deionized water. Subsequently the supernatants were carefully removed with a polypropylene Pasteur pipette and filtered through 0.45μm syringe filters. The samples were sonicated for 2 min, followed by centrifugation for 10 min at 5000 rpm. Determination of Zn was carried out by FAAS as described in Table 3 or by ICP-MS as described in section 3.1 (Table 1). Blanks were treated in the same way.

Table 3: Instrumental parameters for determination of Zn by flame atomic absorption spectrometry

Wavelength (nm)	213.9
Lamp current (mA)	5.0
Bandpass (nm)	1.0
Air/acetylene flow-rate (l min^{-1})	11/1

Variable volume extraction

Varying amounts of dried and ground iceberg lettuce and pumpkin seeds (0.1 - 1.0 g) were accurately weighed into 50 ml tubes and 25 ml of extractant (deionized water) was added, yielding *V*/*m* ratios in the range of 25 - 250 ml g^{-1}. All samples underwent ultrasound-assisted extraction as described before, starting with those with high *V*/*m* ratios and low expected concentrations of measured elements. After the extraction the samples were immediately centrifuged at 3500 rpm for 15 min and the supernatants carefully removed with a polypropylene Pasteur pipette and filtered through 0.45 μm syringe filters. Extracts were analyzed the same day with ICP-MS (Agilent 7500ce, Palo Alto, USA) under instrumental conditions as presented in section 3.1 (Table 1).

3.4 Speciation analysis of Zn

Speciation of Zn in water extracts was performed by low (ultra filtration) and high (size exclusion chromatography) resolution separation techniques.

3.4.1 Ultra filtration

For ultra filtration Amicon® ultra-15 centrifugal filter devices (Millipore) with cellulose low binding membrane were used. The experimental approach is schematically presented in Figure 5. A sample (0.5 g) was weighed in a 50 ml falcon tube and subjected to ultrasound-assisted extraction as described before. One part of filtered sample solution was used directly for total element analysis while the other part was divided over four Amicon filter devices with Nominal Molecular Weight Limit (NMWL) of 3kDa, 10kDa, 30kDa and 100 kDa.

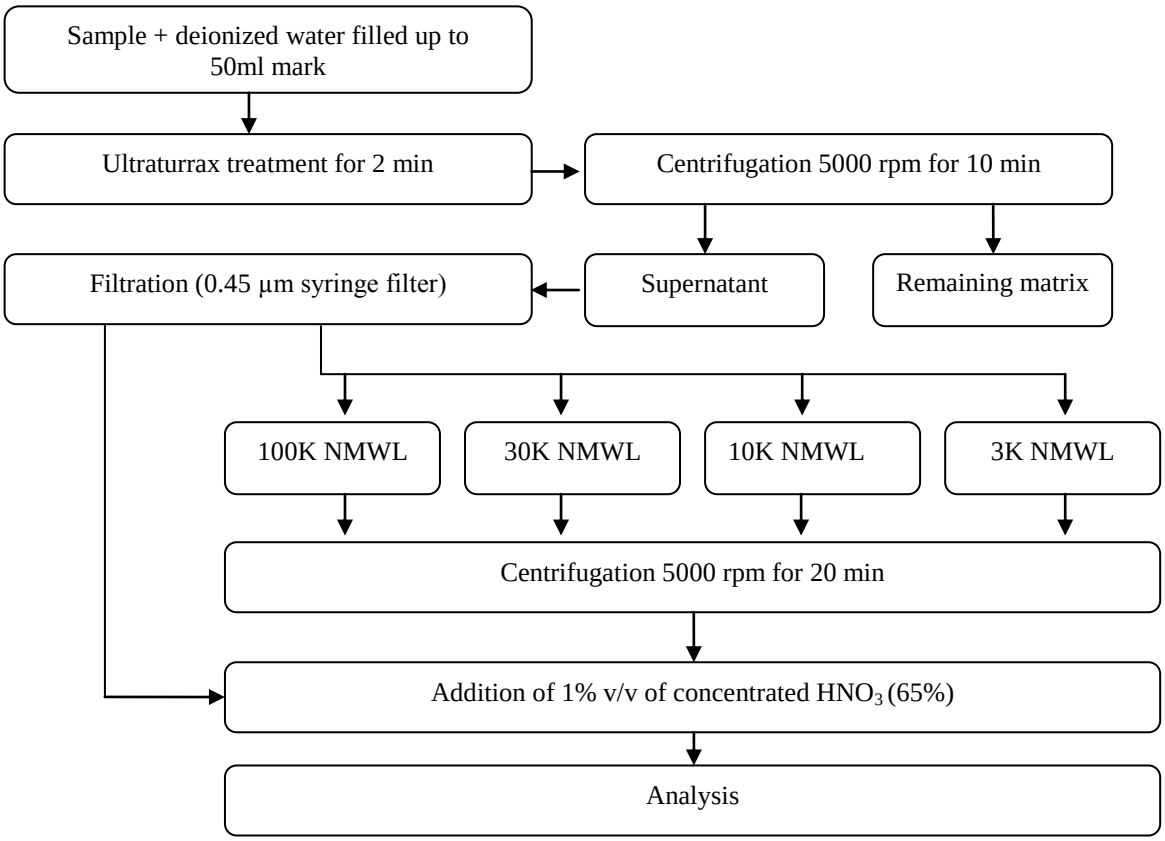


Figure 5: Ultrafiltration – experimental approach

3.4.2 Size Exclusion Chromatography

Sample preparation. Since the chemical composition of pumpkin seeds is complex and consists for 50–70% of lipids (Alfawaz, 2004), a suitable sample cleanup step is essential prior to analysis. For this purpose a procedure as described in the literature (Wuilloud, 2004b) was applied: ground pumpkin seeds (4 g) were subjected to extraction with a 20 mL chloroform–methanol (2:1 *v*/*v*) mixture by thoroughly mixing them together for 15 min. The extract was filtered off on a 0.45µm membrane filter and the residue on the filter was dried at

room temperature. Ground iceberg lettuce was subjected to extraction in the mobile phase directly. Initially two SEC columns, a Superdex 75 column (300 mm × 10 mm) for resolution of compounds from 3 to 70 kDa and a Superdex Peptide column (300 mm × 10 mm) for separation of LMW compounds (0.1 – 20 kDa).

Column calibration. The columns were calibrated with appropriate calibrants (Cytochrome C (12,284 Da), Metallothionein standard (10,000 Da) Aprotinin (6,500 Da), Vitamin B_{12} (1,355.4 Da), Glutathione-oxidized (612 Da), Trycine (179.2 Da) and Glycine (75.1 Da)) and using DAD and ICP-MS as detectors (see below). The calibration was done at the same conditions (Table 4) as used for sample separation by injecting a mixture of all calibrants. The void volume of the columns was determined with Bluedextran (2,000 kDa), also at the same conditions.

Off-line measurements. Separation with subsequent off-line measurement was performed as a preliminary procedure for on-line measurement. Initially only the separation conditions on Superdex 75 were optimized with a tunable UV/VIS spectrometer regarding UV wavelength, flow rate, injection volume and mobile phase. For off-line measurement fractions of 1 ml were collected with a fraction collector, acidified with HNO_3 (65%) and stored at room temperature until analysis next day. Analysis of metals was performed with a HP 4500 ICP-MS using the instrumental conditions given in Table 4. For calibration purposes standard solutions were prepared from a 1000 mg l^{-1} multielement solution (ICP Multielement Standard IV, Merck) in the concentration range 0 – 500 μg l^{-1} in the mobile phase used for separation.

On-line measurements. Since the Superdex Peptide column gave in general a better separation, this particular column was chosen for the remaining on-line experiments. An Agilent Technologies HP 1100 high performance liquid chromatographic system equipped with a G1322A degasser, G1311A quaternary pump, G1313A Auto sampler and G1315A DAD detector was used in combination with the already mentioned HP 4500. As buffers two different solutions (Table 4), related to the extraction procedure, were used. The SEC separation of the elemental species was monitored by DAD and ICP–MS, coupled in series. For this purpose the outlet of the SEC column was interfaced with the DAD which in turn was connected to the liquid sample inlet of the nebulizer. The instrumental operating conditions were as given in Table 4.; for DAD dectection the wavelengths 210, 230 or 250 nm were

used. The percentage distribution of the element among different molecular size fractions was evaluated by relating the area of a particular peak to the total area under the chromatogram.

Table 4: Instrumental conditions for ICP-MS and SEC

SEC CONDITIONS	*Off-line measurements*	*On-line measurements*
Column	Superdex 75 10/30	Superdex peptide 10/30
Resolution range	3000 - 70,000 Da	100 - 20,000Da (optimum 100 – 7,000Da)
Mobile phase	0.01 mol l^{-1} Tris–HCl (pH 7.4)*	0.03 mol l^{-1} Tris–HCl (pH 7.4)
	0.03 mol l^{-1} Tris–HCl (pH 7.4)*	0.03 mol l^{-1} Tris–HCl (pH 2.5)
Flow rate (ml min^{-1})	1.0	0.5
Injection volume (µl)	100, 200	100
ICP-MS CONDITIONS		
RF power (W)	1300	1300
Plasma gas flow rate (l min−1)	15.0	15.0
Auxiliary gas flow rate (l min−1)	1.0	1.0
Carrier gas flow rate (l min−1)	0.98	0.98
Acquisition time (s)	0.2	1.0

*Various combinations with NaN_3 and DTT

3.5 Zn bioaccessibility

To estimate the bioaccessibility of Zn and its species in the human digestion tract (stomach and small intestine) a PBET test protocol as described by Ruby et al. (1996), with some modifications according to Oomen et al. (2003), was performed. The experimental approach is schematically presented in Figure 6. Samples were weighed in 50 ml polyethylene centrifuge tubes in which the whole PBET test protocol was completed. Both pH adjustments were done with a Pasteur pipette and the aid of a magnetic stirrer. Since ICP-MS solutions are in general acidified, additional centrifugation (4000 rpm for 5 min) and filtration (0.45 µm) was necessary for the small intestinal step as bile salts precipitate at low pH values. Measurements were done employing the HP 4500ce, Palo Alto, USA at operating conditions as described in section 3.1 (Table 1).

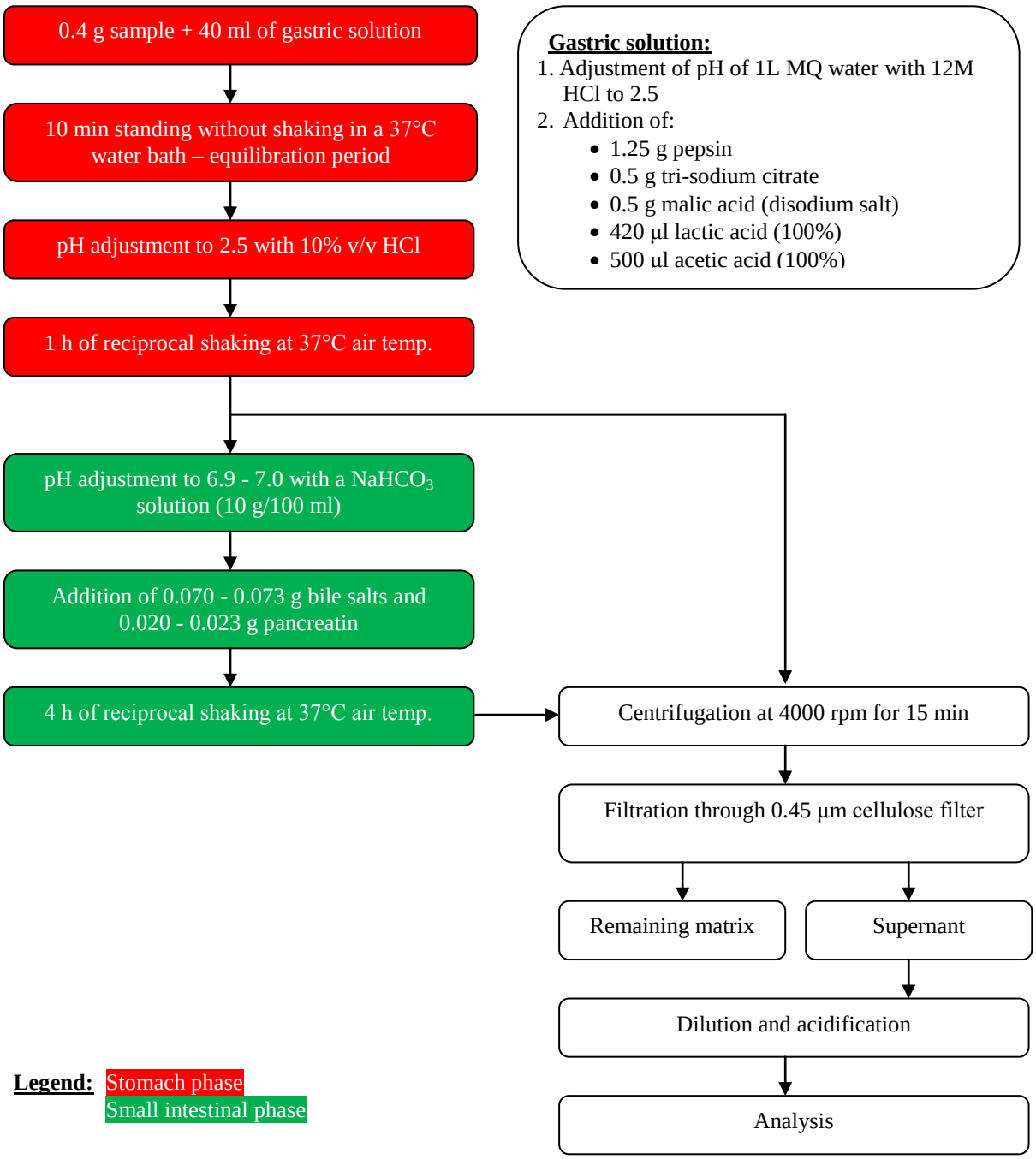


Figure 6: PBET test protocol

4 RESULTS AND DISCUSSION

4.1 Total Zinc

Vegetables and fruits are two plant food groups which contain Zn in a wide concentration range. Seeds contribute in general significantly to the nutrition of the human population in many parts of the world and pumpkin seeds are often consumed directly as a snack food in many cultures throughout the world (Alfawaz, 2004). Pumpkin seeds are one of most concentrated vegetarian sources regarding Zn (WHO, 2009) while iceberg lettuce generally grows quite quickly and is therefore a good catch crop which is readily available in supermarkets throughout the year. Its popularity is also determined by its low cost and long shelf life and minimally prepared lettuce mixes are especially popular (Nicolle et al., 2004; Rubinskiene et al., 2008). The total amounts of Zn found in pumpkin seeds and iceberg lettuce (Table 5) are in good agreement (same order of magnitude) with literature findings (Alfawaz, 2004; Scherz & Kirchhoff, 2006; Glew et al., 2006; Vegetarian society, 2009; WHO, 2009; Juranovic et al., 2002; Kabata-Pendias & Mukherjee, 2007). Concentrations of seven other elements are given as well to serve as background information to explain possible interactions with Zn (see below); please beware that these measured concentrations are indicative only due to non-optimized measurement conditions. For quality control purposes also a standard reference material (SRM NIST 1547: Peach leaves) was analyzed. The recovery for Zn was 97.2% of the certified value (Tab. 5).

Table 5: Total amounts of Ca, Cd, Cu, Fe, K, Mg, Mn and Zn in iceberg lettuce and pumpkin seeds

Element	**Sample**				**Standard Reference Material****	
	Iceberg Lettuce		Pumpkin seeds			
	Conc. (µg/g)	RSD (%)	Conc. (µg/g)	RSD (%)	Certified (µg/g)	Measured (µg/g)
Ca*	1,723.9	0.8	67.4	2.2	15,600 (+/- 200)	N.M.
Cd	3.8	5.0	2.7	0.1	0.026 (+/- 0.003)	N.D.
Cu	37.3	1.3	12.1	6.4	3.7 (+/- 0.4)	2.7
Fe	81.3	1.4	120.5	2.2	218 (+/- 14)	195.2
K*	70,316	1.7	9,258	3.5	24,300 (+/- 300)	N.M.
Mg*	3,734	0.8	5,386.4	2.1	4,320 (+/- 80)	4046
Mn	48.8	1.4	46.0	1.0	98 (+/- 3)	91.4
Zn	62.0	1.5	91.2	2.6	17.9 (+/- 0.4)	17.4

* Indicative values ** SRM NIST 1547: Peach leaves
N.M. – Not measured
N.D. – Not detected

The Zn content in plants is influenced by their age and vegetative stage. The age of the plants has a significant influence on the status of minor and trace elements. The trace element

content of the plants generally decreases from the end of April to the middle of June, in the case of Zn to half the amount found in April (Anke, 2004). The highest Zn content can be frequently observed in young plants (Mestek et al., 2002). Values from 15 to 100 µg g^{-1} in dry matter are considered to be normal. Less than 20 µg g^{-1} in leaves is taken as a deficit while values higher than 400 µg g^{-1} can already be toxic (Kabata-Pendias & Pendias, 2001; Peganova & Eder, 2004). On the other hand, early harvest of seeds means up to 10 times lower concentrations of antinutrients which are natural or synthetic compounds that interfere with the absorption of nutrients (Akwaowo et al., 2000). However, it needs to be stressed that the content of trace elements and also other compounds may vary considerably depending on soil conditions, climate and genetic factors.

