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Fractionation of phosphorus and trace elements species in soybean flour and common white bean seeds by size exclusion chromatography–inductively coupled plasma mass spectrometry

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Abstract

Soluble species of phosphorus, sulfur, selenium and eight metals (Mn, Fe, Co, Ni, Cu, Zn, Mo and Cd) in soybean flour and common white bean seeds were investigated by size exclusion chromatography (SEC) and inductively coupled plasma mass spectrometry (ICP-MS). Samples were extracted by 0.02 mol l^{-1} Tris–HCl buffer solution (pH 7.5). Fractionation of sample extracts by preparative scale SEC was accomplished using a Fractogel EMD BioSEC column (600×16 mm) and 0.02 mol l^{-1} Tris–HCl buffer solution (pH 7.5) as mobile phase (flow rate: 2 ml min^{-1}). A 2-ml sample was injected. Contents of elements in chromatographic fractions were determined by AAS, ICP-AES and ICP-MS. The elution profiles of P, Fe, Co, Ni, Cu, Zn and Mo in both samples were similar. Main species of Co, Ni, Cu, Zn and Mo were found in the low molecular weight region (2–5 kDa), whereas Fe is predominantly bound to high molecular weight compounds (180 kDa). The dominant phosphorus fraction was detected in the medium molecular weight region (10–30 kDa) and the other fraction in the low molecular weight region. Isotachophoretic analysis of chromatographic fractions revealed that the main phosphorus compound in the medium molecular weight region is phytic acid. SEC on Superdex 75 and Superdex Peptide columns (300×10 mm) was performed in on-line hyphenation with ICP-MS. The same mobile phase was used with a flow rate of 0.5 ml min^{-1}; volume of injected sample was 200 μl. Element specific chromatograms were obtained by continuous nebulization of effluent into ICP-mass spectrometer measuring intensities of $^{47}(PO)^{+}$ and $^{48}(SO)^{+}$ oxide ions and ^{55}Mn, ^{57}Fe, ^{59}Co, ^{62}Ni, ^{65}Cu, ^{66}Zn, ^{82}Se, ^{95}Mo and ^{114}Cd nuclides. Chromatographic profiles of elements are generally analogous to those obtained with a Fractogel column, but better chromatographic resolution of separated species was achieved so that slight differences between samples were revealed. Estimated molecular weights of major phosphorus species in soybean flour and common white bean seed extracts are 6 and 3.6 kDa, respectively, whereas those of minor phosphorus species in both samples are 0.7 kDa. Traces of phosphorus were also detected in the high molecular weight region (130 kDa). Chromatograms of P, Ni, Cu, Zn and Mo compounds in both extracts are similar but not identical. Molecular weights of major Cu and Zn species are ~1 and 0.4 kDa for soybean flour and white bean seeds, respectively. In cases of Mn, Fe, Co and Se, the element profiles of soybean flour and white bean seed extracts are significantly different. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Soybean flour; Size exclusion chromatography; Inductively coupled plasma mass spectrometry; Phosphorus; Trace elements

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1. Introduction

The identification and determination of individual species of minerals and trace elements in foods is of great importance in food chemistry and nutrition science [1]. The different chemical binding forms of elements act in different ways on the course of chemical reactions in food and they can of course have different biological availabilities and effects.

Most contemporary analytical methods for trace element speciation in biological samples are based on the combination of effective separation methods (namely liquid chromatography and electrophoresis) and element specific detection by atomic absorption spectrometry (AAS) [2–5], inductively coupled plasma atomic emission (ICP-AES) [6–9] or mass spectrometry (ICP-MS) [9–21]. A comprehensive review of these methods was recently published by Szpunar [22]. Even the application of the most advanced methods for element speciation in biological fluids and plant extracts needs some care because of the low stability of the native species of some elements. Therefore size exclusion chromatography (SEC) [5,7,12–14,16–21] is frequently applied as a moderate separation technique, namely coupled with ICP-MS. This technique makes it possible to adapt the pH and ionic strength of the mobile phase to the properties of a sample with respect to solubility and stability of its components. Interactions between separated compounds and the gel are only slight so that alteration of separated species does not usually occur during separation. Although SEC is less effective in separation compared with other liquid chromatographic techniques, its application in speciation analysis of highly complex matrices is usually necessary at least as the first separation step [23,24].

In this paper the coupling of SEC and ICP-MS was applied for speciation of elements in extracts of soybean flour and common white bean seeds. Both foods represent important sources of vegetable proteins. The amounts of major and trace essential elements in these legumes are rather high, but it is well known that the bioavailability of some elements such as iron and zinc from legumes is low. This paper describes the molecular weight distribution of element species in the plant extracts and relationships among binding forms of individual elements.

2. Experimental

2.1. Samples and sample preparation

2.1.1. The samples

Defatted soybean (*Glycine max*) flour and common white bean (*Phaseolus vulgaris*) seeds were obtained from the market. The samples were milled in a vibrational mill under liquid nitrogen. The total contents of elements as well as their extractable portions (for extraction conditions see below) are given in Table 1.

2.1.2. Preparation of sample extracts

A 1-g sample of finely powdered sample was extracted with 50 ml of 0.02 mol l^{-1} Tris–HCl buffer solution, pH 7.5, by 1 h shaking in a polypropylene flask. The buffer solution was previously purified by passing it through the column filled with Chelex 100 resin (see below). The suspension was centrifuged and the clear supernatant was used for further analyses.

2.2. Analytical methods

2.2.1. Determination of elements

The total contents as well as the extractable amounts of elements were ascertained by microwave digestion (Uniclever digestion unit, Plazmatronika, Wroclaw, Poland) with nitric acid and analysis by flame AAS (Na, Mg, K, Ca, Fe), ICP-AES (P) and ICP-MS (other elements). An atomic absorption spectrometer Avanta P (GBC Scientific, Dandenong, Australia), a PU 7000 ICP-spectrometer (Philips Scientific, Cambridge, UK) and an Elan 6000 ICP mass spectrometer (Perkin-Elmer/Sciex, Norwalk, CT, USA) were used. Details are given elsewhere [25,26].

