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Comparison of zinc species in two specimens of edible plants and their fate in the human gastrointestinal tract*

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Abstract: The objectives of this work were to get information on Zn species in two different specimens of edible plants (pumpkin seeds and iceberg lettuce) and simulation of their behavior in the human gastrointestinal tract. An array of analytical techniques was used to aid in this research: ultrasound-assisted variable volume extraction, size exclusion chromatography (SEC), and physiologically based extraction tests (PBETs); elemental detection was performed by inductively coupled plasma-mass spectrometry (ICP-MS). Results show that pumpkin seeds and iceberg lettuce have different Zn species fingerprints (in water extracts) with a high (ca. 70 %) low-molecular-weight fraction (ca. 500 Da) in iceberg lettuce and a high (ca. 60 %) intermediate/high-molecular-weight fraction (10–20 kDa) in pumpkin seeds. When these Zn species are subjected to conditions simulating the human stomach (pH ~ 2) complete scrambling to their basic ionic form (Zn^{2+}) takes place. Under conditions simulating the digestion in the intestines (pH ~ 7) formation of insoluble Zn complexes occurs, especially for pumpkin seeds, which may be related to antinutrients like naturally present phytate, leading to reduced Zn bioaccessibility in the small intestine.

Keywords: analytical protocols; nutrition; physiologically based extraction test; Zn speciation.

INTRODUCTION

Zn was already reported in the 1960s as an essential micronutrient for human health [1]. Because of specific chemical properties, Zn is extensively involved in cellular and subcellular metabolic processes of the human body. Zn may act as a cofactor of numerous enzymes, as a structural element of enzymes, proteins, and biomembranes, as an initiator of transcription and gene expression processes, and as a part of other biological functions without the risk of oxidation damage [2]. Lately, Zn is receiving increased attention as a public health problem because of adverse health effects which are primarily associated with the consumption of food with low Zn content or unavailable forms of Zn. Recent estimates from

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the FAO (Food and Agriculture Organization) based on food balance data from 176 countries suggest that approximately 20 % of the world's population is at risk of Zn deficiency [3]. Hence, Zn is recognized as a nutritional problem worldwide, both in developed and developing countries.

While biological functions of essential elements are quite well understood, the chemical form in which they occur in different food items is often unknown. Elemental speciation at the macromolecular level is important in physiology, biochemistry, and nutrition. Differences in valency state, complexation by inorganic and organic ligands, and the occurrence of covalent organometallic species determine the availability and toxicity of metals, consequently having high importance in food chemistry and also in clinical and biological disciplines [4]. Identification of the elemental species is not only important for gaining insight into metal-involving biochemical processes on the cellular level [5], but is also critical for a better understanding of the nutritional aspects. In contrast to some toxic trace elements, limited information is available on the physicochemical forms of Zn in edible plants, and information on their fate upon digestion in the gastrointestinal tract is largely lacking although this is of prime importance from a bioaccessibility and bioavailability point of view, especially for people who are pure vegetarians (plant-based diet only). Furthermore, the essential 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 [6].

A limitation of many studies on chemical analysis of trace elements in food is that they often do not take into consideration their bioaccessibility and/or bioavailability. Bioavailability is generally defined as the proportion of the ingested component that reaches the systemic circulation and is available for use in metabolic processes [7], while the fraction of the component that is mobilized from the matrix into the digestive juices is defined as the bioaccessible [8]. There are numerous in vivo approaches to study bioavailability, although each of them with its own limitations. However, in vitro methods for screening of bioaccessibility and bioavailability based on simulation tests are simple, although limited in information retrieval. Simulation of bioaccessiblity processes taking place in the gastric and intestinal phases is related to extractability at low pH (~1) and potential complexation with chelating agents at high pH (~7), respectively. Soluble chelates seem to increase the Zn solubility and its absorption [9].

The focus of this work is on characterization of Zn species in two specimens of edible plants (pumpkin seeds and iceberg lettuce) and investigating their bioaccessibility in the human gastrointestinal tract by simulation tests. Two different plant specimens were compared to explore similarities/differences in plant-related Zn species. Plant specimens of particular nutritional importance were chosen, viz. pumpkin seeds as representatives of one of the most concentrated vegetarian sources of Zn and iceberg lettuce as a fast grower that is readily available in supermarkets throughout the year.

