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Manganese speciation in human milk using size exclusion chromatography combined with strong anion exchange chromatography and inductively coupled plasma mass spectrometry detection

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Human milk was investigated concerning Mn speciation. In a first step subfractions were produced by centrifugation and analysed for manganese by inductively coupled plasma atomic emission spectrometry (ICP-AES). It turned out that Mn concentrations were approximately 3 μg L^{-1} (human milk), 2.85 μg L^{-1} in the defatted fraction, 0.25 μg L^{-1} in the pellet fraction and 2.6 μg L^{-1} in the low molecular weight (LMW) -supernatant fraction. The defatted fraction was investigated further by on-line coupling of size exclusion chromatography (SEC) to inductively coupled plasma mass spectrometry (ICP-MS). The columns had either a separation range between 10 and 150 kDa or 100–2000 Da. It is shown that manganese was found predominantly in the LMW fraction. The mainpeak in LMW-SEC eluted at 95 min, which was assigned to masses around 300 Da due to the mass calibration of the TSK column. Subsequent investigations analysed respective SEC-fractions by strong anion exchange (SAX)-ICP-MS and compared chromatograms with those of Mn standard compounds. Inorganic manganese species and Mn–citrate complex were identified. Some further Mn compounds of low concentration were seen, but could not be assigned to specific standard compounds.

Introduction

Human milk is the first food for humans and it serves as the sole source for all the nutrients including trace elements (trace element species) required for the biological functions and growth during early stages of life. Element content and speciation in human milk is therefore of importance for a healthy development of the newborn.

Manganese is a trace element known to activate many enzymes involved in metabolic processes. In many manganese 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 for sugar regulation. Mn is one of the key elements for enzymes in energy production and increases the level of anti-oxidative protection, especially in mitochondrial Mn-superoxide dismutase.[1–3] Manganese is involved in utilization of vitamin B1 and vitamin E and it is required for normal bone growth or for avoiding clotting defects. On the other hand manganese is used as an anti-knock agent in gasoline, resulting in increased Mn-blood levels as monitored for Canadian children.[4] Increased Mn levels are known for damaging the central nervous system, resulting in motor abnormalities and psychic disorder.[1,3,4]

In human milk manganese is a trace element at very low concentration. Dependent on the stage post partum Mn concentrations between 2 and 5 μg L^{-1} for transitory and mature milk or up to 10 μg L^{-1} for colostrum are published for the European region.[5–8] The environment and nutrition is reported to have some influence.[9] In a former study[7,8] an investigation on a rough Mn distribution was performed distinguishing five fractions from human milk showing different molecular size ranges. These authors reported that 28% of Mn is attributed to the void fraction (>2000 kDa) and 30% to the ''non-proteic fraction'', which was assumed to contain mostly low molecular weight compounds. The resting Mn was spread over the other fractions at low amounts.

In this study for the first time a speciation approach is applied to manganese species in human milk, using a two-dimensional chromatographic separation combined with element specific detection. An intense quality control was additionally applied to the complete analysis including two independently working detectors (where possible). Speciation was performed based on definitions according to reference 10. Pooled human milk (''HM'': 3rd day after delivery from different mothers) was investigated. Human milk as well as subfractions, namely the defatted fraction (''DFF''), the latter subfractionated in pellet (protein) fraction (''PF'') or the supernatant (''LMW'') fraction were taken for manganese speciation investigations. This experiment aimed for a first rough determination of Mn distribution in HM. The DFF was further analyzed by SEC-ICP-MS using first a high molecular weight separation range and then a low molecular weight separation range, both with UV-detection and on-line ICP-MS detection of the ^{55}Mn isotope, to obtain more detailed information on the size of Mn compounds. Alternatively, SEC-fractions were collected from several runs and pre-concentrated by freeze drying. The fractions were subject to SAX-ICP-MS for further characterisation and possibly identification. SAX was used also for standard separations of commercial Mn compounds for elucidating retention times of Mn species. This two-dimensional analytical scheme (SEC + SAX) allowed the monitoring and characterization of the Mn species in human milk as well as the identification of some.

