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Speciation analysis of elements in cereal flours by liquid chromatography–inductively coupled plasma mass spectrometry

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Abstract

Phosphorus, manganese, iron, cobalt, nickel, copper, zinc, and molybdenum species fractions were studied in maize and rye flour. Total and extractable contents of elements were determined by ICP–MS. Extracts of flours were prepared using 0.02 mol/l Tris–HCl buffer solution (pH 7.5) and 70% (v/v) ethanol as extractants, respectively. Both types of extracts were analysed by size exclusion chromatography (SEC) using a Superdex 75 HR 10/30 column on-line coupled to an ICP–MS. The 0.02 mol/l Tris–HCl buffer solution, pH 7.5 served as the mobile phase. Cobalt, nickel, zinc and molybdenum compounds were found in the low molecular mass region (<1 kDa). The main fraction of phosphorus compounds was found in the 3.5 kDa region. Remaining phosphorus compounds were detected in the high-molecular (>150 kDa) and the low-molecular mass (<1 kDa) regions. Minor amounts of copper and phosphorus (in both samples), zinc (in maize flour) and molybdenum (in rye flour) were found in the high molecular mass region (45 to >150 kDa). When the flours' extracts are spiked with cupric ions, practically all copper is bound to low molecular mass compounds. The low molecular mass fractions of unspiked flours' extracts obtained by SEC were further separated by reversed phase chromatography using a Kromasil C4 column and 10% methanol as the mobile phase.
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1. Introduction

Detailed understanding of nutritional significance of essential chemical elements in various foodstuffs is not possible without the consideration of the elements' chemical forms occurring in food and the ways of their alterations. Element speciation analysis can gain worthwhile information that could be interpreted with respect to elements' mobility, availability or biological effects. Element speciation analysis in biological matrices is mostly based on hyphenation of a separation technique (chromatography or electrophoresis), an element detection technique (AAS, ICP–OES, ICP–MS) and molecular identification technique (ESI–MS, MALDI-TOF–MS) (Szpunar, Łobinski, & Prange, 2003). Many articles devoted to the speciation analysis of both toxic and some essential elements in various food commodities appeared recently. The majority of them are focused on mercury and arsenic speciation in fish and other aquatic organisms (Chiou, Jiang, & Danadurai, 2001; Krystek & Ritsema, 2005; Rattanachongkiat, Millward, & Foulkes, 2004; Slejkove, Bajc, & Doganoc, 2004; Snell, Stewart, Sturgeon, & Frech, 2000; Storelli, Storelli, Giacominelli-Stuffler, & Marcotrigiano, 2005; Villa-Lojo,

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Alonso-Rodríguez, López-Mahía, Muniategui-Lorenzo, & Prada-Rodríguez, 2002; Zheng & Hintelmann, 2004) and selenium speciation in selenium-enriched plants, onion-like vegetables, nuts or food supplements (Dietz, Sanz Landaluze, Ximenez-Embun, Madrid-Albarran, & Camara, 2004; Encinar, Sliwka-Kaszynska, Polatajko, Vacchina, & Szpunar, 2003; Kannamkumarath, Wrobel, Wrobel, Vonderheide, & Caruso, 2002; Kotrebai, Tyson, Uden, Birringer, & Block, 2000; McSheeny et al., 2000; McSheeny, Pohl, Szpunar, Potin-Gautier, & Łobiński, 2001; Mounicou, McSheeny, Szpunar, Potin-Gautier, & Łobiński, 2002; Vonderheide et al., 2002; Wrobel, Wrobel, & Caruso, 2002).

Considerably less attention has been paid to elements' speciation in nutritionally most important food of plant origin (Koplík, Borková, Mestek, Komínková, & Suchánek, 2002; Schöppenthau, Nölte, & Dunemann, 1996). Most surprisingly cereals representing the main constituents of the human diet are almost neglected in the majority of element speciation studies. Their production, cultivation area, and consumption exceed those of any other crop. From a nutritional point of view the cereal products are excellent sources of carbohydrates and relatively good sources of proteins and certain vitamins and mineral elements (Souci, Fachmann, Kraut, Scherz, & Senser, 2000). Cereals supply more than half of energy for human nutrition and in many developing countries they provide more than two-third of the total diet. As feed, cereals also contribute greatly to the production of animal proteins. Owing to poverty of people in developing countries the diet of a substantial part of the human population is composed mainly of cereals. Moreover, many people are vegetarians because of their own reasons.

The chemical composition of cereals and nutritional consequences of prevailing consumption of cereals were extensively studied (Lorenz & Lee, 1977). One of the most frequent nutritionists' objections against the diet based strictly on cereals is that essential mineral elements intake is unsatisfactory and the mineral elements availability is low (Hunt, 2003). Since 1980 attempts had been made to carry out speciation analyses of iron, zinc and copper species in extracts of cereal samples (Dunemann & Schwedt, 1986; O'Keeffe, Dunemann, Theobald, & Svehla, 1995; Thiele & Schwedt, 1985). Articles dealing with selenium speciation in wheat flour (Diaz Huerta, Hinojosa Reyes, Marchante-Gayón, Fernández Sánchez, & Sanz-Medel, 2003) and arsenic speciation in rice (Heitkemper, Vela, Stewart, & Westphal, 2001) appeared recently. Nevertheless information about chemical status of trace elements in cereals is still limited. Therefore, the present study of the element species fractionation is focused on cereal flours. The reason why just maize and rye flours were selected consists in contrasting protein compositions of the both grains.

