Pure Appl. Chem., Vol. 78, No. 1, pp. 79–90, 2006.
doi:10.1351/pac200678010079

Trace element speciation in human milk*

Bernhard Michalke‡

GSF National Research Center for Environment and Health, D-85764 Neuherberg/Munich, Germany

Abstract: This paper summarizes speciation results in human milk samples. Information is presented about why these elements were speciated, which techniques were applied, and which speciation results were gained in human milk. We focus on a few selected elements, such as Zn, Se, I, and Mn. Each of these elements is regarded as an "essential" element and of specific importance for newborns. At the end of each element section, we attempt to extract the overall speciation information from the different literature sources. In short, for Zn it can be concluded that this element is bound predominantly to low-molecular-weight (LMW) compounds. Several papers identify the major Zn species as Zn-citrate. A few minor species are identified as well. The pattern of Se speciation seems to be dependent on the nutritional intake. Selenium speciation in milk from slightly Se-depleted regions shows Se bound in the LMW range, ligands were partly identified. In Se-rich regions, Se seems to be associated with proteins, e.g., glutathione peroxidase. The major I species in human milk is iodide, as found by several groups. Other I species, however, were seen as well. The results of Mn speciation from different groups agreed that Mn is to a considerable amount in the LMW fraction. Again, citrate seems to play an important role as ligand.

Keywords: trace elements; element speciation; human milk; hyphenated techniques; zinc; iodine; selenium; manganese.

INTRODUCTION

The determination of trace elements has gained outstanding importance in life and nutritional sciences. Elements present even at minimal concentrations in respective matrices, in fact, can exert fundamental influence on vital functions proportionally to their amounts. The study of physiological or pathophysiological processes in the human body requires the determination of elements in the range of $\mu g\ l^{-1}$, $ng\ g^{-1}$, and even $pg\ g^{-1}$. Essentially, higher concomitant amounts of organic and inorganic components make the determination of trace elements rather difficult independent of whether the investigated matrix is from a living organism or of food origin. Moreover, the complexity of the process that leads from the first step of trace element analysis to the final statement of bioavailability and subsequently biological implications necessitates close collaboration between analytical chemist, and nutrition- and life scientist.

In this framework, during the last two decades analytical chemists and nutrition scientists increasingly realized that total concentrations of chemical elements cannot give, in general, information about mobility, bioavailability, and the eventual impact of elements on biological organisms. Only the knowledge of the chemical species of the elements can provide an understanding of chemical and biochemical reactions, bioavailability, and subsequent paths of metabolism, thus leading to more informa-

*Paper based on a presentation at the 2nd International Symposium on Trace Elements in Food (TEF-2), Brussels, Belgium, 7–8 October 2004. Other presentations are published in this issue, pp. 65–143.

‡E-mail: bernhard.michalke@gsf.de

tion about toxicity or essentiality. It is also worth stressing in this context that new trace elements and some of their species are being added to the list of those that are known or suspected to be essential.

The necessity of and reasons for element speciation in nutrition and specifically in human milk were realized and reported early in speciation literature. Human milk usually is the first diet for the infant. It supposedly meets all the needs of the newborn, especially for providing essential trace elements, as well as relevant proteins and enzymes or all the other organic components, for example, reported in respective literature such as Schaub [1]. Trace elements and organic compounds chemically interact quickly or are already linked together when being secreted by the mammary gland. Thus, elements usually appear as parts of macromolecules (proteins, enzymes, hormones, etc.) and show their impact on biochemistry as respective element species (binding forms) or according to their oxidation state. The quality and quantity of the relevant element species in a food matrix rather than the total element concentration are highly responsible for the bioavailability, for example, in human milk, and finally the biochemical (probably essential or toxicological) impact of an element [2]. Problem-related speciation analysis appears to play a key role in effectively assessing the role by elements in the food. Terms related to speciation were developed and defined from the increased understanding of needs in speciation investigations resulting from the growing experience in this field.

Still, the regular way for elemental speciation—also in human milk samples—is the use of separation and detection methods already established elsewhere, which have to be combined in novel ways and modified according to the relevant speciation problems. Combination and hyphenation of separation techniques and element- or molecule-selective detection systems are generally the basis for speciation analysis. Still, many methodological developments are necessary, mostly for hyphenation and quality-control strategies. Investigations of quality control showed that during sampling, sample preparation, and storage, separation and detection changes in "original species information" can occur easily. Such conceptual errors, generally realized, are relevant for speciation investigations in human milk, too [3,4]. The speciation of elements in human milk historically was performed already at the very beginning of speciation attempts. These early investigations contributed considerably to establishing such speciation concepts, though they could not always provide the complete quality-control-based speciation strategies.

SOME ELEMENTS SELECTED FOR SPECIATION IN HUMAN MILK

In the subsequent section, we focus on a few elements speciated in human milk. The examples refer to a limited selection of those elements mostly of interest especially from the viewpoint of essentiality. We will give descriptions of the different approaches from respective references on why this element was chosen for speciation in human milk, which techniques were used for separation and detection of element species, identification of species, means of quality control, and finally a short overview of the results from different references are present.

