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Speciation of zinc in pumpkin seeds (*Cucurbita pepo*) and degradation of its species in the human digestive tract

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This article is dedicated to the memory of the late Professor Janez Štupar.

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ABSTRACT

Pumpkin seeds are one of the foodstuffs recommended in diets which do not contain other Zn-rich sources. The main objectives of this work were to get information on Zn and its species in pumpkin seeds, and their possible degradation in the human gastrointestinal tract, indicative of Zn bioaccessibility. A sequential analytical approach was applied, focusing on total Zn, spatial Zn distribution, extractability, speciation and bioaccessibility of Zn and its species. It was shown that water extracts of pumpkin seeds exhibit a specific Zn species fingerprint with *ca.* 30% of a low-MW fraction (0.5–2 kDa) and ca. 60% of an intermediate/high-MW fraction (10–20 kDa). Digestion of Zn species under simulated stomach conditions proved that Zn species identified in plant extracts were completely decomposed to Zn^{2+}. The subsequent digestion under intestinal conditions showed that Zn becomes less accessible, indicating that antinutrients like naturally present phytate may be responsible for complex formation in the small intestines, thus reducing the potential for Zn bioavailability.

1. Introduction

Trace elements play an important role in the functioning of life on our planet. Their (essential or toxic) effects are very often related to a particular physicochemical form in which the element is present. While some of the biological functions of essential elements are quite well understood, the chemical form in which elements naturally occur in plants is often unknown. A biological requirement for Zn was first identified by Raulin (1869) when the common bread mould (*Aspergillus niger*) was found unable to grow in the absence of Zn. In the 1920s Zn was shown to be essential for higher plants (Sommer & Lipman, 1926), while some years later (Todd, Elvehjem, & Hart, 1934) Zn was already reported to be essential for rats. Meanwhile, most attention was focused on the toxicity of Zn, in the general belief that Zn deficiency could not occur in humans, as Zn was supposed to be ubiquitous and plentiful in our diets. Nevertheless, in the 1960s Zn was reported as an essential micronutrient for human health as well (Prasad, Halsted, & Nadimi, 1961) and today Zn deficiency is recognised as a nutritional problem worldwide, present in developed and developing countries.

One of the recent estimates from FAO (Food and Agriculture Organisation) based on food balance data from 176 countries is suggesting that approximately 20% of the worlds population is at risk of Zn deficiency (Sandstead & Au, 2007). The causes of Zn deficiency for humans can be the consequence of consumption of food with a low Zn content or unavailable forms of Zn, but also be the result of diseases that impair intestinal absorption and/or increase intestinal Zn losses. Because of specific chemical properties, Zn is extensively involved in cellular and sub-cellular metabolic processes of the human body. Zn may act as a co-factor of numerous enzymes, as a structural element of enzymes, proteins and biomembranes, as an initiator of transcription and gene expression processes (binding of zinc fingers to DNA) and as a part of other biological functions without the risk of oxidation damage (Hambidge, 2000). Because of the involvement of Zn in these numerous processes Zn deficiency can lead to several disorders of the human body which have been well documented elsewhere (Simon-Hettich, Wibbertmann, Wagner, Tomaska, & Malcolm, 2001). Since there is no particular body store or storage organ from which Zn would be readily available (90% of the body's Zn is in the form of a slowly exchanging pool, mainly in bone and muscle) an adequate supply of Zn from food is a basic necessity (Conor, 2004). Furthermore the

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uptake is also reported to depend on the Zn compounds present in the diet (Günther & Kastenholz, 2005).

The primary source of most metals in food is the soil from which food is produced and there is an intimate relationship between soil and the metals which are consumed and incorporated in the human body (Conor, 2004). Nevertheless, soils in some parts of the world are depleted in Zn and consumption of locally grown foods can result in endemic Zn deficiency. Soils formed on limestone and sandstone tend to have low Zn concentrations, in contrast to soils developed on clay, shale, or igneous rock (Alloway, 2008), if inputs from agricultural activities are neglected. We need to be aware that the Zn content of soils and the phytoavailability are critical for plant growth (Alloway, 2008) and crucially govern the transport pathway from the abiotic to the biotic environment.

Identification of the elemental species leads to a better insight in metal-involving biochemical processes at the cellular level (Lobinski, Moulin, & Ortega, 2006) and is critical for understanding the nutritional aspects. In the field of speciation analysis of trace elements in edible plants a clear trend exists towards hyphenating highly selective separation techniques with very sensitive elemental detection techniques. For the measurement of extractable Zn from plants the selectivity is usually achieved by size-exclusion chromatography or ion-exchange chromatography, while techniques like FAAS, GFAAS and ICP-MS assure a high sensitivity for elemental detection. An upcoming trend is the use of soft ionisation techniques as in HPLC-ESI-MS (for direct molecular identification of elemental species in sample extracts) and MALDI-MS (for localised elemental speciation in laser desorbed sample material) (Hill, 2007).