EXPERIMENTAL

A slightly modified "sequential" analytical approach as described in detail before [10] was used in this work and is summarized below.

Sample preparation

Both pumpkin seeds and iceberg lettuce were purchased from local markets in Ljubljana, Slovenia, in one single batch. For pumpkin seeds, we processed a total of 300 g of seeds, and for iceberg lettuce the leaves of three complete plants with a total fresh weight of 1410 g were processed. This processing included washing with Milli-Q water (18.2 MΩ cm) and air-drying (48 h at 40 °C), followed by grinding in an agate ball mill (iceberg lettuce leaves) or grinding in a domestic grinder (pumpkin seeds). All the equipment for sample preparation was washed with 10 % *V*/*V* HNO_3 and rinsed with Milli-Q water prior to preparation.

Determination of total Zn

Ground samples were weighed (0.5 g) in poly(tetrafluoroethylene) (PTFE) vessels in triplicate. Samples were digested in a microwave (Milestone Ethos 1 Advanced Microwave Digestion Labstation) using HNO_3 (7 mL 65 % *V/V*) and H_2O_2 (1 mL 30 % *V/V*) using 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. Standard solutions for calibration were prepared from a stock multielement solution (ICP Multielement Standard IV, Merck) in the concentration range 0–500 μg L^{-1} with a matrix resembling the digests as closely as possible. To correct for potential instrumental drift, internal standardization was used by spiking both the standard and sample solutions with Y, Sc, Ge, and Gd (50 μg L^{-1}).

Aqueous extraction of Zn and its species

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. Ultrasound-assisted aqueous extraction was performed with an ultrasonic homogenizer (LABSONIC® M, Sartorius, Gottingen, Germany; 100 W, 30 kHz frequency) equipped with a titanium probe [80 mm × ∅ 3 mm]). 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 analyzed the same day.

Determination of Zn species

Both plant samples were subjected to the aqueous extraction procedure; prior to extraction of Zn species from the pumpkin seeds a clean-up step (removal of lipids) was applied [11]: 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. For separation of the extracted Zn species, a Superdex Peptide HR 10/30 column (Pharmacia Biotech, Sweden; separation range, 0.1–20 kDa) fitted with a pre-column polyetherether ketone (PEEK) filter (0.22 μm) was used. The column was calibrated with selected calibrants [cytochrome C (12 284 Da), aprotinin (6500 Da), vitamin B_{12} (1355.4 Da), glutathione-oxidized (612 Da), trycine (179.2 Da) and glycine (75.1 Da)] under sample separation conditions using a DAD (G1315A, Agilent Technologies HP 1100). To verify the complexation of Zn^{2+} with phytochelatins, PC2–6 were purchased from AnaSpec (Freemont, CA). The outlet of the size exclusion chromatography (SEC) column was directly interfaced with the inlet of the inductively coupled plasma-mass spectrometry (ICP-MS) nebulizer. The percentage distribution of Zn among different molecular size fractions was evaluated by relating the area of a particular peak to the total area under the chromatogram. For quantification, the peak areas for the respective Zn species were compared to the area obtained upon injection of the sample in the absence of the column and pre-column under identical instrumental conditions. To retrieve the areas from the Zn elution profiles, the peak fitting tool in Origin 8.1 SR4 (OriginLab Corporation, Northampton, USA) was used.

Determination of Zn species bioaccessibility

To estimate the bioaccessibility of Zn and its species in the human digestion tract (stomach and small intestine) a modified version [10] of the original enzymatic-assisted physiologically based extraction test (PBET) protocol [8] was used. The PBET is an in vitro test method for predicting the bioaccessibility of metals from a solid matrix. It considers gastrointestinal tract parameters of the human body, including stomach and small intestinal pH and their chemistry. Samples were weighed in 50-mL "Falcon" tubes in which the whole two-step PBET procedure with the respective gastric (pH = 2.5) and

intestinal (pH = 7.0) phase simulants was performed in a reciprocal shaker at 37 °C. After extraction, all PBET extracts were acidified (1 % *V*/*V* HNO_3) prior to analysis to prevent unwanted sorption. 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 a pH of 2. Because of potential interferences, standards were prepared in the digestion fluids.

