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Speciation of challenging elements in food by atomic spectrometry

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ABSTRACT

The review addresses trends in speciation analysis of challenging – rather rarely examined despite their importance for human health – elements in foodstuffs with special attention prior to sample preparation. Elements of interest are cobalt, iodine, manganese, iron, zinc, copper and molybdenum belong to the group of elements still appealed for searching their speciation despite extremely small contents in foodstuffs.

Advantages and weaknesses of recommended procedures are overviewed and discussed, highlighting state-of-the-art speciation methodologies developed so far in the field.

Contents

1. Introduction 18
2. Microelements of interest 19
3. Analytical problems in the speciation of challenging elements 20
4. Sample preparation – extraction from solid materials 20
5. Isolation from liquid materials 26
6. Analytical procedures 27
7. Conclusions 30
References 30

1. Introduction

Food products contain varying amounts of minerals, and more than 60 elements are typically found in them. Usually, they are classified into two groups: the major salt components and trace elements. The first includes K, Na, Ca, Mg, Cl, S, P and C. Among the trace elements, which are usually present in amounts less than 50 parts per million (ppm), one can find essential nutritive elements (Fe, Cu, I, Co, Mn, Zn, Cr, Ni, Si, F, Mo and Se); nonnutritive but nontoxic elements (Al, B and Sn) and nonnutritive, toxic elements (Hg, Pb, As, Cd and Sb) [1]. The nutritional value of a food containing a given mineral depends not only on its content, but also on its bioavailability for humans.

The examination of trace element speciation in food is extremely important for understanding the biological activity. Unfortunately, some trace elements are extensively studied, such as Se, As and Hg, while others do not attract the attention of analysts (here, one can find elements discussed in the review). The number of papers concerning speciation of the first mentioned elements in food products have increased significantly and reached a growing rate of 90 papers per year. Elements from the second class of elements, e.g., Co, I, Mn, Fe, Zn, Cu and Mo, have received little attention thus far (Fig. 1). Moreover, many papers apparently offering methods for the examination of their speciation actually describe fractionation procedures, allowing only the classification of an analyte or a group

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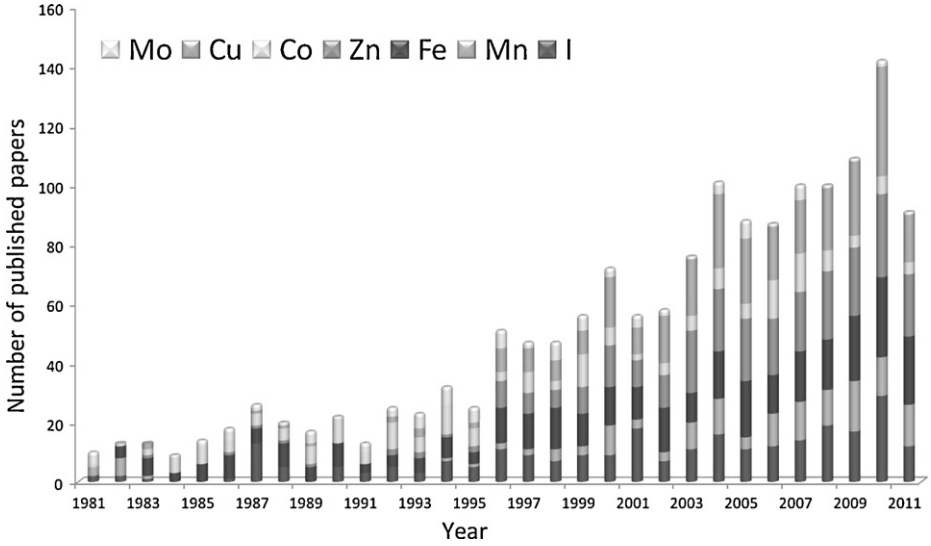

Fig. 1. Evolution of the number of published papers concerning selected elements in food per year from 1981 to 2011. Distribution of research papers as a function of the element considered.

of analytes from certain samples according to physical (e.g., size, solubility) or chemical (e.g., reactivity) properties [2].

The aim of this paper is to present state-of-the-art methods for the analysis of speciation of elements important for human health but belonging to the group of "challenging" elements for analysts.

2. Microelements of interest

Co is an integral part of the only metal-containing vitamin, vitamin B12 [3], which includes a family of compounds containing cobalamin found in meat [hydroxocobalamin (OHCbl) and 5′-deoxy-5′-adenosylcobalamin (AdoCbl, coenzym B12)] and milk [hydroxocobalamin and methylcobalamin (MeCbl)] products. Other sources of cobalamins are food supplements and fortified foods, which primarily contain cyanocobalamin [4]. Typical levels of cobalamins in food range from 3 to 250 ng g^{-1}, and fortified cereals contain as much as 300 ng g^{-1} [5]. Knowledge concerning the bioavailability of vitamin B12 from various food sources is rather limited [6], despite its importance for human metabolism, the production of red blood cells and maintenance of the central nervous system.

I, a constituent of the thyroid gland, performs an important function in the synthesis of the thyroid hormones thyroxine (T4) and triiodothyroxine (T3), which are responsible for thermoregulation, metabolism, reproduction, growth and development, blood circulation, and muscle function, as well as the control of the oxidation-to-reduction ratio in cells [7–9].

The recommended dietary allowance (RDA) of I is 150 mg per day in the United States and from 150 to 200 mg per day in European countries [10]. The supplementation of foodstuffs (e.g., iodized salt [11], milk or meat [12]) with I is commonly practiced to prevent I deficiency disorders, although the element is plentiful in the oceans and marine animals. Seaweeds accumulate exceptionally high quantities of I from the sea [13]. The I level in breast milk is known to be affected by the maternal diet, thereby affecting infant nutrition. Infant formula typically must be supplemented with I [14].

The toxicity and bioavailability of I depend on the character of its species. The inorganic forms, iodide and iodate, are less toxic than elemental iodine or some organically bound forms of I [15]. In addition, the bioavailability of organically bound I [monoiodotyrosine (MIT) and di-iodotyrosine (DIT)] is less than that of mineral iodide [16].

Fe acts as a cofactor for many enzymes and is involved in oxygen transport and electron transfer [17]. Daily requirements for Fe are 8–18 mg for humans [18], and it is potentially toxic in overdose amounts due to its pro-oxidant activity.

The absorption of iron in humans depends on its oxidation state [19]; ferrous salts are better absorbed than ferric salts, which are poorly soluble in the gut. However, Fe(III) may be reduced to the more soluble Fe(II) form in the gut by the action of gastric hydrochloric acid and reducing agents like ascorbic acid [20]. It is generally accepted that only soluble, non-heme iron can be absorbed [21]. Absorption of Fe from human milk is significantly better than from cow's milk or various infant formulas [22]. Some food components, like phytates, phosphate, polyphenols containing alkyl groups, oxalic acid, casein, phosphoprotein, albumin and minerals, such as Ca, Cu, Zn and Mn, decrease the fractional Fe absorption [23,24].

The form of Fe used for food supplementation significantly determines its uptake by humans. Ferrous sulfate is very well absorbed, but it can be responsible for the discoloration and oxidation of food products. The preferred species of Fe used for the enrichment of flour is the elemental form, which is less likely to change foods [1].

Zn is the activation factor for several enzymes (e.g., carboanhydrase, alkaline phosphatase), it stabilizes the structures of RNA, DNA and ribosomes, and it influences the metabolism of certain hormones, including insulin and gonadotropin [25,26].