2. Experimental

2.1. Instrumentation

All ICP–MS measurements were carried out with an ICP mass spectrometer Elan 6000 (Perkin–Elmer, Norwalk, CT, USA) equipped with a glass Meinhard nebuliser, a cyclonic glass spray chamber and a Gilson 212 peristaltic pump for sample aspiration. Sample decomposition was performed in a microwave decomposition unit UniClever (Plazmatronika-Service, Wroclaw, Poland). pH values of buffer solutions were measured by a pH 03 instrument (Labio, Prague, Czech Republic).

Three liquid chromatographic systems were applied: (1) size exclusion chromatography with ultraviolet and on line ICP–MS detection; (2) preparative scale size exclusion chromatography; (3) reversed phase chromatography with ultraviolet and on line ICP–MS detection. The systems (1) and (3) consisted of high-pressure pump Varian Inert 9012 (Varian, Walnut Creek, CA, USA), an analytical column (Superdex 75 HR 10/30, 300×10 mm, produced by Amersham Pharmacia Biotech, Uppsala, Sweden, preceded by a glass Superformance column 150×10 mm, produced by Merck, Darmstadt, Germany, packed by chelating resin Chelex 100 in NH_4^+ form, or PEEK column Kromasil C4 250×4.6 mm $\times$ 5 μm, preceded by PEEK guard column kromasil C4 10×4.6 mm $\times$ 5 μm, produced by Supelco, Bellefonte, PA, USA, respectively), an UV detector Varian 9050 and two Rheodyne 9010 injectors. The first injector (equipped with a 100 μl sample loop) was inserted between the pump and the column and served for sample injection. The other one (equipped with a 500 μl loop) was inserted between the column and the UV detector and served for post-column injection of calibration standards of elements. PEEK or PTFE capillaries (internal diameter 0.25 mm) connected all parts of the systems. The chromatographic system (2) consisted of a high-pressure pump LCP 4020 (Ecom, Prague, Czech Republic), the Superformance column packed by Chelex 100, an injector Rheodyne 9010 equipped with a 2 ml PEEK sample loop, a glass column Fractogel EMD Bio SEC (dimensions 600×16 mm, optimal fractionation range 5–1000 kDa, Merck) and an UV detector HP 1050 (Hewlett–Packard, Waldbronn, Germany).

2.2. Standards and reagents

Tris (hydroxymethyl) aminomethane (Tris) for preparing of the mobile phase and extractant solution was purchased from Fluka (Neu–Ulm, Germany). Methanol and ethanol both of analytical grade were obtained from Merck. Hydrochloric acid (30%) and nitric acid (65%) were both of Suprapur® grade (Merck). Cobalt, copper, indium, iron, manganese, molybdenum, nickel and zinc

stock solutions ($\rho = 1000$ mg/l) were obtained from Merck, too. Phosphorus stock solution ($\rho = 1000$ mg/l) was prepared by dissolving of appropriate amount of ammonium dihydrogenphosphate (purity 99.999%) (Aldrich Chemical Co., Milwaukee, WS, USA) in deionised water. A set of peptide and protein standards obtained from Sigma (St. Louis, MO, USA) was used for calibration of molecular mass estimation by SEC. Distilled deionised water (Milli-Q, Millipore, Bedford, MA, USA) was used for preparation of all solutions.

2.3. The samples and preparation of samples extracts

Semi coarse maize flour and wholemeal rye flour were obtained from the market. Proximate compositions of maize and rye flour were 13.1% and 11.1% of moisture, 0.8% and 1.2% of ash, 7.8% and 7.4% of protein and 2.5% and 1.4% of fat, respectively. These analyses were accomplished according to standard methods (Kirk & Sawyer, 1991).

Extracts of samples were prepared using 0.02 mol/l Tris–HCl buffer solution, pH 7.5 or 70% (v/v) ethanol as extractants. The buffer solution was purified by passing through the glass column packed with Chelex 100 resin before use. This procedure effectively removes traces of transition metals. Two grams of the flour and 50 ml of the extractant were shaken in a 100 ml polypropylene bottle for one hour. Then the suspension was centrifuged and the clear supernatant was used for the analyses of total element content as well as speciation.