2.2.2. Preparative scale SEC

A Superformance glass column (16×600 mm) with Fractogel EMD BioSEC (S) (Merck, Darmstadt, Germany) was applied. The chromatographic system consisted of an HPP 5001 high pressure pump (Laboratorní přístroje, Prague, Czech Republic), a glass scavenger column (10×300 mm) packed with Chelex 100 resin (Merck) in the NH_4^+ form, a Rheodyne 9010 injector with 2-ml PEEK sample

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

Element	Soybean flour		Common bean	
	Total content ($\mu g\ g^{-1}$)	Extractability (%)	Total content ($\mu g\ g^{-1}$)	Extractability (%)
Na	4.0±0.1	112±16	5.0±0.2	106±19
Mg	2990±30	75±9	1610±20	66±2
P	7160±220	69±6	3850±10	74±2
K	24000±340	108±6	14100±50	86±2
Ca	3160±15	37±3	2060±30	25±3
Fe	140±5	33±6	101±5	44±2
Mn	41.7±0.2	68±4	18.2±0.2	52±2
Co	0.100±0.002	82±6	0.120±0.002	80±3
Ni	8.85±0.30	90±10	1.00±0.18	98±7
Cu	16.1±0.1	71±6	6.74±0.01	80±7
Zn	52.9±0.4	78±4	31.8±0.3	79±3
Se	0.71±0.02	85±14	0.11±0.02	88±8
Mo	3.17±0.02	82±8	5.59±0.19	93±3
Cd	0.106±0.002	46±3	<0.0006	N.D.

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

loop, the Fractogel column and a Hewlett-Packard 1050 UV–Vis detector. The mobile phase was 0.02 mol l^{-1} Tris–HCl, pH 7.5, and the flow rate was 2.0 ml min^{-1}. The Chelex 100 column was used in order to remove trace metal impurities from the mobile phase. Absorbance of the effluent was monitored at 280 nm. A set of peptide and protein standards of molecular weights from 0.3 to 669 kDa was used for determination of molecular weight of eluted compounds. These standards were obtained from Sigma (St. Louis, MO, USA). A total of 60 fractions of 2 ml each were collected into polypropylene tubes to be submitted to further analyses, i.e. determination of elements by ICP-AES and ICP-MS, phosphorus compounds, total proteins and carbohydrates. Phosphorus determination in fractions was accomplished without any dilution. For ICP-MS analysis the fractions were spiked with internal standard (indium, final concentration 20 $\mu g\ l^{-1}$) and diluted with water up to 10 ml.

2.2.3. Determination of phosphorus compounds

Selected fractions of sample extracts obtained by SEC on the Fractogel column with elevated phosphorus content (measured by ICP-AES) were submitted to isotachophoretic analysis [27] to determine *ortho*-phosphate and phytic acid (*myo*-inositol-hexakis (dihydrogenphosphate)) contents. The analysis was performed using electrophoretic analyzer EA 100 (Villa-Labeco, Slovakia) with column coupling (pre-separation 0.8×90 mm, analytical 0.3×90 mm). Solutions containing 0.01 mol l^{-1} HCl+0.0055 mol l^{-1} 1,3-bis[tris(hydroxymethyl)methylamino] propane (BTP)+0.1% chydroxypropylmethyl cellulose (HPM) and 0.005 mol l^{-1} morpholinethanesulfonic acid (MES)+0.001 mol l^{-1} BTP served as leading and terminating electrolytes, respectively. Applied driving currents were 250 and 50 μA in pre-separation and analytical capillary, respectively. Conductivity measurement and ultraviolet absorption (λ=254 nm) were used for detection.

2.2.4. Determination of proteins and carbohydrates

The protein content in chromatographic fractions was determined spectrophotometrically by the Bradford method [28] with Coomassie Brilliant Blue G-250. Bovine serum albumin was used as the calibration standard. Determination of total carbohydrates in fractions was accomplished by spectrophotometric measurement (λ=420 nm) of colored products formed by heating of the sample with orcinol (3,5-dihydroxytoluene) in the presence of sulfuric acid [29]. D-Glucose (50–250 μg) was used for calibration.

2.2.5. On-line hyphenation: SEC–ICP-MS

Two size exclusion columns (dimensions 10×300 mm) with different fractionation ranges were applied, namely a Superdex 75 HR 10/30 (optimum fractionation range 3–70 kDa) and a Superdex Peptide HR 10/30 (optimum fractionation range 0.1–7 kDa) both produced by Amersham Pharmacia Biotech (Uppsala, Sweden). The apparatus consists of a Varian Inert 9012 high pressure pump, a glass scavenger column (10×150 mm) packed with Chelex 100 resin (Merck) in the NH_4^+ form, a Rheodyne 9025 injector with 200-μl PEEK sample loop, the Superdex column and a Varian 9050 UV–VIS detector operated at 280 nm. All parts of the apparatus were connected by PEEK or PTFE capillaries. A buffer solution of 0.02 mol l^{-1} Tris–HCl, pH 7.5, with a flow rate of 0.5 ml min^{-1} served as the mobile phase. A set of peptide and protein standards of molecular weights from 0.3 to 158 kDa were used for determination of molecular weights of eluted compounds.

The chromatograph was connected to the cross-flow nebulizer of an ELAN 6000 ICP-mass spectrometer by a 0.5×300-mm PTFE capillary through a four-way PTFE valve. In the course of instrument stabilization the peristaltic pump delivered water through the second channel of the valve to the ICP-MS nebulizer. Before sample injection into the chromatographic system the valve was switched to deliver effluent from the column to the nebulizer. Analysis was started simultaneously in both instruments. The operating conditions of ICP-MS are given in Table 2.

