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Application of size-exclusion chromatography–inductively coupled plasma mass spectrometry for fractionation of element species in seeds of legumes ☆

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Abstract

Fractionation of soluble species of P, Mn, Fe, Co, Ni, Cu, Zn, Se and Mo in pea and lentil seeds was made by on-line hyphenation of size-exclusion chromatography (SEC) and inductively coupled plasma mass spectrometry. Seed samples were extracted with 0.02 mol l^{-1} Tris–HCl buffer solution, pH 7.5. SEC was performed on Superdex 75 and Superdex Peptide columns (300×10 mm) with the same buffer solution as the mobile phase. Monitoring of oxide ion $^{47}(PO)^{+}$ was used for detection of phosphorus compounds. Other elements were detected as ions of ^{55}Mn, ^{57}Fe, ^{59}Co, ^{62}Ni, ^{65}Cu, ^{66}Zn, ^{82}Se and ^{95}Mo nuclides. Elements in individual elution zones were quantified using external calibration. Complete chromatographic recoveries of elements were found in cases of phosphorus, nickel and copper. Substantial parts of manganese and zinc, as well as traces of cobalt, selenium and molybdenum are retained on the column. Injection of EDTA solution removes these elements from the column. Chromatographic profiles of pea and lentil samples are very similar for all elements except Mo. Main element species in the high-molecular-mass region (approx. 190 000 rel. mol. mass unit) were detected in case of Fe. Low-molecular-mass species (<2000 rel. mol. mass unit) as major element forms are typical for Cu and Zn.

Keywords: Phosphorus; Trace elements

1. Introduction

Trace element speciation analysis in biological samples [1] consists of separation, detection and identification of individual compounds of the element. Data concerning chemical speciation of elements present in food are needed for more accurate evaluation of biological availability or toxicity of the element in the diet. Therefore speciation analysis gives new dimension to research of trace elements in food chemistry, biochemistry and nutrition science [2]. Speciation analysis represents a rather difficult analytical task. The most advanced methods are therefore applied to obtain full information about species identity and quantity. Recently hyphenated techniques became the main analytical tool of speciation analysis in biological matrices [3]. Liquid chromatography or capillary electrophoresis in combination with element-specific detection methods

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represents a frequently applied approach to element speciation. Element-specific detection is mostly based on atomic absorption spectrometry (AAS), inductively coupled plasma optical emission (ICP-OES) or mass spectrometry (ICP-MS).

Size-exclusion chromatography (SEC) is especially suitable for separation of element species of limited stability being frequently encountered in protein-rich matrices. These species include, e.g., metalloproteins or low-molecular-mass chelates of transition metals. The main advantages of SEC [3,4] are simplicity of application, compatibility of mobile phase composition with specific demands of certain biological sample and possibility of estimation of molecular mass of element species. On the other hand SEC is less effective in separation compared with other liquid chromatographic techniques. Therefore SEC is often applied as the first method for separation of the fraction containing metallobiomolecules of interest followed by another separation step with element-specific or species-specific detection or even mass spectrometric identification [5]. Nevertheless even SEC itself can give valuable data, e.g., in the case of element species fractionation in biological fluids or food samples. SEC with element-specific detection was successfully applied, e.g., to element species fractionation in blood and its compartments [6–8], milk whey [9] or extracts of vegetable food [10,11]. Our previous article [12] was focused on element species fractionation in soybean flour and common white bean seeds. Element behavior during chromatography as well as their distribution among high- and low-molecular-mass substances are comprehensively described here. Quantities of elements in fractions obtained by on-line SEC–ICP-MS were only approximately estimated by peak area normalization. The methodology was improved and a more accurate quantification of elements in species fraction was achieved. The quantification technique used is based on external calibration. The comparison of this method with isotope dilution as well as the uncertainty estimation were performed recently [13].

This article is devoted to description of fractionation and quantification of element species in pea and lentil seeds by SEC–ICP-MS. Experiments were focused on phosphorus and essential trace elements.

2. Experimental

2.1. Samples and sample preparation

Samples of pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.) seeds were obtained from a market. Before analysis the samples were milled to obtain low diameter particles ($\leq$0.5 mm). Their proximate compositions were 11.4 and 10.8% of moisture, 2.98 and 2.56% of ash, 20.8 and 24.0% of protein and 2.82 and 2.91% of fat for pea and lentil, respectively. These analyses were accomplished according to standard methods, see, e.g., Ref. [14]. Extracts of legume seeds for speciation analysis by SEC–ICP-MS were prepared by agitation of 1 g of sample with 50 ml of 0.02 mol l^{-1} Tris–HCl buffer solution, pH 7.5 for 1 h and centrifugation.