RESULTS AND DISCUSSION

Plants constitute important components of ecosystems as they provide the transport pathways from the abiotic to the biotic environment, which for pure vegetarians translates into an intimate connection between soil and the human body. Regarding physico-chemical forms of Zn in edible plants, recently some more attention has been focused on different nuts [11,12], although insight into their fate upon digestion in the gastrointestinal tract was not revealed. On the other hand, the speciation of Zn in leafy vegetables is more extensively studied [13].

Total Zn

The total amounts of Zn found in pumpkin seeds (92.1 μg g^{-1}) and iceberg lettuce (62.0 μg g^{-1}) are in good agreement with research findings by others [14–16]. While the age of plants has a significant influence on the status of minor and trace elements [17], the highest Zn content can frequently be observed in young plants. However, it needs to be stressed that the content of trace elements and also other compounds may vary considerably depending on natural (soil conditions, climate, and genetic factors) and anthropogenic (agricultural practice, new cultivars, different fertilizers) parameters. The transport of trace elements in plants and their parts 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 [18]. In general, Zn belongs to the group of elements that is moderately mobile, from roots to above-earth parts, and according to Alloway it has already been generally accepted that Zn is transported in the plant either as Zn^{2+} or bound to organic acids [19].

Comparison of Zn species in aqueous extracts

Ultrasound-assisted extraction aids in the extraction process as it facilitates and accelerates the mechanical effect of breaking up the matrix, causing smaller particles to be produced, thereby exposing more surface area to the extractant and enhancing the homogenization.

Water-soluble fraction

The quantitative distribution of Zn between the water-soluble and -insoluble fractions of pumpkin seeds and iceberg lettuce was investigated (Fig. 1). 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 that show a degree of binding, i.e., elements that are not irreversibly bound to the sample tissue, show an extraction-dependency with volume. This phenomenon has been extensively studied by Van Elteren et al. [20] 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, Zn shows a significantly lower water soluble fraction in pumpkin seeds (34.6 % of total Zn) than in iceberg lettuce (60.2 % of total Zn) which may be the consequence of Zn incorporation in macromolecules, especially in proteins, where it plays also a structural role and forms strong complexes with polar groups containing O, N, S [21]. In some cases, it is bound so tightly that

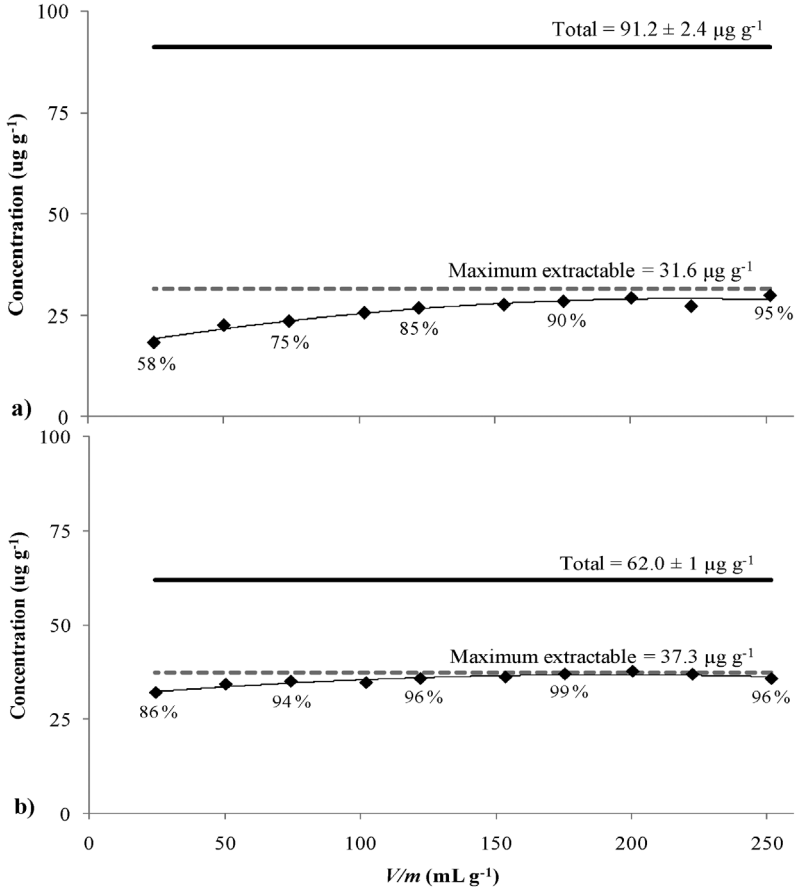

Fig. 1 Extraction yields (in µg g^{-1}) of water-soluble Zn (related to total and maximum extractable) as a function of the *V*/*m* ratio (mL g^{-1}) in (a) pumpkin seeds and (b) iceberg lettuce.