As described in Ref. [30] measurement of oxide ions $^{32}S^{16}O^+$ can be used for the determination of sulfur by ICP-MS. This principle was applied for the detection of sulfur and phosphorus compounds. The intensities of $^{47}(PO)^+$ and $^{48}(SO)^+$ ions were measured. Sensitive detection of oxide ions requires a somewhat higher nebulizer argon flow. Optimization of nebulizer argon flow for detection of sulfur and phosphorus was accomplished by measuring $^{47}(PO)^+$ and $^{48}(SO)^+$ intensities during nebulization of 10 μg ml^{-1} solutions of sulfur and phosphorus (prepared from H_2SO_4 and $NH_4H_2PO_4$, respectively) in the mobile phase (Fig. 1). The plot shows two maxima both for phosphorus and sulfur. The value corre-

Table 2
Operating conditions of ICP-MS used in on-line hyphenation of SEC–ICP-MS

Common parameters		
R.f. power: 1075 W		
Ion lens mode: autolens on (optimized to maximum of ^{9}Be, ^{59}Co and ^{115}In)		
Sweeps per replicate: 30		
Duration of one replicate: 6.00 s		
Replicates per sample: 600		
Analyte	Dwell time (ms)	Integration time (ms)
First group (nebulizer Ar flow: 0.775 l min^{-1})		
^{55}Mn	25	750
^{57}Fe	25	750
^{59}Co	25	750
^{62}Ni	26	780
^{65}Cu	26	780
^{66}Zn	26	780
^{95}Mo	26	780
Second group (nebulizer Ar flow: 0.775 l min^{-1})		
^{59}Co	47	1410
^{82}Se	47	1410
^{114}Cd	47	1410
Third group (nebulizer Ar flow: 1.05 l min^{-1})		
^{43}Ca	47	1410
^{47}(PO)	47	1410
^{48}(SO)	47	1410
^{49}(SO)	47	1410

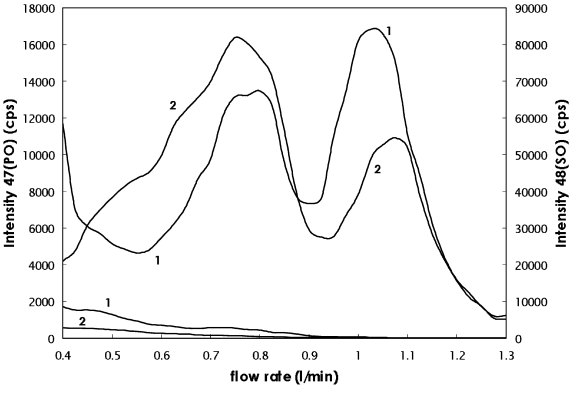

Fig. 1. Optimization of nebulizer Ar flow for ICP-MS detection of phosphorus (1) and sulfur (2) measured as $^{47}(PO)^+$ and $^{48}(SO)^+$ (upper curves: nebulization of P or S, 10 μg ml^{-1}; lower curves: nebulization of 0.02 mol l^{-1} Tris–HCl, pH 7.5).

sponding to second maximum was chosen with respect to minimum background signal.

3. Results and discussion

3.1. Behavior of plant extract components on SEC columns

The high extractability of a number of elements (Ni, Mo, Se, Co, Zn, Cu, P) from soybean flour and common white bean seeds (Table 1) allows speciation by chromatographic methods. On the other hand the information obtained by this approach is of only limited value in the case of elements with moderate (Cd) or low solubility (Fe, Ca). The pH value of the extraction solution was selected with respect to solubility of 11S and 7S globulins representing the main proteins of seeds of leguminous plants [31]. The same solution also served as a mobile phase. This is in accordance with the finding of earlier studies [20,21] that recommend 0.01–0.07 mol l^{-1} Tris–HCl buffer solutions as suitable mobile phases for SEC–ICP-MS analysis of plant extracts.

Fig. 2 shows the chromatograms of soybean flour and white bean extracts obtained by preparative scale SEC using the Fractogel column. Proteins and peptides are detected at the wavelength used (280 nm). Moreover the proteins were determined by the Bradford method in chromatographic fractions. Approximately 90% of proteins extracted from both materials were found in the high molecular weight region (35–320 kDa), while the remaining proteins

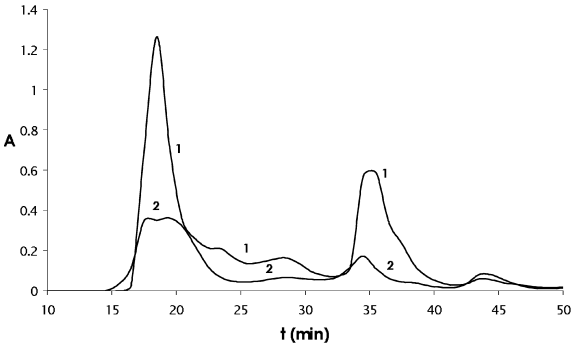

Fig. 2. UV (280 nm) chromatograms of soybean flour (1) and white bean extracts (2) on the Fractogel column.

were detected in the 10–35-kDa region. Chromatographic recovery of total proteins was 99%. Compounds not detected by UV absorption are represented by carbohydrates. In both cases the determination of total saccharides (including the saccharide part of glycoside and glycoprotein molecules) in chromatographic fractions revealed that the majority of saccharides (80–90%) are eluted between 37 and 47 min, i.e. in the low molecular weight region (<1–3 kDa). This elution zone probably corresponds to oligosaccharides representing most carbohydrates in legumes [31]. Some saccharides were also detected in the high molecular weight region (55–400 kDa). Chromatographic recovery of total saccharides was 98%.

As far as the chromatographic behavior of elements is concerned, there is a substantial difference between the group of alkali elements (Na, K, Mg, Ca) and other elements. The behavior of transition metals, phosphorus and selenium, is normal. This means that these elements are eluted within the interval of dead and total volume of the column. In the case of plant extract analysis using the Fractogel column, it was found that compounds of these elements are eluted between 14 and 45 min, whereas alkali and alkali earth metals are eluted from 130 to 290 min. This means that alkali elements are retarded on the column via non-size exclusion effects [32], such as adsorption or ion-exchange. This retention can be moderated in the case of alkali metals by increasing the ionic strength of the mobile phase (e.g. when ten times higher concentration of Tris is used, the retention time of Na and K is shifted to ~55 min; the retention of divalent metals is not influenced). The differences in element behavior can be explained by the different chemical forms of elements present in plant extracts. Transition metals as well as selenium and phosphorus are present in plant extracts in bound forms as organic chelates and covalent compounds, whereas alkali elements occur mostly as cationic species which are retarded in the column. As the mobile phase is alkaline, some functional groups on the surface of column support can be negatively charged and so the gel support may act as an ion-exchanger. This explanation is supported by the fact that strong retention of both alkali and transition metals on columns was observed

when samples containing inorganic compounds of these elements were injected. After injection of such samples the column can be effectively cleaned only by repeated injection of EDTA solution. Generally the same is valid in the case of Superdex columns.