2.4. Determination of total content of elements

Samples of flours (0.5–0.8 g) or flours' extracts (10 ml) were decomposed by pressurized microwave digestion in PTFE vessels with 3 ml of HNO_3 for 10 min. The decomposition took place under controlled conditions (max. microwave power 135 W per one vessel, max. pressure 4.0 MPa). The temperature of sample during decomposition reached approx. 220 °C. The obtained sample digests were transferred into 50 ml calibrated flasks and made up to the mark. Elements' concentrations in decomposed samples were determined by ICP–MS using external calibration technique. Indium added to all samples, blanks and standard solutions ($\rho = 20$ μg/l) served as internal standard. Analyses were performed separately for phosphorus and metals. Details of the analytical method were given earlier (Fingerová & Koplík, 1999; Koplík et al., 2002). Combined uncertainties of total element contents were estimated considering values of results repeatability as well as other sources of uncertainty (calibration, spectral and non-spectral interferences) according to standard rules (Ellison, Rosslein, & Williams, 2000). Then expanded uncertainties were calculated using coverage factor two.

2.5. Size exclusion chromatography–inductively coupled plasma mass spectrometry

Chromatographic analyses were accomplished using Superdex 75 10/30 column and 0.02 mol/l Tris–HCl, pH 7.5 as the mobile phase. The experimental design was described in our previous paper (Koplík, Mestek, Komínková, Borková, & Suchánek, 2004).

2.6. Preparative scale size exclusion chromatography

Isolation of low molecular mass fractions from larger amount of cereal flours extracts was performed by SEC using a Fractogel EMD BioSEC column and the Tris–HCl buffer as mobile phase (flow rate 2 ml/min). Two milliliters of extract were injected into the column and the elution of separated compounds was detected by monitoring of absorbance at the wavelength of 280 nm. When fraction collection was carried out the UV detector was disconnected from the system and the individual fractions were taken just behind the column one per minute. Then the fractions were analysed off line by ICP–MS and those containing majority of zinc were submitted to sub-fractionation by reversed phase chromatography.

2.7. Reversed phase chromatography–ultraviolet spectrometry–inductively coupled plasma mass spectrometry

The arrangement of chromatographic apparatus was analogous to SEC/ICP–MS hyphenation. The UV detector, operated at 220 nm, and the ICP–MS one were connected in a series. 10% (v/v) methanol was used as the mobile phase. The flow rate was 0.4 ml/min. The delay between UV and ICP–MS detectors was ascertained by injection of glutathione solution and recording both absorbance signal and ICP–MS signal of sulphur (measured as $^{48}(SO)^{+}$ intensity).

3. Results and discussion

3.1. Total and extractable contents of elements

The total contents of phosphorus and trace elements in both flours as well as the portion of elements extractable by Tris–HCl aqueous buffer and diluted ethanol are summarized in Table 1. The found total contents of elements are quite comparable with the published data (Jorhem & Sundström, 1993; Varo, Nuurtamo, Saari, & Koivistoinen, 1980). The first step of element speciation analysis of a solid sample consists in extraction of soluble compounds. One could presume that a substantial portion of elements content present in cereal grain is associated with proteins and

Table 1
Total and extractable contents of elements in maize and rye flour

Element	Maize flour			Rye flour		
	Total content[a] (μg/g)	Percentage[b] extractable by		Total content[a] (μg/g)	Percentage[b] extractable by	
		Tris–HCl	70% EtOH		Tris–HCl	70% EtOH
P	932 ± 80	65 (12)	9 (3)	1525 ± 110	57 (5)	10 (2)
Mn	2.81 ± 0.25	55 (5)	2 (0.5)	23.5 ± 1.6	24 (2)	2 (1)
Fe	9.89 ± 1.23	20 (8)	<5	21.0 ± 1.5	18 (4)	<2
Co	0,0025 ± 0,0003	70 (20)	<5	0.0030 ± 0.0005	64 (25)	<5
Ni	0.064 ± 0.009	104 (8)	75 (7)	0,058 ± 0.008	46 (12)	30 (4)
Cu	0.76 ± 0.06	35 (8)	38 (3)	3.02 ± 0.20	51 (3)	20 (1)
Zn	9.44 ± 0.75	43 (6)	9 (3)	24.1 ± 1.6	35 (5)	7 (1)
Mo	0.107 ± 0.007	60 (9)	2 (1)	0.59 ± 0.04	65 (4)	5 (2)

[a] Mean ± expanded uncertainty (coverage factor = 2, $n = 8$).
[b] Mean value, standard deviations are given in parentheses ($n = 3$).

peptides. Results of our previous studies of element species in legumes (Koplík, Borková et al. (2002); Koplík, Pavelková et al. (2002)) showed a significant role of these compounds in the binding of trace metals. Most of the studied elements were effectively extracted from various legumes by Tris–HCl buffer, pH 7.5. As the protein composition of cereals is concerned the circumstances are much more difficult compared to legume proteins. An appreciable portion of cereal proteins, that involves prolamins and glutelins, is insoluble in slightly alkaline aqueous buffer. This was the reason why 70% (v/v) ethanol was used as extractant, too. On the other hand the full Osborne fractionation of proteins (Belitz, Grosch, & Schieberle, 2004) could not be applied because some extractants used in this procedure are either not tolerated by ICP–MS (sodium chloride solution) or may cause breakdown of original element species such as metal–ligand chelates (acidic or strongly alkaline solutions).