it can be removed only with severe chemical treatment. From Fig. 1 it can also be seen that the extraction yield is indeed *V*/*m* ratio-dependent for both cases and that for pumpkin seeds the interactions between target Zn and sample matrix are stronger than for iceberg lettuce.

Size fractionation by SEC-ICP-MS

Because of the capability of ICP-MS to detect metals at very low levels in chromatographic effluents, the coupling of SEC to ICP-MS has already been widely applied for elemental speciation in foodstuffs of animal as well as of plant origin. It should be noted that coelution of a metal and a particular biomacromolecule is not an ultimate proof but only an indication that they belong together [22].

Contradictory to findings by Waldner and Günther [23], highlighting the occurrence of analogous low-molecular Zn species in different vegetables (kohlrabi, Chinese cabbage, chard, leek, spinach, Jerusalem artichokes), from Fig. 2 we can clearly see differences in the occurrence of low-molecular Zn species (<20 kDa) in our two plant specimens. The size-related Zn fractions (in % of the extracted Zn and % of the total Zn) for aqueous extracts of iceberg lettuce and pumpkin seeds are summarized in Table 1. Evaluation of the data shows us that iceberg lettuce has a high low-molecular-weight Zn fraction (ca. 500 Da, peak 5a) and pumpkin seeds a high intermediate Zn fraction (12 384–20 000 Da, peaks 2b–6b). In spite of the differences, it seems that some Zn fractions may be comparable, potentially having the same complexing ligands, as shown by similar molecular weights: 508 and 1503 Da for iceberg