This behavior of alkali and alkali earth metals on Fractogel and Superdex columns is advantageous for ICP-MS detection of other elements. Neither ICP-MS determination of elements in chromatographic fractions nor on-line ICP-MS detection are distorted either by non-spectral or spectral interferences. Owing to the substantial retention of alkali metals, the ICP-MS detection of other elements takes place under stabilized sensitivity conditions. Potassium is present in extracts in such high amounts that this could result in serious suppression of analyte intensity or spectral interference on ^{55}Mn (caused by $^{39}K^{16}O^{+}$) or on ^{95}Mo (caused by $^{40}Ar^{39}K^{16}O^{+}$) [33]. Nevertheless it is necessary to thoroughly rinse the column by the mobile phase before each injection in order to avoid co-elution of analytes from the present sample and alkali elements from the previously injected sample.

3.2. Speciation of elements by preparative scale SEC

The elution profile of phosphorus compounds contained in soybean flour and white bean extracts obtained on the Fractogel column is shown in Fig. 3. Total concentrations of phosphorus in individual fractions are plotted against retention time. Soybean flour and white bean samples show good similarity of phosphorus distribution among fractions. Traces of phosphorus were detected between 16 and 20 min corresponding to the presence of high molecular weight species (probably phosphoproteins). The major phosphorus peaks were detected between 26 and 32 min (apex at 28.5 min) in the medium molecular weight region (10–30 kDa). The minor phosphorus peaks with apexes at 35.5 and 34.5 min for soybean flour and white bean, respectively, correspond to low molecular weight species (<5 kDa).

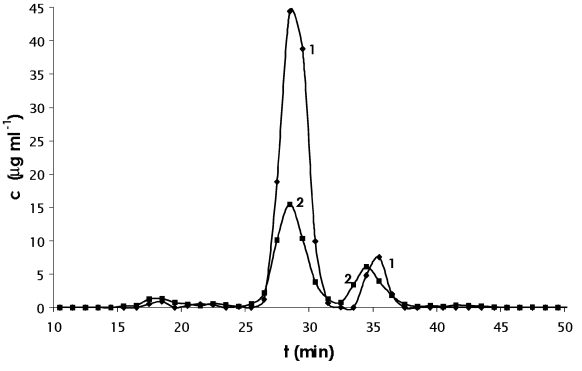

Fig. 3. Phosphorus elution profiles of soybean flour (1) and white bean (2) extracts on the Fractogel column.

Further analysis of phosphorus-containing fractions of soybean flour extract by capillary isotachophoresis revealed that phytic acid is a dominant phosphorus compound corresponding to the major phosphorus peak, while *ortho*-phosphate prevails over phytic acid in the minor phosphorus peak. The mass balance of phosphorus showed that phytic acid accounts for 71% of total phosphorus in the major phosphorus peak (Fig. 3), whereas *ortho*-phosphate accounts for only 6%. In contrast, in the minor phosphorus peak the *ortho*-phosphate phosphorus is three times more abundant than phytate phosphorus.

Phytic acid represents the main phosphorus compound of seeds of legumes and cereals and some other foods of plant origin [34]. It is known that phytic acid is a strong metal chelator forming many salts and complexes with metal ions. Some of these compounds have low solubility so that the presence of metal phytates in foodstuffs is supposed to be one of the causes of decreased bioavailability of essential elements such as zinc, calcium or iron. Although the molecular weight of phytic acid alone is only 0.66 kDa, its detection in the 10–30-kDa region is correct because in neutral or slightly alkaline solutions the anions of phytic acid can be conjugated to positively charged functional groups of protein macromolecules [34].

Figs. 4 and 5 show manganese, iron, cobalt, nickel, copper, zinc and molybdenum elution profiles of extracts of soybean flour and white bean obtained by chromatography on the Fractogel column. Except for manganese, the element specific profiles of soybean flour extract are analogous to those of white bean extract. The major peaks of cobalt, nickel,