Tris–HCl buffer solution (0.02 mol/l, pH 7.5) dissolves the majority of phosphorus, cobalt and molybdenum compounds from both flours. Nickel compounds are completely extracted from maize flour. Iron compounds present in cereal flours are of low solubility. In this point cereals are similar to legumes (Koplík, Borková et al. (2002); Koplík, Pavelková et al. (2002)). Extractabilities of other trace elements vary approx. from 30% to 50%. Diluted ethanol is a less effective extractant than Tris–HCl buffer for most elements. Only in the case of rye flour comparable copper amounts are extracted by both solutions.

3.2. Element species fractionation and quantification by SEC and SEC/ICP–MS

Extracts of both flours in both extractants were analysed by SEC on Superdex 75. Chromatograms obtained by UV detection at 280 nm are shown in Fig. 1. The compounds separated strictly on the basis of molecular exclusion should be eluted within the

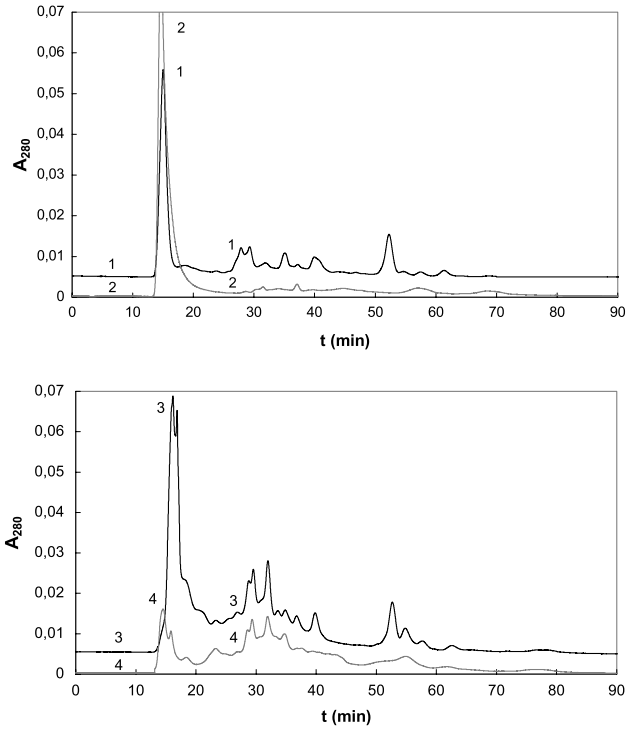

Fig. 1. Size exclusion chromatograms with ultraviolet detection of maize (1, 2) and rye (3, 4) extracts on Superdex 75 column; curves 1,3-Tris–HCl buffer extracts, curves 2,4-ethanol extracts.

interval from 15 to 33 min. There are many elution zones in this region. Appreciable differences between maize and rye flour composition as well as solubilities of their components in both extractants are obvious from Fig. 1. The main peaks in chromatograms of buffer extracts (curves 1 and 3) belong to proteins–albumins and globulins (retention times: 15.1 min for maize flour extract; 16.2 and 16.9 min for rye flour extract). Moreover, minor amounts of some medium molecular mass compounds (estimated M_r approx. 5-6 kDa–retention times 23.8 and 23.4 min for maize and rye, respectively) and low molecular mass

compounds (estimated $M_r \leqslant 1$ kDa, retention times 27.9, 29.4, 32.0 and 26.9, 28.8, 29.6, 32.0 min for maize and rye, respectively) are eluted too. Prolamins and some albumins are the main compounds extracted from maize by 70% (v/v) ethanol (see a dominant peak at $t_R = 14.7$ min). It corresponds to the fact that maize is a cereal crop with the highest proportion of prolamins among all protein fractions. Only traces of low molecular mass compounds were detected in the ethanol extract of maize flour. On the other hand the peaks of ethanol soluble proteins from rye flour (ethanol extract – curve 4, retention times 14.6 and 15.9 min) are considerably lower than those of hydrophilic proteins (aqueous buffer extract – curve 3). This is in accordance with the low proportion of prolamins among rye proteins (Belitz et al., 2004). A comparison of elution curves 3 and 4 in the low molecular mass region shows that some rye flour components are soluble both in ethanol and aqueous buffer solution.

Some components of sample extracts are eluted at longer retention times than 33 min as a result of their interactions with the gel matrix. Molecular mass of these compounds cannot be estimated at all. The existence of non-size exclusion effects was recognised early (Gelotte, 1960) and was encountered many times in practical applications of SEC in speciation analyses (Gardiner & Delves, 1994; Koplík, Pavelková et al. (2002); Michalke, 2002; Szpunar, 2000).

Coupling of ICP–MS with SEC enabled element specific detection of eluted compounds. Both buffer and ethanol extracts of flours were analysed. The chromatograms of Tris–HCl buffer extracts of flours are given in Figs. 2 and 3. The amounts of elements bound to individual element species fractions together with apparent molecular masses of the species fractions are summarized in Table 2.