In contrast to elements like, e.g., As, Hg, Se or Pb, limited information is available on the physicochemical forms of Zn in edible plants. After uptake from soil, Zn in plants does not undergo valency changes and its predominant forms might be low molecular weight complexes, storage metalloproteins, free ions and insoluble forms associated with the cell walls (Brown, Cakmak, & Zhang, 1993). Others suggest also reducing sugars, amino acids and compounds which contain sulphur as possible Zn ligands (Walker & Welch, 1987). Recently some more attention has been focused on nuts and several authors have reported Zn species similar to the ones mentioned above in a variety of nuts, after acid/base extraction (Wuilloud, Kannamumarath, & Caruso, 2004) or aqueous extraction (Naozuka, Marana, & Oliveira, 2010). Additionally, the bioavailability of Zn species present in foodstuffs in the human gastrointestinal tract has been simulated by *in vitro* physiologically-based extraction tests (PBET) similar to those which were primarily developed for human health risk assessment after ingestion of soil (Ruby, Davis, Schoof, Eberle, & Sellstone, 1996).

In this work the focus is on Zn in pumpkin seeds (*Cucurbita pepo*). Seeds in general contribute significantly to the nutrition of the human population in many parts of the world, and pumpkin seeds which are often consumed directly as a snack food, provide one of the most concentrated vegetarian sources of Zn and are as such recommended (WHO, 2009), especially in diets which do not contain other Zn-rich foodstuffs. There is limited information available on Zn and especially on its species in pumpkin seeds and the fate of Zn species upon ingestion is largely unknown although of prime importance from a bioavailability point of view. A "sequential" analytical approach will be followed to elucidate these issues by gradually "delving" deeper into the pumpkin seed matrix, emphasising the nutritional aspects.

2. Experimental

A "sequential" analytical approach as shown in Fig. 1 was applied by gradually focusing on more elaborate procedures. All reagents were of analytical-reagent grade unless mentioned otherwise and standard solutions were prepared in Milli-Q water (18.2 MΩ cm). Prior to analytical treatment seeds were washed with Milli-Q water, followed by drying in an oven at 40 °C for 48 h. For some experiments seed coats and kernels of pumpkin seeds were studied separately after careful separation using a scalpel. Subsequently they were ground in a domestic grinder. Prior to SEC-ICP-MS analysis a cleanup step (removal of lipids) as described in the literature (Wuilloud et al., 2004) was applied: ground pumpkin seeds (4 g) were subjected to extraction with a 20 ml chloroform–methanol (2:1 *V/V*) mixture for 15 min followed by filtering (0.45 μm) and drying the residue at room temperature.

2.1. Determination of total Zn after MW digestion

Ground pumpkin seeds or their constituents (seed coat and kernel) were weighed (0.5 g) in PTFE vessels. Seven millilitres of HNO_3 (65% *V/V*) and 1 ml of H_2O_2 (30% *V/V*) were added to the PTFE vessels, followed by microwave digestion (Milestone Ethos 1 Advanced Microwave Digestion Labstation; Milestone S.r.l., Sorisole (BG) Italy), according to a standard digestion protocol (gradual temperature increase to 200 °C [in 15 min] after which the temperature was kept constant [for 15 min]). Digests were diluted with Milli-Q water; blank solutions were prepared in the same way. A

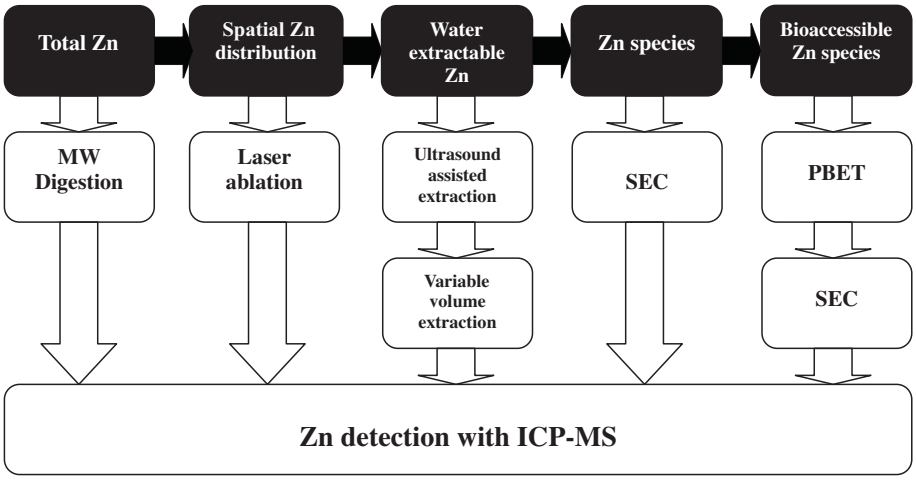

Fig. 1. Analytical approach for determination of Zn and its species in pumpkin seeds. MW = microwave; SEC = size exclusion chromatography; PBET = Physiologically-Based Extraction Test.