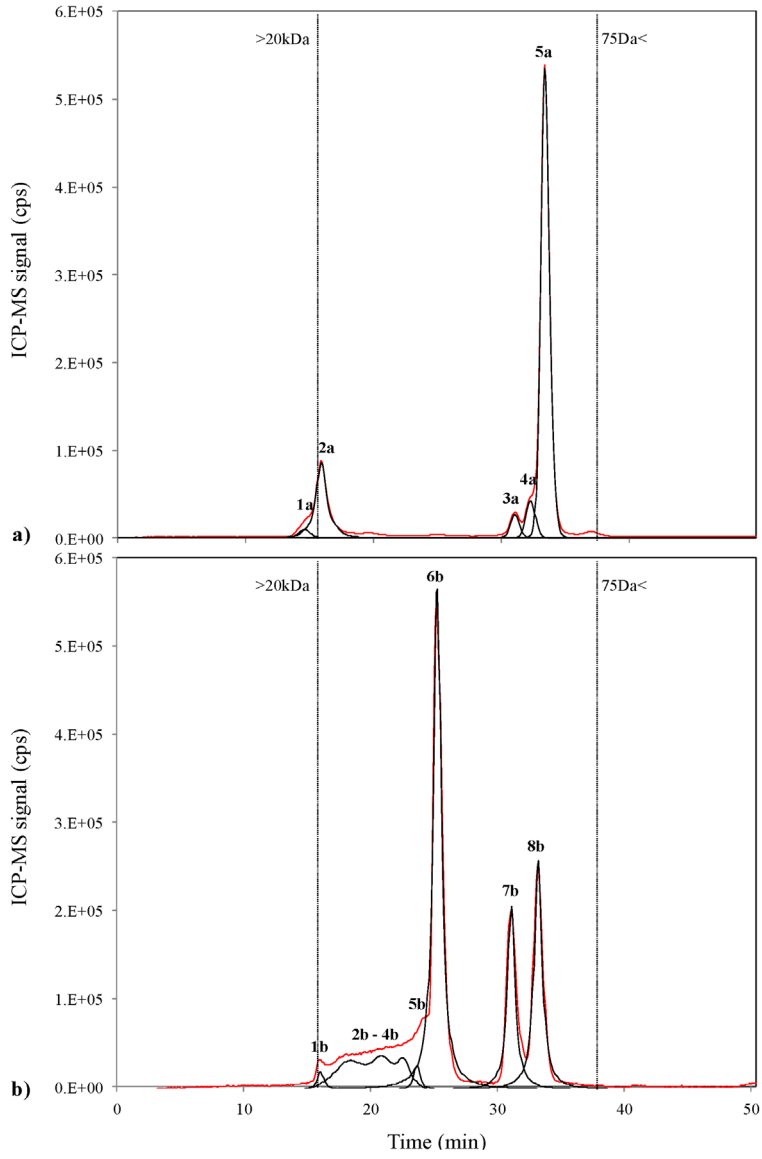

Fig. 2 Fractionation profiles of Zn for aqueous extracts of (a) iceberg lettuce and (b) pumpkin seeds after separation on a Superdex peptide column and elution with an aqueous buffer (0.03 mol L^{-1} Tris-HCl, pH = 7.4). The chromatogram was deconvoluted using a multiple peak fitting routine to identify the peaks in the chromatogram (Table 1); the fit quality can be seen from the correlation coefficients (R^2) of 0.999 and 0.985 for iceberg lettuce and pumpkin seeds, respectively. The useable separation range of the column is indicated by the markers >20 kDa and <75 Da.

lettuce and 649 and 1673 Da for pumpkin seeds. Even though the match is not perfect, maybe due to highly varying amounts of organic material which may slightly affect the chromatographic behavior and thus the absolute retention time, it is close enough to suggest that these samples may have similar ligands responsible for Zn-binding. Günther and Waldner [24] gave evidence that Zn in several vegetal samples is predominantly present as species with a molecular mass lower than 5 kDa, whereas a fraction of 1–34 % is bound to molecules with a molecular weight above 30 kDa. Although Walker and

Table 1 Molecular size distribution of Zn given as fractions (%) of the extractable Zn and the total Zn in iceberg lettuce and pumpkin seeds after SEC-ICP-MS; the extractable Zn concentrations in water were taken from Fig. 1 at a *V*/*m* ratio of 100 mL g^{-1}.

Iceberg lettuce				Pumpkin seeds			
Peak	MW (Da)	Fraction of extractable Zn (%)	Fraction of total Zn (%)	Peak	MW (Da)	Fraction of extractable Zn (%)	Fraction of total Zn (%)
1a	>20 000	2.2	1.2	1b	>20 000	1.3	0.4
2a		21.2	11.8	2b–4b		22.2	6.3
3a	1503	5.2	2.9	5b	12 384–20 000*	1.6	0.5
4a	811	7.5	4.2	6b		41.2	11.6
5a	508	64.1	35.8	7b	1673	14.5	4.1
				8b	649	19.2	5.4

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

Welch [25] indicated phytochelatins (PCs) as potential ligands to form complexes with metals in lettuce, in this work only one peak in iceberg lettuce could be positively matched with a PC from a test group of synthetic PCs (PCs 2–6). In the size exclusion chromatogram, PC2 overlapped perfectly with peak 3a of the aqueous iceberg lettuce extract (not shown) although the certified molecular weight of PC2 (540 Da) and calculated molecular weight of peak 3a (1503 Da) are dissimilar, probably due to the fact that the PC2 molecule in a standard solution has a more linear form and in real samples matrices in the presence of complexing heavy metals are a more globular form. Ergo, PC2 is probably not the compound responsible for the occurrence of peak 3a in the iceberg lettuce chromatogram.

The high intermediate Zn fraction identified in pumpkin seeds, but not in iceberg lettuce, may be comparable to the Zn species identified in earlier work: Naozuka et al. [12] found Zn species with molecular weights of 11.3–12.3 kDa in the water-soluble fraction of Brazil nuts, and Wuilloud et al. [11] found Zn species with molecular weights of 12–13 kDa and 1.3 kDa in NaOH/HCl extracts of both sunflower and Brazil nut samples. Peak 6b (Table 1) eluted closely to the calibration standard with the highest molecular weight (12 384 Da) may correspond to isoforms of the water-soluble sulfur-rich 2S-albumin identified by Drenovics et al. [26] in brazil nuts, also known as soluble storage proteins in pumpkin seeds [27]. The Zn fraction associated with peaks 2b–4b (Fig. 2) is characterized by an undefined hump in the chromatogram, suggesting that unstable Zn ligands may have disintegrated during separation, although little evidence is available to prove this suggestion.