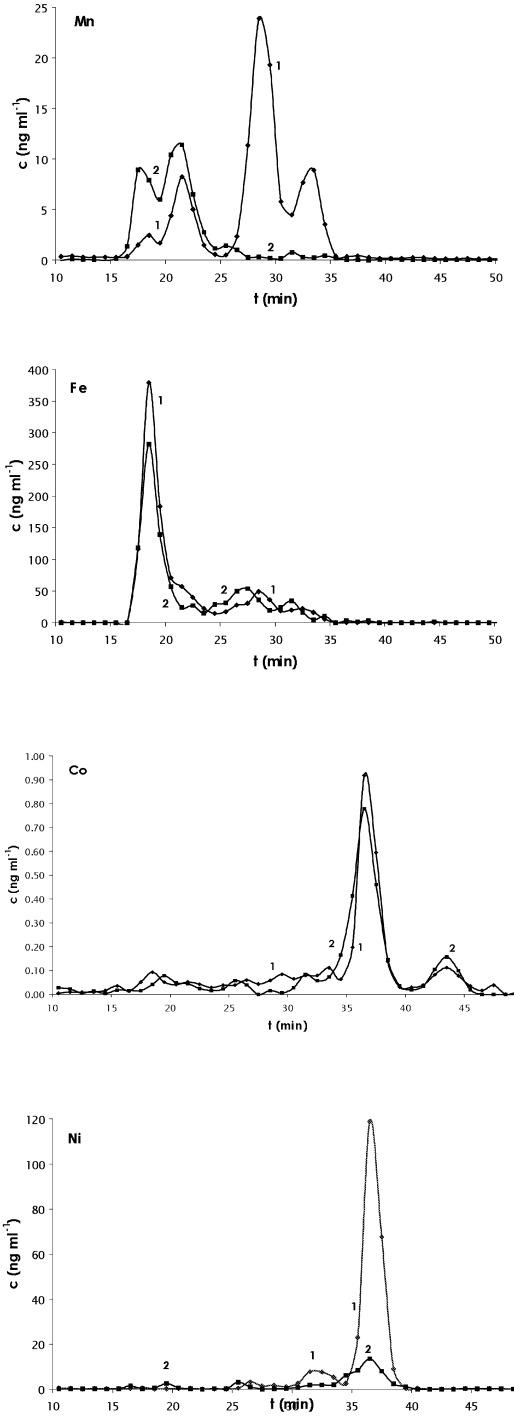

Fig. 4. Manganese, iron, cobalt and nickel elution profiles of soybean flour (1) and white bean (2) extracts on the Fractogel column.

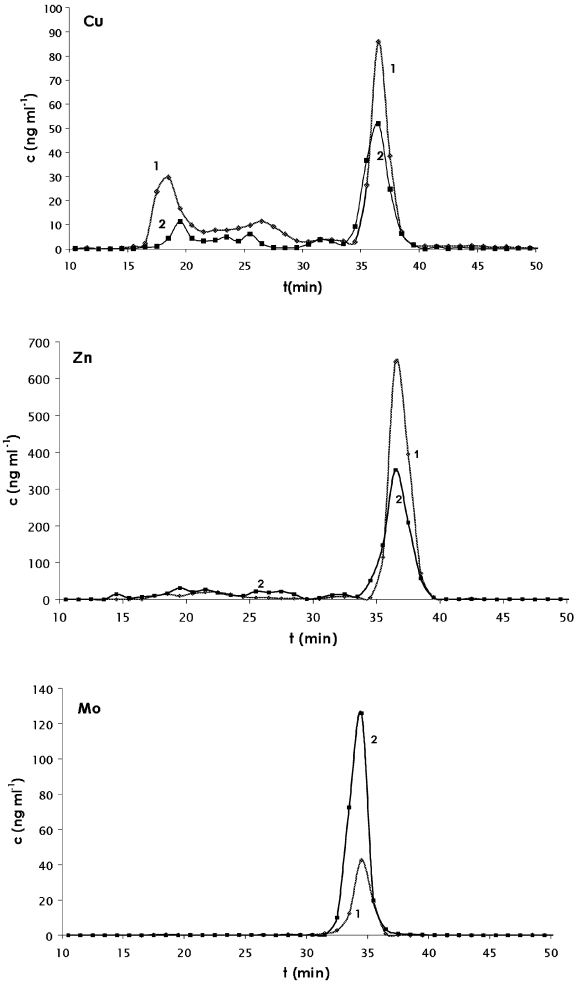

Fig. 5. Copper, zinc and molybdenum elution profiles of soybean flour (1) and white bean (2) extracts on the Fractogel column.

copper and zinc were found between 34 and 40 min (apex at 36.5 min) corresponding to low molecular weight species (2–5 kDa). Molybdenum was eluted practically in a single peak between 32 and 37 min (apex at 34.5 min) corresponding to a molecular weight of ~5 kDa. In medium and high molecular weight regions only traces of cobalt, nickel, zinc and molybdenum were found. On the other hand in both samples copper shows significant minor peaks in the

high (150–180 kDa) and medium (30–40 kDa) molecular weight regions.

Iron was predominantly detected between 16 and 22 min (apex at 18.5 min) corresponding to high molecular weight (180 kDa) species. Similar results concerning iron speciation in soybeans were obtained by Yoshida [35] using SEC on Sephadex 50 and Sephadex 100 columns and 0.01 mol l^{-1} Tris–HCl (pH 7.4) as a mobile phase. According to Ref. [35] the molecular weight of the main soluble iron compound is >100 kDa and the bond between ligand and iron is very stable. Some specific metalloproteins, such as lipoxygenase or protein non-specific iron chelators, account for iron binding in soybeans. Our results showed that chromatographic fractions of soybean flour extract containing most of the phytic acid also contain an insignificant amount of iron. It is therefore obvious that phytic acid does not participate substantially in iron binding in the soluble portion of soybeans. Since only half of the phytic acid content of soybean flour is found in the extract, it can be presumed that an insoluble residue may contain some iron in the form of ferric phytate.

Manganese elution profiles seem to be the most complex of all the elements studied. In both samples two high molecular weight species (~200 and 90 kDa) were detected. In the case of soybean flour extract, the major manganese peak appeared in the medium molecular weight region (20 kDa) with another peak in the lower region (7 kDa). The major manganese peak coincides with the major peak of phosphorus. Yoshida [36] reported that manganese is present in aqueous soybean extract in three different forms: a phytate-binding form, a low-molecular weight ligand-binding form and a manganoprotein with apparent molecular weight of 60 kDa. We found by repeated analyses that the molecular weight distribution of manganese among fractions alters during aging of extract, while profiles of other elements are not time dependent up to 1 day. Data presented in Figs. 3–5 refer to sample extracts injected immediately after preparation. The manganese species instability is due to association or dissociation of manganoprotein molecules, which are known to be formed by several sub-units [37], or to changes of manganese oxidation state in an alkaline solution.

3.3. Speciation of elements by on-line hyphenation: SEC–ICP-MS

Superdex 75 and Superdex Peptide columns were applied for this purpose. The corresponding chromatograms of soybean flour and white bean extracts obtained by detection at 280 nm are shown in Fig. 6. In general the elution curves are analogous to those in Fig. 2 but in the case of the Superdex columns the resolution of separated compounds is better. As far as the separation of metal species is concerned the best resolution in most cases was achieved using the Superdex 75 column.