Chromatograms of phosphorus compounds extracted by Tris–HCl buffer from both flours contain four elution zones. Traces of phosphorus were detected in a high molecular mass region for both samples. In maize flour extract the main phosphorus fraction is found in a medium molecular mass region (approx. 4 kDa), while the minor fraction belongs to a low molecular mass (<0.5 kDa) region. This pattern of phosphorus compounds is very similar to those found earlier in various legume samples (Koplík, Borková et al. (2002); Koplík, Pavelková et al. (2002); Schöppenthau et al., 1996). On the other hand in the case of rye flour extract the overwhelming majority of phosphorus is found in a low molecular mass (<0.5 kDa) fraction. Sum of phosphorus quantities of individual eluted fractions gives the extractable phosphorus content for both samples, thus phosphorus recoveries during SEC are 102% and 98% for maize and rye flour, respectively.

On the other hand the recoveries of manganese were very low (approx. 3% for both samples). As a result of

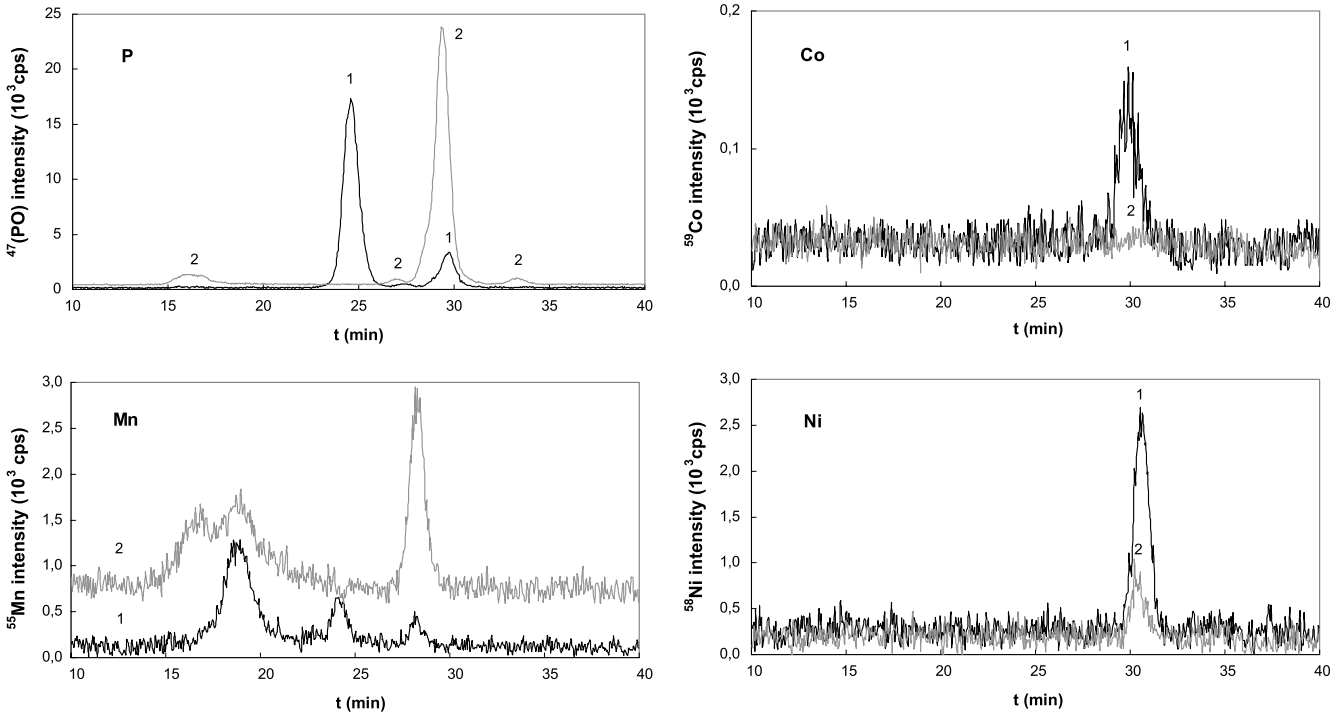

Fig. 2. Chromatograms of phosphorus, manganese, cobalt and nickel compounds of maize (1) and rye (2) flours extracts in Tris–HCl buffer obtained by SEC/ICP–MS using the Superdex 75 column.