The recommended dietary allowance of Zn is 8–11 mg per day, and the tolerable upper intake level (UL) is 40 mg/day, as established by the Institute of Medicine in 2001 [27].

The greatest Zn contents were found in shellfish (approximately 400 ppm). In cereal grains, Zn is found in the range of 30 to 40 ppm. When acidic foods like fruit juices are stored in galvanized containers, an amount of the element sufficient to cause Zn poisoning may be dissolved. In meat, Zn is tightly bound to the myofibrils and has been speculated to influence the water-binding capacity of meat [1].

Cu plays an important role as a cofactor for crucial enzymes [28], including cytochrome c oxidase, Cu/Zn superoxide dismutase and ceruloplasmin, tyrosinase, lysyl oxidase, dopamine-monooxygenase and peptidyl glycine a-amidating monooxygenase (required for the modification of neuropeptide hormones) [29].

The recommended dietary allowance for Cu is approximately 1 mg per day, and a range of 1–3 mg is accepted as a safe level of

intake [30]. The primary sources of Cu are seeds, grains, nuts, beans (mainly in the germ and bran), shellfish and liver [31].

In humans with normal levels of intake, 55–75% of the element is absorbed from food and actively recycled between the digestive tract, body fluids and tissues (particularly the liver). Adults excrete approximately 1 mg of Cu daily, with bile being the primary route for Cu excretion [31,32].

Mn activates many enzymes involved in metabolic processes; it is needed for protein and fat metabolism, healthy nervous and immune systems, and for blood sugar regulation [33]. Mn is involved in the utilization of vitamins B1 and E and is required for normal bone growth [34].

The human dietary requirement for Mn is 2–3 mg per day, while the typical daily intake is 5.4–12.4 mg [35]; therefore, the diets of most people in developed countries provide sufficient amounts of Mn, and food supplementation is not necessary. This essential element [36] is not easily absorbed, but it is present in a wide range of foods. The greatest Mn concentrations are found in foods of vegetal origin, especially wheat and rice, but also in tea, soybeans, eggs and nuts [37–39]. The concentration range from none to toxic levels is very small for this element; therefore, analytical methods designed for its measurement must be highly precise and accurate [40].

Mo primarily functions as a cofactor for xanthine oxidase, aldehyde oxidase and sulfite oxidase in mammals and is regarded as an essential trace element in human nutrition [41]. The biologically active form is a cofactor for at least four human enzymes. In nutritional supplements, Mo is usually present in the form of molybdate.

The mean daily intake of this element has been estimated to be 180 μg per day [42,43]. Its main sources are cereals, vegetables, legumes and milk. In breast milk, the Mo content decreases from the first day of breastfeeding (15 $\mu g\,L^{-1}$) to a constant level after 2–3 weeks (4 $\mu g\,L^{-1}$) [44].

3. Analytical problems in the speciation of challenging elements

There is a lack of information about speciation analysis of the elements of interest, and the available information is rather scattered (Table 1). The primary goal of this review is to present the limitations of high performance separation techniques [mainly high performance liquid chromatography (HPLC) and capillary electrophoresis (CE)] coupled with atomic spectrometric techniques [e.g., electrothermal or graphite furnace atomic absorption spectrometry (ETAAS or GFAAS), inductively coupled plasma optical emission spectrometry (ICP-OES) or mass spectrometry (ICP-MS)] in element speciation analysis, which are likely responsible for the current state of the field.

The most important and problematic step in the analytical procedure is sample preparation. Foods, like other biological materials, have complicated matrices, and direct analysis is difficult and often impossible. The preparation of food samples depends on their nature: whether they are solid or liquid and the concentration of fats. Therefore, it is often necessary to extract species from solid materials or to isolate them from liquids.

The primary mistake made during sample preparation and analysis is with the stability problem of complexed species; it is important to avoid this problem for labile metal complexes. The most common issues occur with changing the oxidation state, methylation, hydrolyzation and the action of microorganisms. Microorganisms are able to split some complex bonds, thereby changing or disturbing the profile of the material. Biomethylation could cause the creation of volatile forms that may be lost during sample preparation [45].

In addition, it is important to remember that during sample preparation, sample dilution can cause the oxidation of the labile forms of the analytes [46], and freezing can cause the denaturation of proteins and the deactivation of enzymes during the mechanical and physical process of forming ice crystals [47].

4. Sample preparation – extraction from solid materials

Depending on the sample's matrices and the elements of interest, extraction, dissolution, leaching or solid–liquid extraction can be used for the isolation of analyte species from solid material, but the procedure used must preserve intact species of the analytes. It is important to realize that despite the chosen conditions (alkaline, acidic or enzymatic), only soluble species are typically able to be extracted [85]. The most useful technique for sample preparations of foods from plant origin is solvent extraction (water or buffer) with centrifugation (Table 1).

The extraction should be conducted to separate the analytes from the matrix without losses or pollution of the sample and without changes to the original form of the analytes in the sample. The choice of the technique for the extraction and the type of extractant used should be made with the following considerations: the chemical properties of the analytes, their chemical forms, the matrix and the applied technique. For the extraction of inorganic forms and metaloorganic compounds, acids or complexing agents are used, and organic solvents are applied for metaloorganic compounds with high molecular weight (HMW). This type of extraction is often conducted in combination with an ultrasonic bath to increase the effectiveness and the speed of the extraction. Checking the recovery with an investigation of the efficiency of the extraction is important in analytical speciation.

Extractions from solid samples that avoid losses or contamination and without alterations to the species are extremely difficult. Mass balances and recovery rates (species spikes) must be determined. Compromises are often necessary between sufficient recovery (strong attack) and preservation of the species [86].

For the isolation of Cu, Fe, Mn and Mo species from fruits and vegetables (e.g., apples or carrots), water or buffer extractions (e.g., Tris–HCl) were recommended [62]. Fruits were washed, peeled, sliced and blended. Homogenates were centrifuged, and aliquots of the obtained supernatant were analyzed. For the analysis of the water-insoluble fraction, enzymatic extractions (commercial enzymatic preparations containing pectinases, hemicellulases and cellulases) were used.

One of the problems in speciation analysis is changing the oxidation state of the element; it has been shown [20] that most of the methods used thus far for speciation analysis of the Fe content in foods modify the oxidation state of the element. Water and buffer extractions are recommended as the most appropriate extractant for the isolation of Fe species (Table 1). During the sample preparation for Fe speciation, the use of chelating agents like EDTA and/or citric acid should be avoided. In addition, hydrochloric acid and reducing agents, such as ascorbic acid, can reduce Fe(III) to the more soluble Fe(II) form and result in false results.

In a study devoted to the speciation of Cu, Zn and Mn in different edible nuts [61], the efficiency of extraction with alkaline (0.05 $mol\,L^{-1}$ NaOH) and acidic (0.05 $mol\,L^{-1}$ HCl) solutions was examined. The use of an alkaline extractant yielded an 82.6–95.2% recovery of the elements. For the acidic solutions, the recovery was only 23.3–34.9%, which may have been due to the isolation of only low molecular weight (LMW) compounds [87]. Nuts have significant amounts of fat, and the alkaline extractions yielded good results. The alkaline extractions may change the species of the elements due to the complex chemical composition of nuts (50–70% lipids, 10–20% proteins and 10–20% carbohydrates), and the sample preparation step may therefore require such a medium. In the chromatographic profiles obtained for acidic (HCl) and basic

Table 1
Procedures recommended for speciation analysis of elements of interest in food products.