Zinc speciation in human milk

Zn speciation had earlier been of importance in human milk speciation. Zn is an essential trace element especially for newborns. Babies need Zn predominantly for cell proliferation, and DNA, RNA, and protein synthesis [5,6]. There are several essential enzymes regulated or activated by Zn. Breast milk was found to contain lower concentrations of Zn compared to formulas. However, breast-fed newborns often showed a more suitable and balanced Zn status than non-breast-fed ones [7,8]. Both findings together clearly pointed to differences in Zn speciation in the different nutrition of the babies.

Early investigations thus were focused on specific Zn ligands in human milk. In a series of publications, Lönnerdal et al. contributed considerably to Zn speciation.

Already, in 1980 this group [7] isolated a low-molecular-weight (LMW) Zn binding ligand from human milk. They used size-exclusion chromatography (SEC) and ultrafiltration for separation with an

eluent containing $NaBH_4$ to reduce charged groups. The second-dimension separation was completed by ion-exchange chromatography to identify the Zn ligands. SEC separation resulted in two minor Zn peaks >70 000 Da, but the major signal around 600–650 Da was identified as citrate. Its presence was additionally confirmed by a citrate-specific assay (enzymatic test) and IR spectroscopy. Concentrations determined in this fraction were 1830 μg/l for Zn (total) and 0.99 mM for citrate.

Based on these results, LMW compounds were believed to be of greater importance in binding essential metals and in transport or function in the animal/human body. The presence of Zn-citrate enhances the availability of Zn to newborns. On the other hand, Zn in cow's milk seems to be 10 times more concentrated but bound to casein. It then gets inaccessible for infants.

These results were further corroborated by Lönnerdal et al. in 1982 [8]. In the same paper, especially the "modified SEC" separation mode, using high Zn concentrations in the eluent for cation saturation of charged groups of the stationary phase was critically investigated. In analogy, a critical investigation on gel effects during SEC separations was systematically performed by Arnaud et al. [3]. These authors checked adverse effects of different SEC gels and compared the results with ultrafiltration.

Possible Zn species were found to be albumin and citrate. Recovery for different gels ranged from 84–107 %. Acetate buffers resulted in a shift of Zn between species. Identification using only SEC retention times was realized to be prone to errors due to charged gel effects. Independent on buffers, however, citrate was always the predominant Zn ligand in human milk. Other Zn ligands were albumin and lactoferrin (with more Zn found at albumin), which was in coincidence with Brätter [9] and Lönnerdal [7]. Martin et al. [10] as well as Blakeborough et al. [11] have also identified Zn citrate.

In the beginning of the 1990s, two papers from Michalke and Schramel focused on Zn speciation in human milk [4,12]. The authors used a simple process by centrifugation of pooled human milk samples (7–14 d) for fat removal and generation of a LMW fraction and high-molecular-weight (HMW) fraction. HMW or LMW fractions were separated by SEC (using columns with the respective separation range). Zn ligands were identified according to retention times and additionally in a second dimension using isoelectric focusing of the collected Zn proteins and using a citrate-specific determination kit (enzymatic test from Boehringer Mannheim, no. 139076) for citrate identification in the LMW chromatogram. The results on Zn species found are summarized in Table 1 (data summarized from Michalke et al. [12,13]).

Table 1 Overview of Zn speciation in human milk, including identification protocol, Zn- and ligand concentrations.

Zn species	**Concentration in HMF (g/l)**	**Species concentration from literature (g/l)**	**Zn concentration per species (μg/l)**
Lactoferrin	2.81 ± 0.5	2–3.5	25 ± 2.1
Casein	4.22 ± 0.8	4–5	69 ± 4.4
Albumin	0.42 ± 0.1	0.3–0.5	33 ± 3.3
Metallothionein	0.01 ± 0.001	0,01	18 ± 2.5
Citrate Analyzed on LMW SEC column	0.52 ± 0.008	0,01	3181 ± 2.5
Total protein	9.6 ± 1.4	8–12	
Total citrate	0.50 ± 0.008	0.25–0.4	
Total Zn		Literature 1500–7500	Measured 3363 ± 274

In 1995, a work by Negretti de Brätter et al. [14] investigated Zn speciation in human milk whey samples from Venezuela using SEC separation combined with induced neutron activation analysis (INAA), or inductively coupled plasma–atomic emission spectrometry (ICP–AES) detection. Total Zn was determined at 2.4–5.2 mg/l. For speciation, these authors used SEC separation combined with INAA, or ICP–AES detection. The Zn profile showed a predominant elution of Zn in the region around 67 kDa, a considerable amount between 10 and 1 kDa, and nearly no Zn below 1 kDa.

Similarly, in 1998 the same group [15] investigated elution pattern of several trace elements from human milk. They found the major elution of Zn at the terminating volume (compounds ≤10 kDa) shown in Fig. 1. Further identification proved the main Zn species to be citrate. Three further Zn species were seen in peaks not totally resolved, which were assigned to casein (approx. 8 %), and albumin. There was an adverse effect observed between Zn-citrate and Se concentration in human milk. High selenium supplementation to the mother resulted in high Se concentration in respective milk linked to lower total Zn and Zn-citrate in this milk.