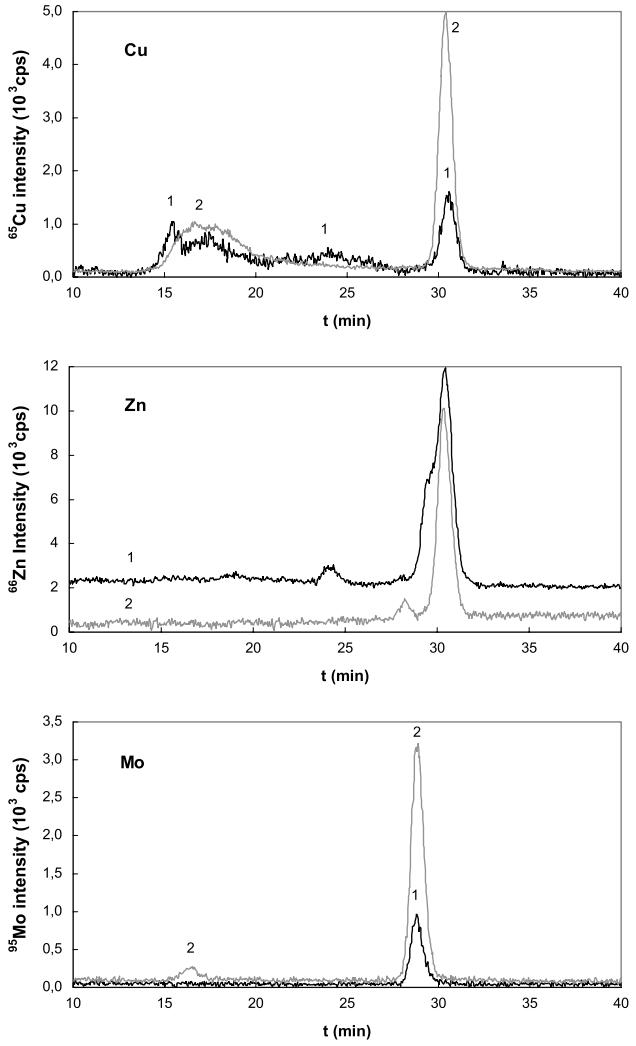

Fig. 3. Chromatograms of copper, zinc and molybdenum compounds of maize (1) and rye (2) flours extracts in Tris–HCl buffer obtained by SEC/ICP–MS using the Superdex 75 column.

low stability of manganese compounds most of manganese is liberated from original soluble species probably as inorganic cations Mn^{2+} or Mn^{3+} that are adsorbed on the gel support of the SEC column. Analogous behaviour of manganese was found in case of soybean flour (Koplík, Pavelková et al. (2002); Schöppenthau et al., 1996). The negligible portions of manganese bound in stable chelates are found in three fractions ranging in apparent molecular mass from 0.7 to 120 kDa. Iron is not eluted at all because soluble iron compounds are unstable.

Both cobalt and nickel compounds are detected as a single peak in the low molecular mass region (<0.5 kDa). No cobalt was eluted from SEC column in case of rye flour. Recoveries of nickel for both samples and recovery of cobalt for maize flour extract approach 100%.

A chromatogram of copper compounds from maize flour extract consists of four elution zones. Two high molecular mass fractions (>150 and 94 kDa) contain approx. one half of soluble copper content. The remaining copper is contained in the low molecular mass fraction (<0.5 kDa) and in a wide elution zone (2–20 kDa). Practically all copper (99%) from maize flour extract is recovered. In the case of rye flour extract copper is distributed almost regularly between the low molecular mass (<0.5 kDa) fraction and a wide high molecular mass (45 to >150 kDa) zone. Copper (93%) present in rye flour extract is recovered. That means that labile complexes or ionic species represent approx. 7% of soluble copper content.

An appreciable part of zinc is not recovered during SEC. Zinc recoveries of 56% and 45% were found for maize and rye flour extract, respectively. The remaining zinc is bound in labile species. Stable zinc compounds in maize and rye flour extract are distributed into three and two fractions, respectively. Major zinc fractions from both samples containing more than 80% of recovered zinc were found in the low molecular mass (<0.5 kDa) region.

Molybdenum compounds were eluted mainly in a low molecular mass region (0.5 kDa). Minor amount of molybdenum was found in a high molecular mass region (140 kDa) in the case of rye flour. Molybdenum recoveries approach to 100%.

Fig. 4 shows chromatograms of phosphorus and copper compounds extracted from both flours by 70% (v/v) ethanol. Elution curves of other elements are not shown, as they are analogous to those of copper or do not contain any peak. Phosphorus compounds are detected both in high and low molecular mass region. Chromatographic recovery of phosphorus compounds approaches 100% for both samples. In spite of lower extractability of phosphorus compounds by ethanol the portion of ethanol soluble high molecular phosphorus species is much higher than the content of Tris–HCl buffer-soluble ones (see Figs. 2 and 4). Owing to the very low solubility of nucleic acids in ethanol it can be concluded that the ethanol soluble high molecular mass phosphorus compounds are mostly phosphoproteins or phytic acid–protein complexes and the abundance of phosphoproteins in prolamin fraction is higher than that in the albumin and globulin fraction.

Chromatograms of copper, zinc and nickel ethanol soluble compounds show a dominant peak or even a unique one in the low molecular mass region (<0.5 kDa), i.e., at the same position as the corresponding peaks found in Tris–HCl buffer extracts. While the recoveries of copper and nickel are approx. 95%, in the case of zinc only one third of ethanol soluble content is recovered during SEC. No peaks were detected for molybdenum compounds in maize flour extract and for cobalt compounds in both samples.