Table 1
ICP-MS operational parameters for determination of (a) Zn in digests and extracts, (b) Zn localised distribution and (c) Zn species in aqueous extracts after chromatographic separation.

Instrumental parameter	
(1a) ICP-MS Agilent 7500ce	
RF power (W)	1500
Plasma gas flow rate (l min^{-1})	15.0
Auxiliary gas flow rate (l min^{-1})	1.0
Carrier gas flow rate (l min^{-1})	0.8
Makeup gas flow rate (l min^{-1})	0.25
Sampling depth (mm)	8.0
Acquisition time (s)	0.2
ORS, He flow rate (ml min^{-1})	5
ICP-MS Hewlett–Packard 4500	
RF power (W)	1300
Plasma gas flow rate (l min^{-1})	15.0
Auxiliary gas flow rate (l min^{-1})	1.0
Carrier gas flow rate (l min^{-1})	0.98
Sampling depth (mm)	6.0
Acquisition time (s)	0.2
(1b) Laser ablation system (NewWave Research UP 213)	
Wavelength (nm)	213
Ablation mode	Raster or crater
Shot repetition rate (Hz)	20
Fluence (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 carrier gas flow rate (l min^{-1})	0.95
Ar make-up gas flow rate (l min^{-1})	0.75
ICP-MS Agilent 7500ce	
RF power (W)	1500
Plasma gas flow rate (l min^{-1})	15.0
Auxiliary gas flow rate (l min^{-1})	1.0
Sampling depth (mm)	8.0
Acquisition time (s)	0.25
(1c) SEC (Agilent Technologies HP 1100)	
Liquid chromatographic system	Degasser (G1322A); Quaternary pump (G1311A); Auto sampler (G1313A); DAD (G1315A)
Column	Superdex peptide 10/30
Resolution range	100–20,000 Da (optimal 100–7000 Da)
Mobile phase	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})	0.5
Injection volume (μl)	100
ICP-MS Hewlett–Packard 4500	
RF power (W)	1300
Plasma gas flow rate (l min^{-1})	15.0
Auxiliary gas flow rate (l min^{-1})	1.0
Carrier gas flow rate (l min^{-1})	0.98
Acquisition time (s)	1.0

quadrupole ICP-MS instrument (Agilent Technologies 7500ce ICP-MS, Palo Alto, CA) in ORS (Octopole Reaction System) mode was used for determination of Zn in the digests using instrumental conditions as given in Table 1a. Standard solutions for calibration were prepared from a stock multielement solution (ICP Multielement Standard IV; Merck, Darmstadt, Germany) in the concentration range 0–500 μg l^{-1} with a matrix resembling the sample solutions as closely as possible. To correct for potential instrumental drift, internal standardisation was used by spiking both the standard and sample solutions with Y, Sc, Ge and Gd (50 μg l^{-1}).

2.2. Determination of the spatial Zn distribution by LA-ICP-MS

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) is a sensitive, solid sampling technique for spatial distribution analysis of elements, both for lateral and depth profiling purposes on the micrometre scale (Lobinski et al., 2006). An Agilent 7500ce quadrupole ICP-MS interfaced with a laser ablation device UP213 (New Wave Research, Fremont, CA) was used with typical operational conditions as presented in Table 1b. The laser ablation device contained a frequency-quintupled Nd:YAG laser (wavelength, 213 nm; pulse width, 4 ns) with a motorised stage and a low volume, fast washout laser ablation chamber, a so-called SuperCell. The elemental distribution in the sample was investigated by depth profiling on seeds with seed coat and with seed coat removed.

2.3. Aqueous extraction of Zn and its species

Ultrasound-assisted extraction was performed with an ultrasonic homogeniser (LABSONIC® M, Sartorius, Gottingen, Germany; 100 W, 30 kHz frequency) equipped with a titanium probe [80 mm × 3 mm]). Varying amounts of dried and ground pumpkin seeds (0.1–1.0 g) were accurately weighed into 50-ml Falcon tubes and 25 ml of extractant (Milli-Q water) was added. The samples were sonicated for 2 min, followed by centrifugation for 15 min at 1900*g*. The supernatants were filtered through 0.45-μm syringe filters, extracts were acidified (1% *V/V* HNO_3) and analysed the same day using instrumental conditions as presented in Table 1a.