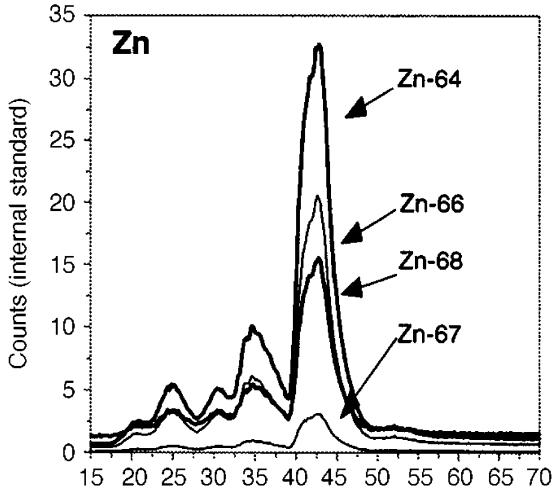

Fig. 1 Zn speciation in a human milk sample. The Zn elution pattern is seen produced from an SEC column which is directly coupled to an ICP–MS detector. As Zn isotopes may be interfered, four Zn isotopes are monitored in parallel. Only when the natural isotope ratio is maintained the peak can be considered as a Zn signal without a relevant interference. From Brätter et al. 1998, with permission from the Royal Society of Chemistry.

Finally, in recent work by Bocca et al. [16], the total Zn content and binding pattern was determined in human milk of different polluted regions from Italy in some analogy to the Negretti de Brätter paper. These authors found an elution pattern with five Zn protein fractions: >2000 kDa, assigned to caseins aggregates; 500–2000 kDa, assigned to immunoglobulines; 100–500 kDa, assigned to albumin; 2–100 kDa, assigned to (probably) α-lactalbumin; and, finally, compounds smaller than 2 kDa. The authors concluded that Zn is associated predominantly with α-lactalbumin and also with citrates

Summarizing the results on Zn speciation, nearly all investigations found Zn-citrate to be the predominant Zn species in human milk. Several further species were found of minor importance, but 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.

Selenium speciation

During the last two decades, Se attracted the focus of interest in human milk and Se speciation gained general importance in human health investigations. Se is an essential trace element, and sufficient Se supplementation can protect against several heart diseases and is discussed in relation to cancer prevention [12,17]. Detoxifier effects of Se are proven and described widely [2,4,12,18]. Se deficiency is related to a series of diseases [17,19–21]. Furthermore, the thyroid metabolism is impaired because many deiodinases are Se proteins [22]. Se deficiency is most critical for the brain and growth of infants [22]. Newborns initially show serum Se levels of around 80 μg/l [3] or slightly decreased ones (50–60 μg/l [19]), which decrease during the first half-year of their life and reach the starting values only after 12 months [17,18]. Furthermore, the total Se concentration in breast milk decreases from colostrum (20–80 μg/l [23]) to mature milk to a range of 5–18 μg/l [18,23–25], slightly varying between lactating women and depending on nutrition [15]. But the bioavailability of Se is dependent on its binding form (species) [26,27]. Therefore, Se speciation based on clear identification and quantification of the species in human milk was deemed necessary.

Few groups worked on this topic during the last 15–20 years. In a pioneering work, Brätter et al. [9] coupled an SEC column to on-line digestion followed by hydride generation (HG)–ICP–AES detection. They used the HG device for matrix separation and increased the detection sensitivity, whilst the on-line digestion was needed for getting species available for HG. Detection limits of 1–2 μg/l (Se) were achieved. They found Se associated to LMW compounds. In 1995 [14], they repeated the experiments with human milk samples from the seleniferous region of Venezuela. They used SEC separation and ICP–mass spectrometry (MS) detection paralleled with Se quantification using INAA for quality control. The column was mass-calibrated with defined proteins isolated from human milk for providing exact species size information. The results showed some differences to former investigations. Now, Se was predominantly associated with HMW compounds. The continuation of the work in 1998 [28] used human milk samples from three different countries (Portugal, Germany, and Venezuela). Se species eluted mostly between 20 and 45 min, with peak maxima at 25, 35, and 40 min. These retention times were assigned to proteins such as glutathione peroxidase. Furthermore, these authors found variations in the elution pattern between samples from different countries. This result was related to the different Se intake from mothers living in different regions.

Michalke and coworkers published a series of papers on Se speciation in human milk, too, with special respect to quality control [29–31]. All papers together completed a Se speciation scheme shown in Fig. 2. Generally, they used pooled human milk samples between the 7^{th} and 14^{th} day after delivery. A first methodical paper provided techniques for clear identification of Se species using different capillary electrophoresis (CE) methods as a secondary separation for identification. First, defatted human milk samples were SEC-fractionated, and the size-characterized Se-containing fractions were analyzed by two different capillary zone electrophoresis (CZE) methods or by a CZE- and a capillary isoelectric focusing (cIEF) method. Se was determined using electrothermal vaporization (ETV)–ICP–MS in SEC fractions and even in CE fractions, which were gathered after consecutive SEC- and CE separation. Using this set-up, a two-dimensional purification and identification via SEC retention time and CE migration time in series was possible, as well as a direct assignment of Se to the (two-dimensionally) purified Se compound [29,30]. Based on these methodical developments, investigations were performed in more detail with a higher sample number. In this follow-up study, the Se concentrations per species were determined in the LMW range as 2.5 (± 0.2) (Se-carrying glutathione, GSeH) μg/l, 3.1 (± 0.3) μg/l (Se-cystamine, SeCM), 5.2 (± 0.4) μg/l (Se-cystine, SeC), and 1.1 (± 0.1) μg/l (Se-methionine, SeM). The results were confirmed by a set of quality-control means. The results are in accordance with Brätter et al. 1988 [9], but contradictory to results of the same group in 1995 (Negretti de Brätter et al. [14]). It should be noted that Michalke and Schramel [30] and Brätter et al. [9] used samples from the slightly Se-depleted Germany, whilst the group of Brätter in 1995 and partly in 1998 [14,15] predominantly analyzed samples from the seleniferous areas of Venezuela.