Table 2
Results of SEC/ICP–MS analyses of Tris–HCl buffer extracts of maize and rye flour

Element	Maize			Rye		
	t_R (min)	Apparent M_r (kDa)	Element's content in the fraction (μg/g)	t_R (min)	Apparent M_r (kDa)	Element's content in the fraction (μg/g)
P	16.0	180	4	16.3	160	62
	20.6	3.5	510	26.9	1.2	14
	27.4	1	10	29.4	<0.5	760
	29.7	<0.5	92	33.3	n.e.	20
Mn	18.7	52	0.038	16.9	120	0.048
	24.1	4.3	0.008	19.0	46	0.079
	28.3	0.7	0.003	28.3	0.7	0.071
Co	29.9	<0.5	0.002	–	–	–
Ni	30.5	<0.5	0.070	30.4	<0.5	0.023
Cu	15.5	>150	0.044	17.2	45–>150[a]	0.68
	17.4	94	0.081	–	–	–
	24.1	2–20[a]	0.066	–	–	–
	30.5	<0.5	0.072	30.4	<0.5	0.75
Zn	19.1	45	0.06	–	–	–
	24.1	4.4	0.07	28.3	0.7	0.57
	30.5	<0.5	2.16	30.4	<0.5	3.23
Mo	–	–	–	16.6	140	0.019
	28.8	0.5	0.062	28.9	0.5	0.328

n.e., not estimated.
[a] Broad elution zone.

A low molecular mass molybdenum species (0.5 kDa) is detected in ethanol extract of rye flour, but the mass balance gives only 60% recovery.

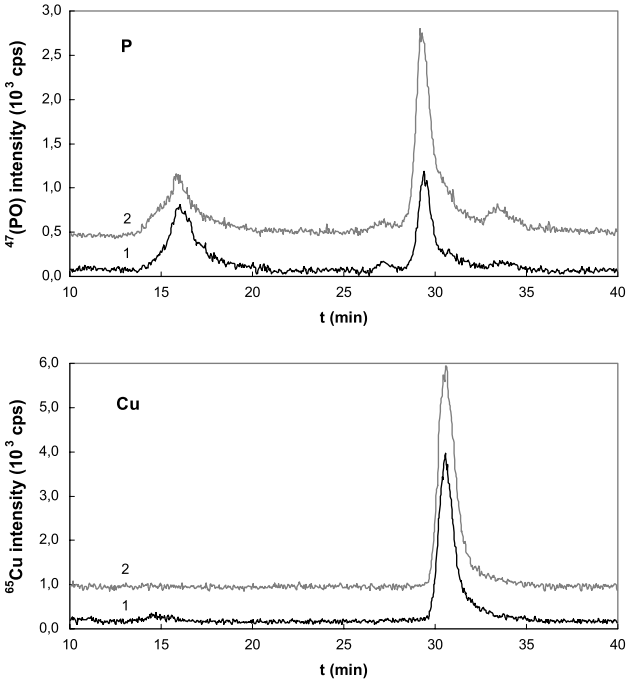

Fig. 4. Chromatograms of phosphorus and copper compounds of maize (1) and rye (2) flours extracts in 70% (v/v) ethanol obtained by SEC/ICP–MS using the Superdex 75 column.

3.3. Further investigation of low molecular mass species fraction

An appreciable part of soluble copper, zinc and molybdenum content and all cobalt and nickel from both samples were found in the low molecular mass region (<1 kDa). Our previous experiments (Koplík et al., 2004) showed that metal chelates contained in the corresponding fraction of legume seeds are more stable than other soluble metal compounds. Moreover, a transfer of copper from medium and high molecular mass fractions into a low molecular mass fraction was observed to occur during storage of peas. One could assume that in particular this fraction of soluble components of plant seeds contains a strongly chelating substance and has high metal binding capacity. In order to verify this assumption the extracts of flours in Tris–HCl buffer were spiked with various levels of cupric ions and the samples were submitted to SEC/ICP–MS analysis. An illustration of these experiments is given in Fig. 5 showing copper elution profiles of unspiked and spiked rye flour extract. As expected, practically all added copper appears in the low molecular mass region. An increase of copper content in high molecular mass fraction is negligible. The mass balance of copper showed that copper recovery from rye flour extract is dramatically reduced when more than 1000 ng/ml of Cu is added, which is caused by saturation of metal binding compounds. Copper concentration in copper-saturated solution is approx. 17 fold higher than the original copper

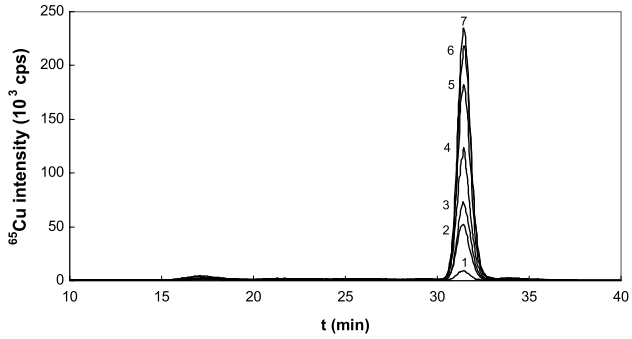

Fig. 5. SEC/ICP–MS of original and copper-spiked extract of rye flour in Tris–HCl buffer solution 1– original sample, curves, 2–7 – 200, 300, 500, 1000, 1500 and 2000 ng/ml of Cu added.

content. Copper-spiked maize flour extracts show analogous elution profiles. The saturation of maize flour extract occurs approx. at 500 ng/ml of Cu added, which is 50 fold higher content than the original one.