2.4. Determination of Zn species by SEC-ICP-MS

After the cleanup step ground seeds were subjected to extraction in the mobile phase solution as described before for the aqueous extraction (see Section 2.3). A Superdex Peptide HR 10/30 column (Pharmacia Biotech, Sweden; separation range, 0.1–20 kDa) fitted with a pre-column PEEK filter (0.22 μm) was applied for separation of the extracted Zn species. The column was calibrated with selected calibrants (cytochrome C (12,284 Da), aprotinin (6500 Da), vitamin B_{12} (1355.4 Da), glutathione-oxidised (612 Da), trycine (179.2 Da) and glycine (75.1 Da)) under sample separation conditions using a diode array detector (G1315A, Agilent Technologies HP 1100). The size exclusion chromatography (SEC) separation of the elemental species was monitored by ICP–MS. For this purpose the outlet of the SEC column was interfaced to the liquid sample inlet of the nebuliser. The instrumental operating conditions are given in Table 1c. Detailed study of Zn elution profiles was performed using the integration tool in Origin 7.5 SR4 (OriginLab Corporation, Northampton, MA).

2.5. Determination of Zn species bioaccessibility using PBET

To estimate the bioaccessibility of Zn and its species in the human digestion tract (stomach and small intestine) a PBET protocol (Fig. 2) as described by Ruby et al. (1996), with some modifications according to Oomen et al. (2003), was performed. Samples were weighed in 50-ml Falcon tubes in which the whole PBET procedure was performed under conditions as described in Fig. 2. To prevent unwanted sorption of Zn to container walls all PBET extracts were acidified (1% *V/V* HNO_3). This made additional centrifugation (5 min at 2500*g*) and filtration (0.45 μm) of the small intestinal phase extracts necessary as bile salts precipitate at pH = 2. Measurements were performed employing the ICP-MS at operating conditions as given in Table 1a.

3. Results and discussion

3.1. Total Zn

The total amount of Zn found in pumpkin seeds (91.2 μg g^{-1}) is in good agreement with literature findings (Juranovic, Breinhoelder, & Steffan, 2003; WHO, 2009). Values from 15 to 100 μg g^{-1} in dry