2.2. Analytical methods

2.2.1. Determination of total content of elements

Decomposition of seed samples and the respective extracts was accomplished by pressurized microwave digestion in PTFE vessels with a $HNO_3+H_2O_2$ mixture using a Uniclever digestion unit (Plazmatronika, Wroclaw, Poland). The concentrations of elements were determined by ICP-OES (phosphorus and iron) and ICP-MS (other elements). A PU 7000 ICP spectrometer (Philips Scientific, Cambridge, UK) and an ELAN 6000 ICP-MS system (Perkin-Elmer/Sciex, Norwalk, CT, USA) were used. Details on analytical methods are given in a previous paper [15]. The accuracy of the analytical results was verified by analysis of NIST standard reference material (SRM) 1515 Apple Leaves and SRM 1570a Spinach Leaves.

2.2.2. Size-exclusion chromatography

Two SEC columns (dimensions 300×10 mm for both) were applied, namely Superdex 75 HR 10/30 (optimum fractionation range 3000–70 000 rel. mol. mass unit) and Superdex Peptide HR 10/30 (optimum fractionation range 100–7000 rel. mol. mass unit) both produced by Amersham Pharmacia Biotech (Uppsala, Sweden). Molecular masses of eluted compounds were estimated by column calibration using a set of amino acid, peptide and protein

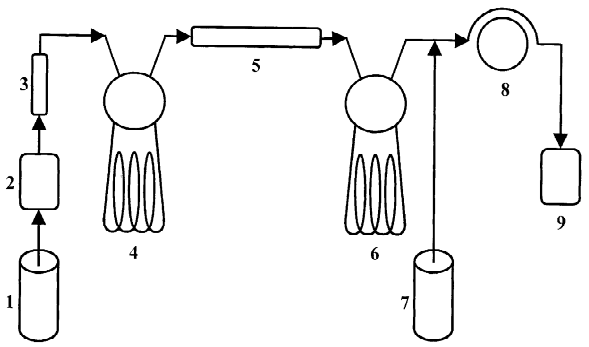

Fig. 1. Scheme of the SEC–ICP-MS apparatus; 1—mobile phase reservoir, 2—high-pressure pump, 3—Chelex 100 column, 4—first injector (for sample injection), 5—Superdex column, 6—second injector (for standard solutions injection), 7—reservoir of internal standard solution, 8—peristaltic pump, 9—ICP-MS system.

standards (obtained from Sigma, St. Louis, MO, USA) falling within the interval of molecular masses from 100 to 440 000 rel. mol. mass unit.

A buffer solution of 0.02 mol l^{-1} Tris–HCl, pH 7.5 served as the mobile phase. A high-pressure pump Varian Inert 9012 was used for mobile phase delivery. The flow-rate was 0.5 ml min^{-1}. Traces of transition metals were on-line removed from the mobile phase by passing through a glass column (150×10 mm) packed with Chelex 100 resin (Merck, Darmstadt, Germany) in the NH_4^+ form. The samples were injected into the Superdex column by a Rheodyne 9025 injector with a 100-μl polyether ether ketone (PEEK) sample loop (true volume 111 μl). The second injector—a Rheodyne 9025 with a 100-μl PEEK sample loop (true volume 103 μl)—was inserted between the Superdex column and detector. The latter injector served for post-column injection of calibration solutions of elements. At the end of apparatus a detector (optionally UV–Vis detector Varian 9050 operated at 280 nm or an ICP-MS system) was attached. All parts of apparatus were connected by PEEK or PTFE capillaries (0.25 mm I.D.). The back pressure of the chromatographic system ranged from 1.4 to 1.7 MPa. In case of ICP-MS detection the flow of effluent (0.5 ml min^{-1}) was joined together with a flow of internal standard solution (50 μg l^{-1} of In). The mixed flow (approx. 1.3 ml min^{-1}) was delivered by a peristaltic pump Gilson 212 to the cross-flow nebulizer of the ICP-MS system. The scheme of the whole apparatus is shown in Fig. 1.