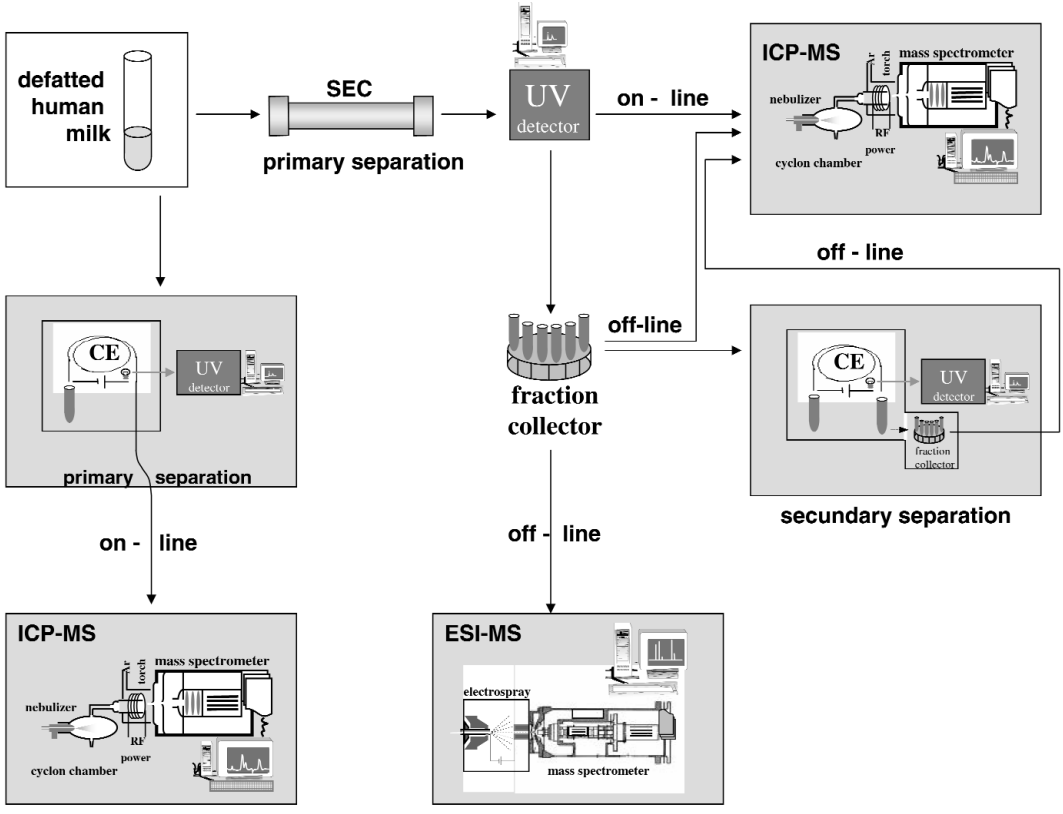

Fig. 2 Overview of a completed Se speciation scheme with special respect to quality control. A multidimensional speciation approach is realized by SEC–ICP–MS followed by SEC–CE–ICP–MS and SEC–ESI–MS. In parallel, two approaches were set up, CZE–ICP–MS and cIEF–ICP–MS.

Finally, Michalke and Schramel analyzed Se speciation in human milk using CE coupled to ICP–MS for getting results completely independent from a SEC fractionation step. Free-zone electrophoresis was employed as well as cIEF [31,32]. These experiments confirmed the findings from the SEC–CE–ETV–ICP–MS approach of 1997. These fast techniques were used to elucidate the trends of Se species in human milk at different lactation states [13]. It turned out that protein-associated Se decreases rapidly with increasing lactation state from ca. 3.8 µg/l to below the limit of detection (LoD). This is similar to the glutathione-bound Se (from ca. 8 µg/l to ca. 2 µg/l) whilst SeC increases from 2 to 4.5 µg/l. This may be of specific importance due to the central role of SeC in Se metabolism of humans. SeCM has a maximum of 4 µg/l around the 10^{th} day postpartum. The concentration of SeM remains low, around 1 µg/l.

Summarizing the results on Se speciation, it is striking that data are available from samples either from low Se regions or very high Se regions.