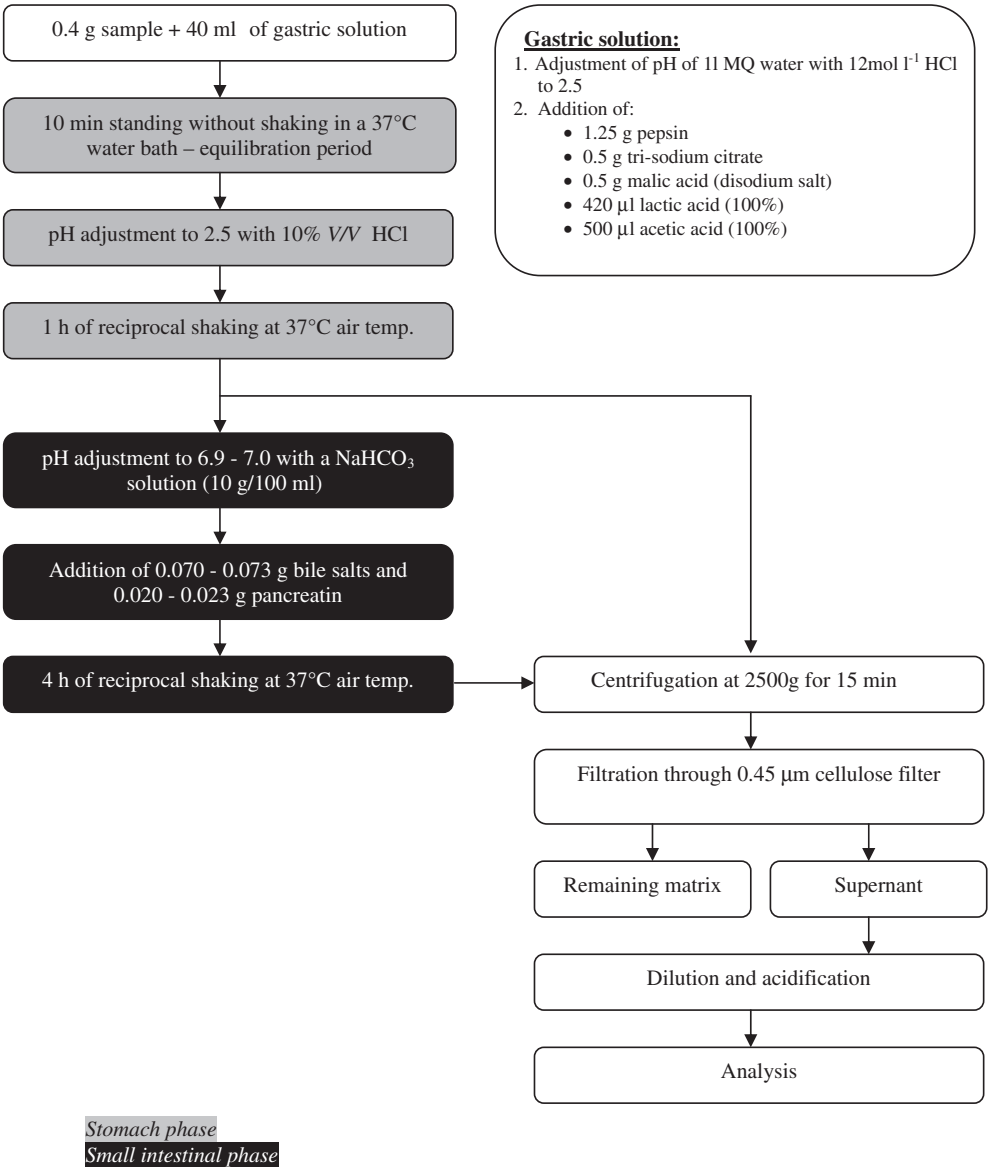

Fig. 2. PBET test protocol.

matter are considered to be normal, depending on age and vegetative stage of the plant and influenced by soil and climate conditions. Values higher than 400 µg g^{-1} can already be toxic (Peganova & Eder, 2004). ICP-MS measurement of Zn in pumpkin seed digests is prone to matrix interferences where dilution gives rise to higher and more accurate Zn concentrations comparable with those obtained with the method of standard additions. These matrix interferences depend on the absolute concentration of interferents present and can be caused by changes in sample transport and ionisation efficiency, clogging of sampling orifices and coulombic repulsion in the interface and ion-optic train (Evans & Giglio, 1993). Upon dilution of the matrix the concentration of the interferant drops to levels where matrix effects are alleviated (McClenathan, Ray, & Hieftje, 2001). To circumvent any possible risk of biased results due to matrix interferences, the method of standard additions was applied routinely in the remainder of this work. Since consumers of pumpkin seeds can choose between the snack food variant with fully or partially removed seed coat, the Zn distribution in seed coat and seed kernel was investigated separately. When measuring the individual constituents of the seed (see Table 2) it can be observed that the kernel and the seed coat of the pumpkin seed contain roughly similar absolute amounts (in µg) of Zn; since the mass ratio of kernel:seed coat is *ca.* 2, the Zn concentration ratio of kernel:seed coat is *ca.* 0.5. This is unexpected as the seed coat is supposed to contain insignificant amounts of Zn, which is (in the case of legume and cereal seeds) mostly accumulated in the embryo and endosperm of the seed (Longnecker & Robson, 1993). To this end the distribution of Zn in pumpkin seeds was investigated more precisely by LA-ICP-MS.

3.2. Spatial Zn distribution

The LA-ICP-MS approach was reported for the study of essential element accumulation in leaves of a Cu-tolerant plant (Wu, Chen, & Becker, 2009) and for studying the distribution of nutrient and toxic elements in cross-sections of tobacco tissues (Becker, Dietrich, Matusch, Pozebon, & Dressler, 2008). Investigation of the spatial distribution of Zn by depth profiling on pumpkin seeds with and without seed coat (see Fig. 3a) shows that the seed coat

Table 2
Zn concentrations and mass balance of Zn in whole pumpkin seed, kernel and seed coat based on triplicate analysis.

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
Seed coat only	2.1619	139.7	0.3	302.0	51

contains almost no Zn and confirms that the Zn concentration in a thin layer directly under the seed coat, the so-called endosperm envelope (usually tightly compressed against the seed coat), is much higher than in the kernel. The endosperm is a collection of stored food the young plant will use as it begins to germinate, or grow, and is known to contain high levels of Zn (Longnecker & Robson, 1993). Since the high spatial resolution data generated by LA-ICP-MS show a clear Zn distribution pattern accentuating