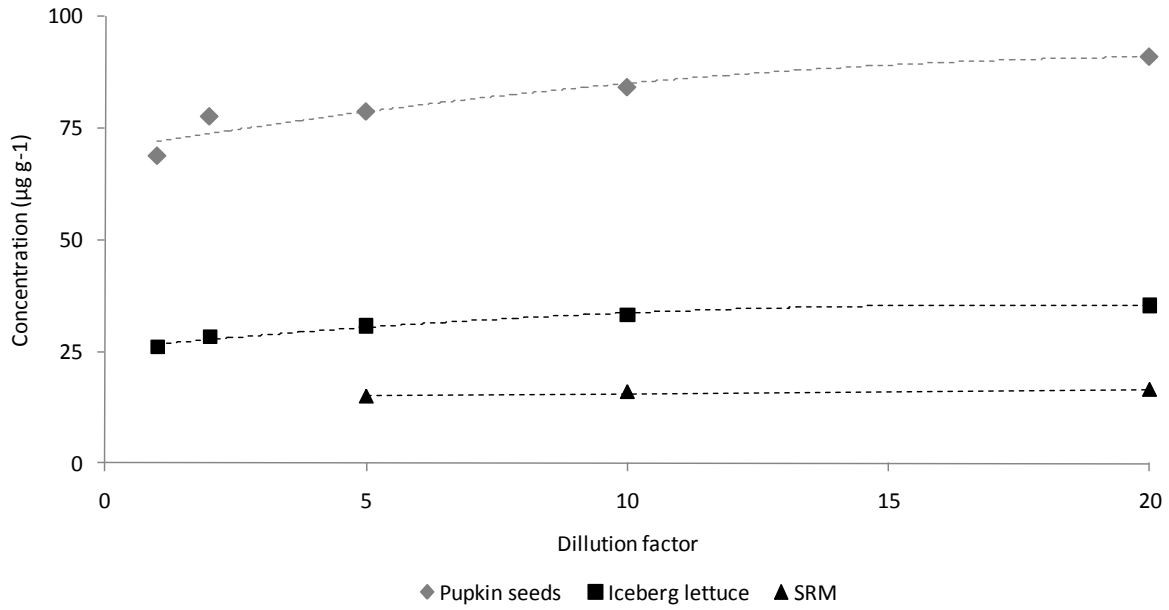


Figure 7: Zn concentration (ug g^{-1}) depending on dilution factor after total digestion (SRM NIST 1547: Peach leaves)

Zn concentration measurements by ICP-MS were investigated for potential matrix interferences. In general undiluted samples were prone to yield lower Zn concentrations than expected as a result of matrix interferences (Fig. 7). These interferences could be overcome by at least 20-fold dilution of the sample as illustrated (Fig. 8) by the sample graphs (standard addition) being parallel to the standard (external) calibration graph. However, to circumvent any possible risk of biased results due to matrix interferences the method of standard addition was applied routinely. In both situations presented (Figs. 7 and 8) digests from standard reference material (SRM NIST 1547: Peach leaves) were analyzed.

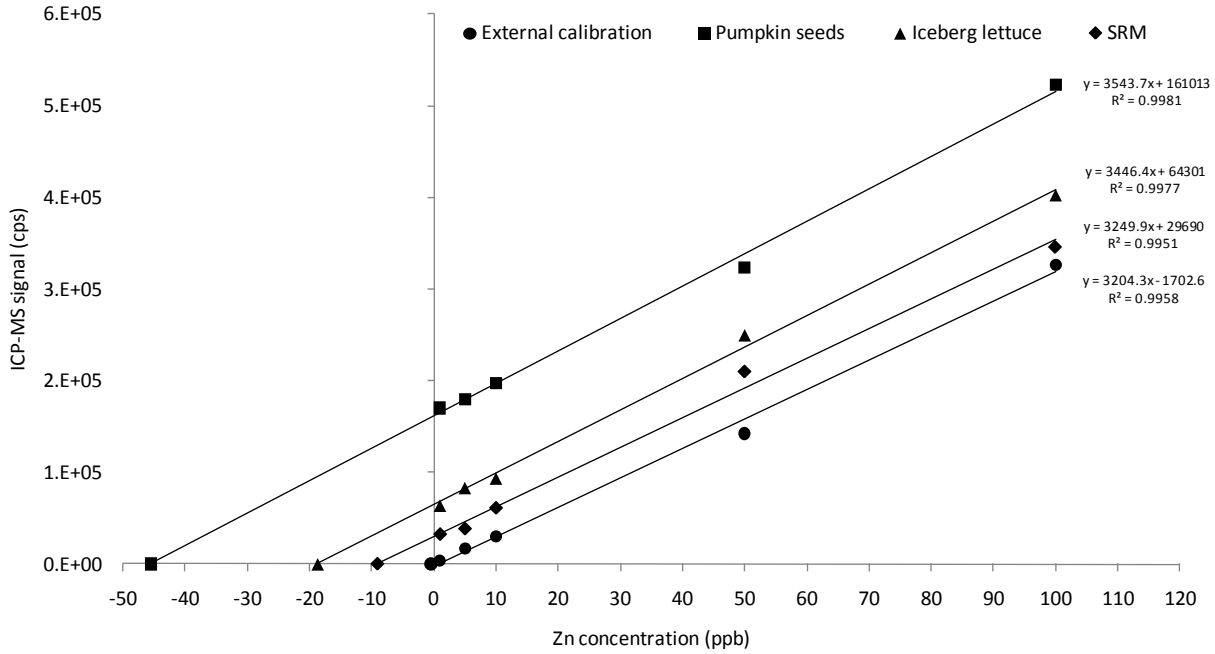


Figure 8: Standard addition of Zn after total digestion and dilution factor 20 (SRM NIST 1547 : Peach leaves)

In Table 5 not only Zn is mentioned but also some other elements which have the ability to interact with Zn in plants or in the human body. It has been postulated (Hill & Matrone, 1970) that elements with similar properties will act antagonistically to each other as a result of their competition for binding sites on proteins that require metals as cofactors. The major difference observed between both samples is for K and Ca which are "denser" in iceberg lettuce than in pumpkin seeds, while other elements, except Mg, are present in the same order of magnitude as Zn. When we want to interpret complex interactions of Zn with other elements from a nutritional point of view a problem arises as this field of research mostly deals with investigations based on supplementing a single element to the regular nutrition. Another thing to consider is the ratio between elements consumed; especially divalent cations like Ca, Cd, Fe, Cu and Mg have the ability to inhibit uptake of Zn in the human body (Conor, 2004). Fe is of special interest because of frequent use in the form of supplements. Whittaker (1998) presents several studies where the effects of Fe on Zn absorption are described but many of the results appear to be conflicting as some studies show that high concentrations of Fe can have a negative effect on the absorption of Zn in human adults if Zn and Fe are given in solution while if they are given in a meal, such an effect is not observed. Ca, especially in the presence of phytate, can also limit the Zn uptake from food (Wood & Zheng, 1997). Vegetables, which are Ca rich, include lettuce and asparagus. Both species store also large amounts of Mg (Anke, 2004). We should be aware that numerous other interactions of Zn

with trace and minor elements may occur. There are also other naturally occurring components of the diet which can even have a bigger effect on the absorption of Zn in the human body; they will be discussed in detail later.

While we can buy different varieties of pumpkin seeds in food stores, among which also those without husk, we are interested how the Zn concentration in the whole seed compares to the concentration in the husk and kernel separately. From results presented in Table 6 it seems that Zn is more concentrated in the husk than in the kernel. To verify the distribution of Zn in pumpkin seeds in detail a LA-ICP-MS scanning technique was applied having a spatial resolution (lateral and depth) in the order of micrometers.

Table 6: Zn concentrations and mass balance of Zn in whole pumpkin seed, kernel and husk

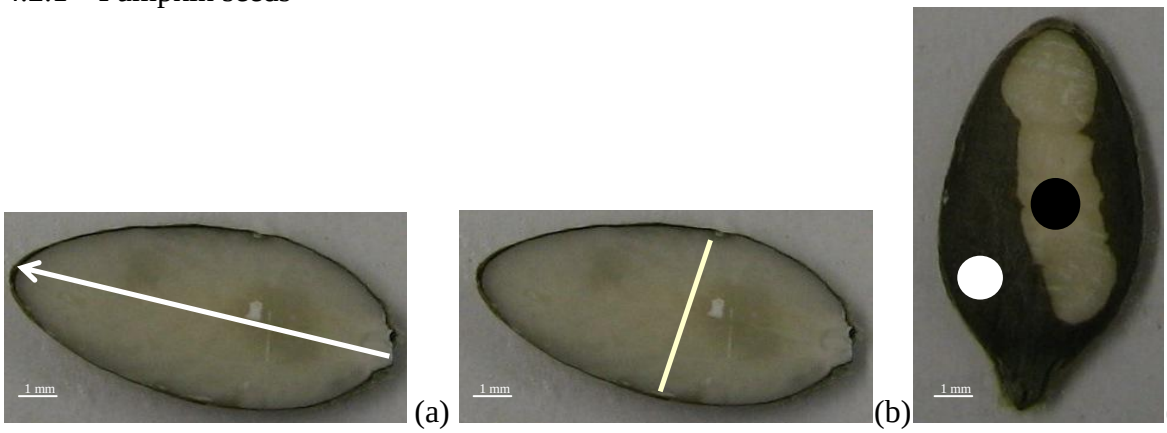
Sample	Weight (g)	Zn concentration		Zn mass balance	
		($\mu g\ g^{-1}$)	RSD (%)	(µg)	%
Whole seed	6.5085	91.2	2.6	592.6	100
Kernel only	4.3466	62.3	3.6	270.8	46
Husk only	2.1619	139.7	0.3	302.0	51

4.2 Spatial Zn distribution

The transport of trace elements in plants and their organs depends on several factors such as plant species, growth season, morphology of the leaves' surface, pH, valency state, competing cations, chelating ligands, chemical form of an element and formation of insoluble salts (Kabata-Pendias & Pendias, 2001). In general Zn belongs to the group of elements which are moderately mobile from roots to above-earth parts. According to Alloway (2008) it has already been generally accepted that Zn is transported in the plant either as Zn^{2+} or bound to organic acids.

LA-ICP-MS is a sensitive, solid sampling microanalytical technique which can yield an image of the elemental distribution of the sample's surface. The technique is well-established in the field of geological and metallurgical sample analysis (Lobinski et al., 2006), while its application for the imaging of soft tissues is just emerging (Becker et al. 2005; Becker et al., 2008; Wu et. al., 2009a; Wu et al., 2009b). Direct elemental imaging in plant tissues is often more reliable than bulk analysis because it is not affected by sample preparation which may alter the element distribution (Becker et al., 2008). In addition, elemental imaging enables the correlation of tissue distribution with biochemical functions (Lobinski et al., 2006). Minimization of the variability that occurs in the sampling process with LA-ICP-MS is possible with the usage of an internal standard. In order to verify the influence of variability during the sampling process on the ion intensity, the signal of ^{13}C was measured as well. The ICP-MS signal of the element is therefore presented as the element / ^{13}C ratio (Figs. 10 – 12 and 14 – 15). The ^{13}C isotope has already been successfully used as an internal standard for mapping purposes in soft tissues (Caumette et al., 2007; Becker et al. 2008).

4.2.1 Pumpkin seeds



(a) (b) (c)

Figure 9: Pathways of the laser beam for a lengthwise scan (a), a crosswise scan (b) and a depth profile of the pumpkin seed interior/exterior (c); the white and black dots in (c) denote the depth profiling locations on the seed with husk and with husk removed, respectively. The beam diameter was in all instances 100 µm

The spatial Zn distribution in the case of pumpkin seeds was studied in three different directions (Fig. 9). The decision for depth profiling on the seed with/without husk was made to confirm the findings of the lateral scans which indicate a strong increase of the Zn concentration towards the periphery of the seed and to verify the distribution of Zn in pumpkin seeds obtained by bulk analysis (Table 6).

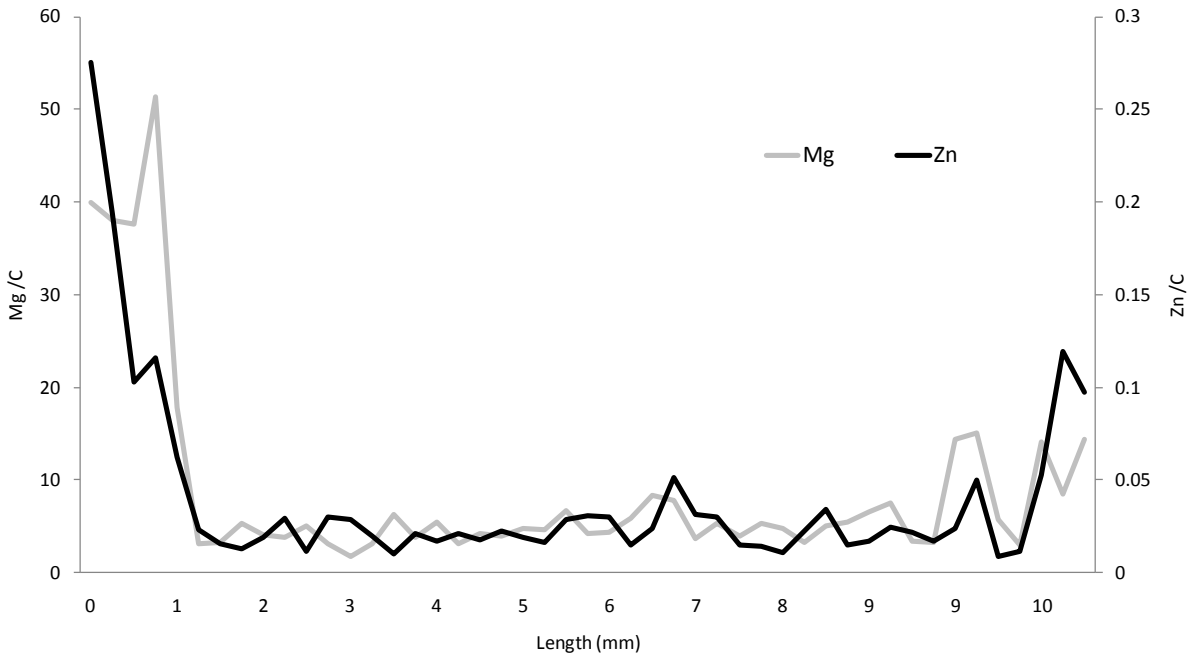


Figure 10: Lengthwise scan of pumpkin seed interior for Zn and Mg at a scanning speed of 50µm s^{-1}

In Fig. 10 significantly higher concentrations of Zn can be observed at the stem[6] of the seed then in other locations. The Zn concentration slightly increases again when the laser beam is nearing the end of its path, indicating a slightly higher Zn concentration under the husk on the other side. The same distribution pattern can be observed for minor elements like Mg (Fig. 10) and K, Ca, Fe and Mn (not given). The decrease of the concentration as a function of the laser path was the highest in the case of Mn. Surprisingly, Cu, as the element nearest to Zn in the periodic table, and therefore having similar chemical properties, did not show such an obvious lateral distribution pattern as was expected. Antagonistic/synergistic elemental behaviour may be studied by comparing spatial elemental distribution profiles obtained by simultaneous measurement of an array of elements by LA-ICP-MS. In Table 7 Pearson correlation coefficients are presented to relate the elements in a lengthwise scan across the sample (pumpkin seeds and iceberg lettuce). We can conclude that Zn is highly synergistic with Mg, K, Mn (correlation coefficient >0.8). A crosswise scan of the pumpkin seed interior showed no significant fluctuations in apparent concentrations. The distribution of Zn and also of other elements seems to be rather uniform in that direction.

Table 7: Pearson correlation coefficients to relate Ca, Cd, Cu, Fe, K, Mg, Mn, Zn in pumpkin seeds and iceberg lettuce upon lengthwise scanning by LA-ICP-MS.

	Ca		Cd		Cu		Fe		K		Mg		Mn		Zn	
	PS	IL	PS	IL	PS	IL	PS	IL	PS	IL	PS	IL	PS	IL	PS	IL
Ca	1.00	1.00														
Cd	-0.21	0.13	1.00	1.00												
Cu	0.30	0.47	-0.81	0.01	1.00	1.00										
Fe	0.76	0.46	-0.20	0.25	0.41	0.41	1.00	1.00								
K	0.79	0.72	0.02	0.19	0.25	0.37	0.69	0.30	1.00	1.00						
Mg	0.41	0.31	-0.02	0.36	0.29	0.09	0.42	0.19	0.73	0.39	1.00	1.00				
Mn	0.68	0.49	-0.02	0.11	0.42	0.35	0.76	0.68	0.70	0.41	0.58	0.23	1.00	1.00		
Zn	0.67	0.47	0.18	0.26	0.54	0.35	0.70	0.48	0.81	0.43	0.82	0.54	0.83	0.52	1.00	1.00

Note: PS-pumpkin seeds; IL-iceberg lettuce

[6] In this work the expression ”stem“ is used to mark the specific growth area of the seed (beginning of the laser path for lateral scanning). In botanic terminology the expression »Hypocotyl« is also used.