Selenium speciation in samples from the low Se regions clearly point to Se being associated with LMW compounds. These findings were confirmed by only-SEC and only-centrifugation approaches and by multidimensional set-up. The latter even identified the species GSeH, SeCM, SeC, and SeM. The results of samples from high Se regions (provided by "only-SEC approaches") showed various peaks, one of them assigned to glutathione peroxide.

Iodine speciation

Iodine is known to be an essential micronutrient, which is utilized by the thyroid gland for the biosynthesis of the thyroid hormones thyroxin (T4) and tri-iodothyronine (T3) [33–35]. These hormones strongly influence an extended range of biochemical reactions. Immune defense and antibody production are dependent on reliable thyroid function and availability of T4 and T3 hormones [36,37]. Knowledge of the status of the different I species (such as T4, T3, or iodide) in body fluids can give information about malfunction of the thyroid gland and may explain also other T4/T3-correlated metabolic abnormalities [35]. Thus, in the past, investigations were performed on I speciation in human body fluids in general, predominantly using chromatographic methods [38]. Furthermore, lack of I supplementation to newborns has been discussed to result in slower brain development up to severe damage to the central nervous system [39]. It is supposed that there is an I transporter and a peroxidase enzyme involved in I accumulation in mammary glands [40]. These facts make I speciation important to a matter of human milk investigations.

The group of Brätter et al. investigated I speciation in human milk in 1998 and 2000 [15,39]. In 1998 they monitored I at ^{127}I on-line by ICP–MS after SEC separation. It turned out that 80 % of I in human milk was present as iodide. In 2000, a distribution profile of I fractionated by SEC was also shown. The speciation of I in human milk whey showed a predominant iodide peak as well as six further peak maxima in the HMW range (5–300 kDa). Total I in European breast milk samples was determined at 95 ± 60 µg/l.

Michalke investigated I speciation in breast milk as well [13]. Total I varied according to lactation state, beginning at 60 µg/l at day 2 (postpartum) reaching 100 µg/l at day 3, and decreasing to 80 µg/l (day 6) or 60 µg/l constantly from days 9 to 60. A prefractionation by centrifugation showed I being associated with fat at approximately 30 % throughout, and 70 % to the LMW fraction. Characterization of I species from milk whey (pooled human milk, days 7–14) was done using SEC–ICP–MS and strong anion exchange (SAX)–ICP–MS. The SEC fractionation (TSK HW 40 column, 500 × 20 mm) showed predominantly iodide with ca. 37 µg/l as well as two more I species with 1.5 and 1 µg/l I having retention times pointing to T4 and T3 hormones. SAX–ICP–MS experiments confirmed iodide to be the major I species in human milk. A follow-up study employing CZE–ICP–MS showed iodide as the main I species, but T4 hormone was found in addition. Figure 3 demonstrates the electropherogram.

Summarizing the results, on I speciation in breast milk the few papers agree on iodide being the predominant I species in this matrix. Nutritional influence and even dependency is seen directly for iodide as well as indirectly from Se supplementation to the mother, then influencing the concentration of organic I species.

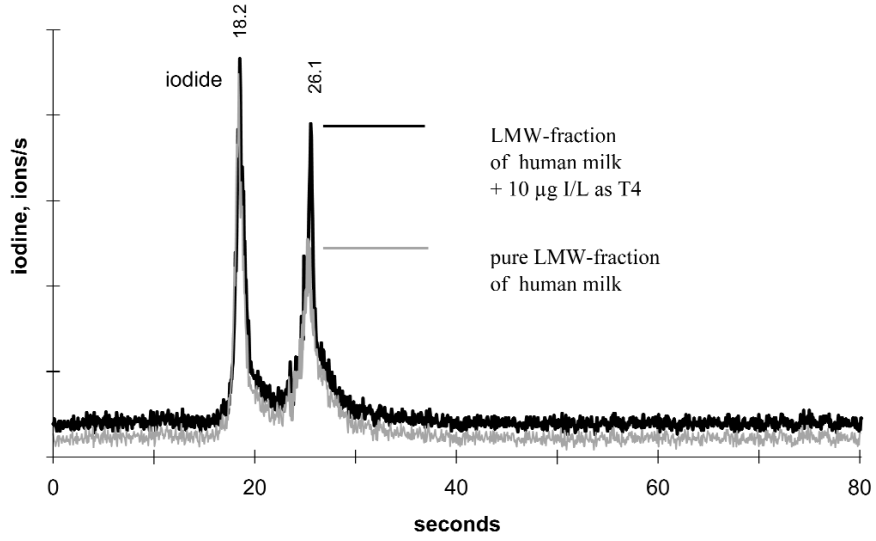

Fig. 3 Iodine speciation of a human milk sample by CZE–ICP–MS. Iodide and T4 hormone are detected. The T4 standard addition clearly identifies this hormone. From B. Michalke, *J. Anal. At. Spectrom.* **123**, 14, 1297–1302 (1999), with permission from the Royal Society of Chemistry.