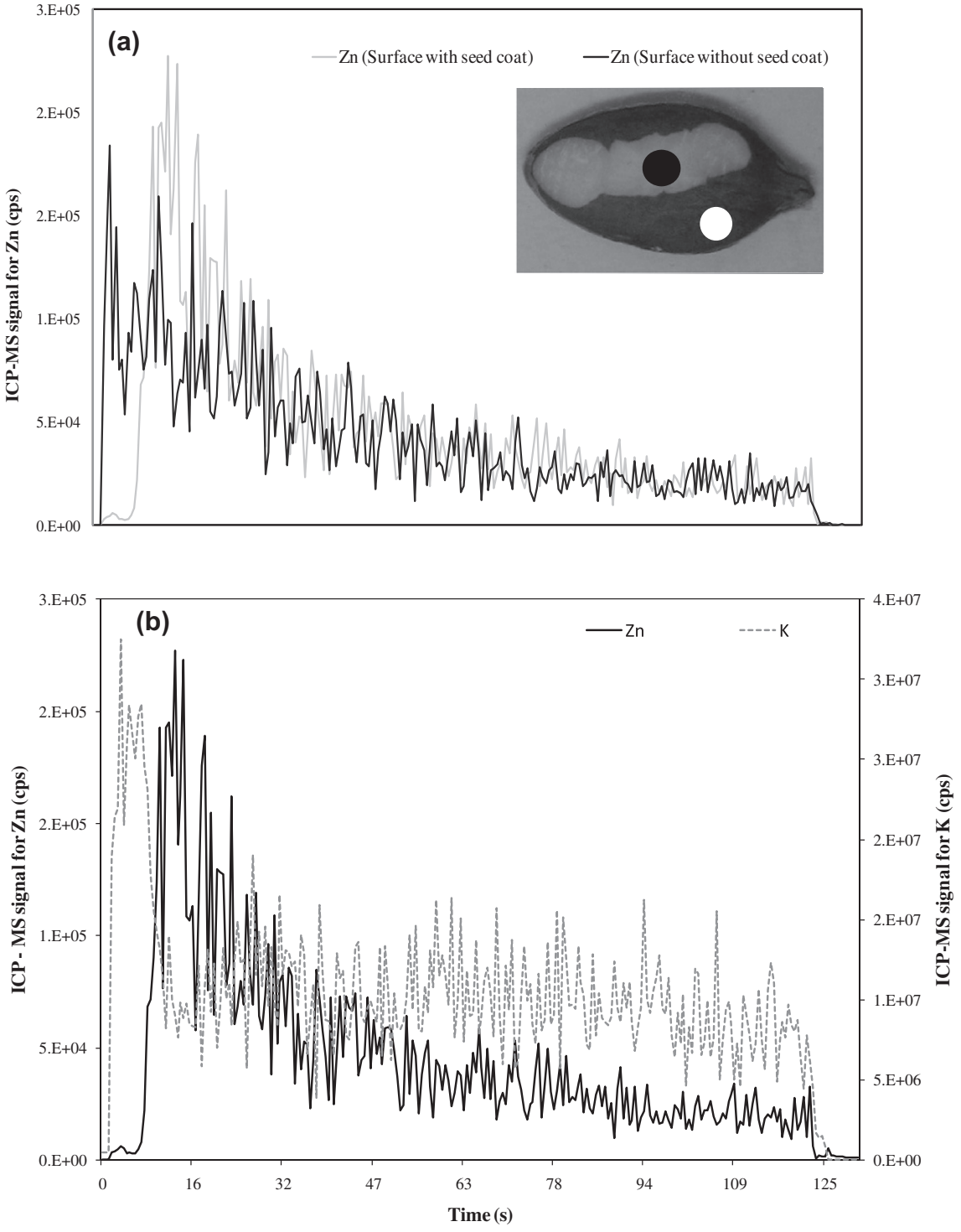

Fig. 3. Depth profiling profiles for (a) Zn on the pumpkin seed surface with seed coat (white dot) and with seed coat removed (black dot); (b) Zn and K on the seed surface with the seed coat (see a). The white and black dots denote the depth profiling locations and are not related to the laser beam diameter. The laser beam diameter was in all instances 100 μm.

the high levels in endosperm, the low spatial resolution data obtained in Table 2 might be erroneous, as the crude separation of seed coat from kernel can also have removed parts of the endosperm with the seed coat. Subsequently, this may have led to Zn levels in seed coat which are too high. Although LA-ICP-MS was applied to gain more insight into the distribution of Zn in pumpkin seeds, it was also shown that the separation of seed coat from kernel is rather erratic, leading to unwanted Zn losses. Also 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 Fig. 3b we can see that e.g., K behaves differently from Zn with a distribution profile showing a higher density in the seed coat than in the deeper layers, probably because of storage reasons.