Table 1
ICP-MS operating conditions used for SEC–ICP-MS analyses

Data acquisition: 30 sweeps per replicate, 1000 replicates per sample, duration of one replicate 3.00 s
Ion lens mode: autolens on (optimized to maximum of ^{9}Be, ^{59}Co and ^{115}In)
The first group:
Analytes (dwell times, ms): ^{55}Mn (10), ^{57}Fe (10), ^{58}Ni (10), ^{59}Co (12), ^{62}Ni (10)
Other ions (dwell times, ms): ^{39}K (4), ^{115}In (10), ^{118}Sn (10)
RF power: 1000 W
Nebulizer Ar flow: 0.875 l min^{-1}
The second group:
Analytes (dwell times, ms): ^{65}Cu (10), ^{66}Zn (10), ^{82}Se (14.5), ^{95}Mo (10)
Other ions (dwell times, ms): ^{83}Kr (14.5), ^{115}In (10), ^{118}Sn (10)
RF power: 1000 W
Nebulizer Ar flow: 0.875 l min^{-1}
The third group:
Analytes (dwell times, ms): $^{47}(PO)$ (17)
Other ions (dwell times, ms): ^{43}Ca (17), ^{46}Ti (17), ^{115}In (17), ^{118}Sn (17)
RF power: 1025 W
Nebulizer Ar flow: 0.975 l min^{-1}

RF, radio frequency.

Table 2
The total contents of elements and the percentages extractable to 0.02 mol l^{-1} Tris–HCl, pH 7.5, buffer solution

Element	Pea		Lentil	
	Total content	Extractability	Total content	Extractability
	($\mu g\ g^{-1}$)	(%)	($\mu g\ g^{-1}$)	(%)
P	4380±70	76±6	3380±50	66±4
Mn	10.2±0.2	68±5	14.3±0.3	49±2
Fe	53.5±1.9	49±4	81.5±2.2	41±7
Co	0.060±0.001	75±6	0.071±0.003	80±5
Ni	1.41±0.04	100±3	1.89±0.14	96±6
Cu	7.56±0.13	78±7	8.52±0.16	65±4
Zn	32.6±0.6	81±3	42.1±0.5	64±7
Se	<0.02	Not determined	1.03±0.04	91±12
Mo	2.64±0.05	100±4	12.7±0.5	62±8

All data are expressed as mean±SD of triplicate determination.

The duration of the SEC–ICP-MS analysis was 50 min. In the course of the first 10 min after injection of sample into Superdex column three standard solutions of elements were consecutively injected to the flow of mobile phase using the second injector. The obtained peaks of analytes were integrated and used as calibration data for quantification of elements in fractions separated by SEC. The isotopes selected for elements detection were ^{55}Mn, ^{57}Fe, ^{59}Co, ^{58}Ni, ^{62}Ni, ^{65}Cu, ^{66}Zn, ^{82}Se and ^{95}Mo. Measurement of oxide ion $^{47}(PO)^{+}$ was used for detection of phosphorus according to Ref. [12]. The signal of ^{115}In was monitored as internal standard. The main ICP-MS operating conditions used for SEC–ICP-MS analyses are summarized in Table 1. Three groups of analytes (first: Mn, Fe, Co, Ni; second: Cu, Zn, Se, Mo; third: P) were analyzed in separate chromatographic runs. Monitoring of other ions necessary for checking of interfering elements levels or correction of isobaric nuclides were included in ICP-MS methods, too. Dwell time values for individual analytes were adapted to obtain a replicate time of 3.00 s.

3. Results and discussion

High contents of essential elements are amongst the main nutritional features of legume seeds. Determined total contents of elements are summarized in Table 2. Soluble species were extracted from seed samples by Tris–HCl buffer solution. The pH value of extractant was chosen with respect to solubility of