Chromatograms of phosphorus species obtained by SEC–ICP-MS of plant extracts on both columns are given in Fig. 7. The phosphorus elution curves are very similar to those obtained using the Fractogel column (Fig. 3). The main phosphorus peaks ob-

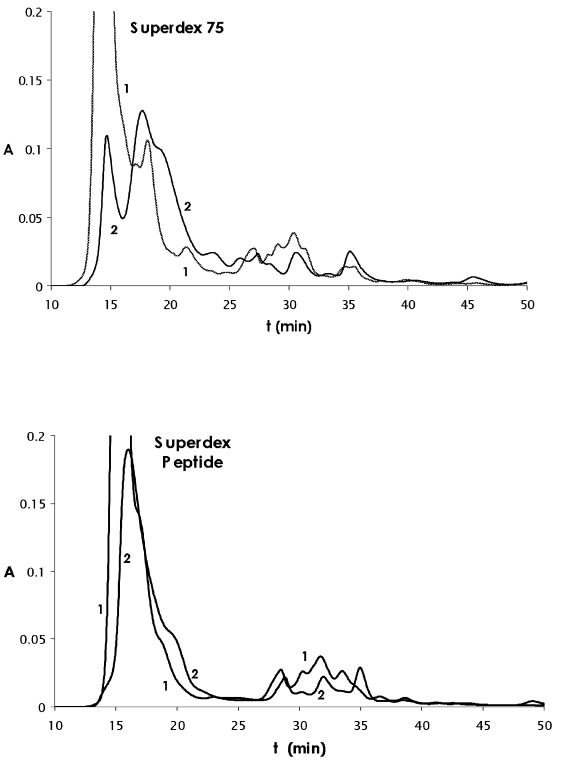

Fig. 6. Chromatograms of soybean flour (1) and white bean (2) extracts on the Superdex 75 and Superdex Peptide columns obtained by UV detection (λ=280 nm).

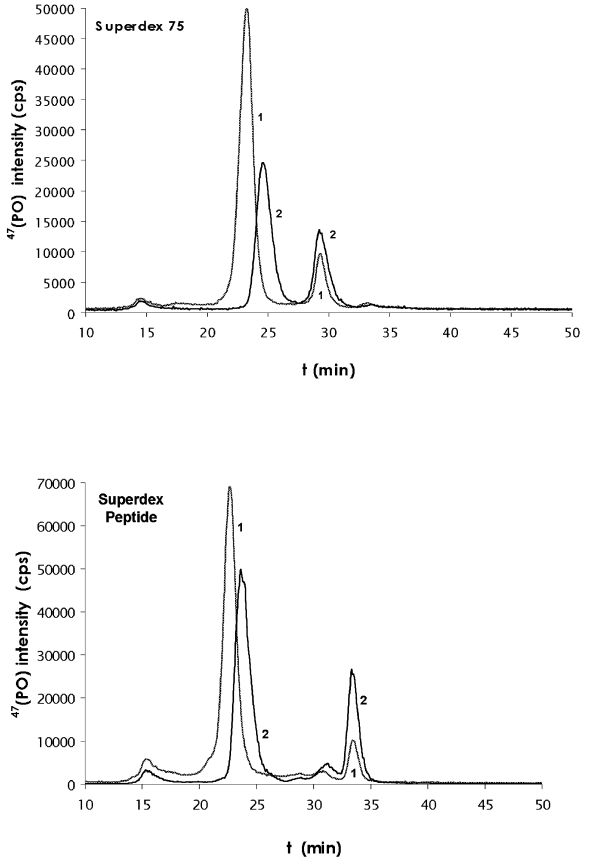

Fig. 7. Chromatograms of phosphorus compounds of soybean flour (1) and white bean (2) extracts obtained by SEC/ICP-MS using the Superdex 75 and Superdex Peptide columns.

served in the chromatograms in Figs. 3 and 7 probably represent the same fraction. On the basis of Superdex 75 calibration, the molecular weights of the main phosphorus compounds were estimated to be 6 and 3.6 kDa for soybean flour and white bean, respectively. The molecular weight of phosphorus compounds corresponding to the minor peak was estimated to be 0.7 kDa for both samples. Using the Superdex Peptide column two low molecular species were detected. Their molecular weights were estimated to be 0.1 and 0.3 kDa. Traces of phosphorus were also detected in the high molecular weight region (>70 kDa). In another study by Schöppenthau [7] of phosphorus, sulfur, manganese, zinc and