Manganese speciation

Mn is a trace element known to activate many enzymes involved in metabolic processes. In many Mn enzymes, it is the key element at the active sites. Mn is needed for protein and fat metabolism, healthy nerves, and a healthy immune system as well as sugar regulation. Mn is one of the key elements for enzymes in energy production and increases the level of antioxidative protection, especially in mitochondrial Mn-superoxide dismutase [41–43]. Mn is involved in utilization of vitamins B1 and E, and it is required for normal bone growth or for avoiding clotting defects. On the other hand, Mn is used as anti-knock agent in gasoline, resulting in increased Mn blood levels as monitored for Canadian children [44]. Increased Mn levels are known for damaging the central nervous system, resulting in motoric abnormalities and psychic disorder [41,43,44].

In human milk, Mn is a trace element at very low concentration. Dependent on the postpartum stage, Mn concentrations between 2 and 5 µg/l for transitory and mature milk or up to 10 µg/l for collostrum are published for the European region, e.g., [16,45–47]. The environment and nutrition is reported to have some influence on such levels. Only few groups investigated Mn speciation in human milk up to now. Due to the weak binding of Mn to some organic ligands, most Mn species in human milk are considered to be labile. Therefore, typically the first (or sole) analytical step was again an SEC fractionation in most investigations.

In 2000, Bocca et al. published multi-element speciation results, including data on Mn speciation. These data were derived from samples of 60 mothers living in different areas of Italy. The investigation was based on SEC fractionation using a mass-calibrated column (mass calibration between 14.1–2000 kDa). These authors reported total Mn in human milk around 3 µg/l. They found 28 % of Mn attributed to the void fraction (>2000 kDa) and 30 % to the "non-proteic fraction", which was assumed to contain mostly LMW compounds. The remaining Mn was spread over the other fractions at low amounts.

In their multi-element speciation approach in 1998, Brätter et al. [15] report also about Mn SEC fractionation in breast milk compared to cow's milk. The noisy Mn trace shows two peaks, a broader one eluting around 33 min and a sharp main peak at 45 min. The Mn elution between 32–34 min is attributed to Mn-lactoferrin by the authors. Interestingly, the latter peak shows the same retention time as

Zn-citrate in the same paper, from which it can be concluded that citrate might be the Mn ligand as well here, too.

Finally, in 2004 Michalke and Schramel [48] published a multidimensional investigation on Mn speciation in human milk. Total Mn was determined at 2.8 µg/l, which is in accordance with values from the other studies. These authors used prefractionation via centrifugation, gaining a fat, a protein, and an LMW-supernatant fraction, followed from two different SEC fractionations, again followed from SAX–ICP–MS measurements. In this work, it turned out that Mn predominantly was LMW bound (ca. 90 % of Mn), the rest having very low Mn concentration, was spread to bigger molecules. Such HMW peaks were detected around 75 and 107 kDa. Standard addition experiments at the LMW–SEC step as well as in the SEC–SAX–ICP–MS step investigation pointed to Mn-citrate as the most important Mn species in breast milk. Also, inorganic Mn was seen in low amounts. As an example, Fig. 4 shows the consecutive chromatograms of SEC–ICP–MS and SAX–ICP–MS. The findings are in good accordance with Brätter et al.. These authors found Mn lactoferrin (78 kDa) and the predominant species at citrate retention time, whilst Michalke and Schramel found low concentrated Mn compounds around 75 kDa and citrate as the main Mn species. The results of Bocca et al. partly agree as at least 30 % were seen in the LMW (probably citrate?) fraction.

Summarizing the Mn speciation investigations, the papers agree that citrate plays a prominent role. Further Mn compounds seem to be present, the determination of their concentration as well as their clear identification vary somewhat between investigations.