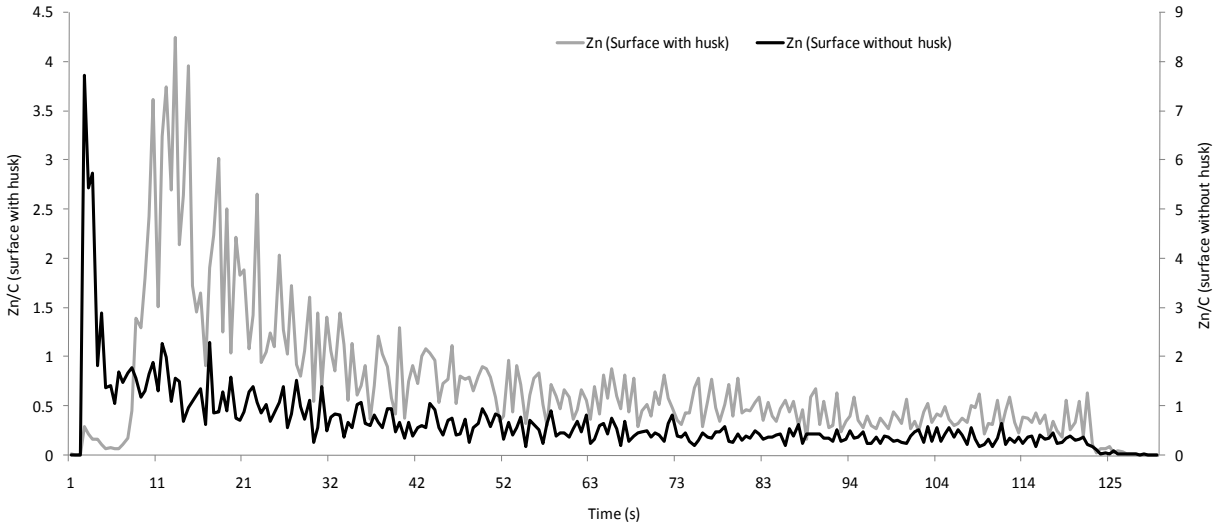


Figure 11: Depth profiling for Zn on the pumpkin seed with husk and with husk removed

From Fig. 11 it can be observed that the husk contains almost no Zn and that the Zn concentration directly under the husk is much higher than in the kernel. This implies that the bulk data on Zn and other elements in husk and kernel (Tab. 6) are dubious. Since the seeds were peeled by hand also the Zn-rich thin layer directly under the husk may have been (partially) removed contributing to higher Zn concentrations in the husk. The same pattern of Zn distribution, with concentrations decreasing from skin to the core, was observed in the apple fruit (Longnecker & Robson, 1993).

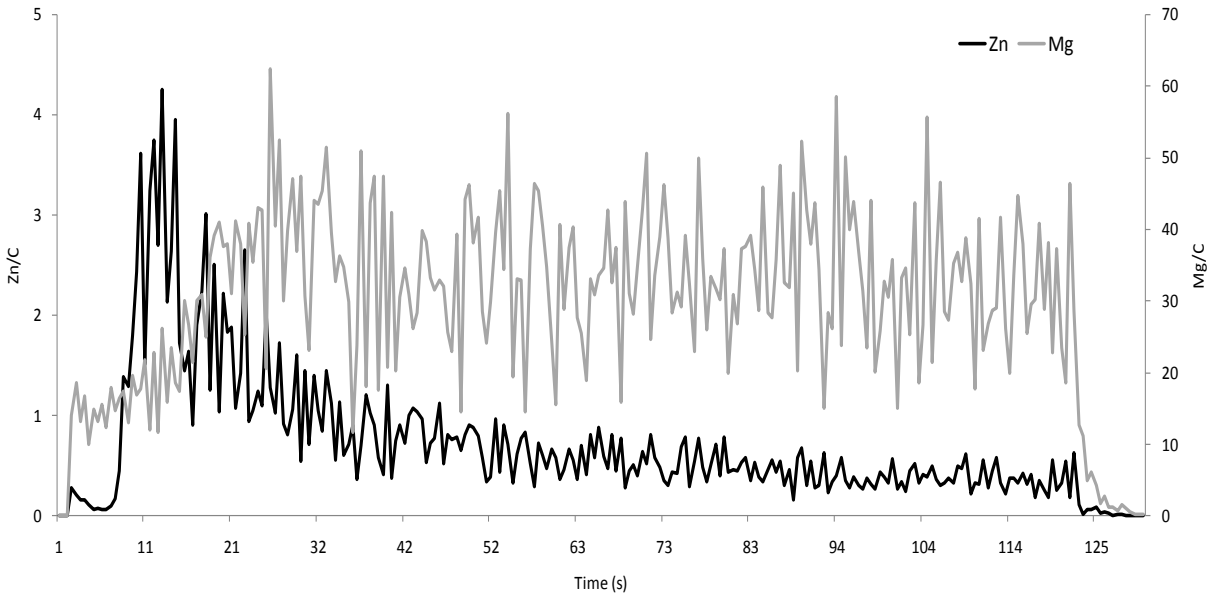


Figure 12: Depth profiling for Zn and Mg on the pumpkin seed with husk

In Fig. 12 Zn and Mg depth profiles are shown, having very little in common as far as correlation is concerned, indicated by a correlation coefficient of < 0.1. Usually young developing tissues tend to have higher Zn concentrations than mature tissues except in the

case of seeds (Longnecker & Robson, 1993). Regarding the accumulation of Zn in different parts of the seed it was already obvious, that Zn accumulates rapidly in the embryonic tissue and is transported to the storage tissue at seed maturity (Longnecker & Robson, 1993). Early accumulation of Zn in the embryonic tissue could be the consequence of the Zn role in protein synthesis.

4.2.2 Iceberg lettuce

Since a single leaf of iceberg lettuce does not fit into the laser ablation chamber only part of the leaf was subjected to microanalysis by LA-ICP-MS. Since the leaf is too thin for depth profiling only lateral scanning was performed (Fig. 13).

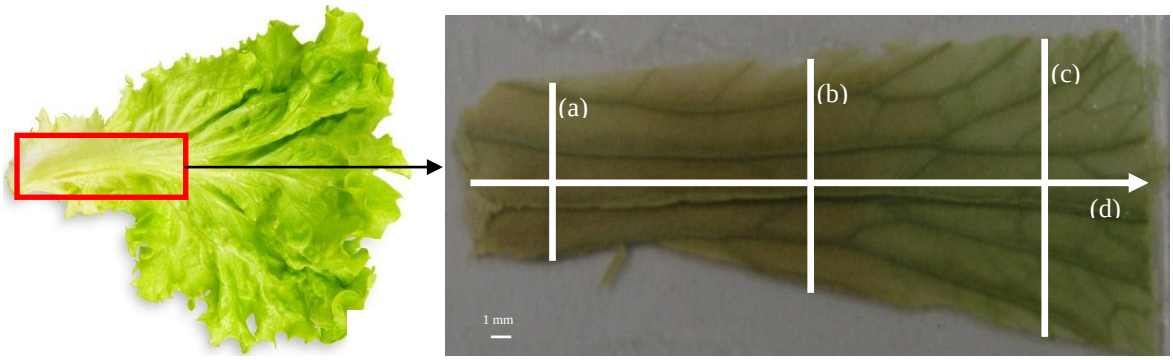


Figure 13: Pathways of the laser beam for crosswise (a-c) and lengthwise (d) scans of an iceberg lettuce leaf

In Fig. 14 a rather uniform Zn distribution can be observed. Since in Zn-deficient plants more Zn is present in the leaf blade[7] then in the petiole[8], while in Zn-sufficient plants Zn is more equally distributed between the leaf parts (Longnecker & Robson, 1993) we can conclude from the lateral scanning studies that the iceberg lettuce investigated was not deficient in Zn. Also other elements from Tab. 5 show the same distribution pattern; for a comparison also K, as a representative minor element, is plotted in Fig. 14.

[7] The expanded portion of a leaf (Botanical dictionary)
[8] The stalk of a leaf, attaching the blade to the stem (Botanical dictionary)

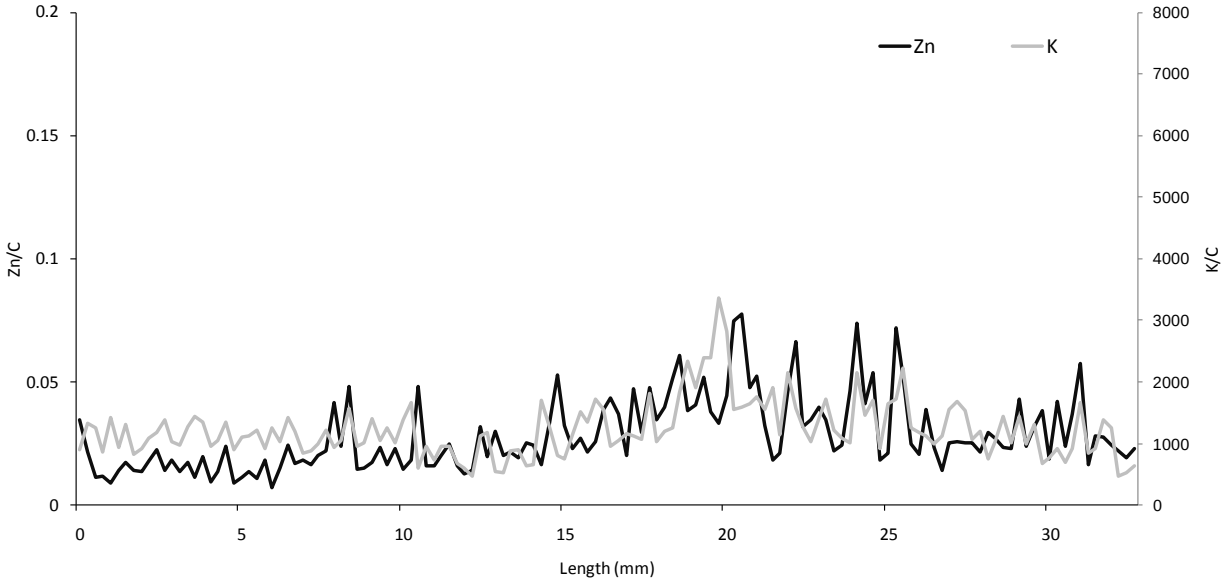


Figure 14: Lengthwise scan of an iceberg lettuce leaf for Zn and K at a scanning speed of 50 µm s^{-1}

The same uniform Zn distribution pattern can also be observed by crosswise scanning of an iceberg lettuce leaf (Fig. 15). The uniform distribution of Zn was a surprise as higher Zn concentrations were expected in the veins of the leaf, but Zn transport in the xylem which is responsible for the transport of water and soluble mineral nutrients from the roots throughout the plant, does not necessarily coincidence with that of water (Longnecker & Robson, 1993). Comparing the correlation coefficients of all the elements given in Tab. 7 there is no significant synergistic or antagonistic behaviour with Zn observed (Pearson correlation coefficient 0.5 or less).

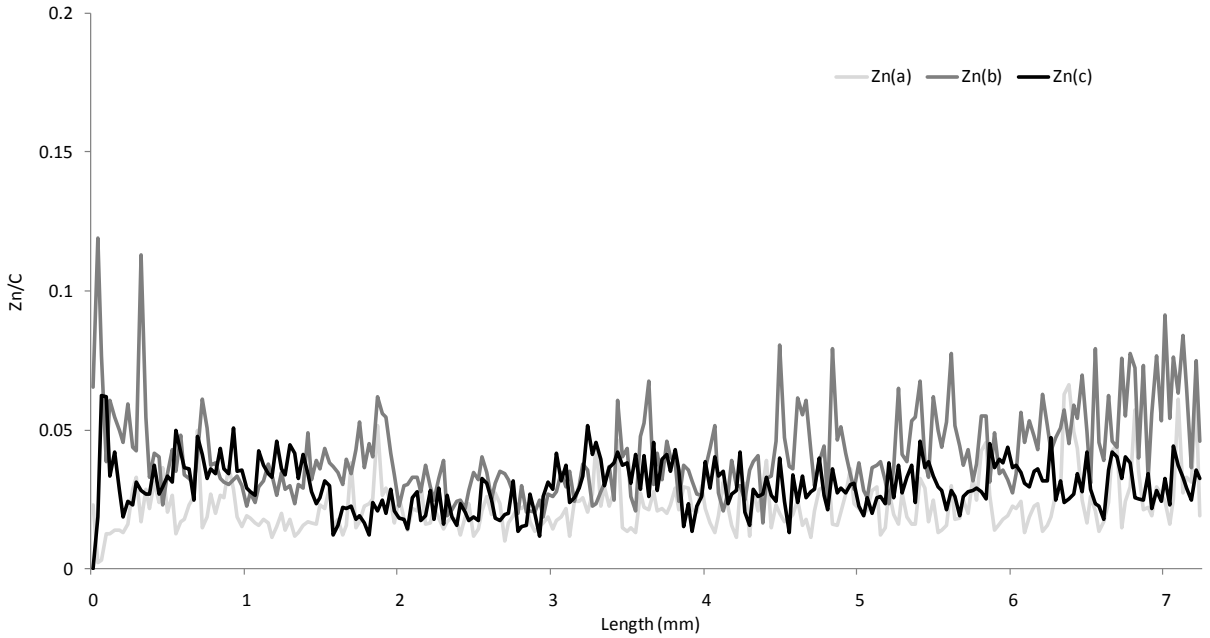


Figure 15: Crosswise scans of an iceberg lettuce leaf according to Fig. 13

It should be noted that for this vegetable (iceberg lettuce) the age of the leaf and the plant status regarding Zn play important roles. In Zn-deficient plants young leaves generally have higher concentrations then older ones, while in the case of an adequate supply, Zn accumulates in the older leaves of plants leading to subsequent higher Zn concentrations in older leaves than in new growth leaves (Longnecker & Robson, 1993). Zn can be partially translocated from old leaves to developing organs when needed (Alloway, 2008). According to Kabata-Pendias & Pendias (2001) the bioavailability of trace elements (Fe, Mn, Cu and Zn) from aerial sources through the leaves, including foliar application of fertilizers, may also have a significant impact on the element concentration. However, a fraction of the adsorbed elements may already be washed off from the foliage by rainwater. Differences in wash-off behavior are element-dependent with easy removal of Pb indicating a superficial deposit and more difficult wash-off indicating a greater penetration into the leaf as for Zn and Cu (Kabata-Pendias & Pendias, 2001).

Although LA-ICP-MS was applied to gain more insight into the distribution of Zn and other elements in pumpkin seeds and iceberg lettuce, from a nutritional point of view this is less relevant as humans in general consume the whole seed/plant. However, for plants/fruits we only partially consume, more detailed studies on translocation of elements during growth and subsequent ageing upon storage may be used as an indication for nutritional value.

4.3 Zinc species

4.3.1 Water soluble fraction

Acording to Nies (2004) an element must be soluble in water to be available for life. In speciation analysis of solid samples, including solid foodstuffs, the first task is to dissolve as many of the elements of interest as possible to make them accessible in most of the subsequent analysis steps like ultrafiltration, chromatography, etc. According to Günther and Von Bohlen (1990) this "initial speciation step" is an important part of the whole speciation procedure but it should be as simple as possible and not too aggressive to ensure the integrity of the species (Michalke, 2004). For the purpose of elemental speciation various extraction techniques are applied, using mild to strong acidic or basic extraction conditions (Wuilloud et al., 2004a; Wuilloud et al., 2004b). Extraction with water may be not so efficient but prevents structural changes, denaturation of proteins and destruction of protein-metal complexes (Makarov & Szpunar, 1998). A preparative cellular decomposition step is necessary in most cases which, due to the structure and composition of cellular walls in plants, often require more drastic methods like homogenization by electric dispersion, so-called liquid shearing (Günther & Von Bohlen, 1990) or Ultra-Turrax. Ultrasound is therefore of great help in the pre-treatment of solid samples, while it facilitates and accelerates the mechanical effect of breaking up the matrix and causing smaller particles to be produced, thereby exposing more surface area to the extractant (Rostagno et al., 2003) enhancing the homogenization (Luque-Garcia & Luque de Castro, 2003). According to a literature review by Santos and Capelo (2007) ultrasound assisted extraction is mainly used as an alternative to microwave-assisted extraction. For the purpose of this work an ultrasound probe was employed to accelerate extraction of the water-soluble Zn fraction from pumpkin seeds and iceberg lettuce by direct immersion into the solid-liquid mixture. It should be noted that the ultrasonic power generated in this setup is at least up to 100 times greater than that generated by an ultrasonic bath (Santos & Capelo, 2007).