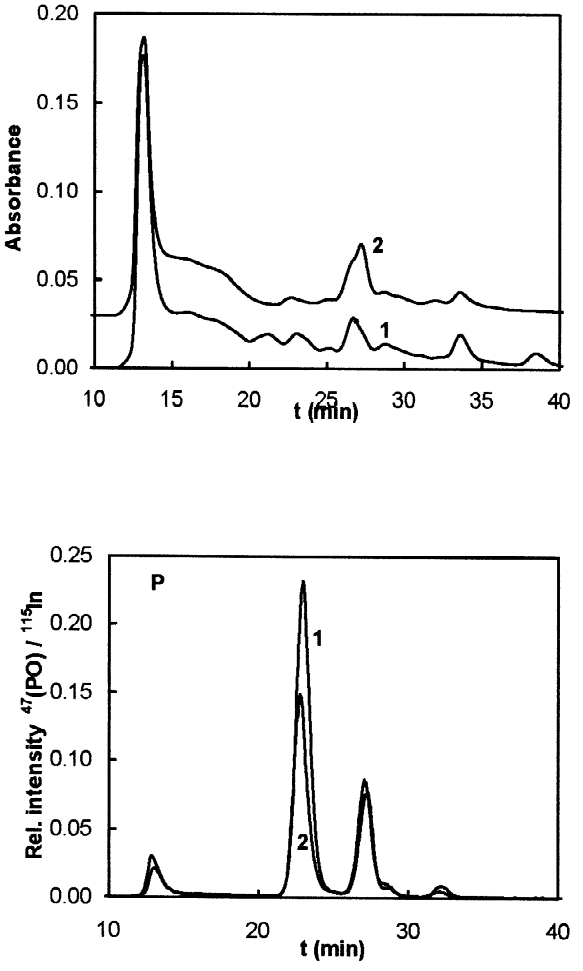

Fig. 2. Chromatograms of pea (line 1) and lentil (line 2) extracts on Superdex 75 column with UV (λ=280 nm) detection of organic compounds and ICP-MS detection of phosphorus species.

11S and 7S globulins representing the main proteins of seeds of leguminous plants [16]. Extractabilities of elements expressed as percentages of total element content are also given in Table 2.

The Superdex 75 and Superdex Peptide columns were applied to SEC–ICP-MS of legume seed extracts. Major chemical elements in extracts of legumes (K, Mg, Ca) do not interfere with ICP-MS detection of phosphorus and trace elements as alkali elements are retained on the column and are not eluted together with other elements. This fact is discussed in more detail in a previous paper [12]. In order to remove alkali elements originating from the previous sample the column must be rinsed at least by 40 ml of mobile phase before the next injection. As the quality of separation is concerned the separation of element species except phosphorus is better on the Superdex 75 column. However, it cannot be assumed that element peaks in chromatograms (see Figs. 2–4) represent individual chemical species in terms of chemical structure. The chromatograms of extracts on Superdex 75 column obtained by UV absorption and ICP-MS detection of P, Mn, Fe, Co, Ni, Cu, Zn, Se and Mo are shown in Figs. 2–4. Retention times, estimated apparent molecular masses of eluted species and element quantities corresponding to individual peaks are summarized in Table 3. In cases of low-molecular-mass species the molecular masses were more correctly estimated from retention times on the Superdex Peptide column.

Element quantities eluted by SEC were compared with known amounts of elements present in injected sample extract and chromatographic recoveries were