Experimental

Chemicals

Toyo Pearl TSK HW 55 F and TSK HW 40 S, were both obtained from Toso Haas, Stuttgart, Germany and served as stationary phases for SEC columns. The material was slurried and packed into the respective column bodies under 4 mL min^{-1} flow rate. NaOH, NaCl, HNO_3, NH_4-acetate/acetic acid, $NaHCO_3$ and Na_2CO_3 (each suprapure grade) were

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purchased from Merck, Darmstadt, Germany. TMAH (25% suprapure) was delivered from TAMA Chemicals, Osaka, Japan. $Argon_{liq}$ was purchased from Messer, München, Germany. An Ar vaporizer at the tank provided Ar gas.

For mass calibration of SEC columns several compounds were used: γ-globulin, lactoferrin, β-lactoglobulin, lysozyme, and metallothionein, thyroxin, *N*,*N*′-bis(*t*-BOC)-L-cystine, *N*-CBZ-L-glutamic acid, citric acid and methionine were purchased from Sigma-Aldrich, Deisenhofen, Germany.

Standard compounds

The manganese standard compounds arginase, isocitrate dehydrogenase, prolidase, pyruvate carboxylase, oxalate oxidase, concanavalin A, galactosyl transferase, $MnCl_2$ and $Mn(NO_3)_2$ were obtained from Sigma-Aldrich, Deisenhofen, Germany. Stock solutions were prepared by weighing 100 mg material into polyethylene vials and dissolving the powder with 10 mL Milli-Q water (18 MΩ cm^{-1}, Millipore, Eschborn, Germany). Working solutions were prepared daily by further dilution with Milli-Q water. A stock solution of Mn-citrate standard was produced by dissolving $MnCl_2$ (10 mg Mn L^{-1}) in surplus citric acid solution (100 mg L^{-1}, Sigma Chemicals, Deisenhofen, Germany). Working solutions were prepared by appropriate dilution with Milli-Q water according to desired Mn concentration.

Sampling

Sampling of the human milk was carried out as described in ref. 11 using a manual pump. The tubing of the manual pump and the sampling vessels were cleaned with HNO_3 for removing possible Mn contamination and then with Milli-Q water for removing HNO_3. No commercially available motorized pumps were used to avoid contamination from stainless steel parts. After sampling, the human milk samples were pooled (different women, third day after delivery) and frozen immediately for storing at −20 °C.

Sample preparation

After thawing, 1 ml of the pooled human milk was taken for pressure digestion[12] and Mn quantification in the whole milk by ICP-AES. The determined Mn concentration was taken as 100% for mass balances. 10 mL aliquots of human milk samples were divided into subfractions *via* centrifugation, using a "Biofuge 17 RS" from Heraeus, Osterode, Germany. For defatting the milk the conditions were 1000 × *g*, 10 min, 8 °C, providing the fat layer on the top. The fat was carefully removed and the remaining fraction pipetted into another vial. The defatted milk was used either for subsequent SEC fractionation or for further subdivision into protein pellet and low molecular weight supernatant (25800 × *g*, 30 min, 8 °C). The supernatant was used for Mn determinations by ICP-AES, the pellet was resuspended in Milli-Q water (3 mL) and then also measured for Mn by ICP-AES. Another 3 mL of the defatted milk was used for Mn determination in the DFF fraction by ICP-AES whilst 2 mL aliquots served as samples for subsequent SEC investigations.