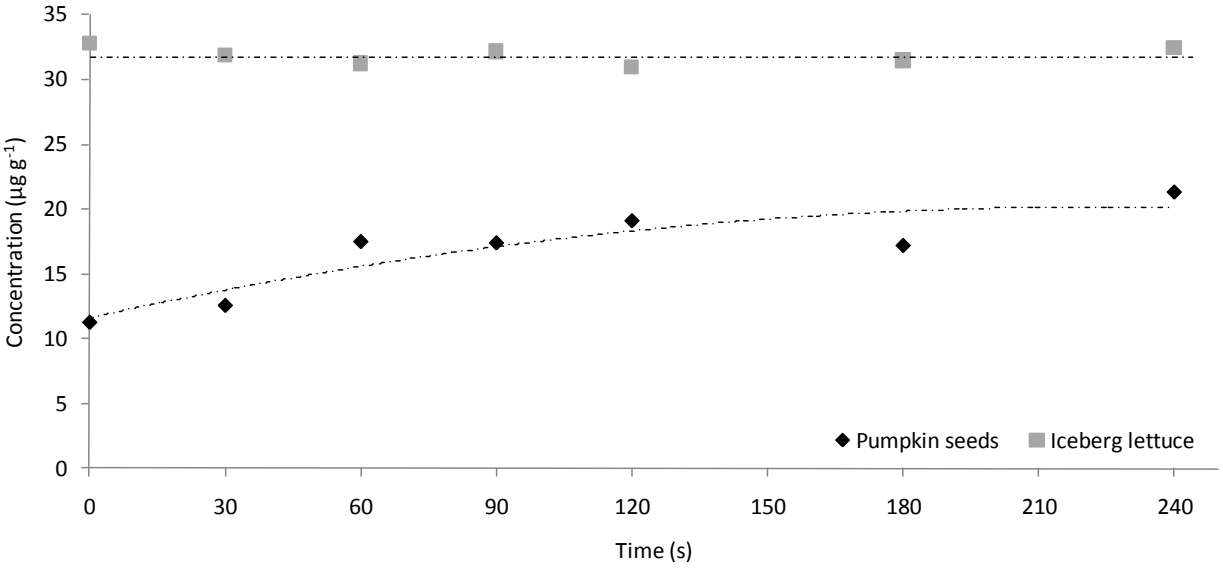


Figure 16: Determination of the optimal sonication time for Ultra-Turrax treatment of pumpkin seeds and iceberg lettuce (the average of duplicate measurements is given)

To optimize the extraction, sample-water mixtures (V/m = 100 l kg^{-1}) were subjected to ultrasound as a function of sonication time (Fig. 16). The first sample (t = 0) was not sonicated but only shaken by hand for a few seconds; others were sonicated for 30-240 s in 30 s intervals. After sonication the suspensions were centrifugated (10 min at 5000 rpm) to clear the extracts and the supernatants were then carefully removed with a polypropylene Pasteur pipette and filtered through 0.45µm syringe filters before measurement. In the case of iceberg lettuce the extraction was practically independent of sonication time, while in the case of pumpkin seeds an optimum extraction was reached in 60 - 120 s (Fig. 16). The discrepancy between both samples may be a consequence of more effectively ground iceberg lettuce than pumpkin seeds (see also section 3.1) due to the fact that pumpkin seeds could not be ground in an agate ball mill because of their high fat content (Alfawaz, 2004). It has been demonstrated by Filgueiras (2000) that particle size can have a significant effect on the extraction efficiency with an ultrasound probe. This suggests that grinding in the case of iceberg lettuce must have led to (partial) break down of cellular walls. It was decided to sonicate both samples routinely for 120 s in the remainder of the work. These short sonication times prevent the extraction temperature to rise thereby circumventing a change in the physico-chemical characteristics of the species (Santos & Capelo, 2007).

Ultrasound assisted extraction was compared to cryogenic extraction, i.e. a sample-water mixture in a 50 ml falcon tube alternatingly frozen (in liquid nitrogen, 77*K*) and thawed (in warm water, 310*K*) three times. The results are presented in Tab. 8 and demonstrate a better

extraction efficiency for ultrasound assisted extraction in the case of pumpkin seeds while for iceberg lettuce no significant difference is observed. This is additional confirmation that cellular walls of iceberg lettuce were broken down already by grinding of the sample with a ball mill.

***Table 8: Comparison of water soluble Zn by two different extraction approaches* (*V/m=100* l kg^{-1})**

Sample	Extraction approach	Concentration (µg/g)	RSD (%)
Pumpkin seeds	Ultra-Turrax	25.7	2.1
	Cryogenic	15.7	2.4
Iceberg lettuce	Ultra-Turrax	36.2	1.6
	Cryogenic	35.4	1.8

According to Szpunar et al. (1999) most of the studies regarding speciation of metals have focused on the water-soluble fraction (aqueous buffer) that contains 10-20% of the elements present in the sample. Günther & Kastenholz (2005) presented Ultra-Turrax homogenization data on Zn and some other elements for more than 20 commercially available vegetable and fruit samples. Zn in cytosol[9] ranged between 31 and 87% in most plants and was 31 % for white cabbage while values for iceberg lettuce ranged between 71 and 87%. Concerning animal foods, some studies on zinc binding components in muscle tissues of slaughter animals and mollusk tissues have also been conducted. Results for the extraction of Zn from muscles of beef, pork, lamb and chicken showed that only 30% of the Zn is water soluble (Scherz & Kirchhoff, 2006).

In this work the quantitative distribution of Zn and some other elements between the water soluble and water insoluble fractions of pumpkin seeds and iceberg lettuce was investigated. Measurement of the water soluble Zn and Mg fractions of both types of plant samples are shown in Fig. 17; they are comparable with literature data (Günther & Kastenholz, 2005) and yields are in both cases higher than in samples of animal origin (Scherz & Kirchhoff, 2006). Zn shows a significantly lower water soluble fraction in pumpkin seeds than in iceberg lettuce which may be the consequence of a much higher protein content in pumpkin seeds (40%, Alfawaz, 2004) than in iceberg lettuce (< 2 %, nutritional data). According to Brown et al. (1993) Zn is incorporated in many macromolecules, especially in proteins, where it plays also a structural role and forms strong complexes with polar groups containing O, N, S and in some cases it is bound so tightly that it can be removed only with severe chemical treatment.

[9] Cytosol or intracellular fluid is found inside cells.

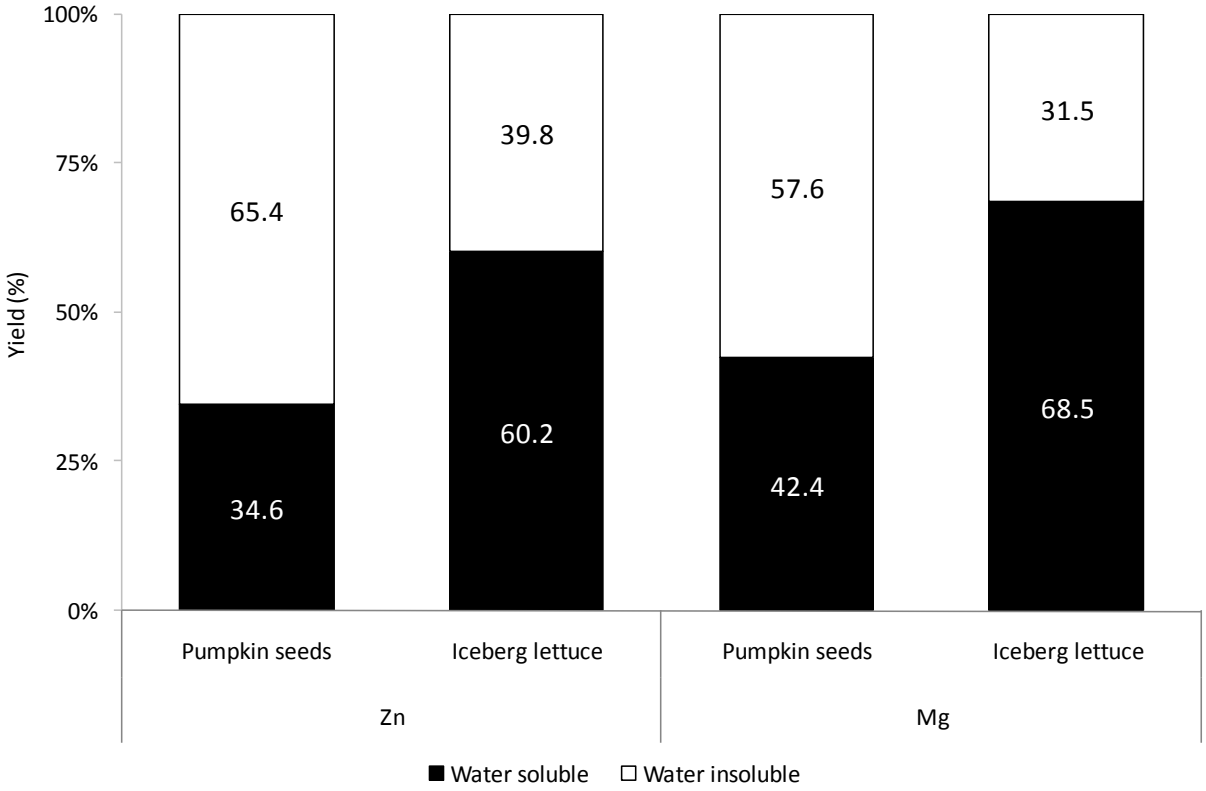


Figure 17: The water soluble fraction of Zn and Mg after extraction of pumpkin seeds and iceberg lettuce with deionized water using ultra sound assisted extraction (V/m = 100 l kg^{-1})

Results obtained for Zn are comparable with these for other elements like Mg (Fig. 17), Ca and Cu (not given); despite differences in plant chemistry it seems that the water soluble fractions of these elements do not differ significantly. The physico-chemical form of Zn in the remaining solid residue is unclear but will be further elaborated in section 4.5 on solubilization of Zn in pumpkin seeds and iceberg lettuce in a simulated human gastro-intestinal tract.

4.3.2 Extractable Zn

It is often assumed that a certain mass of sample in a certain volume of extractant suffices to extract the element fraction related to that extractant composition completely. However, this is true for readily extractable elements only; elements which show a degree of binding, i.e. elements which are not irreversibly bound in the sample tissue, show an extraction dependency with volume. This phenomenon has been extensively studied (van Elteren and Budič, 2004; van Elteren et al., 2007) under the assumption of reversible adsorption and desorption processes during extraction. With a simple linear sorption isotherm the available pool of elements in a certain extractant may be deduced by measuring the elements released as a function of the *V*/*m* ratio. By extrapolation to an infinite *V*/*m* ratio the maximum

extractability of the elements in a certain extractant may be found (Fig. 18). The following dependencies are valid:

$$ma_0 = c_1 V + ma_1 \qquad (1)$$

with a_0, c_1 and a_1 being the maximum extractability (μg g^{-1}), equilibrium element concentration in extract (μg ml^{-1}) and the remaining equilibrium element concentration in sample (μg ml^{-1}), respectively. Substitution of the linear sorption isotherm $K=a_1/c_1$ into Eq. (1) we get a dependence of c_1 with V/m:

$$c_1 = a_0(K + V/m) \qquad (2)$$

Eq. (2) may be rewritten and linearized to:

$$1/c_1 = (V/m)/a_0 + K/a_0 \qquad (3)$$

From Eq. (3) a_0 and K can be found from the slope and intercept, respectively. The extractability Y (%) is a function of the V/m ratio as may be seen from the following equations:

$$Y = 100(V/m * (V/m + K)) \qquad (4)$$

Extraction of Zn in water clearly follows a linear sorption isotherm according to Eq. 3 for both sample types (Fig. 19). From these linear graphs we may deduce the maximum extractable Zn concentrations (a_0) and the equilibrium constants (K): a) pumpkin seeds, a_0 = 31.6 μg g^{-1}, K = 20.9 l kg^{-1}; iceberg lettuce, a_0 = 16.1 μg g^{-1}, K = 4.9 l kg^{-1}. Since the K for iceberg lettuce is lower than for pumpkin seeds easier extraction is guaranteed; a K value of 0 implies readily extractable elements. This is seen from the extraction yields (Y) in Fig. 18, which are 85.5% and 58.1% for iceberg lettuce and pumpkin seeds, respectively, at V/m = 25 l kg^{-1}, and evidences that for pumpkin seeds interactions between target Zn and sample matrix are stronger than for iceberg lettuce. The equilibrium constant is a function of different extraction parameters such as extractant composition, temperature, shaking time, shaking frequency, shaking amplitude, etc. (van Elteren et al., 2007). For ultrasound assisted

extraction this translates into sonication time, sonication frequency and sonication amplitude which were discussed before (see section 4.3.1).

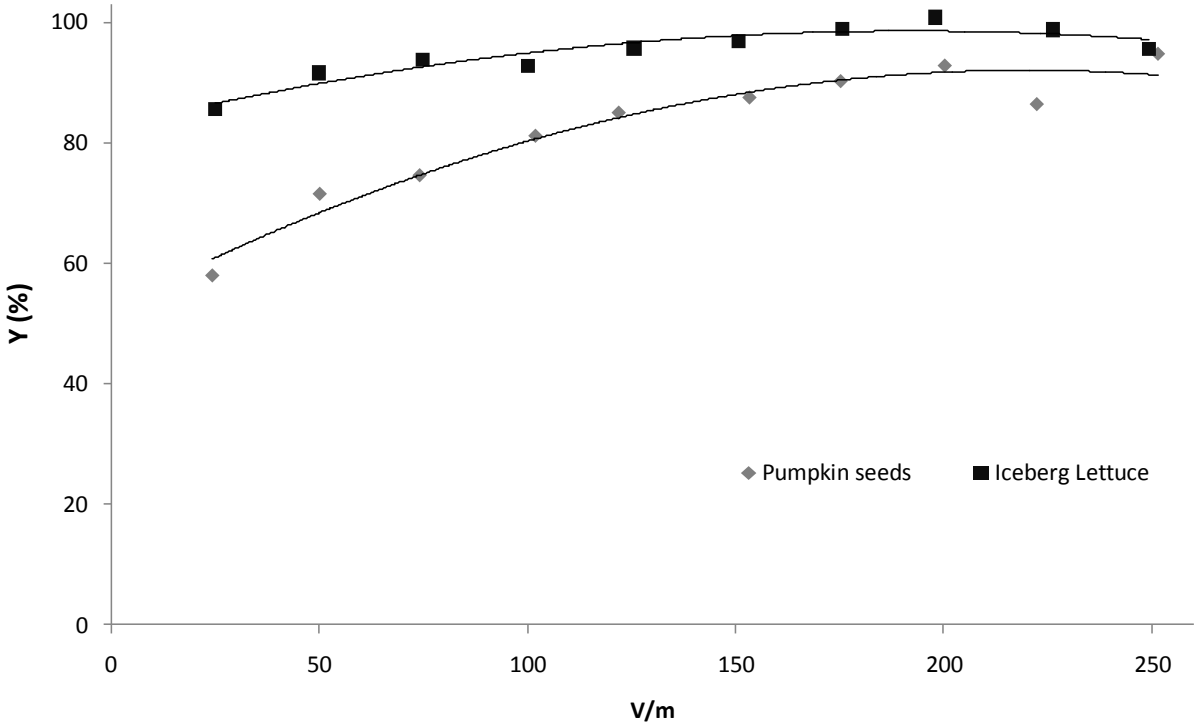


Figure 18: Extraction yield (Y) of water soluble Zn as a function of V/m ratio in pumpkin seeds and iceberg lettuce

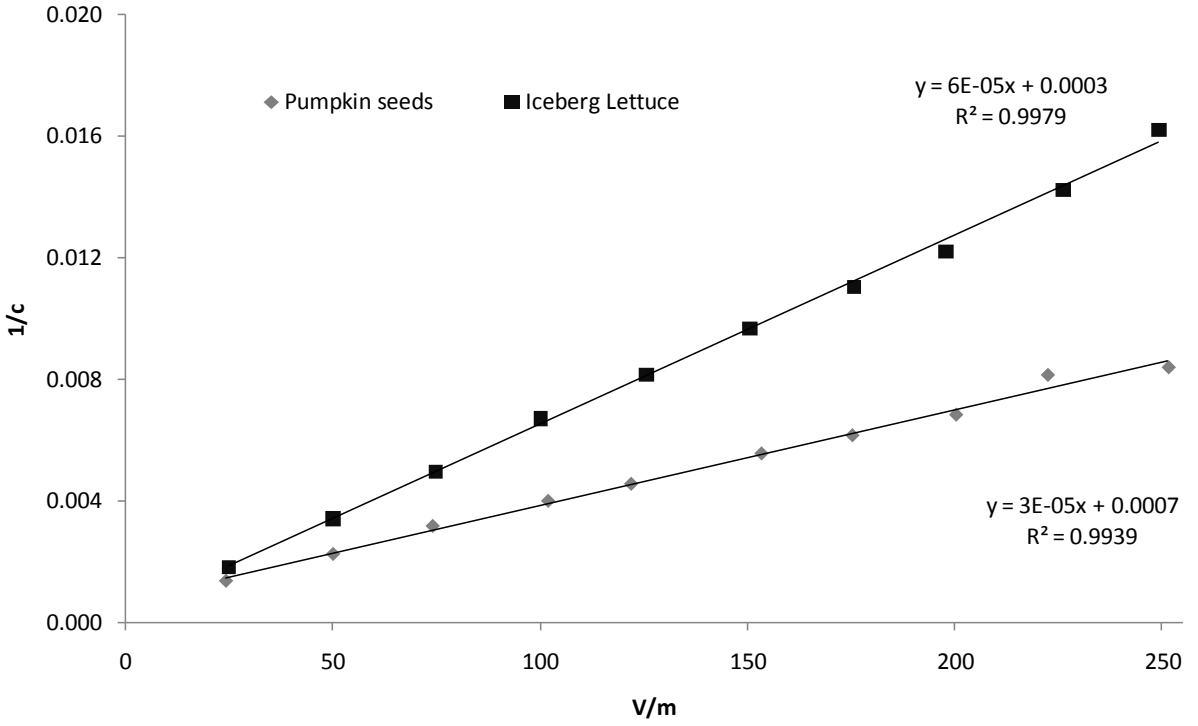


Figure 19: Linear sorption isotherms for water soluble Zn from pumpkin seeds and iceberg lettuce