In addition to proteins and peptides, plants often contain a large number of low-molecular-weight compounds that play an important role in the handling of metals in plants as well. Among them, some organic acids like citrate, amino acids like histidine, and phosphate derivatives are of particular interest [28].

Comparison of Zn species bioaccessibility

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 [29]. There is general agreement that the uptake of essential trace elements from food depends on the chemical form in which an element is present. If an element in food is present in an insoluble form it is unlikely to be absorbed from the gastrointestinal tract and will be excreted [30]. Consequently, uptake of the element from the diet varies depending on food preparation procedure, actual mixture of different foods consumed, and host factors. The release of Zn in a simulated gastrointestinal environment (PBET) used in this work gave evidence of significantly higher extraction yields (Table 2) after enzymatic-assisted

Table 2 Zn concentration in digests after a simulated gastrointestinal digestion of iceberg lettuce and pumpkin seeds.

Sample	After stomach phase only			After stomach and small intestinal phase		
	Conc. (μg g^{-1})	RSD (%)	% of total Zn	Conc. (μg 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

extraction than after ultrasound-assisted extraction. Comparison of the Zn elution profiles in the SEC-ICP-MS chromatograms (Fig. 3) of pumpkin seeds and iceberg lettuce extracts with these of a Zn^{2+} standard solution (column recoveries of 98, 102, and 104 % for iceberg lettuce, pumpkin seeds, and pure Zn^{2+}, respectively) confirms complete decomposition of previously indicated Zn species to Zn^{2+} during the simulated gastric phase.

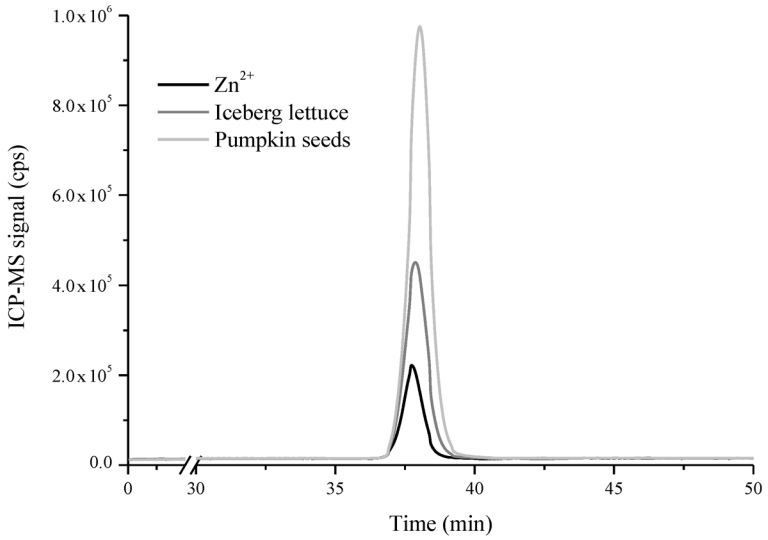

Fig. 3 Fractionation profiles of Zn for extracts of pumpkin seeds and iceberg lettuce after a simulated stomach digestion step compared to Zn^{2+} in stomach digestion fluid after separation on a Superdex peptide column eluted in an aqueous buffer (0.03 mol L^{-1} Tris-HCl, pH = 2.5).

After the chyme passes from the stomach into the neutral pH environment of the duodenum and small intestine, complexation of Zn^{2+} with ligands that have "survived" the stomach phase may occur, potentially leading to reduced bioaccessibility as discussed further. From Table 2, it is clear that the enzymatic-assisted extraction procedure in both samples releases the majority of Zn in the stomach phase (80–90 % of total Zn). Nevertheless, a small portion remains unextractable despite the severe extraction conditions, most likely because of its role as a structural element [21]. An aberration in this extraction behavior can be seen for pumpkin seeds where the amount of Zn after the stomach and intestinal phases is lower than that of the stomach phase only, while for iceberg lettuce no significant differences can be observed. This phenomenon may be associated with the presence of phytate (a predominant storage form of phosphorous) in pumpkin seeds, which is known to effectively bind minerals. Phytates accumulate in the seeds during the ripening period and since they are negatively charged under

physiological pH (6–7) they are able to bind divalent cations like Zn^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Cu^{2+} and reduce the bioaccessibility of Zn^{2+} in humans [31].