The production of defatted human milk, as well as gaining subfractions by centrifugation using the conditions described above, had been developed in former studies[13] and turned out to provide successfully the required fractions in several following investigations.[14,15]

Instruments and operation

Chromatography. For size exclusion chromatography (SEC) a BIORAD ECONO SYSTEM (pump and UV detector, 232 nm) was used as the eluent delivery system, equipped with a 2 mL sample loop. SEC columns were used for a separation range (according to the manufacturer) of approx. 10–150 kDa (500 × 20 mm ID, stationary phase: Toyo Pearl TSK HW 55 F for high molecular weight (HMW) separation) or for a separation range of about 100–2000 Da (500 × 16 mm ID, stationary phase: Toyo Pearl TSK HW 40 S for LMW separation). The whole chromatographic system was metal-free. In both cases 10 mM NH_4-acetate/acetic acid, pH 6.3 served as eluent at a flow rate of 1 mL min^{-1}. 10 mM NH_4-acetate/acetic acid, at pH 6.3 turned out to show several advantages for Mn speciation: At this pH sticking of Mn compounds to the SEC-stationary phases was minimal although the ionic strength of the buffer was low. The low concentration of buffers was mandatory to avoid crusting and/or clogging of the nebulizer, spraychamber and cones at ICP-MS. The buffer constituents chosen cause no problems for plasma detectors such as ICP-MS or ICP-AES (in contrast to *e.g.* phosphate buffers) and are even suitable for electrospray ionization (ESI) mass spectrometry. The latter technique initially was planned for identification of "unknown" Mn compounds. Unfortunately, the concentration of such compounds after two-dimensional chromatography was finally below the limits of determination from ESI-MS.

The column effluent was routed directly to ICP-MS for on-line Mn measurements or to a "Fraction Collector 100" (Pharmacia, Freiburg, Germany). Fractions were collected in 5 min intervals, frozen at −20 °C and subsequently freeze dried (Heraeus-Christ, type "Beta", Osterode, Germany). The controlled sample temperature in the freeze dryer was set to 4 °C to minimize the risk of species degradation or transformation during freeze drying. The freeze dried fractions of three SEC separations were dissolved in 2.5 mL Milli-Q water and measured for Mn by ICP-AES. The elution profiles were compared to on-line chromatograms. The sum of Mn from fractions was compared with the Mn amount injected on the column (calculated from the Mn concentration in HM and the injection volume). The Mn amounts were used for calculating mass balances.

Further investigations of resuspended SEC fractions were performed by SAX chromatography. Here the eluent delivery system was a "Beckman Gradient Pump No. 127" (Beckman, Munich, Germany), completely metal free (PEEK), equipped with a SAX separation column (Dionex AS 11, Dionex, Idstein, Germany). The system operated at 0.75 mL min^{-1} flow rate using a gradient elution with 10 mM NH_4-acetate/acetic acid, pH 6.3, serving as eluent A, 0.8 mM NaOH was used as eluent B and 10 mM NaOH as eluent C. The gradient is given in Table 1. These conditions turned out to provide a suitable separation of the Mn standard compounds.

ICP-MS. An ELAN 5000, Perkin Elmer (Sciex, Toronto, Canada) was employed for on-line determination of ^{55}Mn in the graphic mode. It was equipped with a Meinhard nebulizer and a cyclon spraychamber (Perkin Elmer).

The RF power was set to 1200 W, the plasma gas was Ar at a flow rate of 15 L min^{-1}, whereas the nebuliser gas was Ar at 800 mL min^{-1}. The dwell time was set to 300 ms. These parameters were the optimal conditions for this instrument.