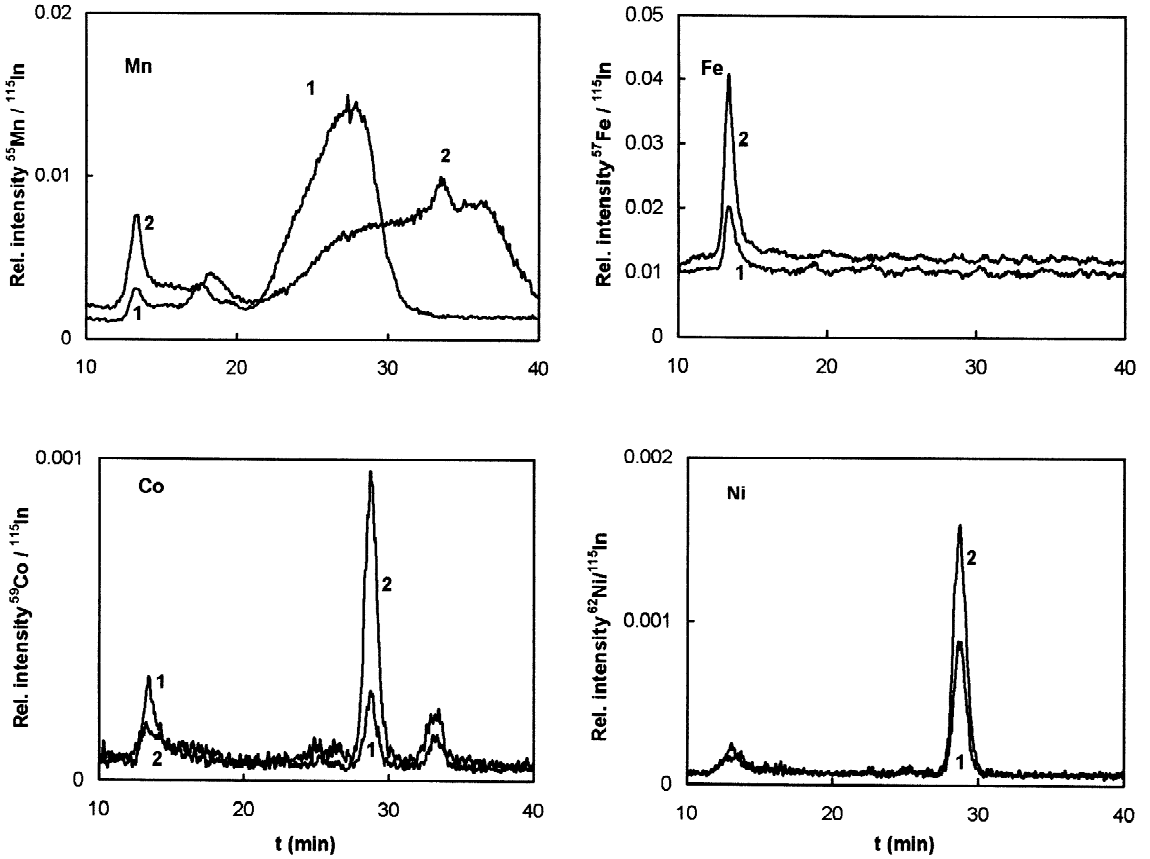

Fig. 3. Chromatograms of manganese, iron, cobalt and nickel containing compounds in pea (line 1) and lentil (line 2) extracts obtained by SEC–ICP-MS using the Superdex 75 column.