4.3.3 Size fractionation by ultrafiltration

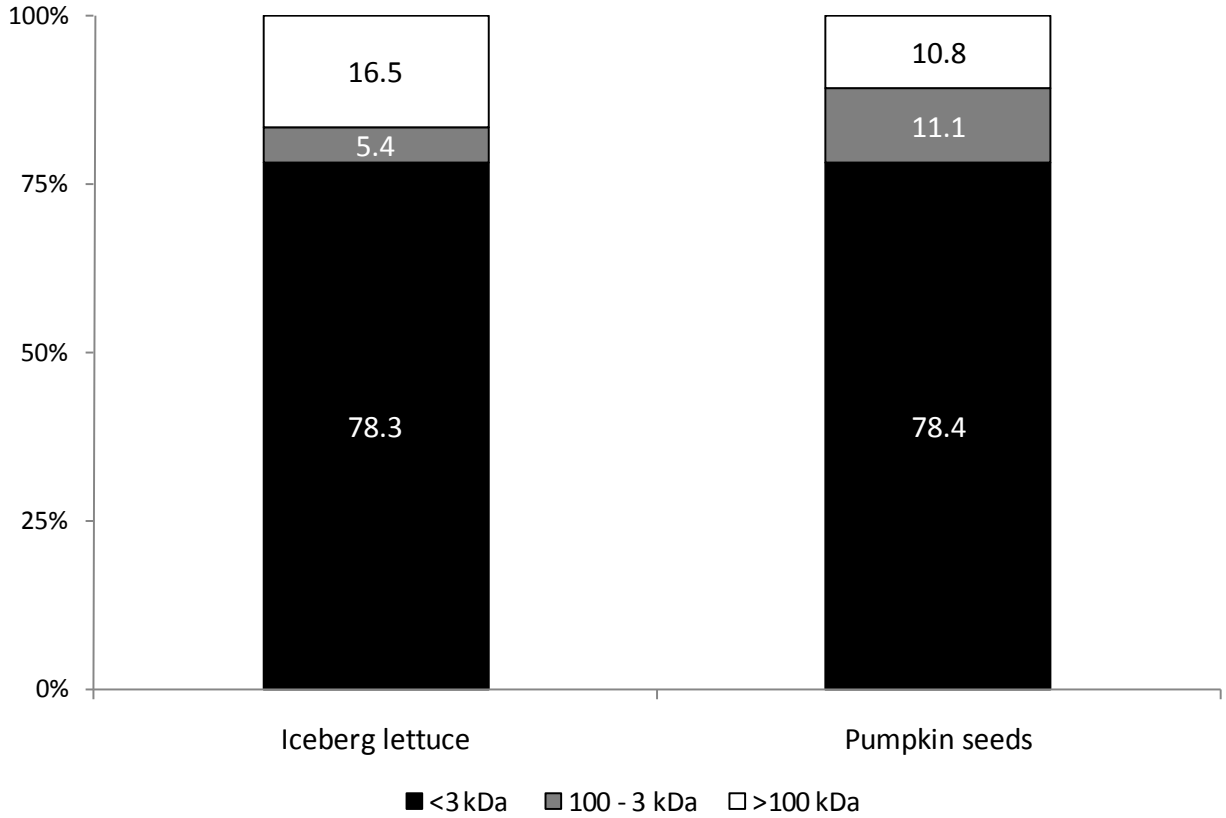


Figure 20: Size fractionation of water soluble compounds using ultrafiltation

Size fractionation results presented in Fig. 20 are comparable to those obtained by Günther and Waldner (1992) who show that Zn in plant cytosol is predominantly bound to molecules with a MW <5,000Da whereas a fraction of 1-34% is bound to molecules with a MW >30,000 Da. This also agrees with findings by Walker and Welch (1987) which show a low molecular Zn-containing fraction in lettuce leaf using a combination of different separation techniques; the major Zn-binding fraction had a MW of 1250Da and represented 73% of the total soluble Zn. This is similar to our data with 78% of the total soluble Zn present in the MW fraction <3,000 Da. For such low molecular weights the question arises if there is any "free" or ionic Zn is present. Brown et al. (1993) report that such Zn ranges from 5.8% in tomatoes to 6.5% in alfalfa which suggests that in our samples "free" or ionic Zn might be similar. Although the similarity in size fractionation results for our two samples looks surprising, from a botanical point-of-view this is not so unexpected as biological functions for different plants are comparable. We should be aware that separation cut-offs in ultrafiltration are based on globular molecules which may result in exclusion of linear molecules depending on the direction they cross the membrane (Michalke, 2004). For this reason the later applied size

exclusion chromatography (section 4.3.4) is a much better technique with a size fractionation which is not discrete but continuous.

4.3.4 Size fractionation by Size Exclusion Chromatography

As already mentioned before (see section 1.5.2), an improvement of ultrafiltration in terms of resolution can be obtained by SEC, at the same time tolerating raw sample extracts (Szpunar & Lobinski, 2003). SEC in combination with ICP-MS, in either off- or on-line measurement mode, has already been presented with its advantages and disadvantages (see section 1.5.4). For the separation two Superdex columns (Superdex 75 [range, 3-70kDa] and Superdex peptide [range, 0.1-20 kDa]) were chosen because of their tolerance to a wide pH range (1-14), high resolution ability and inertness towards binding of metals. The stationary phase material - composite of cross-linked agarose and dextran - is much less prone to metal losses than silica-based columns, especially in the presence of low ionic strength eluents (Szpunar & Lobinski, 2003).

4.3.4.1 Off-line measurements

SEC separation with subsequent off-line measurement was performed as a preliminary step to optimize the separation conditions. As water mostly prevents structural changes like denaturation of proteins and serious destruction of protein-metal complexes, an aqueous buffer was a logical choice, the more since water was already used as an extractant. Various aqueous buffers (0.01M Tris + 0.1% NaN_3; 0.01M Tris + 0.1% NaN_3 + 0.1% DTT; 0.03M Tris + 0.1% DTT;) were tested for their suitability as a mobile phase. DTT is added due to its antioxidative properties while NaN_3 increases ionic strength and also acts as an antioxidant. Regarding Zn binding components from lettuce and pumpkin seeds no significant resolution differences were observed using different buffer eluents. This is a known phenomenon in SEC where the mobile phase is mostly selected on its ability to solubilize the analyte of interest and not such much for its role in the separation process. However, it may have significant impact on the ICP-MS selectivity/sensitivity as will be discussed later. Addition of sodium azide (NaN_3) to the mobile phase prevents possible oxidation of proteins, bacterial growth in the column, decreases retention on column, inhibits certain enzymes and does not interact with proteins or change their chromatographic behaviour but unfortunately decreases the ICP-MS sensitivity (24.8% in our case) and limits the use of detection by UV/VIS as the mobile

phase is non-transparant in the wavelength range 200-250 nm. The reduced sensitivity is counteracted by preparation of Zn standards in the mobile phase used for separation.

Injection volumes need to be carefully established as there is a delicate balance between resolution (small volume for optimal peak separation) and sensitivity (large volume for optimal sensitivity). In the case of complex samples injection volumes less than 0.5% of the column bed volume do not improve resolution (GE Healthcare, 2009). To this end injection volumes of 100-200 µl (corresponding to 0.4-0.8% of the column bed volume) were used for separation on a Superdex 75 column (1 ml min^{-1}; off-line detection) or a Superdex peptide column (0.5 ml min^{-1}; on-line detection). The flow rate of 0.5 ml min^{-1} in on-line detection mode was dictated by the detector to prevent overflow of the ICP-MS nebulizer. To prevent peak tailing a detergent (0.05% TWEEN) was added to the samples before ultrasound-assisted extraction in both the setups with on- and off-line detection. Detergents are also useful as solubilizing agents for proteins with low aqueous solubility. According to the column manufacturer they should not affect the separation.

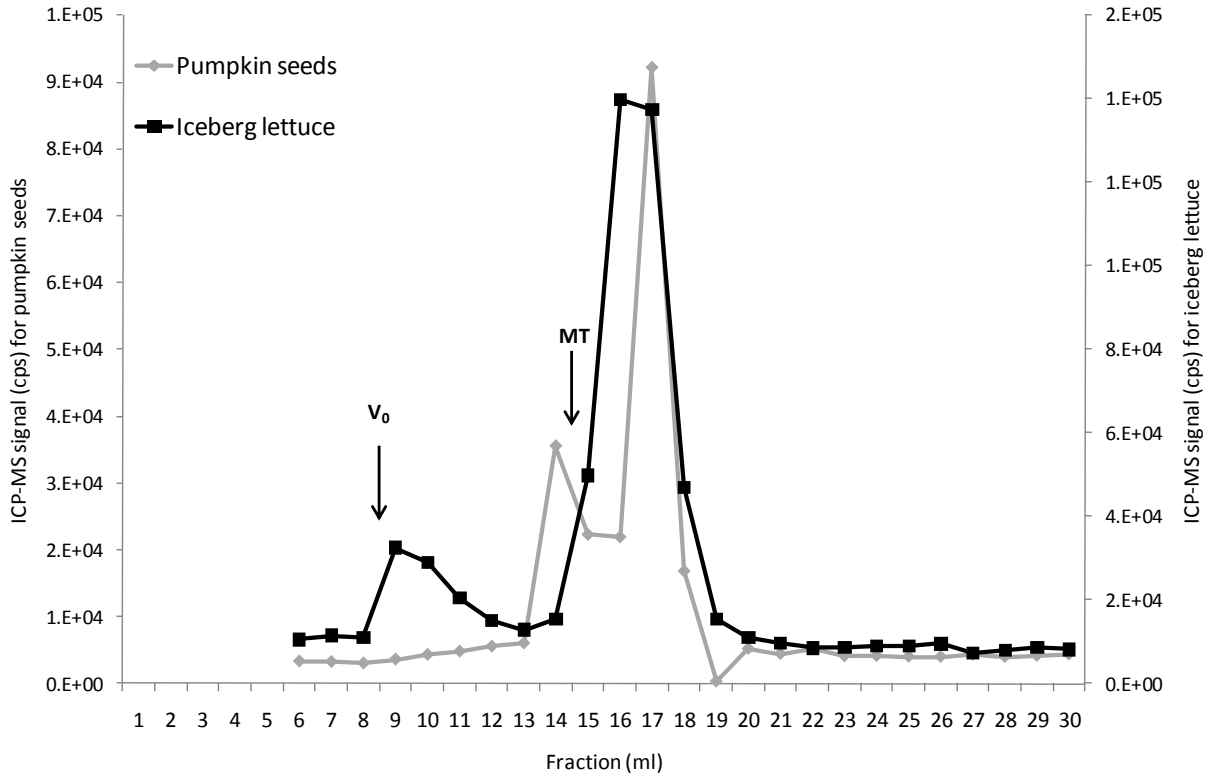


Figure 21: Zn distribution profiles of pumpkin seed and iceberg lettuce extracts (200 ul) after separation on a Superdex 75 column eluted with 0.01M Tris-HCl buffer (0.1% NaN_3, pH = 7.4) at a flow rate of 1 ml min^{-1}. The arrows represent void volume (V_0; 70kDa) and elution volume of metallothionine (MT; 10kDa)

In Fig. 21 Zn elution profiles for iceberg lettuce and pumpkin seed extracts on a Superdex 75 column are given. Comparison shows differences in the high molecular range (>70 kDa; void volume peak) and in the peak eluted just before the MT standard (10kDa), the first related to iceberg lettuce and the second related to pumpkin seeds. Similarity was found in a peak eluted after the MT standard. In both cases the majority of Zn is present in the low molecular range (<10 kDa), which is comparable with results obtained by ultrafiltration (see section 4.3.3). In contrast the UV chromatograms recorded (not given) indicate that in both samples more high molecular (>70 kDa,) than low molecular (<10kDa) compounds are present. The Zn elution profile for iceberg lettuce extract (Fig. 21) is comparable with Zn profiles of other vegetables (spinach, Jerusalem artichoke, leek, celeriac, chard, kohlrabi) as reported by Günther & Waldner (1992), with the high molecular fraction in all cases significantly lower than the low molecular fraction using comparable experimental conditions. Since most of the Zn is present in the low molecular fraction, which can probably not be further resolved due to the rather wide separation range of the Superdex 75 column and the rather crude fractionation, it was decided to apply a column with a lower separation range and on-line Zn detection. A Superdex peptide column with an optimal separation range between 0.1 and 7kDa (exclusion limit: 20kDa), interfaced with the ICP-MS, was used for this purpose.

4.3.4.2 On-line measurements

Because of the capability of ICP-MS to detect metals at very low levels in liquid chromatographic effluents the coupling of HPLC to ICP-MS has been already widely applied for elemental speciation in foodstuffs of animal as well as of plant origin (Szpunar et. al. 1999). According to Szpunar et al. (2003) it should be noted that coelution of a metal and a particular biomacromolecule is only an indication that they belong together. The ultimate proof can be obtained by applying affinity chromatography for the particular protein. Because of the complex nature of metal-biomolecule systems a combination of SEC with other separation techniques like ion exchange and reversed phase chromatography is needed to gain more exact information about the compound separated on SEC. However, one needs to be aware that SEC is a relatively mild separation technique whereas in more “aggressive” separation techniques based on ionic or van der Waals forces the complex species may disintegrate on the column.

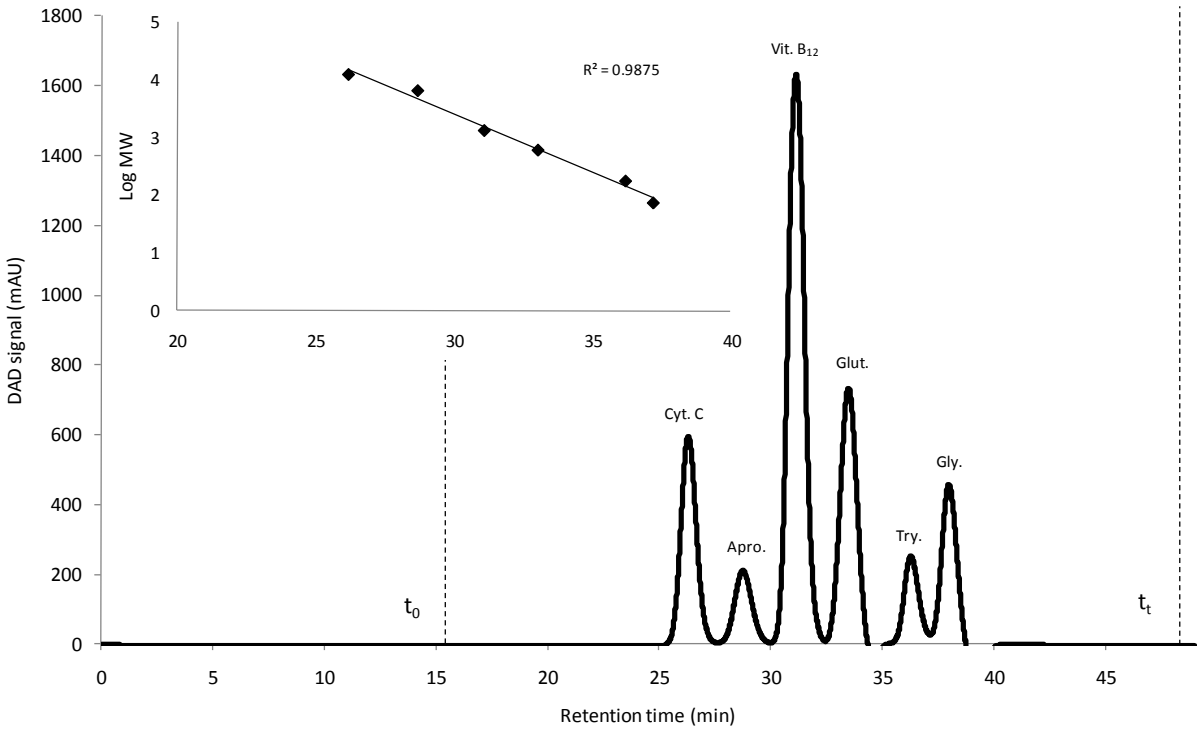


Figure 22: Calibration of the Superdex peptide column with a standard mixture (100 µl) of Cytochrome C (MW=12,384), Aprotinin (MW=6,512), Vitamin B_{12} (MW=1355.4), Gluthation-oxidized form (MW=612), Trycine (MW=179.2) and Glycine (MW=75.07) prepared and eluted in 0.03 M Tris-HCl (pH = 7.4) at a flow rate of 0.5 ml min^{-1}

When presenting chromatographic data, elution volume as well as retention time can be used (Mayer, 2004); in the remainder of this work retention times were used to compare DAD and ICP-MS signals. The dead time (t_0) was determined with bluedextran (MW=2,000kDa) and was found to be 15.4 min. The total analysis time (t_t) was calculated to be 48 min from the column bed volume (24 ml) and the flow rate (0.5 ml min^{-1}). The calibration chromatogram (Fig. 22) closely resembles the calibration chromatogram presented by the column manufacturer. Calibration was performed at the same conditions (flow rate, composition of mobile phase and injection volume) as used in the analysis of samples. To keep the ICP-MS sensitivity as high as possible and to be able to record the DAD signal at 210 nm, a mobile phase with 0.03M TRIS, but without NaN_3, was used. Since the ICP-MS signal is recorded later than the DAD signal the delay was experimentally measured by injection of vitamin B_{12} (having Co as its central element) and comparing the time-shift in the peaks obtained at 210 nm (DAD) and ^{59}Co (ICP-MS). It was found that in practice the delay was insignificant (< 3s). Although for the analysis of samples the DAD signal was scanned at three different wavelengths simultaneously (210, 230 and 250 nm) we will only show the 210 nm traces in Figs. 23-25 since they are the most sensitive towards peptide bonds.