3.3. Water soluble fraction

For elemental speciation it is essential that the species of interest are extracted from the sample with an extractant which keeps the integrity of the species intact, preventing structural changes, denaturation of proteins and destruction of protein–metal complexes (Makarov & Szpunar, 1998). For this reason water was used although the extraction efficiency may be lower compared with, for example, mild to strong acidic or basic extractant (Wuilloud et al., 2004). Ultrasound-assisted extraction aids in the extraction process as 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 and enhancing the homogenisation. To optimise the ultrasound-assisted extraction, sample–water mixtures were subjected to sonication for different time intervals; an extraction plateau was reached in *ca.* 120 s using 1 g of sample in 100 ml of Milli-Q water. It is often assumed that a certain mass of sample (m, in g) in a certain volume of extractant (V, in ml) suffices to extract the elemental 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, Šlejkovec, Kahn, & Goessler, 2007) under the assumption of reversible adsorption and desorption processes during extraction. With a simple linear sorption isotherm the available pool of an element in a certain extractant may be deduced by measuring the elements released as a function of the volume-to-mass (V/m) ratio. Following this approach the maximum extractable Zn concentration from pumpkin seeds was 31.6 μg g^{-1}. From Fig. 4 it can be seen that the extraction yield is indeed desorption-controlled with an extraction efficiency of 58% and 95% (of the maximum extractable Zn concentration) at V/m = 25 ml g^{-1} and V/m = 250 ml g^{-1}, respectively.

3.4. Size fractionation by size exclusion chromatography

SEC is a gentle separation method and does normally not result in loss of elemental species or on-column alterations. Consequently, SEC plays a dominant role in the separation of labile and weak metal complexes. The main advantage of SEC-ICP-MS can be seen in its simplicity, limited formation of artefacts during the separation and the low detection limits achievable (Mestek, Kominkova, Koplik, Borkova, & Suchanek, 2002). Various trace metals, including Zn, in a variety of nuts, have been extensively studied by SEC-ICP-MS (Naozuka et al. 2010; Wuilloud et al., 2004). In Fig. 5 a SEC-ICP-MS chromatogram is shown of a pumpkin seed extract (extractant: 0.03 mol l^{-1} TRIS–HCl, pH = 7.4; V/m = 100 ml g^{-1}). Even though the separation run was extended to 50 ml, with the intention to control the elution of all fractions, no peaks were observed after the total column bed volume (24 ml).

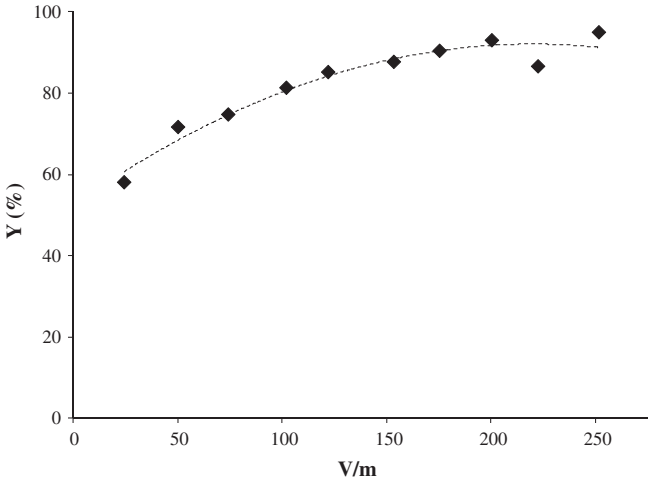

Fig. 4. Extraction yield for water soluble Zn as a function of the V/m ratio in pumpkin seeds calculated via $Y = 100 \times ([V/m] \times ([V/m] + K)$ and $K = a_1/c_1$, where Y = extraction yield; c_1 = equilibrium element concentration in extract (μg ml^{-1}); a_1 = remaining equilibrium element concentration in sample (μg ml^{-1}); K = equilibrium constant.

Zn species associated with peak number 3 (Fig. 5) may be comparable to the Zn species with molecular weights of 11300, 11,900 and 12,300 Da found by Naozuka et al. (2010) in the water-soluble fraction of brazil nuts. They were identified as isoforms of the water-soluble sulphur-rich 2S-albumin by Dernovics, Giusti, and Lobinski (2007) and are known as storage proteins in pumpkin seeds. Similar results were obtained by Wuilloud et al. (2004) for NaOH and HCl extracts of different nut samples 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 amongst a MW fraction of 12,000–13,000 Da and a lower MW fraction of 1300 Da, comparable to peak number 4 (Fig. 5). The fraction under peak number 5 (Fig. 5) has a molecular weight very close to Zn-glutathione in oxidised form (612 Da) indicating that glutathione may play a role as a Zn-binding ligand in pumpkin seeds. Glutathione is the most abundant 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, Gara, Asard, & Horemans, 2002). Michalke (2004) already indicated that low molecular weight complexes in plants 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 though the Zn fraction between peak 1 and 2 (Fig. 5) is characterised by an undefined hump, suggesting that unstable Zn-ligand may 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 or Zn was bound nonspecifically to unknown ligands with free binding sites for Zn.