Element	Other elements	Matrix	Technique	Solvent used	Separation technique	Chromatographic conditions	Detection technique	Limit of detection	Ref.
Sample preparation					Separation and detection				
Foods of plant origin									
Co		Green coffee	Solvent extraction	Water	–		ETAAS, ICP-OES	4 μg kg^{-1}	[48]
		Commercial vitamin B12 preparations	Solvent extraction	Water	CE		ICP MS	50 μg L^{-1}	[49]
	Fe, Zn, Cu, Mn, Mo	Soybean flour	Solvent extraction	Tris–HCl	SEC	Column: fractogel column with elevated phosphorus content, mobil phase: 0.02 mol L^{-1} Tris–HCl, pH 7.5, flow rate: 2.0 mL min^{-1}	ICP MS	–	[50]
	Zn, Mn	Wine	Ultracentrifugation		–		ICP MS	0.15 μg L^{-1}	[51]
I		Seaweed	Solvent extraction	NaOH or HCl or Tris–HCl	SEC	Column: superdex 75 HR 10/30 Mobile phase: 0.03 mol L^{-1} Tris–HCl, pH 8.0 Flow rate: 0.6 mL min^{-1} Injection volume: 100 μL	ICP MS	–	[13]
					IC	Column Ion Pac AS-11 anion exchange column (250 mm × 2.0 mm i.d. × 13 mm) Mobile phase: 0.005 mol L^{-1} sodium hydroxide Flow rate: 0.3 mL min^{-1} Injection volume 20 μL			
					RPLC	Column Alltima C18 (150 mm × 4.6 mm, 5 mm) Mobile phase (A) 0.01 mol L^{-1} Tris–HCl (pH 7.3): (B) 0.01 mol L^{-1} Tris–HCl (pH 7.3) and 50% MeOH Flow rate: 0.5 mL min^{-1} Injection volume 50 μL			
		Microalgae	Solvent extraction	Tris–HCl, SDS	SEC	Column: Superdex-75 (10 × 300 mm × 13 μm) Mobile phase: Tris–HCl, pH 7.0 Flow rate: 0.75 mL min^{-1} Injection volume: 50 μL	ICP MS	–	[52]
					AEC	Column: Agilent (4.6 mm × 150 mm) Mobile phase: 20.0 mM NH_4NO_3, pH 5.6 Flow rate: 1.0 mL min^{-1} Injection volume: N/A			
Fe		Beverages	Solvent extraction	1-(2-pyridylazo)-2-naphthol (PAN) in chloroform	–		FAAS	9 μg L^{-1}	[17]
		Legumes	Solvent extraction	Water	–		UV–vis spectrophotometry, AAS	–	[20]
		Wine	Ultracentrifugation		–		UV–vis spectrophotometry, FAAS	Fe(II) 0.22 μg L^{-1} Fe(III) 0.72 μg L^{-1}	[53]
		Fruit juices	Ultracentrifugation				UV–vis spectrophotometry	–	[54]
	Zn, Cu	Rye and oat flakes	Solvent extraction	Tris–HCl	SEC	Column: Superdex 75 10/300 GL Mobile phase: 0.02 mol L^{-1} Tris–HCl, pH 7.5 Flow rate: 1.2 mL min^{-1} Injection volume: 100 μL	ICP MS		[55]

Table 1 (*Continued*)

Element	Other elements	Matrix	Technique	Solvent used	Separation technique	Chromatographic conditions	Detection technique	Limit of detection	Ref.
Sample preparation					Separation and detection				
	Cu, Mn	Beer	SPE	Acetic acid buffer	SEC	Column: Superdex 75 HR 10/30 (300 mm × 10 mm id) Mobile phase: 0.2 M acetic acid buffer, pH 4.4 Flow rate: 1.0 mL min^{-1} Injection volume: 150 μL	ETAAS	1.7 μg L^{-1}	[56]
	Zn, Cu	Mushrooms	Solvent extraction	NaOH or HCl	SEC	Column: Superdex 75 HR 10/30 (300 mm × 10 mm id) Mobile phase: 10 mmol L^{-1} CAPS buffer, pH 10.0 Flow rate: 0.7 mL min^{-1} Injection volume: 100 μL	UV ICP MS	–	[57]
	Cu	Cashew nuts	Solvent extraction	Tris–HCl	SEC	Column: Superdex 75 10/300 GL Mobile phase: 0.2 mol L^{-1} Tris–HCl buffer, pH 7.5 Flow rate: 0.5 mL min^{-1} Injection volume: 100 μL	ICP-OES	–	[58]
	Zn, Cu, Mn	Nuts and seed	Solvent extraction	Tris–HCl	SEC	Column: Superdex 75 10/300 GL Mobile phase: 0.2 mol L^{-1} Tris–HCl buffer, pH 7.5 Flow rate: 0.5 mL min^{-1} Injection volume: 200 μL	MALDI TOF MS	1.4 μg kg^{-1}	[59]
Zn		Pumpkin seeds	Solvent extraction	Water	SEC	Column: Superdex Peptide 10/30 (300 mm × 10 mm id) Mobile phase: 0.03 mol L^{-1} Tris–HCl (pH 7.4), 0.03 mol L^{-1} Tris–HCl (pH 2.5) Flow rate: 0.5 mL min^{-1} Injection volume: 100 μL	ICP MS		[60]
	Cu, Mn	Nuts	Solvent extraction	NaOH	SEC	Column: Superdex peptide HR 10/30 Mobile phase: 50 mmol L^{-1} Tris–HCl buffer pH 8.0 Flow rate: 0.6 mL min^{-1} Injection volume: 100 μL	UV ICP MS	–	[61]
	Cu	Apple	Solvent extraction	Water, enzyme	SEC	Column: Superdex 75 HR 10/30 (300 mm × 10 mm id) Mobile phase: 30 mmol L^{-1} ammonium formate buffer, pH 5.2 Flow rate: 0.6 mL min^{-1} Injection volume: 20–100 μL	ICP MS	–	[62]
Cu	Mn	Nuts	Solvent extraction	NaOH	SEC	Column: Superdex peptide HR 10/30 Mobile phase: 50 mmol L^{-1} Tris–HCl buffer, pH 8.0 Flow rate: 0.6 mL min^{-1} Injection volume: 100 μL	UV ICP MS	–	[61]
		Apple	Solvent extraction	Water, enzyme	SEC	Column: Superdex 75 HR 10/30 (300 mm × 10 mm id) Mobile phase: 30 mmol L^{-1} ammonium formate buffer, pH 5.2 Flow rate: 0.6 mL min^{-1} Injection volume: 20–100 μL	ICP MS	–	[62]
		Wine	Ultracentrifugation		–		DPASV	5000 μg L^{-1}	[63]
		Rice	Solvent extraction	HNO_3, NaH_2PO_4, HCl	–		XANES	–	[64]

Table 1 (*Continued*)