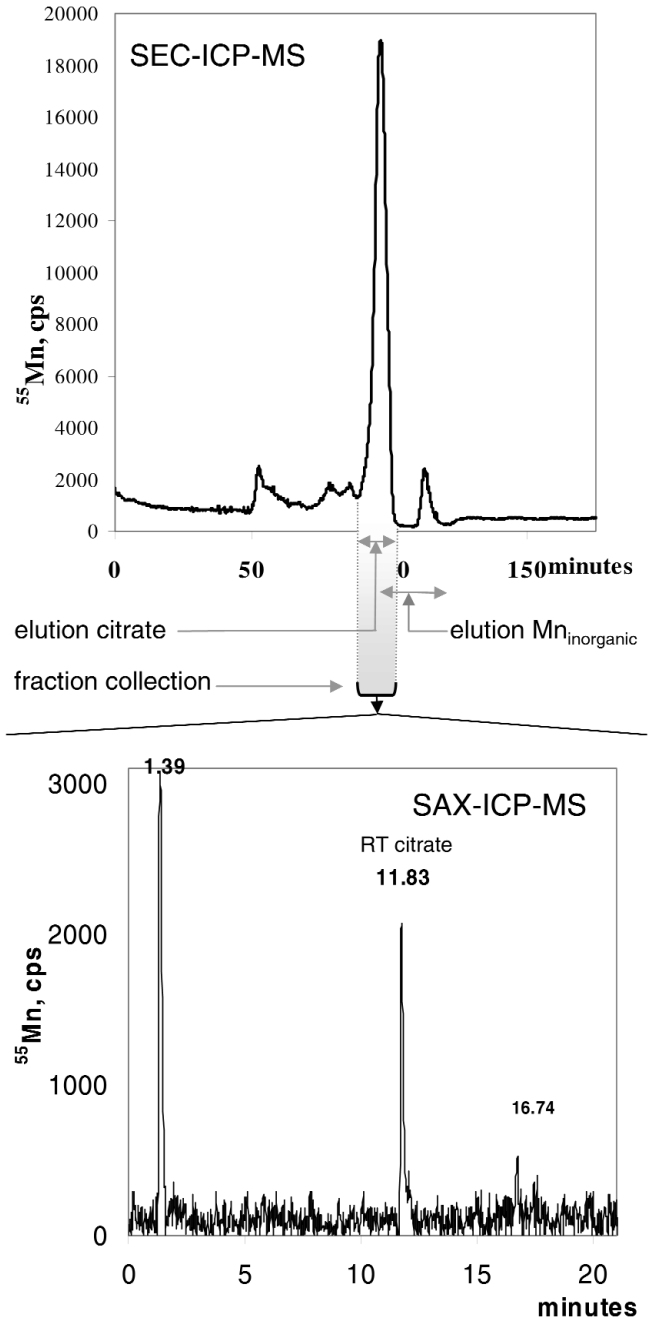

Fig. 4 Two-dimensional speciation of Mn in human milk. First, a separation according to size was performed, followed by SAX–ICP–MS analysis. Citrate and inorganic Mn appeared when using both methods in series.

CONCLUSION

Elemental speciation in human milk is of prime importance in order to understand the bioavailability of trace elements for the newborn and to probably get better information about metabolic pathways. The knowledge gained will be of importance for formula production as well. More speciation approaches used SEC combined with elemental detection, however, multidimensional experiments were increasingly employed. In the case of the elements reported in this paper, the coincidence between papers is often achieved at least for the predominant species.

REFERENCES

1. J. Schaub. *Composition and Physiological Properties of Human Milk*, Elsevier, Amsterdam (1985).
2. D. M. Templeton, F. Ariese, R. Cornelis, L.-G. Danielsson, H. Muntau, H. P. van Leeuwen, R. Łobinski. *Pure Appl. Chem*. **72** (8), 1453–1470 (2000).
3. J. Arnaud, D. Andre, M. C. Bouillet, D. Kia, A. Favier. *J. Trace Elem. Electrolytes Health Dis.* **6**, 81–90 (1992).
4. B. Michalke, D. Münch, P. Schramel. *Fresenius' J. Anal. Chem*. **344**, 306–310 (1992).
5. M. P. Waalkes and P. L. Goering. *Chem. Res. Toxicol.* **3** (4), 281–288 (1990).
6. B. J. Mill and R. D. Lindeman. In *Spurenelemente*, H. Zumkley (Ed.), Georg Thieme Verlag, Stuttgart (1983).
7. B. Lönnerdal, A. G. Stanislowski, L. S. Hurley. *J. Inorg. Biochem*. **12**, 71–78 (1980).
8. B. Lönnerdal, B. Hoffman, L. S. Hurley. *Am. J. Clin. Nutr*. **36**, 1170–1176 (1982).
9. P. Brätter, B. Gercken, U. Rösick, A. Tomiak. In *Trace Element Analytical Chemistry in Biology and Medicine*, Vol. 5, P. Brätter and P. Schramel (Eds.), Walter de Gruyter, Berlin (1988).
10. M. T. Martin, K. F. Licklider, J. G. Brushmiller, F. A. Jacobs. *J. Inorg. Biochem*. **15**, 55–65 (1981).
11. P. Blakeborough, D. N. Salter, M. J. Gurr. *Biochem. Soc*. **209**, 505–512 (1983).
12. B. Michalke, D. Münch, P. Schramel. *J. Trace Elem. Electrolytes Health Dis.* **5**, 251–258 (1991).
13. B. Michalke. *Elementspeziesanalytik in Bio-medizinischen und Umweltproben*, Habilitationsschrift an der technischen Universität Graz (1998).
14. V. E. Negretti de Brätter, S. Recknagel, D. Gawlik. *Fresenius' J. Anal. Chem*. **352**, 137–142 (1995).
15. P. Brätter, I. N. Blasco, V. E. Negretti de Brätter, A. Raab. *Analyst* **123**, 821–826 (1998).
16. B. Bocca, A. Alimonti, E. Coni, M. Di Pasquale, L. Giglio, A. P. Bocca, S. Caroli. *Talanta* **53**, 295–303 (2000).
17. P. D. Whanger. *J. Trace Elem. Electrolytes Health Dis.* **6**, 209–221 (1992).
18. T. W. Westermark. In *Trace Element Analytical Chemistry in Medicine and Biology*, Vol. 3, P. Schramel and P. Brätter (Eds.), pp. 49–70, Walter de Gruyter, Berlin (1984).
19. I. Lombeck, K. Kasperek, H. D. Harbisch, L. E. Feinendegen, H. J. Bremer. *Eur. J. Pediatr.* **125**, 81–88 (1977).
20. S. Bro, H. Berendtsen, J. Norgaard, P. J. Jorgensen. *J. Trace Elem. Electrolytes Health Dis.* **2**, 165–169 (1988).
21. I. Lombeck, K. Kasperek, H. D. Harbisch, K. Becker, E. Schumann, W. Schröter, L. E. Feinendegen, H. J. Bremer. *Eur. J. Pediatr*. **128**, 213–223 (1978).
22. G. Örndahl, U. Sellden, S. Hallin, H. Wetterqvist. In *Trace Element Analytical Chemistry in Medicine and Biology*, Vol. 4, P. Schramel and P. Brätter (Eds.), pp. 597–604, Walter de Gruyter, Berlin (1987).
23. J. R. Arthur, F. Nicol, G. J. Beckett. *Biol. Trace Elem. Res.* **33**, 37–42 (1992).
24. M. F. Picciano and J. A. Milner. In *Composition and Physiological Properties of Human Milk*, J. Schaub (Ed.), pp. 77–86, Elsevier, Amsterdam (1985).