3.5. Bioaccessible Zn Species

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) may lead to interferences in the measurement of Zn by ICP-MS; therefore it is essential to prepare the Zn

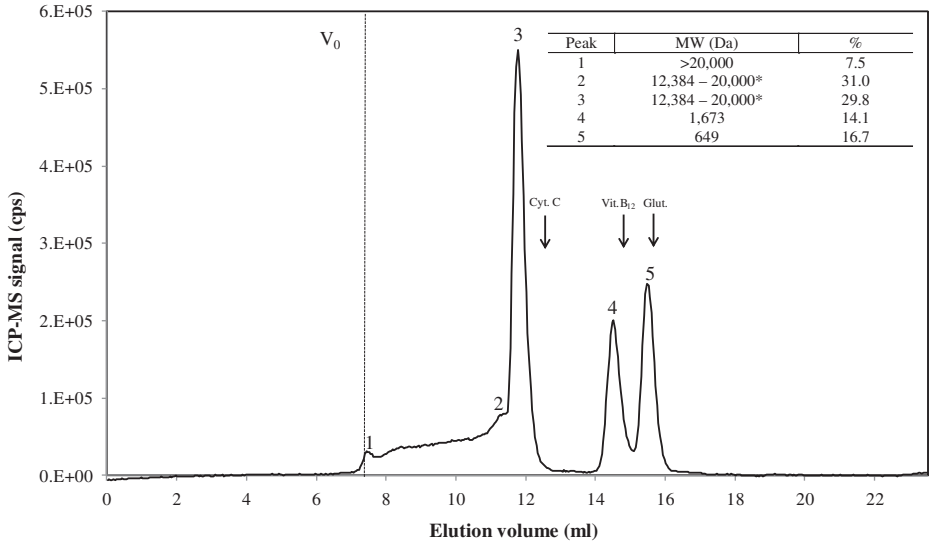

Fig. 5. Fractionation profile of Zn (cps) in pumpkin seed extract after separation on a Superdex peptide column eluted with aqueous buffer (0.03 mol l^{-1} Tris–HCl, pH = 7.4). The arrows represent cytochrome C (Cyt C; 12,384 Da), vitamin B_{12} (Vit. B12; 1355.4 Da) and glutathione in oxidised form (Glut; 612 Da). In the inserted table the chromatographic results are summarised (% refers to the area under the peak with respect to the total area in the chromatogram); peaks denoted with ∗ are out of the calibration range (and also out of the optimal separation range) implying that precise molecular weights can not be reported.

standards in the digestion fluids. Not surprisingly, the release of Zn in a simulated gastrointestinal tract after enzymatic-assisted extraction is significantly higher than after ultrasound-assisted extraction with "gentle" speciation extractants. The comparison of the Zn elution profile of pumpkin seeds extracts with a Zn^{2+} standard solution (not shown) confirms complete decomposition of previously indicated Zn species in the human gastrointestinal tract. It seems that the only Zn species present is Zn^{2+} with column recoveries of 98% and 104% for pumpkin seeds and pure Zn^{2+}, respectively. However, the presence of low-molecular-weight Zn complexes with, for example, single amino acids cannot be excluded with this method. Assessment of the bioaccessibility *via* simulation of the digestive tract shows that the amount of Zn after the stomach phase (79.2% of total Zn) is lower than the amount of Zn after the complete (stomach + intestinal phase) digestion (52.9% of total Zn). This may most probably be due to the presence of phytates which are known to form an insoluble complex with Zn^{2+}. Pumpkin seeds contain phytates which accumulate in the seeds during the ripening period (Kumar, Sinha, Makkar, & Becker, 2010) and can bind Zn^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+} and other divalent cations, where bioaccessibility of Zn^{2+} was reported to be the most impaired in humans. Because of its capacity to immobilise certain elements from the diet, phytate has already been considered as an antinutrient. In several studies, summarised by 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. Phytate–mineral complexes are largely insoluble at the pH of the small intestine and therefore formation of Zn–phytate complexes might also increase faecal losses of endogenous Zn.

4. Conclusions

From the above findings it follows that the "sequential" analytical approach offers excellent opportunities to study the speciation of Zn (and other elements as well) in seeds and other edible plants, gradually unravelling the physicochemical properties of Zn and elucidating the "deteriorating" effect of the gastrointestinal tract on the integrity of the Zn species. In spite of the complete decomposition of Zn species under simulated human stomach conditions, the subsequent extraction step simulating digestion in the intestines shows that Zn is less available in this environment, disproving conclusions of Zn speciation studies done in the past suggesting that low-MW Zn species may have nutritional value. Speciation of essential elements prior to a severe digestion procedure, e.g., as encountered in the human gastrointestinal tract, seems irrelevant, but should be of interest to nutritionists, dieticians and planners in the fields of nutrition and food technology. Of course we should be aware that pumpkin seeds are only one constituent of the 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.

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