Element	Other elements	Matrix	Technique	Solvent used	Separation technique	Chromatographic conditions	Detection technique	Limit of detection	Ref.
Sample preparation					Separation and detection				
Mn		Tea	Solvent extraction	Organic solvents	–		FAAS	–	[65]
		Corn, rice, wheat flour	Solvent extraction	NaOH	–		FAAS	0.75 μg L^{-1}	[66]
		Pine nuts	Solvent extraction	Chloroform/ methanol; sodium hydroxide	SEC	Column: Hiload 26/60 Superdex 30 Prep and 75 Prep Mobile phase: 5o mmol L^{-1} Tris–HCl buffer pH 8.0 Flow rate: 2 mL min^{-1} Injection volume: 2 mL	ICP MS	26 μg kg^{-1}	[67]
					IEC	Column: Dionex AS11-HC Mobile phase: A: 10 mM NH_4-acetate/acetic acid (pH 6.3); B: 0.5 mM NaOH; C: 10 mM NaOH Flow rate: 0.5 mL min^{-1} Injection volume: 75 μL			
		Rye and oat flakes	Solvent extraction	Tris–HCl	SEC	Column: Superdex 75 10/300 GL Mobile phase: 0.02 mol L^{-1} Tris–HCl, pH 7.5 Flow rate: 1.2 mL min^{-1} Injection volume: 100 μL	ICP MS		[50]
Foods of animal origin									
Co		Eggs	Solvent extraction	Water	SEC	Column: Zorbax GF 250 (300 mm × 10 mm, 5 mm) Mobile phase: 30 mM Tris-HNO_3 buffer, pH 7.5 Flow rate: 0.5 mL min^{-1} Injection volume: 100 μL	ICP MS	0.1 μg L^{-1}	[68]
	Fe, Zn, Cu, Mn, Mo	Mussels	Solvent extraction	Tris–HCl, protease inhibitor	SEC	Column: Sephadex G-75 (100 cm × 1 cm id) Mobile phase: 10 mM Tris.HCl, pH 7.4; 5 mM 2-mercaptoethanol (2-MCE), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM NaCl Flow rate: 0.2 mL min^{-1} Injection volume: 1 mL	ICP MS	4.6×10^{-2} μg kg^{-1}	[69]
I		Milk, infant formula	Ultracentrifugation		SEC	Column: Superdex 75 HR 10/30 (10 mm × 300 mm × 13 mm) Mobile phase: 30 mM Tris–HCl buffer, pH 7.0 Flow rate: 0.75 mL min^{-1} Injection volume: 100 μL	ICP MS	1 μg L^{-1}	[12]
		Eggs	Solvent extraction	Water	SEC	Column: Superdex 75 (10 mm × 300 mm × 10 μm) Mobile phase: 30 mM Tris–HCl buffer, pH 7.5 Flow rate: 0.7 mL min^{-1} Injection volume: 100 μL	ICP MS	10 μg L^{-1}	[70]
		Human milk	Ultracentrifugation				ICP MS	–	[71]
		Bovine milk	SPE	$(NH_4)_2SO_4$	–		EINAA-CS	230 μg L^{-1}	[72]
		Milk	Ultracentrifugation		IC	Column: HIC-6A Mobile phase: 3.5 mM L^{-1} Na_2CO_3/1.0 mM L–$^{-1}$ $NaHCO_3$ Flow rate: 1.2 mL min^{-1} Injection volume: 30 μL	ICP MS	3×10^{-3} μg kg^{-1}	[73]
	Fe, Cu	Human milk	Ultracentrifugation		SEC	Column: TSKgel G 2000 SWXL Mobile phase: 0.1 mol L^{-1} Tris–HCl, pH 7.0 Flow rate: 0.5 mL min^{-1} Injection volume: N/A	ICP MS	–	[74]

Table 1 (*Continued*)

Element	Other elements	Matrix	Technique	Solvent used	Separation technique	Chromatographic conditions	Detection technique	Limit of detection	Ref.
Sample preparation					Separation and detection				
Fe		Beef meat	Solvent extraction	HCl/water/acetone (heme Iron) citrate phosphate buffer (non-heme iron) sodium dihydrogen orthophos-phate, potassium cyanide and sodium sulfate	SEC	Column: Progel (TSK Gel G2000SWXL) (300 mm × 7.6 mm, 10 μm, 125 Å) Mobile phase: 0.1 M Tris–HCl, pH 7.2 Flow rate: 1.0 mL min^{-1} Injection volume: N/A	UV ICP MS	2.4 μg kg^{-1}	[75]
		Meat and seafood	Solvent extraction	HCl/water/acetone (heme Iron) Hematin (non-heme iron)	SEC	Column: TSK Gel G2000SW Mobile phase: 0.1 mol L^{-1} Tris–HCl, pH 7.2 Flow rate: N/A Injection volume: N/A	UV ICP MS	– 850 μg L^{-1}	[76]
		Breast milk and infant formula	Ultracentrifugation		–		ETAAS	1.4 μg L^{-1}	[77]
	Zn, Cu, Mn	Porcine liver	Solvent extraction	Tris–HCl, sucrose, ammonium formate	SEC	Column: TSK HW 55 S (50 cm × 8 mm) Mobile phase: 10 mM ammonium acetate/10 mM sodium chloride, pH 7.5 Flow rate: 0.5 mL min^{-1} Injection volume: N/A	ICP MS	–	[78]
					RPLC	Column: C4 column HyPurity (30 mm × 4.6 mm) Mobile phase: 10 mM ammonium acetate, pH 7.5; 10 mM ammonium acetate in 60% methanol, pH 7.5 Flow rate: 0.45 mL min^{-1} Injection volume: N/A	ESI MS		
Zn		Human milk	Ultracentrifugation		SEC	Column: Fractogel TSK HW 55 Mobile phase: Na_2HPO_4/NaH_2PO_4 buffer, each salt in concentrationsof 10 and 50 mmol L^{-1}, pH 5.5, 7.0 Flow rate: 0.5 mL min^{-1} Injection volume: N/A	ICP MS	–	[79]

Table 1 (*Continued*)

Element	Other elements	Matrix	Technique	Solvent used	Separation technique	Chromatographic conditions	Detection technique	Limit of detection	Ref.
Sample preparation					Separation and detection				
		Tod dler milk-based formula	Solvent extraction	TFA	AEC	Column: Mono Q column (50 mm × 5 mm i.d.) Mobile phase: 0.02 mol L^{-1} Tris–HCl, pH 8 Flow rate: 0.5 mL min^{-1} Injection volume: 50 μL	ESI MS MS	115.4 μg L^{-1}	[80]
					RPLC	Column: C18 Hi-Pore (250 mm × 4.6 mm, i.d.; 5 μm particle size) Mobile phase: 0.037% (v/v) TFA, B, 0.027% (v/v) TFA and 80% (v/v) acetonitrile Flow rate: 0.8 mL min^{-1} Injection volume: 100 μL			
	Cu	Mussels	Solvent extraction	Tris–HCl, protease inhibitor	SEC	Column: Sephadex G-75 Mobile phase: 10 mM Tris–HCl, (pH 7.4), 5 mM 2-mercaptoethanol (2-MCE), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 25 mM NaCl Flow rate: 0.2 mL min^{-1} Injection volume: 1 mL	ICP MS	10.3×10^{-2} μg L^{-1}	[81]
					AEC	Column: Mono Q HR 5/5 Mobile phase: (A) 4 mM Tris–HCl, pH 7.4; (B) 250 mM ammonium acetate + 10 mM Tris–HCl, pH 7.4 Flow rate: 1 mL min^{-1} Injection volume: 50 μL			
Cu	Mn	Milk	SPE	EDTA solution	–		ICP-OES	1 μg L^{-1}	[82]
Mn		Human milk	Ultracentrifugation		SEC	Column: TSKHW 55 F (500 mm × 20 mm ID, Mobile phase: 10 mM NH_4-acetate/acetic acid, pH 6.3 Flow rate: 1.0 mL min^{-1} Injection volume	ICP MS	–	[34]
					SAX	Column: SAX separation column Mobile phase: 10 mM NH_4-acetate/acetic acid, pH 6.3, 0.8 mM NaOH, 10 mM NaOH Flow rate: 0.75 mL min^{-1} Injection volume: 1 mL			
	Mo	Human milk	Ultracentrifugation		SEC	Column: TSK Guard column Mobile phase: 0.1 M HEPES, 0.08 M NaCl, pH 6.8 Flow rate: 0.9 mL min^{-1} Injection volume: 100 μL	ICP MS	0.08 μg L^{-1}	[83]
Mo		Milk, infant formula	Ultracentrifugation		SEC	Column: Sephadex gel G25-150 Mobile phase: N/A Flow rate: N/A Injection volume: 1 mL	EAAS	20 μg kg^{-1}	[84]

(NaOH) extraction media, HMW fractions were obtained by the NaOH extract, while the HCl extract yielded only LMW compounds. However, the use of different solvents can be integrated in a multistage extractive scheme to isolate different element forms after each extractive stage, while at other times, the extraction scheme may consist of different extraction steps to remove possible concomitants before isolation of the target elements [88].