In consideration of the prominent role of low molecular mass compounds for the binding of trace elements we aimed at further separation of this fraction. Preparative scale SEC, using a Fractogel column and the same mobile phase, was applied to obtain higher amount of the fraction for the second separation. In general the samples' components are separated on the Fractogel column by a similar way as on the Superdex column. Only unspiked Tris–HCl buffer extract were analysed. The collected fractions of effluent were analysed by ICP–MS. Fig. 6 gives an illustration of zinc elution profiles. The low molecular mass fractions of both samples were collected from 35 to 39 min and analysed by reversed phase chromatography.

Owing to the high polarity of separated compounds a slightly hydrophobic stationary phase (Kromasil C4) was selected. 10% (v/v) methanol was chosen as a suitable mobile phase acceptable for ICP–MS. Detection was carried out simultaneously by UV and ICP–MS. Chromatograms showing the absorbance and ICP–MS signals of individual elements were recorded. Correction of the delay of retention times for ICP–MS detector was made. Fig. 7 shows the chromatograms of the low

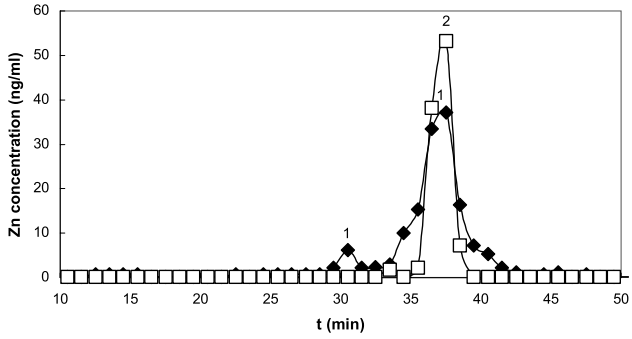

Fig. 6. Zinc elution profile of maize (1) and rye (2) flour extracts on Fractogel EMD Bio SEC column.

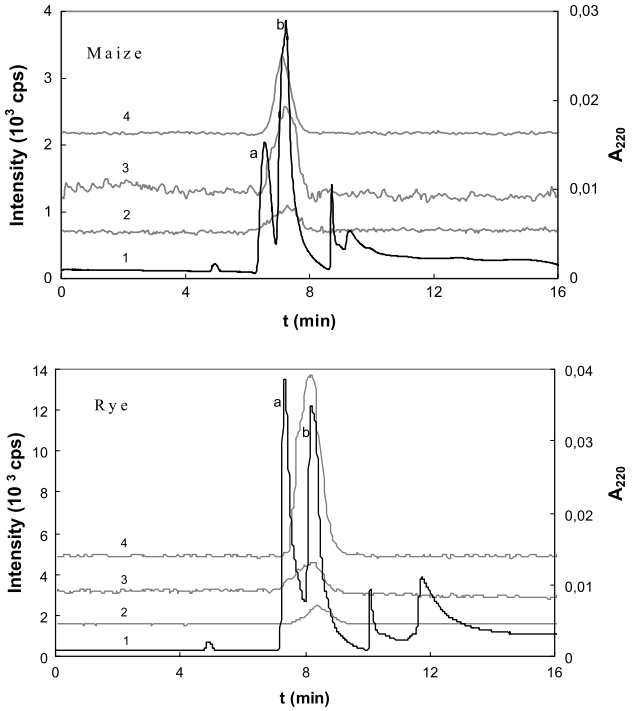

Fig. 7. RP-HPLC/UV/ICP–MS analyses of low molecular mass fractions of maize and rye flours extract 1– absorbance, 2 – Cu, 3 – Zn, 4 – P.

molecular mass fraction obtained from extracts of maize and rye flour. For practical reasons only copper, zinc and phosphorus signals are shown. Individual curves are set off to make the figure clearer. Organic compounds detectable at 220 nm are eluted within 30 min. The most important peaks at the absorbance curve are the sub-fractions designated as *a* and *b*. Each element is eluted in a single peak. The elements peaks are wide but they are more or less overlapped with sub-fraction *b*. Although the co-elution of elements and sub-fraction *b* (or partly *a*) does not prove that the elements are bound to the corresponding organic compounds the presence of a chelating substance in the fractions could be expected. Therefore, an additional research aimed at sub-fractions characterization by various mass spectrometric methods is needed.

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