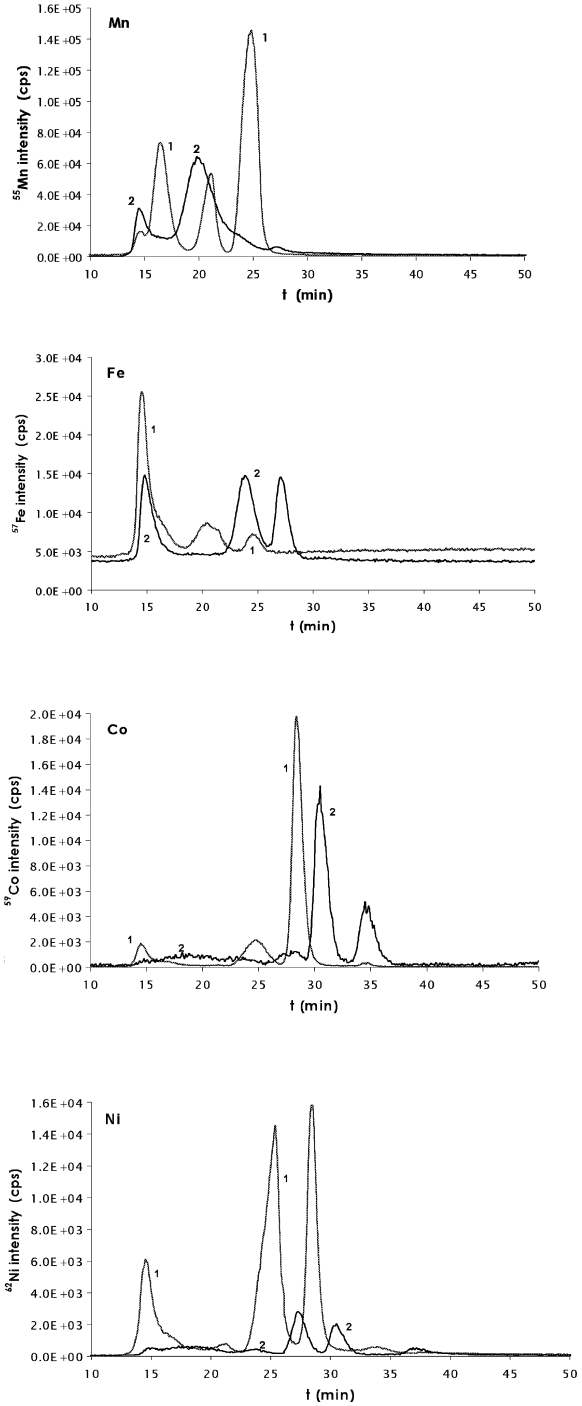

Fig. 8. Chromatograms of manganese, iron, cobalt and nickel compounds of soybean flour (1) and white bean (2) obtained by SEC/ICP-MS using the Superdex 75 column.

copper species in soybean flour, chromatography on a Superdex 75 column with the same mobile phase and ICP-AES detection was applied. Our results are similar to that study [7].

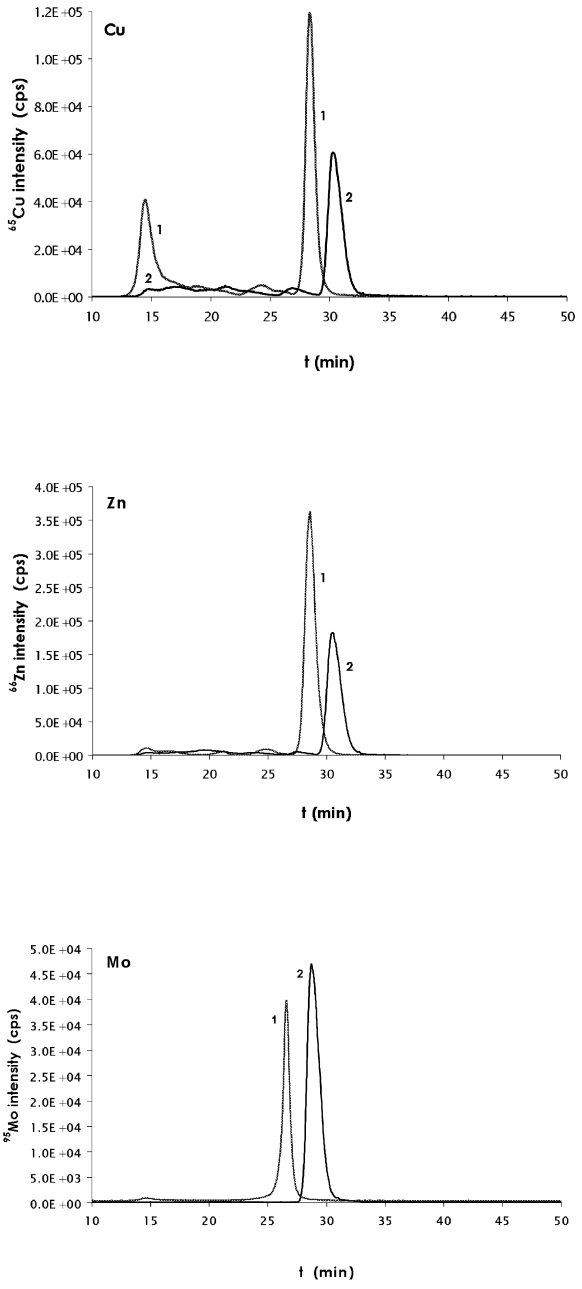

Fig. 9. Chromatograms of copper, zinc and molybdenum compounds of soybean flour (1) and white bean (2) extracts obtained by SEC/ICP-MS using the Superdex 75 column.

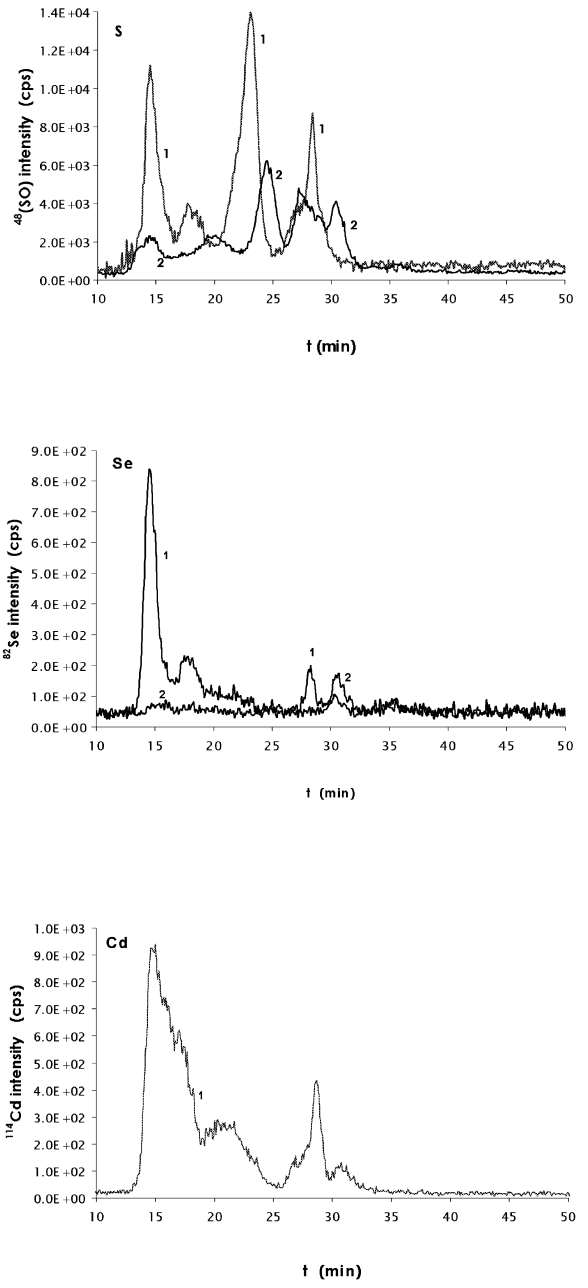

Fig. 10. Chromatograms of sulfur, selenium and cadmium compounds of soybean flour (1) and white bean (2) extracts obtained by SEC/ICP-MS using the Superdex 75 column.