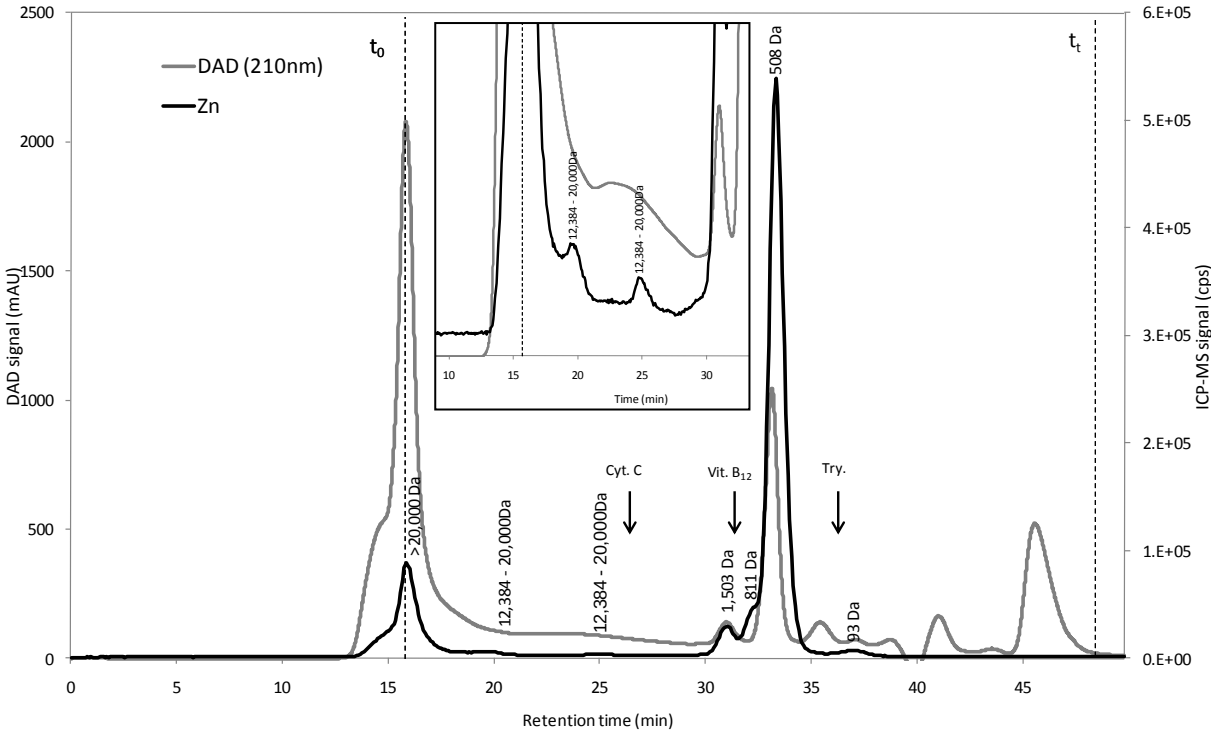


Figure 23: Fractionation profile of Zn (cps) and DAD signal (mAU) in iceberg lettuce extract after separation on a Superdex peptide column eluted in an aqueous buffer (0.03M Tris-HCl, pH = 7.4). The insert shows a part of the chromatogram using a different scaling to reveal the otherwise hidden peaks. The arrows represent Cytochrome C (Cyt C; 12,384Da), Vitamin B_{12} (Vit. B_{12}; 1355.4Da) and Trycine (Try.; 179.2)

The Zn elution profile for iceberg lettuce extract (Fig. 23) with continuous on-line measurement of Zn is comparable to the Zn elution profile with discrete off-line measurement (Fig. 21), although with much higher resolution. Peaks between Cytochrome C (12,384Da) and the column exclusion limit (20,000Da) (Fig. 23 and 25) fall out of the calibration range (also out of optimal separation range of the column, viz. 100-7,000Da) and are therefore not reported. The Zn-related fraction is predominantly present as low-molecular material, in contrast to findings by DAD which seem to indicate that more material is present in the high molecular fraction but most likely without the capability to complex Zn^{2+}. Here are more than 1200 proteins which are predicted to contain, bind, or transport Zn^{2+} (Hänsch & Mendel, 2009). We can only speculate to which ligand(s) Zn could be bound, e.g. Piero et al. (2002) have purified an enzyme protease, named lettucine, from lettuce leaves, which is made up of a single subunit with an apparent molecular weight of 40,000Da. One of the possible compounds could be also be an enzyme Cu–Zn superoxide dismutase with a molecular weight of 32,500Da, which is responsible for antioxidant defense, and where Zn is bound to histidine side chains (Hänsch & Mendel, 2009). In addition to proteins and peptides, a large number of low molecular weight compounds play an important role in the handling of metals in plants.

Among them some organic acids like citrate, amino acids like histidine and phosphate derivatives are of particular interest (Briat & Lebrun, 1999).

Phytochelatins (PCs) are a group of oligopeptides composed of three amino acids (cysteine, glutamic acid and glycine) and synthesized at the cellular level in cytosol. Their role is to detoxify metals during the formation of metal-phytochelatin complex in which the metal is bound to the thiol group of cysteine (Szpunar & Lobinski, 2003), where they can be induced already by exposition to trace levels of essential metals (Cobbett, 2000). The synthesis of the PCs can be stimulated by a range of heavy metal ions including Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+} and Zn^{2+} (Grill et al., 1985). Depending on the plant species and tissue, phytochelatins may assume different lengths, but in general PC_2 (MW=540), PC_3 (MW=773) and PC_4 (MW=1005) are the most abundant ones (Meyer & Rausch, 2008). Comparison of the DAD chromatograms of iceberg lettuce and a group of synthetic PCs, which were analyzed separately at the same experimental conditions, revealed overlap with PC_2 (Fig. 24). However, it should be emphasized that the molecular weight of the iceberg lettuce peak (1,503Da) obtained from the calibration graph (Fig. 22) does not match the certified molecular weight of phytochelatin 2 (540Da). This anomaly may be related to the fact that PCs used in standard solutions are purely synthetic, whereas in the real sample (iceberg lettuce) interactions with numerous other compounds present can occur. Also differences in PC concentration between standard solution and real sample can contribute. Overall a low "match" was seen between Zn and PCs which is not surprising as the samples did not contain any heavy metals on toxic levels which might significantly induce synthesis of PCs. In kohlrabi and Chinese cabbage neither phytochelatins nor other Zn-binding ligands (as described in the literature at that time) match the low molecular Zn species found in the edible parts of these two plants (Waldner & Günther, 1996). Also the peptides isolated by McKenna & Chaney (1995) regarding Cd-Zn complexes from the low molecular or high molecular Zn leaf extracts had an amino acid composition dissimilar to phytochelatins although some report "background" phytochelatin concentrations in plant tissues (Maier et al., 2003).

As citrate is prevalent in plants leaves (Rauser, 1999) Zn-citrate might be a possible candidate for the low molecular weight Zn species although no evidence for the presence of Zn-citrate in lettuce could be found in the literature. However, citrate has a high capacity to chelate metal ions, which has been well documented in the case of Fe, Ni and Cd (Briat & Lebrun,

1999). Since citrate is not a protein, elution on a Superdex peptide column would be necessary to confirm this assumption.

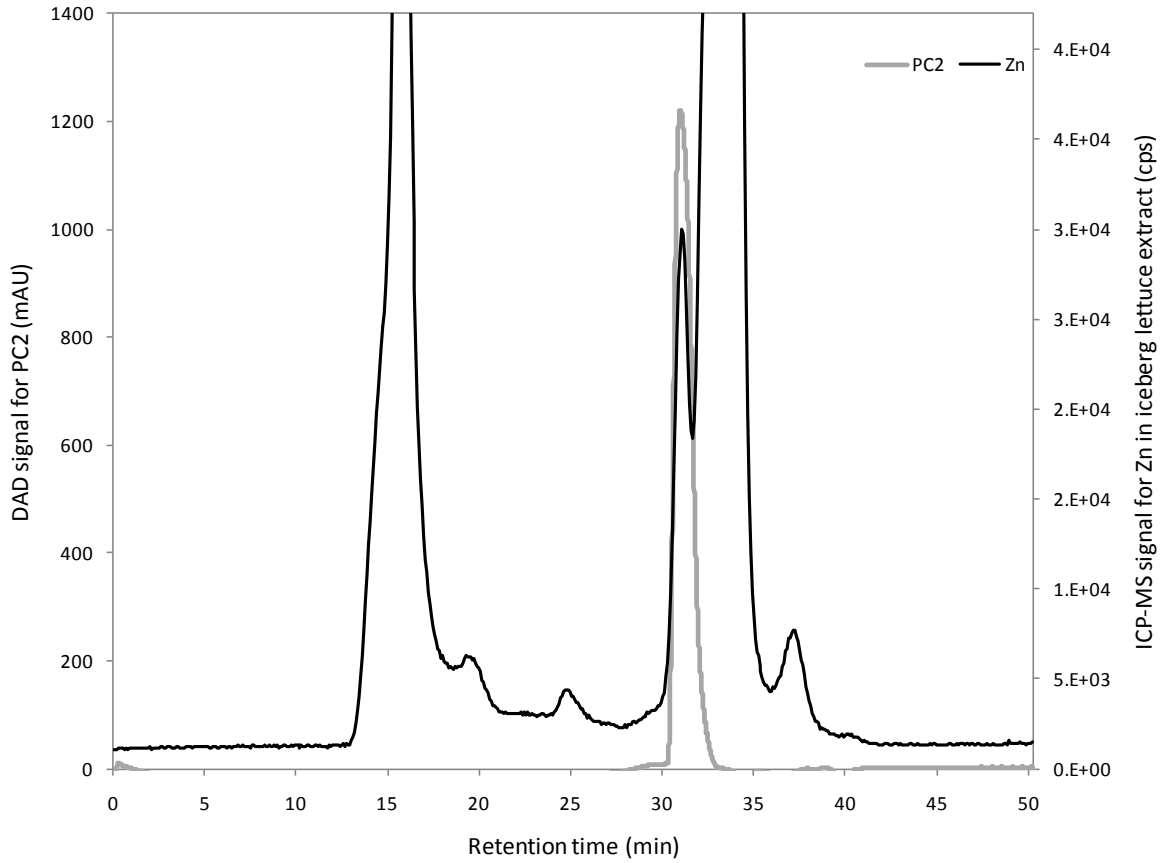


Figure 24:Comparison of phytochelatin 2 (PC_2; DAD signal at 210 nm) and Zn in an iceberg lettuce extract eluted with 0.03M Tris-HCl buffer (pH = 7.4) on a Superdex peptide column

Since pumpkin seeds contain 50–70% of lipids a suitable sample cleanup step is essential prior to analysis as lipids, if not removed, can otherwise degrade the separation. For this purpose a procedure with a wet-chemical extraction step as already described (see section 3.4.2) was applied although a guard column before the analytical column could take care of this problem as well.

As expected, separation with on-line detection yields better resolution than separation with off-line detection as shown from the Zn elution profiles in Figs. 25 and 21, respectively. In spite of the fact that columns with different properties were used the Zn species in the peak close to the retention time of Cytochrome C (Fig. 25) seem comparable to the Zn species in the fraction around 14 ml with a MW of ca. 10,000Da (Fig. 21), while the fractions around 17-18ml having a MW<3000Da (Fig. 21) may be compared to the peaks indicated by 1,673 and 649Da (Fig. 25). The suboptimal resolution of Zn species between the column exclusion

limit and the peak close to Cytochrome C (Fig. 25) seems also visible in the separation with off-line analysis (Fig. 21) although less clear. Zn species in the peak close to Cytochrome C (Fig. 25) may be comparable to the Zn species with molecular weights of 11,300, 11,900 and 12,300Da found by Naozuka et al. (2010) in the water-soluble fraction of brazil nuts. They may be identified as isoforms of the water-soluble sulphur rich 2S-albumin (Dernovics et al., 2007) which are known as storage proteins in pumpkin seeds. Similar results were obtained by Wuilloud et al. (2004b) for NaOH and HCl extracts of different nut samples (almond, black walnut, Brazil nut, cashew, peanut, pecan, pine nut, sunflower and white walnut) showing chromatographic Zn elution profiles in two to three MW fractions, depending on the type of nut studied. For sunflower and Brazil nut samples Zn was distributed among a MW fraction of 12,000–13,000Da and a lower MW fraction of 1,300Da. For the rest of the nut samples an additional fraction was found with a MW of 4,000–4,500 Da. Although the UV-chromatogram (Fig. 25) shows that a significant amount and density of low-molecular organic material (<500Da) is present, this materials does not seem to complex Zn.

Detailed comparison of Zn elution profiles for iceberg lettuce (Fig. 23) and pumpkin seed extracts (Fig. 25) was performed using the integration tool (trapezoidal rule) in Origin 7.5. The Zn size distributions retrieved (in percentage of total) for iceberg lettuce and pumpkin seeds are summarized in Tab. 9. Evaluation of the data shows us that iceberg lettuce has a high (ca. 70%) low-MW Zn-fraction (ca. 500Da) and pumpkin seeds a high (ca. 60%) intermediate Zn-fraction (12,384 – 20,000Da). It seems that some Zn fractions may be comparable, potentially having the same complexing ligands, as shown by similar molecular weights: 508 and 1503Da for iceberg lettuce and 649 and 1673Da for pumpkin seeds. Even though the match is not perfect, maybe due to highly varying amounts of organic material (see UV-chromatograms in Figs. 23 and 25) which may slightly affect the chromatographic behaviour and thus the absolute retention time, it is close enough to suggest that these samples have similar ligands responsible for Zn-binding.

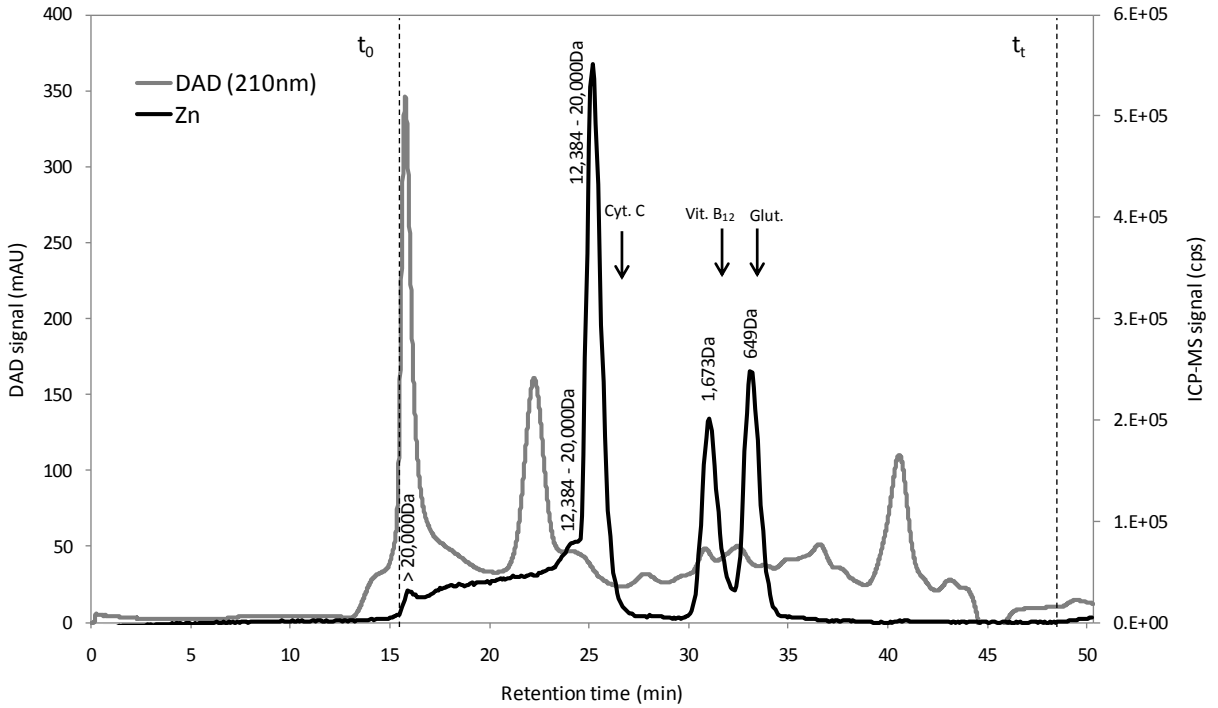


Figure 25: Fractionation profile of Zn (cps) and DAD signal (mAU) in pumpkin seed extract after separation on a Superdex peptide column eluted with aqueous buffer (0.03M Tris-HCl, pH = 7.4). The arrows represent Cytochrome C (Cyt C; 12,384Da), Vitamin B_{12} (Vit. B_{12}; 1355.4Da) and Glutathione in oxidized form (Glut; 612Da)

The Zn peak at 649 Da (Fig, 25) has a molecular weight very close to Zn-glutathione in oxidized form (612 Da) indicating that glutathione may play a role as a Zn-binding ligand in pumpkin seeds. Glutathione is the most abundant and ubiquitous low-molecular weight thiol in fungal, plant and animal tissues, where its relative stability and high solubility in water makes it a particularly adequate electron acceptor or donor in physiological reactions (Potters et al., 2002). According to Meyer & Rausch (2008) glutathione is present in almost all eukaryotic and many prokaryotic cells in two forms (reduced form: GSH; oxidized form: GS-SG) where two GSH molecules form a single GS-SG molecule during oxidation. Glutathione plays its crucial role in cellular detoxification of heavy metals most likely in two ways: as precursor for PC biosynthesis and as a cellular redox sensor due to the decrease of the reduced form of glutathione in response to heavy metal exposure (Meyer & Rausch, 2008). Glutathione is present in low millimolar concentrations in plant cells, which could explain the low UV response in the chromatogram around 33 min (Fig. 25). Under normal conditions glutathione is predominantly present in its reduced form (GSH), with only a small proportion present in its fully oxidized state (GS-SG). The only cellular compartment for which high concentrations of GS-SG have been shown is the endoplasmic reticulum (Meyer & Rausch, 2008).