Iodine is reportedly less stabile in acidic media than alkaline media when using ICP-OES/MS [89,90]. Stark et al. [91] have demonstrated that the presence of iodate and iodide in acid digests leads to the formation of molecular iodine according to the following reaction: $IO^{-3} + 5I^- + 6H^+ \rightarrow 3I_2 + 3H_2O$. This reaction can lead to analyte losses, carry-over, and memory effects, as well as significant sensitivity alterations in pneumatic nebulization ICP-MS [88]. To avoid these problems, some applications apply alkalinization to the acid digests before measurements are taken [13].

Food products of animal origin (e.g., meat, eggs and milk) create more analytical problems than those reported for plant foodstuffs. A strong example of an efficient but species preserving extraction method for Mn species in tissues of animal origin is presented by Diederich and Michalke [92]. The authors improved the extraction method published by Nischwitz et al. [93], who used atmosphere to prevent species oxidation but with very low efficiency. The improved extraction method used cryogenic conditions (+N_2 liquid) under an inert gas atmosphere. The tissue degradation effects were evaluated during long-term storage. The extraction efficiency increased from 17% (−N_2 liquid) to 26% (+N_2 liquid) for Mn in brain extracts and from 28% (−N_2 liquid) to 44% (+N_2 liquid) in liver extracts. For Fe species, the increase was only from 40% (−N_2 liquid) to 44% (+N_2 liquid) in brain, but from 64% (−N_2 liquid) to 74% (+N_2 liquid) in liver. Manganese compounds in biological samples are generally very unstable [94] and decompose easily during sample preparation and analysis. Even the procedure conducted under limited oxygen access could not prevent the oxidation and dissociation of the original manganese species [55].

Enzymatic (protein, pectin, pepsin and lipase) hydrolysis is also recommended for Mn speciation analysis. [95]. However, some of the enzymes (like protease) are not specific and can break the bonds in peptides. As a result, the interesting connections of proteins with elements cannot be observed in the sample. Very often, extractions with sodium dodecyl sulfonate (SDS), which denatures proteins and removes metalloproteinase from animal tissue, are used in meat products [96]. In the speciation analysis of Cu, Zn, Mo and Mn in seafood (mussels and crabs) tissue, the extraction of metal species is preceded by defatting with acetone or chloroform [97,98], or trypsin is used to destroy the proteins [99]. Seafood tissues are also homogenized with Tris–HCl (pH 8.6) containing phenylmethanylsulfonyl fluoride [69,81].

In chicken egg samples, the primary problem during analysis is the high fat content (approximately 10%). For this matrix, water extraction followed by the isolation of fat with organic solvents (acetone or ether) has been found to be the most suitable solution [68,70].

For better efficiency, the authors present multiple extractions. The amount of the element remaining in solid material is reported to depend only on the volume of the solvent apprehended in it (usually in the first cycle), rather than on the number of repeated leachings [100]; multiple extractions do not improve the efficiency of the process.

5. Isolation from liquid materials

Liquid foodstuffs can be divided into two groups for speciation analysis: compounds containing fat (e.g., milk, infant formula) and those without fat in the matrix (e.g., juice, wine and other beverages). In the latter case, the preparation step is usually simple and samples are only diluted after filtration or ultracentrifugation [53,63].

Liquid–liquid extraction (LLE) should extract a large proportion of the element compounds of interest and only a small proportion of all the other matrix elements. The primary advantages of this technique are its simplicity, rapidity and ease of use, but the relatively high cost of the procedure and the loss of reagents, which have the potential to contaminate the water with toxic organic compounds during reagent disposal [101], should be considered. LLE extraction is often used in combination with sonification, microwave irradiation, high pressure, heating, shaking and accelerated solvent extraction. Sonification may increase the efficiency of extraction form plant cells by more than 20% [102]. Despite many advantages, the dangers of using high temperatures and pressures, which might change the species (labile metal species complex), should be considered.

The analysis of fat-containing foodstuffs, especially milk (human or animal) or infant formula, must include a preliminary preparation step. As it is an emulsion containing solid particles, the casein and fat should first be separated from the element-rich aqueous phase (whey) by centrifugation. Next, the species of elements of interest are separated primarily by the use of solid phase extraction (SPE), a procedure proposed for the analysis of I, Fe, Mo and Mn [82,103].

SPE is commonly used as an enrichment technique when low concentrations of analytes need to be recovered and analyzed. SPE can be performed off-line, with the sample preparation separated from the subsequent chromatographic analysis, or on-line by direct connection to the chromatographic system [104]. SPE of the whey allows the separation of anionic and cationic species, when ion-exchanging resin is used as a stationary phase. However, the SPE can partially remove complex-bound metals from bioligands in an uncontrolled manner. Sometimes, the recovery for metals is very low due to the creation of hydrogen bonds between ligands and silanol groups in the SPE columns.

The sample treatment procedures with good recoveries are recommended for fat and milk proteins based on two steps: the centrifugation of a whole milk sample for fat elimination and the centrifugation of the resulting whey after adjusting the pH to 4.6 with acid to precipitate and separate the casein micelles [105,106]. Finally, an ultrafiltration treatment of the whey is necessary to avoid the effect of high-weight molecules remaining in the whey, mainly proteins, for the separation and detection.

The low pH during the precipitation of casein has been proven to release complex-bound metals from many other bioligands, even more when the isoelectric point is reached. The variation in pH between 5.5 and 8.5 clearly demonstrates the advantage of a neutral pH, and water was the most suitable for speciation experiments in human milk (pH 6.6) [79].

The cloud point extraction (CPE) separation method was proposed for the extraction of Fe species by Filik and Giray [107]. In a beer sample, the authors reacted iron(II) with 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (5-Br-PADAP) in the presence of EDTA, yielding a hydrophobic complex, which was then extracted to form a a surfactant-rich phase. In many investigations of beer samples, LLE or SPE are the most widely used techniques, but they are complex and time-consuming. The surfactants provide analytical chemistry new possibilities for separation and preconcentration, which is based on CPE. This method is solvent free and non-polluting [108].

The presented techniques allow the separation and preconcentration of compounds and the matrix of the sample. Removing the matrix can cause a change in the equilibrium of species in the analyzing material. The eluates should be investigated within a short time period, which is the reason that separation techniques should be connected on line with detectors.