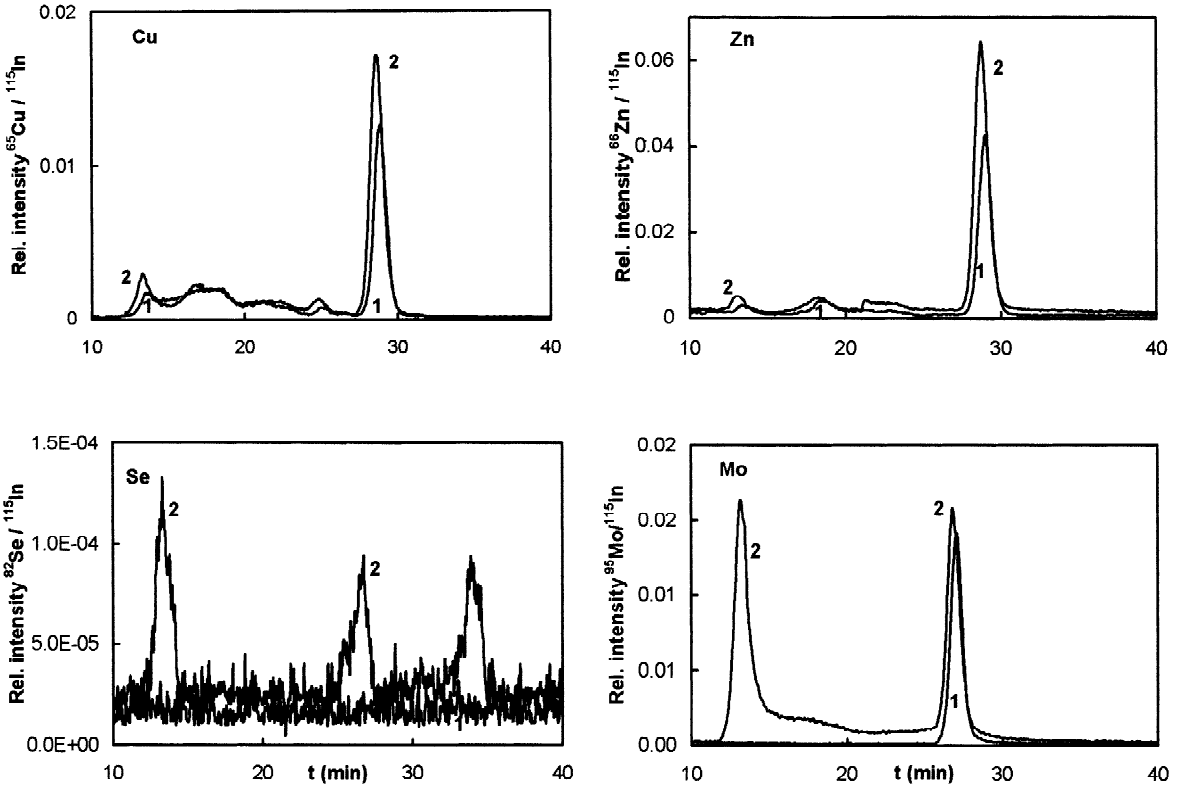

Fig. 4. Chromatograms of copper, zinc, selenium and molybdenum containing compounds in pea (line 1) and lentil (line 2) extracts obtained by SEC–ICP-MS using the Superdex 75 column.

calculated. While recoveries of phosphorus, copper and nickel ranged from 99 to 103%, in the case of zinc only 63 and 65% of the injected amount was recovered for pea and lentil, respectively. Manganese eluted within 10 and 50 min represents approx. 50% of injected amount for both samples. Recoveries of cobalt were 88 and 80% and those of iron were 80 and 102% for pea and lentil, respectively. In case of molybdenum 90 and 80% of the element were recovered. In the course of chromatography of lentil extract 93% of selenium were recovered. Incomplete recoveries of some studied elements were caused by low stability of some element species. The extracted chelates of metals with biological ligands dissociate during chromatography or even during sample preparation. The liberated metal ions are then adsorbed on the gel support of the SEC column. This fact was proved by consecutive injection of 0.002 mol l^{-1} EDTA solution, which removed all adsorbed transition metals from the SEC column. EDTA solution was therefore applied to column cleaning between individual sample analyses. It was observed that the species adsorbed on column ''contaminated'' by previous sample could be eluted with some chelating components of the next sample. Therefore EDTA treatment of the column is necessary before each analysis in order to avoid erroneous results and artifacts.

Elution profiles of phosphorus in pea and lentil extracts are very similar one to another. The estimated molecular masses of the major phosphorus species are approx. 7000 rel. mol. mass unit, whereas those of the minor phosphorus species are <2000 rel. mol. mass unit for both samples. Moreover phosphorus elution profiles show appreciable similarity with those of soybean flour [11,12]. Phosphorus elution profiles of all mentioned samples show dominant phosphorus peak in the medium-molecular-

Table 3
Summary of SEC–ICP-MS analyses of pea and lentil extracts on the Superdex 75 column

Element	Pea[a]				Lentil[a]			
	t_R (min)	$M_r \cdot 10^{-3}$ (rel. mol. mass unit)	Element (ng)	Quantity (%)[d]	t_R (min)	$M_r \cdot 10^{-3}$ (rel. mol. mass unit)	Element (ng)	Quantity (%)[d]
P	13.05	221[b]	575	8	12.85	237[b]	679	13
	22.95	6.5	4900	66	**22.75**	7	2794	54
	27.30	1.4[b] (0.2)[c]	1694	23	27.05	1.5[b] (0.2)[c]	1693	33
	32.20	0.25[b]	200	3	32.10	0.25[b]	103	2
Mn	13.35	198[b]	0.53	3	13.40	195[b]	0.77	5
	17.70	42	0.64	4	18.20	35	0.40	2
	Broad zone				Broad zone			
	21–32	?	6.77	45	22–40	?	7.17	42
Fe	**13.50**	188[b]	46	81	**13.40**	195[b]	86	103
Co	13.40	195[b]	0.044	44	13.25	205[b]	0.017	12
	28.90	0.8[b] (0.4)[c]	0.029	29	**28.70**	0.8[b] (0.4)[c]	0.077	55
	33.50	0.2[b] (0.2)[c]	0.015	15	33.45	0.2[b] (0.2)[c]	0.018	13
Ni	13.00	224[b]	0.84	23	13.05	221[b]	0.63	14
	28.80	0.8[b] (0.4)[c]	2.86	80	**28.75**	0.8[b] (0.4)[c]	4.00	90
Cu	13.85	166[b]	1.25	10	13.30	202[b]	1.33	10
	17.20	50	2.23	17	17.60	44	2.82	21
	18.35	34	1.38	11	21.80	10	1.48	11
	21.45	11	1.39	11				
	25.20	2.5[b] (1.2)[c]	0.36	3	24.85	3	0.71	5
	28.90	0.8[b] (0.4)[c]	6.56	51	**28.60**	0.9[b] (0.4)[c]	7.19	53
Zn	13.50	188[b]	1.42	2	13.20	225[b]	1.90	3
	18.60	31	3.34	6	18.25	35	2.71	4
	21.30	12	0.81	1	21.30	12	2.67	4
	29.00	0.8[b] (0.4)[c]	32.0	55	**28.70**	0.8[b] (0.4)[c]	34.2	52
Se					13.30	202[b]	0.64	32
					26.70	1.7[b] (0.6)[c]	0.61	30
					33.90	0.1[b] (0.1)[c]	0.63	31
Mo					**13.25**	205[b]	8.64	45
	27.05	1.5[b] (0.4)[c]	5.29	90	26.85	1.5[b] (0.4)[c]	6.80	35