Table 9: Molecular size distribution of Zn (% of total area under the chromatogram) in iceberg lettuce and pumpkin seeds after SEC-ICP-MS

Sample	Peak	MW (Da)	%
Iceberg lettuce	1	>20,000	18.1
	2	12,384 – 20,000*	2.1
	3	12,384 – 20,000*	1.6
	4	1503	4.5
	5	811	4.4
	6	508	66.8
	7	93	2.6
Pumpkin seeds	1	>20,000	7.5
	2	12,384 – 20,000*	31.0
	3	12,384 – 20,000*	29.8
	4	1,673	14.1
	5	649	16.7

Peaks out of the calibration range (and also out of the optimal column separation range); precise molecular weights cannot be reported.

The data for iceberg lettuce presented in Tab. 9 show that the sum of the fractions with molecular weights of 1503Da, 811Da and 508Da represents 75.7% of the total soluble Zn, which is comparable with the major Zn-binding fraction of 1250 Da, representing 73% of the total soluble Zn, found by Walker and Welch (1987) in lettuce leaf (it should be noted that the resolution in our case was better).

According to Michalke (2004) organometallic complexes are usually large, high molecular weight compounds, mostly proteins, thereby agreeing with Marmiroli et al. (2005) who state that metal ions are bound to proteins but disagreeing on their molecular size range (low molecular range); furthermore, they indicate that most metal ions present in the shoots of plants are bound to low molecular weight ligands. Metals are sometimes bound non-specifically, and often loosely, which may cause problems in the separation process leading to disintegration of the complex on the column. Even although the HMW Zn fraction in Fig. 25 is characterized by an undefined hump, suggesting that instable Zn-proteins have disintegrated during separation, little evidence is available to prove that suggestion. Upon disintegration, Zn^{2+} ions should have formed giving a Zn response in the low MW range in the chromatogram. Since this response is not observed we may assume that Zn complexes stayed intact on the column which be due to their high affinity to sulphur, nitrogen and oxygen containing ligands (Palmer & Guerinot, 2009). Recent estimates done by Andreini et al. (2008) suggest that close to 10% of all eukaryotic proteins are Zn dependent and the Zn homeostasis system in eukaryotic cells very tightly controls the availability of metal ions in the cell and directs the specific delivery of metal ions to their respective metalloproteins (Palmer & Guerinot, 2009). Key homeostatic processes include, tightly controlled transport of

metal ions across membranes, chelation by low-molecular weight molecules and delivery to target proteins, via metallochaperones (Palmer & Guerinot, 2009). Besides proteins, plants often contain significant concentrations of polysacharides which can bind cations electrostatically (Szpunar & Lobinski, 2003). In the literature not much information about metal coordination to carbohydrates could be found although structurally complex pectic polysaccharide, rahmnogalacturonan-II, found in aqueous extracts, can complex specific divalent cations (Ishii & Matsunaga, 1996; Pellerin et al., 1996).

4.4 Zinc uptake

A limitation of many studies on chemical analysis of trace elements in foods is that they generally do not take into consideration bioaccessibility and/or bioavailability (see also section 1.5.5). The gastrointestinal bioaccessibility, i.e. the metal fraction released from foods as metal ions, metal complexes, etc. in the digestion tract, is presented and discussed here for Zn in iceberg lettuce and pumpkin seeds using the PBET approach (see section 3.5). The nutritional usefulness depends on the absorption efficiency of the released Zn species from the intestine into the blood stream.

Before any reliable PBET study can be undertaken the analytics need to be considered in detail as the harsh matrices (digestion fluids and plant matrices) upon measurement of Zn (and some other elements) by ICP-MS may lead to interferences. Interferences by the digestion fluids is confirmed in Fig. 26 which shows that a Zn calibration graph in water has a higher sensitivity (or slope) than a Zn calibration graph in a solution simulating the combined stomach-intestine phase. Since such a discrepancy is unacceptable in quantitative analysis it is essential to prepare the Zn standards in the digestion fluids. However, the plant matrices themselves may also contribute to potential Zn interferences and to investigate these interferences calibration graphs of Zn in PBET extracts of iceberg lettuce and pumpkin seeds, after a simulated stomach-intestine digestion, were compared to these in digestion fluid alone. From Fig. 27 we can see that all calibration graphs are parallel, suggesting that interferences from the plant matrices are absent. For the remainder of the work the so-called method of standard additions (see also section 4.1) was used routinely to circumvent potential interferences.

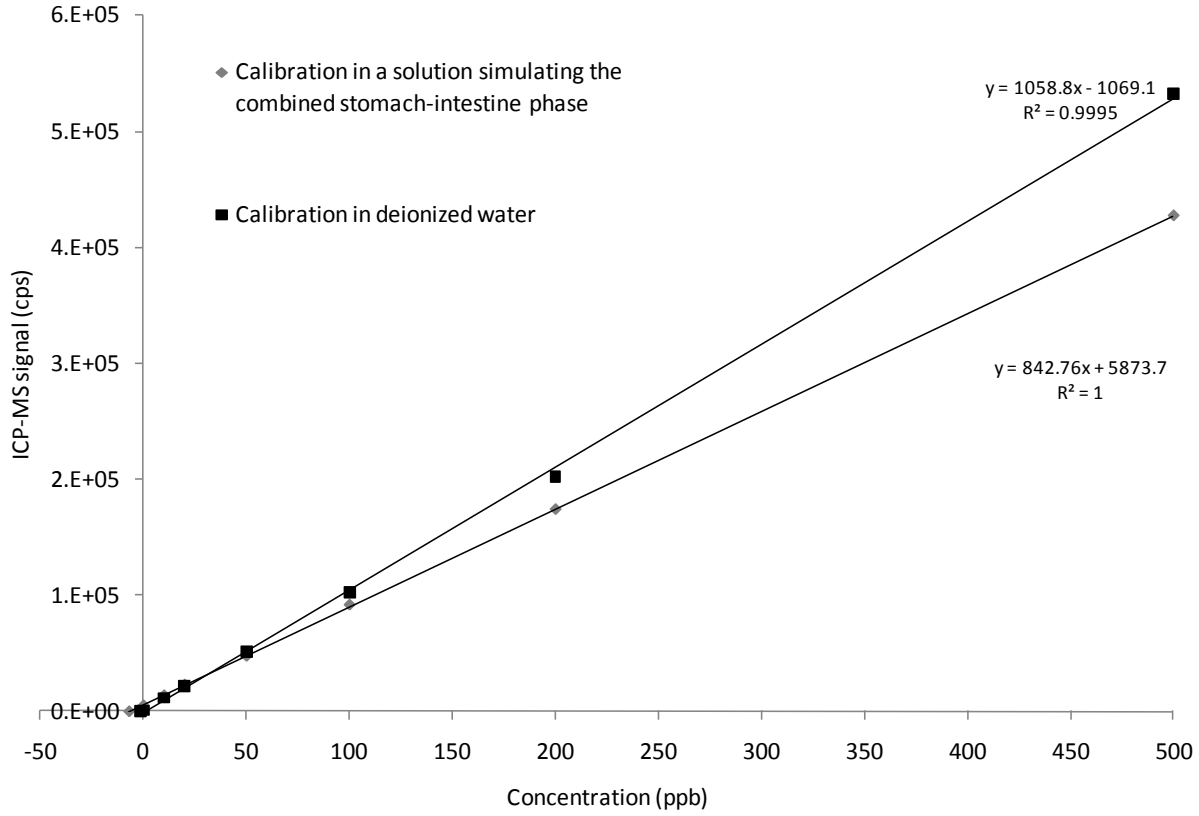


Figure 26: Comparison of Zn calibration graphs in two media (water and digestion fluid)

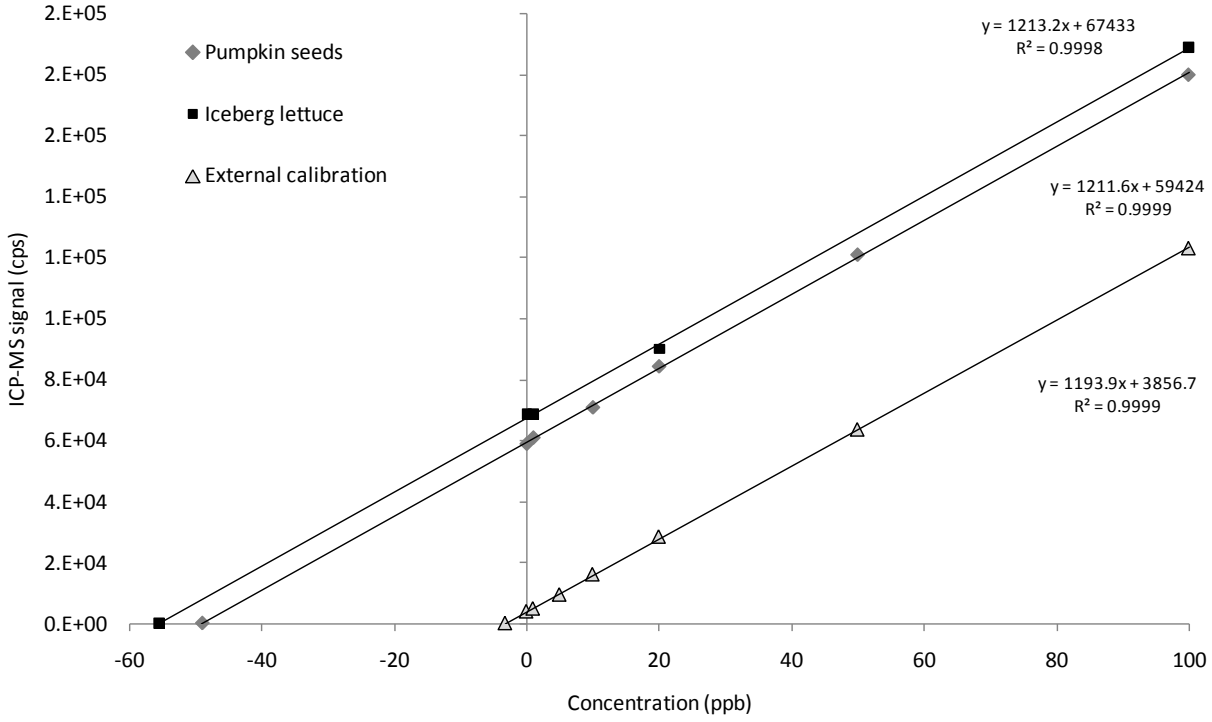


Figure 27: Comparison of Zn calibration graphs in extracts of pumpkin seeds and iceberg lettuce after a simulated stomach-intestine digestion and digestion fluid alone

Results for the release of Zn from iceberg lettuce and pumpkin seeds in a simulated gastrointestinal tract are presented in Tab. 10. It is obvious that the enzymatic-assisted

extraction procedure releases the majority of Zn in the stomach phase (80-90% of total Zn). Since the extraction of Zn species in water using ultrasound-assisted extraction released only 34.6% and 68.5% of the total Zn for pumpkin seeds and iceberg lettuce respectively (see section 4.3.1) it is clear that Zn is bound to proteins which are decomposed when pepsin[10] is present. Interesting is the situation observed in the case of pumpkin seeds where the amount of Zn after the intestinal phase is lower than after the stomach phase. This may be due to the presence of phytate which is known to bind minerals and serves as a storage form of phosphorous.

Table 10: Zn concentration in digests after a simulated gastrointestinal digestion of iceberg lettuce and pumpkin seeds

Sample	After stomach phase			After stomach and small intestinal phase		
	Conc. ($\mu g\ g^{-1}$)	RSD (%)	% of total Zn	Conc. ($\mu g\ g^{-1}$)	RSD (%)	% of total Zn
Iceberg lettuce	53.4	1.8	86.1	55.3	1.6	89.1
Pumpkin seeds	72.2	2.8	79.2	48.2	1.7	52.9

Because of its capacity to bind minerals, phytate has been considered to be an antinutrient. In several studies, summarized by House (1999) and Kumar et al. (2010), it was proposed that phytate reduces the Zn bioavailability to people because of the formation of insoluble salts or even co-precipitation of Zn as a Zn-Ca-phytate complex, where the stability and solubility of the complexes depend on the pH value, the phytate-to-Zn molar ratio and the presence of other compounds in the solution. Pumpkin seeds contain phytates which accumulate in the seeds during the ripening period (Kumar et al., 2010) and can bind Zn^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+} and other divalent cations among which the bioavailability of Zn^{2+} was reported to be the most impaired in humans (Lopez et al., 2002). During digestion dietary phytates dissociate because of the low gastric pH but they can rearrange again to new phytate complexes after the chyme[11] passes from the stomach into the neutral pH environment of the duodenum and small intestine. The newly formed complexes can include considerable amounts of Zn and also other divalent cations like Fe^{2+}, Cu^{2+} and Mn^{2+} (Windisch, 2002). In the current work, besides reduction of Zn-release from 79.2 to 52.9% of total Zn (Tab.10), also reduction of Mn-release was observed, while in the case of Fe and Cu no changes were noticed. Phytate complexes are largely insoluble at the pH of the small intestine and therefore formation of Zn–phytate complexes might also increase fecal losses of endogenous Zn as reported by Windisch and Kirchgessner (1999). An exception is ferric phytate, which is also insoluble at pH values in the range 1.0 - 3.5 (Kumar et al., 2010), explaining why reduction of Fe-release was not

[10] An enzyme which is released in the stomach and that degrades food proteins into peptides

[11] Chyme is the semi fluid mass of partly digested food expelled by the stomach into the small intestine.

observed in this work. Although the amount of dietary phytate commonly present in plant foods does not completely prevent the absorption and utilization of dietary Zn (House, 1999) a significant portion may be "lost" as is suggested from the data in Tab. 10. Since the form in which many minerals in foodstuffs and in the gastrointestinal tract are largely unknown, it is difficult to predict the specific interactions of phytate with the minerals. Hence little attention has been paid to the understanding of the in situ interaction of phytate with nutrients and minerals in the gastrointestinal tract of humans (Kumar et al., 2010).

Because of its ability to complex Ca, oxalate, as a common constituent of plants is also considered to be an antinutrient while transition metals can be co-precipitated with Ca oxalate as well. Therefore vegetarians who consume greater amounts of vegetables will have a higher intake of oxalates than non-vegetarians. Rhubarb, spinach and beet are common high oxalate-content foods (Noonan & Savage, 1999), while iceberg lettuce contains much lower amounts (Altunkaya & Gökmen 2008). Data on the oxalate content in pumpkin seeds were not found but they are considered to be low.

Besides inhibition also promotion of Zn absorption should be mentioned. Some compounds (gluconate, citrate, histidine, cysteine, glycine and aspartate) have shown to enhance the absorption in animal experiments and may also explain why Zn availability is better from protein-rich diets (Peganova & Eder, 2004). Zn absorption from the gastrointestinal tract is not only controlled by inhibitors and promoters but also affected by the Zn status in the human body, where in Zn deficient state more Zn is absorbed. This homeostatic mechanism of Zn was demonstrated by Anke (2004), where a placebo-controlled, double blind study with a daily intake of 6.7 mg in a placebo cluster and 17 mg of Zn in a zinc cluster was done. The latter excreted exactly 10 mg more Zn in feces.

The scope of this work was not only identification and characterization of Zn species in pumpkin seeds and iceberg lettuce but also gastrointestinal bioaccessibility. The data presented in Fig. 28 confirm that complete decomposition of previously indicated Zn species in plants (see section 4.3.4) takes place in the human gastrointestinal tract. It seems that the only Zn species present is Zn^{2+} with column recoveries of 98 and 102% for pumpkin seeds and iceberg lettuce, respectively (injection of pure Zn^{2+} yielded a column recovery of 104%). However, the presence of low molecular Zn complexes with e.g. single amino acids cannot be

excluded; in future research ion-exchange chromatography will be used to confirm the absence of these Zn complexes.

After the chyme passes from the stomach into the neutral pH environment of the duodenum and small intestine, complexation of Zn^{2+} with ligands which have "survived" the stomach phase may occur, potentially leading to reduced bioaccessibility as discussed above. When the bioaccessible fraction reaches the small intestine, the bioavailability primarily depends on absorption processes. Data summarized by Windisch (2002) indicate that a major pathway of absorption is mediated by transport proteins located in the intestinal brush-border membrane[12] which collect Zn and also other elements (Fe, Cu, Mn) as inorganic divalent cations more or less selectively from the mucus layer. Homeostatic control of absorption seems to be based on the expression of these proteins and/or of molecules of the subsequent transport line from the cell lumen towards the blood stream. Zn is further transported to cells bound to proteins, predominantly albumin, α2-macroglobulin and transferrin (Tapiero & Tew, 2003).