Dietary phytates are soluble under acidic conditions in the stomach and precipitate at neutral pH in the intestine [32]. Formation of Zn-phytate complex or coprecipitation of Zn as a Zn–Ca-phytate complex might increase fecal losses of endogenous Zn as reported by others [33]. Although the amount of dietary phytate commonly present in plant foods does not completely prevent the absorption and utilization of dietary Zn [34], a significant portion may be "lost" as is suggested by the data in Table 2. Phytate is not the only compound impairing the trace element absorption in the intestines, but it seems that it is the most effective [32]. Other components like inorganic phosphate, polyphenols, and nondigestible dietary fibers reduce the absorption of trace elements as well. Although dietary phytate is mostly described as an "antinutrient", its positive effects on human health (anticancer, antioxidative, and anticalcification) should not be overlooked [32].

From the bioaccessibility results above, we can calculate the required daily consumption of pumpkin seeds or iceberg lettuce to meet the reference values for Zn intake (10 mg/day and 7 mg/day for men and women, respectively) for adolescents and adults in the general population; in these reference values, the daily losses (2.2 and 1.6 mg for men and women, respectively) and an average absorption rate of 30 % are already taken into account [35]. In Table 3, the calculated daily consumption amounts of pumpkin seeds or iceberg lettuce upon 100 % availability or fractional availability (related to the bioaccessibility values in Table 2) are given. It can be seen that consumption of considerable amounts of the studied plant specimens is necessary to fulfil the daily requirements, implying that supplementation with other dietary products is essential. Especially during outstanding human body status like stress, obesity, trauma, rehabilitation after starvation, dilutional effects of rapid growth (such as the catch-up growth of premature infants), pregnancy, and lactation which already recognizes the essentiality of Zn for the child in the uterus, such supplements are mandatory [36].

Table 3 Required daily consumption of pumpkin seeds or iceberg lettuce necessary to meet reference values for Zn intake [36] under the assumption of 100 % availability (= total Zn) or fractional availability (= 52.9 and 89.1 % of total Zn for pumpkin seeds and iceberg lettuce, respectively; see Table 2).

Sample (total Zn)	Gender	Daily consumption	
		100 % availability	Fractional availability
Pumpkin seeds (91.2 μg/g)	Male	110 g	208 g
	Female	77 g	146 g
Iceberg lettuce (2.4 μg/g)*	Male	4.2 kg	4.5 kg
	Female	2.9 kg	3.2 kg

*Total amount was normalized to fresh weight.

CONCLUSIONS

The results presented and discussed in this work have not only revealed plant-dependent Zn fingerprints in water extracts but also possible degradation patterns in the human gastrointestinal tract, which might be of nutritional importance. Experimental findings confirm that pumpkin seeds are a significantly more abundant source of Zn than iceberg lettuce. While the extraction yield of water-soluble Zn is *V/m* ratio-dependent for both plant specimens, interactions between Zn and sample matrix are stronger for pumpkin seeds. The size-related Zn fractionation gave evidence about different Zn species fingerprints (in water extracts) with a high low-molecular-weight fraction (ca. 500 Da) in iceberg lettuce and a high intermediate-molecular-weight fraction (10–20 kDa) in pumpkin seeds. When these Zn species are subjected to conditions simulating the human stomach, complete decomposition to their basic ionic form (Zn^{2+}) takes place, disproving conclusions of Zn speciation studies done in the past, suggesting that

low-molecular-weight species may have nutritional value. The subsequent extraction step simulating digestion in the intestines further shows that Zn is sometimes less available in this environment. In particular, certain components in pumpkin seeds, possibly phytates, may be responsible for complexation of Zn^{2+} into insoluble form, leading to reduced biosorption and potentially to Zn deficiency. This is particularly important for pure vegetarians as their diet is by definition poor in Zn and rich in compounds depressing utilization of Zn to humans.

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