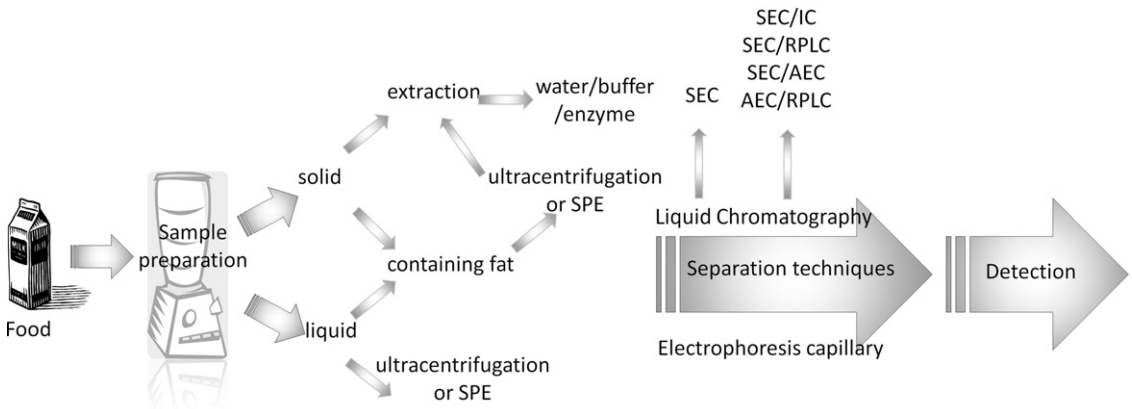

Fig. 2. The pathway of speciation analysis of challenging elements in food samples.

In addition, some instrumental methods, such as those referred to as non-chromatographic methods, require the conversion of some element species to another form that selectively responds to the instrumental technique used. Sample pre-treatment is not necessary when using certain non-chromatographic methods such as X-ray absorption near edge structure (XANES) [64,109].

Fig. 2 illustrates the scenario of speciation analysis with challenging elements from food sample preparations, throw separation mechanisms and finally the best detection (presented in Fig. 3).

6. Analytical procedures

The identification of the variety of elements and their species requires the use of separation steps in the analytical procedures because methods traditionally used for the identification and determination of trace elements do not allow for distinguishing between organic, inorganic and other forms. A wide range of separation techniques, but primarily LC and CE, can be used for the separation of element species in food. Among them, size exclusion chromatography (SEC) plays a special role, as well as ICP-MS, which is the most frequently recommended detection unit.

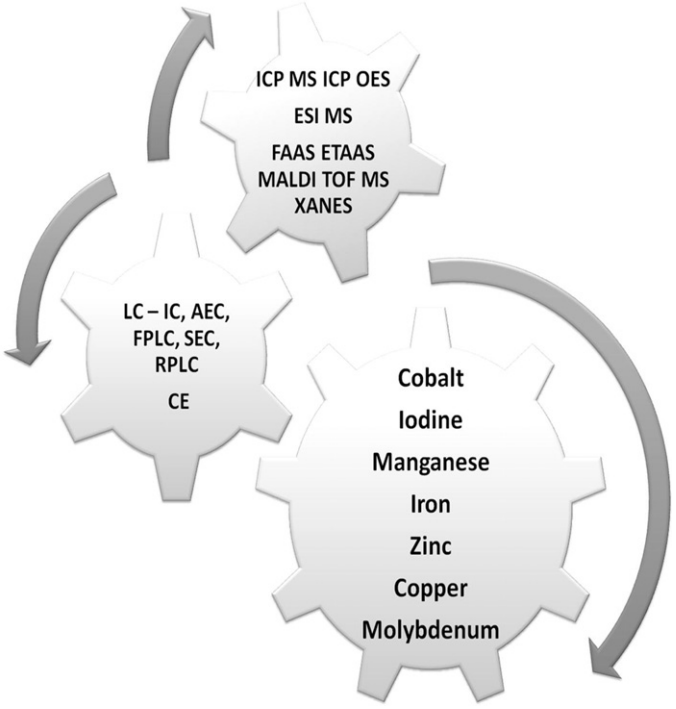

Fig. 3. Analytical techniques used for speciation of challenge elements.

SEC is the one of the most commonly used separation techniques for complexes with bioligands, where complexes are separated on the basis of the retention mechanism sufficient for the molecular weight of the analyte. Elements in food are complexed with various bioligands, and their bioaccessibility depends on this complex; therefore, understanding which ligands are complexed with each element is an important outcome of analytical speciation. The primary advantages of SEC-ICP-MS are its simplicity, the limited formation of objects during the separation and the low detection limits achievable [110]. These are the reasons why SEC techniques are common, but they can only be recommended for fractionation of the element compounds, and they only provide information about groups of compounds. Many authors neglect these limitations and refer to it as speciation analysis, but information about the type of form is provided only by methods like mass spectrometry.

Many stationary phases of LC and buffers or organic modifiers can denature native species. Chelating eluents or ion exchangers may cause recomplexation of free or labile-bound metal species. Additionally, the mobile phases can cause severe alterations in the species (e.g., buffers can stabilize biomolecules but may also alter species equilibrium through complexing tendencies or an input of metal contamination) [111].

The most popular detector used in LC, UV–vis spectrophotometry [112,113], is of limited use in speciation studies. UV–vis spectrophotometry is used only for the differentiation of non-heme and heme Fe species in complex matrices [20,53,75]. The limitation of UV detection is the relatively poor sensitivity and that good detection limits require a high-absorbing UV complex for good sensitivity in the different detection modes.

Element-selective detection plays a significantly more important role in these analyses (e.g., ETAAS, ICP-OES or ICP-MS; Table 1). ETAAS has the advantages of being widely available, highly sensitive and relatively cheap. ETAAS has been used for the speciation analysis of Mn, Mo and Cu in foodstuffs [84]. The detection limits achieved by LC-ETAAS ranged from approximately $\mu g\,mL^{-1}$, and similar values were reported for LC-ICP-OES. GFAAS, the most popular form of ETAAS, is a powerful tool for the routine analysis of trace and ultratrace elements in clinical and biological samples [114]. This technique is low-cost, simple, and small sample volumes are required (5–20 μL). However, this technique is susceptible to spectral and matrix interferences.

Because the concentrations of trace elements in food samples are low (e.g, 10–100 $ng\,g^{-1}$), the use of AAS or ICP-OES detection methods is not suitable for many applications. Better detection capabilities can be achieved using ICP-MS, and a combination of high performance liquid chromatography LC and ICP-MS offers the

best sensitivity for the on-line detection of elements. Additionally, some of these detectors work discontinuously, and it is therefore problematic to use them as chromatographic on-line detectors.

One of the most difficult elements in speciation analysis is ***Fe***. Many authors have presented speciation investigations based only on the extraction and separation procedures of iron species and determine only the content of the element. Speciation analysis of Fe in food focuses primarily on the separation and detection of Fe(II) and Fe(III). The main analytical problem in this case is the low concentration of the element in food, requiring the use of very sensitive methods. To solve this problem in the past, Vis spectrophotometric methods were recommended, especially those based on chelates with 2-(5-bromo-2-pyridylazo)-5-(diethylamino)-phenol (Br-PADAP). This reagent forms a complex with Fe(II), absorbing at 560 and 748 nm [115], and with Fe(III), absorbing exclusively at 560 nm, which allowed the speciation of Fe in wine [53]. A much more sensitive FAAS (Flame Atomic Absorption Spectrometry) procedure for the determination of Fe(II)/Fe(III) [17] was based on the preliminary extraction of Fe(II) with 1-(2-pyridylazo)-2-naphthol (PAN) in chloroform; under developed conditions, Fe(III) does not form a complex with the reagent and remains in the aqueous phase.

Many investigations of Fe are based on the speciation of the element in human milk. One investigation performed the separation on a SEC column instead of conducting the extraction procedure for milk (breast milk and infant formulas). Next, Fe was determined in the separate protein fractions by ETAAS [77]. In defatted whey, Fe(II), Fe(III) and Fe-protein species were found. In breast milk, Fe was bound primarily to proteins with molecular masses of 3 and 76 kDa.