Chromatograms of manganese, iron, cobalt, nickel, copper, zinc, molybdenum, sulfur, selenium and cadmium species on Superdex 75 are given in Figs.

Table 3
Summary of SEC–ICP-MS analyses on the Superdex 75 column

Element	Soybean flour[a]			White bean[a]		
	t_R (min)	M_r (kDa)	Peak area (%)	t_R (min)	M_r (kDa)	Peak area (%)
P	14.5	132[b]	3	14.4	136[b]	3
	23.2	6 (5)[d]	84	24.6	3.6 (3.5)[d]	64
	29.3	0.7[c] (0.7)[d]	12	29.2	0.7[c] (0.7)[d]	33
Mn	14.6	127[b]	3	14.5	132[b]	19
	16.4	67	27	19.8	20	77
	21.1	13	16	27.1	1.5[c] (3.8)[d]	4
	24.8	3.4	54			
Fe	14.6	127[b]	65	14.8	118[b]	30
	20.4	16	24	23.9	4.6	42
	24.6	3.6	11	27.1	1.5[c] (0.75)[d]	28
Co	14.6	127[b]	8	18.8	29	16
	24.8	3.4	16	28.2	1[c] (0.75)[d]	7
	28.4	0.9[c] (0.4)[d]	75	30.5	0.4[c] (0.3)[d]	55
	34.6	0.1[c] (0.1)[d]	1	34.5	0.1[c] (0.1)[d]	22
Ni	14.6	127[b]	19	14.8	118[b]	7
	21.2	12	3	18.8	29	22
	25.3	2.8[c] (0.7)[d]	43	23.7	5	9
	28.4	0.9[c] (0.4)[d]	32	27.3	1.4[c] (0.7)[d]	37
	33.7	0.1[c] (0.2)[d]	3	30.5	0.4[c] (0.3)[d]	25
Cu	14.5	132[b]	32	14.8	118[b]	3
	18.9	28	6	16.9	56	9
	24.2	4.2	5	21.2	12	13
	28.3	1[c] (0.4)[d]	58	26.8	1.7[c] (1.1)[d]	6
				30.5	0.4[c] (0.3)[d]	69
Zn	14.6	127[b]	3	19.5	22	11
	16.4	67	3	23.6	5.2	3
	24.8	3.4	5	27.5	1.3[c] (0.8)[d]	3
	28.6	0.9[c] (0.4)[d]	89	30.5	0.4[c] (0.3)[d]	83
Mo	14.6	127[b]	3	21.8	10	1
	26.6	1.8[c] (0.2)[d]	97	28.7	0.8[c] (0.2)[d]	99
S	14.5	132[b]	24	14.4	136[b]	11
	17.7	42	11	20.1	18	14
	23.1	6.2	42	24.5	3.8	32
	26.8	1.7[c] (0.4)[d]	8	27.2	1.4[c] (0.2)[d]	27
	28.4	0.9[c] (0.1)[d]	15	30.4	0.5[c] (0.1)[d]	16
Se	14.5	132[b]	70			
	17.7	42	21			
	28.3	1[c] (0.4)[d]	9	30.7	0.4[c] (0.2)[d]	100
Cd	15.0	110[b]	62			
	20.3	17	21			
	27.9	1.1[c] (0.9)[d]	5			
	28.7	0.8[c] (0.5)[d]	9			
	30.8	0.4[c] (0.3)[d]	3			

[a] Minor peaks (<2%) of elements (except Co and Mo) were omitted.
[b] Estimation is above the upper limit (70 kDa) of the fractionation range given by the manufacturer.
[c] Estimation is below the lower limit (3 kDa) of the fractionation range given by the manufacturer.
[d] The value of the molecular weight estimated by analysis using the Superdex Peptide column is given in brackets.

8–10. All results of SEC–ICP-MS analyses using the Superdex 75 column including retention time, apparent molecular weight estimation and relative area of the peak are summarized in Table 3. The fractionation range of the Superdex 75 column according to the manufacturer is 3–70 kDa. In cases where the estimated molecular weight of the species was lower than 3 kDa, the value in the table is completed by the value obtained using the Superdex Peptide column (fractionation range 0.1–7 kDa) given in brackets. Both chromatograms were compared and the respective peak in the low molecular weight region was identified on the basis of similar relative peak area. Perfect or reasonable agreement of both estimated molecular weight values was found for phosphorus, cobalt, nickel, copper, zinc and cadmium low molecular weight species. In the case of molybdenum, the differing molecular weight values and the inversion of the elution sequence of the main molybdenum and zinc peaks indicate that some non-size exclusion effects occur during chromatography on the Superdex Peptide column.

It is important to note that the dominant peaks of copper and zinc, which appeared in the low molecular weight region, and the minor peak of cadmium are co-eluted with the minor peak of sulfur compounds. It is known that sulfur containing peptides designated as phytochelatins [38] are the main ligands of these elements in contaminated plants. The structure of phytochelatins can be expressed as $(\gamma\text{-Glu-Cys})_n\text{-X}$ or $(\gamma\text{-Glu-Cys})_n$, where X represents Gly, β-Ala, Ser or Glu and $n=2–7$. Although the presence of phytochelatines was not proven, it is probable that these or similar compounds are binding partners of several trace metals. This could be the cause of the regular occurrence of most copper and zinc in the low molecular weight region.

4. Nomenclature

BTP	1,3-bis[tris(hydroxymethyl)methylamino] propane
HPM	chydroxypropylmethyl cellulose
MES	morpholinethanesulfonic acid
Tris	tris(hydroxymethyl) aminomethane

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