[a] Retention time of dominant peak is designated by bold type.
[b] Estimation is out of optimum fractionation range of the column (3000–70 000 rel. mol. mass unit) given by producer.
[c] In parentheses, the molecular mass value estimated by analysis using the Superdex Peptide column.
[d] Percentage of total amount of the element present in injected volume of seed extract.

mass region and the second one in the low-molecular-mass region. In analogous experiments [12] it was found that the most abundant phosphorus fraction in sample of soybean flour is represented mostly by phytic acid.

Chromatographic behavior of manganese species on SEC columns is anomalous. Approximately 50% of manganese is not recovered at all. Manganese fraction, which is not recovered, is adsorbed on the column. Some species can be found in the high-molecular-mass region. These species represent about 7% of total manganese amount in seed extracts. The remaining manganese is eluted in very broad zones from 21 to 32 min and from 22 to 40

min for pea and lentil, respectively. Some redistribution of manganese from original species probably occurs during chromatography or sample preparation. Manganese is transferred from labile species to other ones of diverse properties. Molecules, which are separated strictly on the basis of SEC mechanism, must be eluted within interval from 12 and 34 min. It is therefore obvious that some manganese compounds are retarded by weak interaction with stationary phase. Therefore an estimation of molecular mass is impossible. It seems that stability of original manganese compounds is limited and sample preparation manner as well as sample age affect the result significantly.

Iron elution profiles of pea and lentil extract contain major peak corresponding to high-molecular-mass iron species (188 000 and 195 000 rel. mol. mass unit) and minor broad peak in molecular mass region from 10 000 to 20 000 rel. mol. mass unit. The latter peak is not visible in Fig. 3 and is not included in the data of Table 3. This fraction of iron was detected only in case of simplified SEC–ICP-MS coupling, when the chromatographic effluent was directly delivered to the nebulizer of the ICP-MS system. The low sensitivity of iron detection is further decreased by dilution of effluent with the flow of internal standard solution. Therefore the small iron peak disappeared.

Main species of copper and zinc were detected in both samples in the low-molecular-mass region (<2000 rel. mol. mass unit). Selenium in lentil extract was detected in three fractions the molecular masses of which range approx. from 100 to 200 000 rel. mol. mass unit. Molybdenum elution profiles of pea and lentil are completely different: in lentil extract the main molybdenum peak appeared in the high-molecular-mass region (approx. 200 000 rel. mol. mass unit) and the other one in the low-molecular-mass region (<2000 rel. mol. mass unit), whereas in pea extract (similarly as in case of white bean and soybean flour [12]) practically all molybdenum is eluted in a single peak in the low-molecular-mass region.

4. Conclusion

SEC–ICP-MS is a simple technique for element species fractionation, which can be applied for number of elements. With respect to low stability of some manganese species, a substantial portion of its species is decomposed during analysis. The experimental design using the post-column injection of calibration standards and the aspiration of internal standard together with column effluent made possible the accurate quantification of elements in separated fractions. Mass balance of elements in the course of chromatography can lead to estimation of the element fraction present in a sample as ionic form or labile complexes. Accurate quantitative data can help to disclose problems in individual cases and make some modification of methodology to avoid artifacts and minimize errors. On the other hand the characterization of species only by retention quantities and estimated molecular mass are insufficient to ascertain species identity. SEC is not efficient enough to separate all species present in a sample as chemical individuals. Therefore an application of multidimensional separation techniques and the use of identification tools such as electrospray ionization MS are needed to reach substantial advance in this field.

Acknowledgements

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