For the improved fractionation of elements in a beer sample, a two-dimensional procedure (SPE and SEC) was developed [56]. Ion-exchange SPE allowed the differentiation of cationic species of other elements from the anionic forms of Fe. SEC analysis demonstrated that the molecular masses of the examined species were on the order of 4–12 kDa. Based on these results, it was suggested that metal ions are complexed by polymeric phenols or phytic acid. For the quantitative study of metal contents, off-line ETAAS was used; the most important aspects are sorption and desorption in the fractionation of Fe. SPE techniques provide information about the metal complexes with polyphenols and labile complexes of Fe.

The recovery of Fe in beer and milk samples after SEC separation was not presented in the two cited investigations, which might explain why the proteins with Fe were found only in this range of molecular masses. Coni et al. [116] studied the SEC-ICP-MS technique for Fe speciation in human milk. They reported five Fe fractions: caseins (>2000 kDa), immunoglobulins (500–2000 kDa), the peak from 100–500 kDa corresponded to albumin or lactoferrin and the peak from 2–100 kDa included lactoalbumin, and the last one (<2 kDa) revealed Fe bound to LMW compounds (likely citrate).

To improve the detection limits in Fe speciation, ICP-OES and MS were coupled with liquid chromatography. The developed procedure allowed the determination of the total Fe in meat and seafood in addition to the identification of non-heme Fe, heme Fe and Fe-containing myoglobin [75,76]. A similar fractionation study was performed for milk using SPE coupled to ICP-OES, and various cationic, anionic, neutral and casein-bound species of Cu and Mn were found [82].

ICP-MS methods are still problematic for the determination of Fe species due to interfering polyatomic ions of $Ar^{40}O^{16}$ on Fe^{56}. Harrington et al. reduced this problem in meat analyses by finding the best compromise with the medium resolution mode on the voltages on the instrument lens [75]. They used SEC coupled to sector field ICP-MS (SF-ICP-MS) detection. The results were validated using measurements of the total iron concentration in the sample and in the extracts. In addition, Sanz Medel and co-workers [117] suggested the use of H_2 as a collision gas to reduce the background interference.

In all research, a very important part of the work is to check the chromatographic recovery of the elements, especially for Fe, because in many investigations, the recovery decreased and approximately one quarter of the soluble Fe was transformed into ionic forms [55,57,59]. The absorption and desorption of Fe during analytical procedures should be controlled.

Speciation analysis of ***Mn*** is extremely difficult due to its very low concentration in foodstuffs, and few papers have been published so far in this field. Additionally, the on-line ICP-MS detection in food fractions is unclear, either from non-spectral or spectral interferences. Due to the substantial retention of alkali metals, the ICP-MS detection of other elements takes place under stabilized sensitivity conditions. In food products, some elements (e.g., potassium) are present in high amounts and can change the results for spectral interferences [on ^{55}Mn ($^{39}K^{16}O$) or on ^{95}Mo ($^{40}Ar^{39}K^{16}O$)] or analyte intensity. Other spectral interferences for ^{55}Mn are $^{37}Cl^{18}O$, $^{23}Na^{32}S$ and $^{38}Ar^{16}O^{1}H$. For the fractionation of soluble species of Mn (and multielemental detection – Fe, Co, Zn and Mo) present in soybean flour and common white bean seeds, SEC has been recommended. The contents of elements in chromatographic fractions were determined using ICP-MS. Most Mn species were found in the low molecular mass region (2–5 kDa), whereas Fe was predominantly bound to high molecular mass compounds (180 kDa). The Mn elution profile was the most complex; its species were detected in the medium molecular mass region (20 kDa), as well as in the low mass region (7 kDa). These results are inconsistent with previously published findings, and the authors suggest that changes in the manganese oxidation state during the sample preparation may have been responsible for the profile [50].

Compared with the relatively simple analytical methodology discussed above, multidimensional chromatography coupled to atomic spectrometric detection offers much more information [118–120]. In this study a metallomics analytical approach has been applied for the first time to the study of manganese species in pine nuts, with the aim of identifying known and unknown Mn-binding biomolecules in this food. The speciation of Mn in pine nuts was examined using SEC-IEC-ICP-MS [67]. The ICP-MS instrument was equipped with a reaction cell; hydrogen was introduced into the octopole cell as the reaction gas. Using strong anion-exchange chromatography (AEC), some solvents in the mobile phase could change element species; to avoid this situation, 10 mM NH_4-acetate/acetic acid (pH 6.3) was mixed with 0.8 mM/0.1 mM NaOH. The identification of $MnCl_2$ andMn-citrate was confirmed by analysis of the standards, as well as by nano electrospray ionization with direct infusion quadruple time-of-flight mass spectrometry (nESI QqTOF-MS) analysis. The latter allowed the elucidation of the structure of the third Mn-containing compound as its complex with isocitrate dehydrogenase. This method offers high specificity for manganese and allows sensitive determinations in pine nuts. An advantage of these procedures was the use of the buffer system to protect the manganese species from changing and the identification of the species by the nESI QqTOF-MS technique.

Multidimensional chromatography was also used in the speciation of Mn in human milk. The very low total Mn concentration with the single species of even lower concentration and the difficulties in the sensitivity of ICP-MS detection (interference on ^{55}Mn) was examined by Michalke and Schramel [34]. After applying the combination of SEC and strong anion exchange (SAX) ICP-MS (two-dimensional) techniques, allowing SEC-fractions, the fractions were then subjected to SAX-ICP-MS for further characterization and possible identification. This two-dimensional analytical scheme (SEC and SAX) allowed the monitoring and characterization of the Mn species in human milk and the identification of some species. The chromatograms reveal LMW bound to Mn (90%

of Mn), while the remaining Mn was widely spread and associated with HMW molecules (peak between 75 and 107 kDa). The conclusion from this investigation was that Mn-citrate is the most important Mn species in breast milk. These results can be compared with results from Bratter et al. [121], who reported Mn-lactoferrin (78 kDa) and citrate species of Mn in human milk.

Nischwitz et al. [78] presented the multidimensional chromatography analysis of Mn species in porcine liver extracts by SEC and RPLC coupled to ICP-MS. The obtained extracts were also investigated by off-line coupled to ESI-MS. In particular, species containing Cu, Zn, Fe or Mn were considered. The optimized conditions for the extraction (three procedures) and speciation of the forms present in the extract allowed the identification of few peaks of compounds containing manganese. Unfortunately, attempts to propose their structures were unsuccessful. As a result, it was suggested that in Tris–HCl extract (found to be the most efficient), after cell fractionations by differential centrifugation, Mn-compounds were mainly cytosolic. However, another unidentified compound found in the mitochondrial fraction, was proposed to potentially contain pyruvate carboxylase (Mn-enzyme present in the mitochondria of liver cells).

The speciation of manganese in liver extracts was also performed by CE-ICP-MS and presented by Michalke [122]. Capillary electrophoresis was recommended as the appropriate technique for the separation of unstable species causing problems in chromatography. The developed method was used for the analysis of liver extracts, and several Mn-containing compounds were identified and quantified (e.g., Mn-transferrin, Mn-albumin and Mn-citrate). The selected technique is not as popular as chromatographic ones [123], but it offers milder separation conditions due to the lack of a stationary phase [124].

The analytical techniques used for the separation and identification of ***I*** species almost exclusively involve ICP-MS detection coupled with various separation techniques, such as RPLC [125,126], IC [73,91], SEC [12,13] and CE [127].