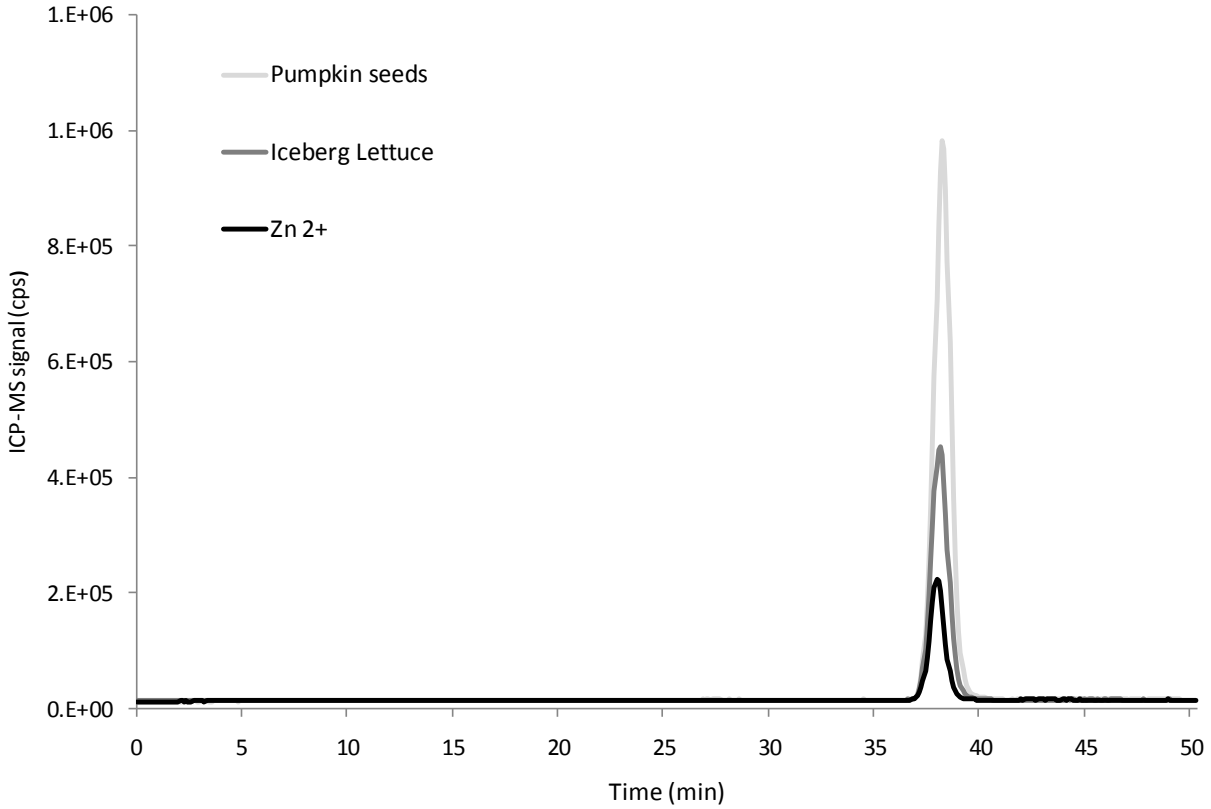


Figure 28: Zn species in pumpkin seeds and iceberg lettuce after a simulated stomach digestion step compared to Zn^{2+} in stomach digestion fluid (0.03M Tris–HCl (pH 2.5)

[12] A region epithelium that possesses densely packed microvilli (microscopic cellular membrane), which greatly increases the surface area of the epithelium and facilitates the absorption in the small intestine.

Although in vivo and in vitro techniques have been used to investigate elemental bioaccessibility and bioavailability, it is likely that no single procedure is perfect for all elements and model systems. It should be stressed that simulation of the human digestion tract is usually, also in the case of the PBET approach, an average of all possible circumstances which can appear in the human digestion tract which are also related to age, sex and societal customs and can vary from individual to individual. How these averages were acquired and which physiological conditions were considered is well presented and discussed by Oomen et al. (2002). One way to study the Zn bioavailability by *in vitro* techniques is based on Caco-2 cells (Campen & Glahn, 1999), which develop microvilli and act similar to small intestinal epithelial cells. This technique as described by Campen and Glahn (1999) was successfully applied for studying the availability of Fe from foods, while for Zn there has been limited use. Studies in this field primarily targeted the factors affecting the uptake, while little effort has been devoted to developing Caco-2 cell systems for bioavailability simulation.

4.5 Vegetarian diet as a risk factor considering zinc deficiency

During passage through the human gastrointestinal tract, element species may be transformed from the original dietary compounds into other species before reaching the final site of absorption (Windisch, 2002). Factors affecting the bioavailability of trace elements to humans in individual foodstuffs have been the subject of numerous research reports. According to House (1999) these factors can be divided into three major groups: diet composition, food preparation and host factors.

The Zn requirement in adults is mainly met by consumption of meat in omnivorous diets, while, a purely vegetarian diet (Nriagu, 2007a) and a constant omnivorous diet based on white poultry meat and finfish does not provide sufficient Zn for adult men (Milne et al., 1983). Vegetarians consume significantly more Zn per kg of body weight (Anke, 2004), while they may need as much as 50% more Zn as non-vegetarians because of the decreased absorption from foods of plant origin through the already mentioned phytate, oxalate, fibers, lignin and other antinutrients (Tapiero & Tew, 2003; Peganova and Eder, 2004).

The trace element content of plants is known to be affected during different environmental effects already presented (see section 1.1.1) but agricultural practice (new cultivars, different fertilizers, etc.) can have a significant effect as well. According to the results presented by

Ekhlom et al. (2007) the trace element density in vegetable foods is now lower then it was 30 years ago and in spite of clearly increased consumption of vegetables, trace element intake decreases. Plant breeding has developed better cultivars for higher yields and resistance to diseases, while the trace element content of these cultivars was of no interest (Ekhlom et al., 2007).

We also need to be aware that Zn requirements increase as age increases as the extent of Zn absorption in the small intestine falls from 33% to 18% (Sanstead et al., 1982). Additional Zn is also needed during outstanding human body status like stress, obesity, trauma and rehabilitation after starvation, pregnancy and lactation which already recognizes the essentiality of Zn for the child in the uterus (Peganova & Eder, 2004) and dilutional effects of rapid growth (such as the catch-up growth of premature infants).

Although plant-based food products may have drawbacks regarding the zinc metabolism they constitute an important source of carbohydrates, dietary fibers, vitamins and also some non-nutrients which influence other biochemical process in the human body. Therefore a mixed diet presents the lowest risk for any of deficiencies regarding trace elements and also other essential compounds needed for normal functioning of the human body as long there are no metabolic disturbances.

5 CONCLUSIONS

The main objectives of this thesis were to get information about i) Zn species in two different plant samples (pumpkin seeds and iceberg lettuce) and ii) degradation of these species in the human gastro-intestinal tract. Both objectives were achieved with a "sequential" analytical approach using more and more involving technical steps (*i* - total digestion analysis; *ii* - LA-ICP-MS; *iii* - ultrasound-assisted variable volume extraction; *iv* - ultrafiltration; *v* - SEC-ICP-MS; *vi* - physiologically-based extraction tests), gradually unraveling the physico-chemical properties of Zn in plant extracts and elucidate the "deteriorating" effect of the gastro-intestinal tract on the integrity of the Zn species.

Results from experimental findings in step *i* confirmed that pumpkin seeds (91 μg g^{-1}) are a more abundant source of Zn than iceberg lettuce (62 μg g^{-1}). Employing LA-ICP-MS for elemental distribution profiles in step *ii* revealed that for pumpkin seeds the Zn concentration directly under the husk was much higher than in the kernel while in the case of iceberg lettuce the Zn distribution in the leaf is more uniform. Because of ICP-MS multi element detection capability also antagonistic/synergistic behavior of Zn with some other elements (Ca, Cd, Cu, Fe, K, Mg) could be observed and may be present due to similar/different binding and/or storage sites in the investigated plant samples. In step *iii* the maximum extractable Zn in water was investigated as a function of the extractant volume-to-sample mass (*V*/*m*) ratio and showed a linear sorption isotherm relationship for both sample types. The maximum amount of Zn extractable from pumpkin seeds and iceberg lettuce was derived to be 34.6 and 42.2% of the total Zn, respectively. Size fractionation of the water extracts by ultrafiltration in step *iv* gave comparable results for both sample types with a major Zn fraction (ca.78%) below 3,000Da, comparable with literature results for other vegetables and plants as well. To improve upon ultrafiltration in terms of resolution, size fractionation by size exclusion chromatography was applied in step *v*. Results show differences in Zn size fractionation related to sample type in the high (>20,000Da) and intermediate (12,000 – 20,000Da) molecular range, although the size fractionation was comparable below 1700Da. Employing physiologically-based extraction tests in step *vi* gave evidence that all Zn species identified in plant extracts decompose in the human gastro-intestinal tract (SEC of the extract behaved identical to a Zn^{2+} standard). In spite of the complete decomposition of Zn species under human stomach conditions, the subsequent extraction step simulating digestion in the intestines shows that Zn is less available in this environment (for pumpkin seeds). This

indicates that anti nutrients like naturally present phytate may be responsible for complex formation in the small intestines, thus reducing the Zn bioaccessibility.

From the above findings it follows that the "sequential" analytical approach offers excellent opportunities to study the speciation of Zn (and probably other elements as well) in plants and the integrity of species upon digestion. From an analytical point of view it is worth mentioning that LA-ICP-MS is a promising microanalytical tool for detailed spatial resolution studies of elements during plant growth and parts we consume. Extraction of Zn species from plants, without disturbing the speciation and maximizing the extraction yield, is a prerequisite in speciation analysis. Using "gentle" extractants as far as pH, ionic strength, etc. is concerned in suitable extractant volume-to-sample mass ratios we can satisfy this requirement. Additionally, SEC as the least speciation disruptive separation technique completes this picture. From a plant point of view it is important to highlight the results obtained with SEC-ICP-MS as the "hidden" information in the chromatograms needs further "exposure" regarding the characteristics of the ligands; techniques like LC-MS/MS may aid in the identification of these ligands. From a nutritional point of view the speciation of essential elements prior to a severe digestion procedure as e.g. encountered in the human gastrointestinal tract seems irrelevant, but should be of interest to nutritionists, dietitians, public health workers and planners in the fields of nutrition and food technology. Of course we should be aware that pumpkin seeds and iceberg lettuce are only two constituents of the heterogeneous human diet and that in this work only Zn was investigated, therefore these results should be considered as an additional piece in the whole plant speciation puzzle. As a general comment, and recommendation, it can be said that a mixed diet still presents the lowest risk for Zn deficiency.

Follow-up. The unexpected "complete" decomposition of all in plant extract identified Zn species in the human stomach, and the fact that Zn bioavailability and Zn affinity is governed by compounds naturally present under conditions in the small intestine, merit further study to reveal the metabolic processes and may yield potential remedies for Zn deficiencies. Also experiments with cell lines based on Caco-2 cells may be helpful in understanding these metabolic processes. From an analytical point of view the Zn species in the solid residues after the extraction are still of interest as they make up a considerable part of the non-extractable Zn.

"You need to know a lot, before you notice how little you know" (Karl Heinrich Waggerl)

6 SUMMARY

Attention was focused for years on Zn toxicity because of the general belief that Zn deficiency could not occur because Zn was assumed to be ubiquitous and plentiful in our diets. About 40 years ago, Zn was recognized as an essential micronutrient for human health. Today, Zn deficiency is recognized as a nutritional problem worldwide. The risk factors for Zn deficiency are primarily environmental in its origin. The causes of Zn deficiency fall in two main categories (i) nutritional causes such as consumption of food items with either low Zn contents or unavailable forms of Zn, and (ii) conditioned deficiency related to diseases and genetic malfunctions that impair intestinal absorption and/or increase intestinal loss of Zn. Plants provide the transport pathway of trace elements from the abiotic to the biotic environment. Uptake of metals by plants reflects their bioavailability in soils and affects plant yield and crop quality to the extent that animal or human health may be jeopardized. In plants themselves, Zn plays a key role as a structural constituent or regulatory co-factor of a wide range of different enzymes and proteins in many important biochemical pathways. While some of the biological functions of essential elements are quite well understood, the chemical form of elements which naturally occur in plants is mostly unknown.

The main objectives of this thesis are to get information on Zn species and their differences regarding sample type (pumpkin seeds and iceberg lettuce) and their possible degradation in the gastro-intestinal tract. A sequential analytical approach is applied focusing on total Zn, spatial Zn distribution, extractability of Zn, speciation of Zn and bioaccessibility of Zn. For determination of total Zn, as a reference point for further analysis, a microwave digestion technique was applied. The study of the spatial Zn distribution was performed by LA-ICP-MS and related to the distribution of other elements. To counteract extraction discrepancies related to V/m ratios a so-called variable volume extraction was applied, which extrapolates the V/m ratio to infinity thereby yielding the maximum extractable amount of water soluble Zn. Speciation of Zn in the extracts was performed by low (ultrafiltration) and high (size exclusion chromatography) resolution separation techniques with the goal to unravel the MW distribution of Zn species. Physiologically-based extraction was used for i) simulation of the release of Zn in the gastro-intestinal tract upon ingestion and ii) investigation of the degradation of the Zn species during the digestion process. Element-specific detectors used were ICP-MS and FAAS.

Results show that pumpkin seeds and iceberg lettuce have different Zn species fingerprints (in water extracts) with a high (ca. 70%) low-MW fraction (ca. 500Da) in iceberg lettuce and a high (ca. 60%) intermediate/high-MW fraction (10,000 – 20,000Da) in pumpkin seeds. When these Zn species are subjected to conditions simulating the human stomach (pH ~ 2) they break down completely, disproving conclusions of Zn speciation studies done in the past suggesting that low-MW Zn species may have nutritional importance. However, these findings open up a wide range of further interesting research possibilities, especially in the case of pumpkin seeds, where results evidence that anti-nutrients (e.g. naturally present phytate) can reduce the uptake of Zn by complexing with Zn^{2+} in the intestines (pH ~ 7).

7 POVZETEK

Toksičnost cinka je bila več let v središču pozornosti predvsem zaradi splošnega prepričanja, da je cinka v človeški prehrani dovolj in da je nevarnost pomanjkanja le-tega zanemarljiva. Pred nekaj več kot 40 leti pa je bilo dokazano, da je cink eden izmed bistvenih mikroelementov, ki so potrebni tudi za ohranjanje zdravja ljudi in živali. Danes je pomanjkanje cinka v prehrani svetovni problem; dejavniki tveganja za pomanjkanje cinka pri človeku, so predvsem okoljskega izvora. Vzroke za pomanjkanje cinka tako delimo v dve kategoriji: (i) prehranski vzroki, kot so uporaba živil z nizko vsebnostjo cinka in/ali vsebnostjo cinka v bionedostopni obliki, ter (ii) pogojno pomanjkanje zaradi različnih bolezni in genetskih okvar, ki zmanjšujejo absorpcijo cinka v črevesju in/ali povečajo njegove izgube. Rastline predstavljajo vmesni člen potovanja mikroelementov iz tal do živali in ljudi. Prevzem kovin v rastline odraža njihovo razpoložljivost v tleh in lahko vpliva tako na obseg kot tudi na kakovost pridelka v tej meri, da predstavlja celo tveganje za pomanjkanje posameznega elementa pri živalih in ljudeh. V rastlinah cink kot strukturni element in/ali kot kofaktor številnih encimov in proteinov v biokemijskih procesih igra ključno vlogo. Medtem ko so nekatere biološke funkcije mikroelementov, ki so v rastlinah naravno prisotni, dobro poznane, je njihova kemijska oblika večinoma neznana.

Glavna cilja naloge sta bila pridobiti informacije o kemijskih zvrsteh cinka in njihovih razlikah glede na tip vzorca (bučna semena in solata kristalka) ter o njihovi morebitni razgradnji v prebavilih človeka. Uporabljen je bil analitski pristop, kjer so si sledili postopki določanja skupnega cinka, porazdelitve cinka znotraj vzorca, ugotavljanje vodotopnega cinka, ugotavljanje cinkovih zvrsti in določanje njegove biodostopnosti. Za določanje skupnega cinka kot referenčne vrednosti za nadaljnjo analizo je bil uporabljen mikrovalovni razkroj. Porazdelitev cinka v povezavi s porazdelitvijo drugih mikro- in makroelementov v vzorcu je bila določena z LA-ICP-MS. Za ugotavljanje učinkovitosti ekstrakcije je bil uporabljen t. i. "variable volume extraction approach". Speciacija cinka v vodnih ekstraktih je potekala z metodo nizke (ultrafiltracija) in visoke (ločitvena kromatografija) ločljivosti. Simulacija fizioloških pogojev človeških prebavil je bila uporabljena za i) ugotavljanje ekstrakcije cinka v prebavilih po zaužitju in ii) ugotavljanje stopnje razgradnje cinkovih zvrsti med prebavo. Kot detektor cinka in tudi ostalih elementov sta bila v vseh primerih uporabljena FAAS ali ICP-MS.

Rezultati kažejo razlike v cinkovih zvrsteh glede na tip vzorca. V solati je tako večina cinka (cca. 70 %) prisotnega v nizkem molekularnem območju (cca. 500Da), medtem ko je v primeru bučnih semen večina cinka (cca. 60 %) prisotna v srednjem in visokem molekularnem območju (10 000–20 000Da). Izpostavljenost teh zvrsti pogojem, podobnim človeškemu želodcu (pH ~ 2), povzroči njihovo popolno razgradnjo, kar je v nasprotju s sklepi nekaterih starejših študij, ki nakazujejo, da ima najverjetneje cink v nizkem molekularnem območju določeno prehrambno vrednost. Vendar pa te ugotovitve po drugi strani odpirajo široko polje nadaljnjih raziskav, še posebej v primeru bučnih semen, kjer rezultati kažejo, da t. i. proti hranila (npr. naravno prisoten fitat) lahko zmanjšajo biodostopnost cinka zaradi kompleksacije z Zn^{2+} v tankem črevesju človeških prebavil (pH ~ 7).

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