25. K. Dörner, K. Schneider, E. Sievers, G. Schulz-Lell, H.-D. Oldings. *J. Trace Elem. Electrolytes Health Dis.* **4**, 37–40 (1990).
26. P. Van Dael and H. Deelstra. In *Trace Element Analytical Chemistry in Medicine and Biology*, Vol. 6, P. Schramel, P. Brätter, B. Ribas (Eds.), pp. 321–330, Consejo Superior De Investigationes Cienificas, Madrid (1994).
27. J. B. Luten, W. Bouquet, M. M. Burggraaf, J. Rus. In *Trace Element Analytical Chemistry in Medicine and Biology*, Vol. 4, P. Schramel and P. Brätter (Eds.), pp. 509–519, Walter de Gruyter, Berlin (1987).
28. P. Brätter, V. E. Negretti de Brätter, S. Recknagel, R. Brunetto. *J. Trace Elem. Med. Biol.* **11**, 203–209 (1997).
29. B. Michalke and P. Schramel. *J. Chromatogr., A* **716**, 323–329 (1995).
30. B. Michalke and P. Schramel. *Biol. Trace Elem. Res.* **59**, 45–56 (1997).
31. B. Michalke and P. Schramel. *J. Chromatogr., A* **807**, 71–80 (1998).
32. B. Michalke. *Spectroscopy* **15** (4), 30–34 (2000).
33. E. Junderwood. *Trace Elements in Human and Animal Nutrition*, 4th ed., Academic Press, New York (1977).
34. A. S. Prasad. *Trace Elements and Iron in Human Metabolism*, Plenum, New York (1978).
35. H. Keller. *Klinisch-chemische Labordiagnostik für die Praxis: Analyse, Befund, Interpretation*, Georg Thieme Verlag, Stuttgart (1991).
36. W. Reinhardt, M. Luster, K. H. Rudorff, C. Heckmann, S. Petrasch, S. Lederbogen, R. Haase, B. Saller, C. Reiners, D. Reinwein, K. Mann. *Eur. J. Endocrin.* **139**, 23–28 (1998).
37. L. M. Robison, P. W. Sylvester, P. Birkenfeld, J. P. Lang, R. J. Bull. *J. Toxicol. Environ. Health, Part A* **55**, 93–106 (1998).
38. G. Knapp, B. Maichin, P. Fecher, S. Hasse, P. Schramel. *Fresenius' J. Anal. Chem.* **362**, 508–513 (1998).
39. P. Brätter, I. N. Blasco, V. E. Negretti de Brätter, A. Raab. *Metal Ions in Biology and Medicine*, Vol. 6, pp. 751–754, John Libbex Eurotext, Montrouge (2000).
40. J. A. Rilema and D. L. Rowady. *Proc. Soc. Exp. Biol. Med.* **215**, 366–369 (1997).
41. W. Zidek. In *Spurenelemente-Grundlagen-Ätiologie-Diagnosen Therapie*, H. Zumkley (Ed.), pp. 140–151, Georg Thieme Verlag, Stuttgart (1983).
42. M. Ashner, K. E. Vrana, W. Zheng. *Neurotoxicology* **20** (2–3), 173–180 (1999).
43. F. C. Wedler and D. J. Kllimis-Tavantzis (Ed.). *Manganese in Health and Disease*, CRC, Boca Raton (1994).
44. R. T. Ingersoll, E. B. Montgomery, H. V. Aposhian. *Neurotoxicology* **20** (2–3), 467–476 (1999).
45. B. Lönnerdal. In *Trace Elements in Man and Elements*, A. M. Roussel, R. A. Anderson, A. E. Favrier (Eds.), pp. 353–358, Plenum, New York (2000).
46. R. M. Parr, E. M. DeMaeyer, V. G. Iyengar, A. R. Byrne, G. F. Kirkbright, G. Schöch, L. Niinisto, O. Pineda, H. L. Vis, Y. Hofvander, A. Omoloulu. *Biol. Trace Elem. Res.* **29**, 51–75 (1991).
47. E. Coni, A. Alimonti, A. Bocca, F. La Torre, D. Pizzuti, S. Caroli. In *Element Speciation in Bioinorganic Chemistry*, S. Caroli (Ed.), pp. 255–283, John Wiley, New York (1996).
48. B. Michalke and P. Schramel. *J. Anal. At. Spectrom.* **19**, 121–128 (2004).