Sanchez and Szpunar [12] used a similar instrumental setup for the fractionation analysis of iodine. In most of the examined samples of animal or human origin, only iodide was found. However, in infant formula, more than 50% of the element was present in HMW species. Bratter et al. [121] used the same technique and monitored I on-line; they reported that 80% of the I in human milk was present as I^-. Similar results were found in an investigation of chicken eggs [70].

A thorough study of I speciation in seaweed demonstrated that it differs substantially within the marine algae family [13]. Experiments were performed with multidimensional chromatography coupled with ICP-MS. Separation techniques were used: SEC for the fractionation species of different molecular masses, AEC for the separation of inorganic forms of -I and RPLC for the separation and identification of low molecular mass -I species. In the examined materials, the main inorganic species of iodide was found. LMW iodine species are proteins bound to iodinated species (identified in hydrolysates as monoiodotyrosine and di-iodotyrosine).

Examination of Zn, Co, Cu and Mo speciation is rarely presented due to the low concentration of these elements in foodstuffs, as well as problems related to the separation of their species. Various trace metals, including Zn, Cu and Mn, have been extensively studied in a variety of nuts by SEC-ICP-MS [59,61]. In many investigations, these elements are studied together in the same extraction and using the same techniques.

Many groups have investigated ***Zn*** species in human milk [121,128,129]; they used SEC with ICP-OES or MS, and their results typically include Zn-citrate, which is the predominant Zn species in human milk. Several species of minor importance were found, with their presence being confirmed by at least two different groups. These Zn species were Zn-lactoferrin, Zn-casein, Zn-albumin, and Zn-α-lactalbumin. The high bioavailability of Zn in human milk is believed to be linked to Zn-citrate.

For the speciation of Zn [80] in CPP (casein phosphopeptides) fractions from a milk-based toddler formula, a combined procedure was proposed: AE-HPLC followed by RPHPLC-ESI-MS with parallel FAAS. The first approach provides information about the m/z of the separated species, and the second reveals the presence of Zn in fractions from the AE column. Fractions of CPP were investigated by AEC, and the identification of CPP was performed using RPLC-ESI-MS. As a result, it was found that zinc could bind to CPP.

In a recent study from 2011, Ovca et al. [60] focused on Zn in pumpkin seeds (*Cucurbita pepo*), and the SEC-ICP-MS technique was used to determine the Zn species. Water extracts of pumpkin seeds exhibited a specific Zn species fingerprint with approximately 30% of a LMW fraction (0.5–2 kDa) and approximately 60% of an intermediate/HMW fraction (10–20 kDa). The digestion of Zn species under simulated stomach conditions proved that the Zn species identified in plant extracts were completely decomposed to Zn^{2+}. The spatial Zn distribution by laser ablation (LA) ICP-MS revealed that the seed coat contains almost no Zn and confirms that the Zn concentrated in a thin layer directly under the seed coat is greater than that in the kernel. LA-ICP-MS is very helpful for use with foodstuffs with different layers, such as nuts, seeds and onions, as it allows researchers to directly analyze each layer for the concentration of elements.

The fractionation of Zn compounds in honey was determined using non-ionic apolar adsorbing resin, Amberlite XAD-16, and a geltype strongly acidic cation exchange resin SPE column. Another set of solutions was subjected to physical fractionation using filtration with membranes of 0.45 μm pore size and a molecular weight cutoff size of 100, 50, 30, 10 and 5 kDa. Both approaches used for the partitioning of Zn enabled the retrieval of information on the fraction of total Zn that was the predominant class of species (59–89%) and, regarding its positive charge and very low molecular size, this species appears to be the most available from honey and absorbable in digestion [130].

Baker and Miller-Ihli [49] investigated ***Co*** in vitamin supplements using CE-ICP-MS. The use of a formic buffer with pH 2.5 in CZE allowed the best separation. The developed procedure offered a one hundred times greater sensitivity than the CZE-Uv/Vis protocol, but it still could not be compared with that achieved using SEC-ICP-MS, where the DL is 0.1 ng mL^{-1}.

Results from a study developed for the examination of Co speciation in chicken eggs [68] indicated that phosphitin from chicken egg yolks and ovalbumin from egg whites can be cobalophilins, which are responsible for the protection of naturally existing vitamin B12 from degradation processes and enzymatic digestion. In many cases, the concentrations of Co in the food extracts were extremely low; this made the Co detection in SEC unattainable, even when using the sensitive technique of ICP-MS.

Cu speciation is often present in multi-elemental speciation analyses. The results presented indicate that Cu is bound to LMW organic molecules, such as phytochelatins [55] in plant foods, and labile complexes, as determined by SPE-ICP-OES analyses performed in milk [82]. Wuilloud et al. [61] assessed the Cu species in nuts; Cu was found to be primarily associated with a fraction with a relative 10–14 kDa (~50%), and the rest of the Cu present was distributed among several LMW fractions. The HCl extracts revealed LMW fractions (1.2–1.5 kDa; 65–77%) in the nuts.

During the ICP-MS analysis of Cu, investigators should remember the interferences with Cu detection using the isotope 63 (e.g., $^{40}Ar^{23}Na$). The absorption of metal (e.g., Cu) by SEC materials (on the column) and the interaction with unsaturated complexing agents from food are also important considerations [131]. The Cu recovery during the analysis of beer was significantly greater and averaged 139 ± 4%, which illustrates why the recovery is such

an important part of the analysis. To obtain detailed information, the recoveries must be determined for each species of interest separately.

Similar fractionations were presented in investigations of beer with elements like Mn, Cu and Fe. A simple method based on dual-column, solid-phase extraction and FAAS for off-line detection was proposed for the chemical fractionation of Cu, Fe and Mn in beer [56,132] and for Cu in wine [133].

Mo speciation was determined in porcine liver and mushrooms using the SEC-ICP-MS technique. Nischwitz et al. [78] presented the three detected Mo peaks; the largest peak revealed a molecular weight of 300–400 kDa, and the other three Mo liver enzymes have been described in the literature: sulfite oxidase (110 kDa) [134], aldehyde oxidase (270 kDa) [135] and xanthine dehydrogenase (approximately 300 kDa) [136].

A single Mo peak corresponding to a LMW fraction of 3–4.15 kDa was observed in mushrooms. The amount of ^{95}Mo associated with that peak was 100% of the total Mo eluted from the SEC column. The acidic extraction was more efficient than the basic extraction. In porcini mushrooms, Mo is likely present as a relatively small organic species. Detailed information about the recoveries has not yet been determined for each species.

7. Conclusions

The speciation analysis of elements (Fe, Mn, I, Co, Cu and Mo) is of high importance for human health, but it is challenging for analysts and is still a challenge for analytical chemistry. Their low concentrations in food, complex chemical behavior and the instability of species are the reasons that only limited information is available about their functions in living organisms. However, despite the limitations of contemporary analytical instrumentation presented in this review, one can predict that technical progress will soon be made in this nearly unexplored research field. Atomic spectrometry techniques will undoubtedly play a crucial role in new methodologies.

The hyphenated techniques, consisting of separation modules (LC or CE) and element selective detection, such as atomic spectrometry techniques, are useful in the improvement of sensitivity and in the identification and quantification of element species. The highly significant issues of separation mechanisms, mobile phase, pH and sample preparation, must be carefully considered to avoid the changing of species and to ensure the exact characterization of a sample. Furthermore, the molecular selective detectors (e.g., ESI-MS) can provide structural information on the analytes.

All the information received from the separation (SEC), determination of the fractions (ICP-MS) and identification are important for the characterization of unknown challenging element compounds in food.

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