Table 1 The gradient programme for SAX chromatography is shown. The eluents are A = 10 mM NH_4-acetate/ acetic acid, pH 6.3, B = 0.5 mM NaOH, C = 10 mM NaOH. These conditions turned out to provide a suitable separation of the Mn standard compounds

Time (min)	Eluent A (%)	Eluent B (%)	Eluent C (%)
0–3	100	0	0
3–4	0	100	0
4–14 gradient elution	0	100 → 0	0 → 100
14–22	0	0	100
22–26 re-equilibration to A	100	0	0

ICP-AES. ICP-AES was used as a second element specific detector whenever possible as a mean of quality control. An ICP-AES "JY 70 plus" system (Jobin Yvon, Long-Jumeau, France) was used for manganese determination in human milk, subfractions and SEC fractions as well as SAX fractions when analysing Mn standard compounds by SAX (no on-line operation possible). Sample introduction was performed by a peristaltic pump (1.5 mL min^{-1}, Abimed, Langenhagen, Germany), connected to a Meinhard nebulizer which was fitting into cyclon spraychamber. The measured element line was: Mn: 257.610 nm The RF power was set to 1000 W, the plasma gas was Ar at a flow rate of 15 L min^{-1}, whereas the nebuliser gas was Ar at 600 mL min^{-1}. Every ten measurements three blank determinations and a control determination of a certified Mn standard was performed. These parameters were the optimal conditions for this instrument.

Ion chromatography (IC). IC was used for chloride and phosphate determination in SEC fractions.

An Dionex DX 500 IC system with suppressor and conductivity detection was employed (Dionex, Idstein, Germany). The separation columns were an AG 4 A pre-column connected to an AS 4 A analytical column (both Dionex). The isocratic elution was achieved with a $NaHCO_3$ (1.7 mM)/Na_2CO_3 (1.8 mM) eluent (degased) at a flow rate of 2 mL min^{-1}.

Quality control

When performing speciation investigations in human body fluids many alterations of species can occur, such as contaminations, losses, stability problems of species during the analytical procedure or identification problems.[17] Therefore, many experiments were planned to overcome these difficulties

1. Generally, contact of samples or eluents with metal (stainless steel) parts was strictly avoided, as this caused contamination and increased the on-line monitoring noise. All surfaces which were in contact with the samples or eluents were metal free—this was also necessary for pump heads, injection syringes and syringe needles—otherwise the Mn noise increased *e.g* in SEC separations, or a considerable Mn peak was observed in the void at 1.4 minutes during SAX separation. Each SEC fraction was screened by SAX-ICP-MS and no Mn peaks were found in those SEC fractions where no Mn elution was seen during SEC. This showed that Mn contamination during the analytical procedure was unlikely.

2. Mn determinations were carried out and mass balances were calculated after the analytical steps by comparing the injected Mn amount with the sum of Mn quantified in fractions (*e.g*. after centrifugation, SEC fractionation). As the detection of the ^{55}Mn isotope may be interferred[18,19] (especially in total Mn determinations without chromatography) the element determinations (off-line) in HM subfractions or SEC fractions were performed additionally by ICP-AES for comparison.

3. The SEC columns were regularly cleaned using a procedure described in ref. 20 and the effluent of the columns was monitored for Mn (mass balance). Briefly: Three rinsing steps on the SEC column were performed, starting, with NaCl, 0.2 M for 90 min, to remove organic compounds/proteins, which were probably sticking to the stationary phase, followed by Milli-Q water for 90 min and finally using the chromatographic eluent (10 mM NH_4-acetate/acetic acid, pH 6.3) for 120 min. Each step was performed at regular flow rates.

4. First estimations on molecular size of the Mn compounds were performed by mass calibration of both SEC columns. The calibration curves are shown in Fig. 1. Several HMW and LMW compounds were injected onto respective SEC columns and retention times were determined by peak maxima in UV detection.

5. Further characterisation of Mn compounds was carried out by a second separation method (SAX) coupled to ICP-MS and comparing retention times of samples and standards.

Analytical concept

Generally, when performing manganese speciation difficulties arise due to the low species concentrations and the various possibilities for contamination. Therefore, a concept was developed of proceeding in gradual steps forward, first gaining only a rough insight about Mn speciation in human milk fractions combined with minimal risk of species alteration, and proceeding finally to a combination of several techniques to obtain more detailed speciation results. Fig. 2 shows the three major steps, beginning with subfractionation of human milk

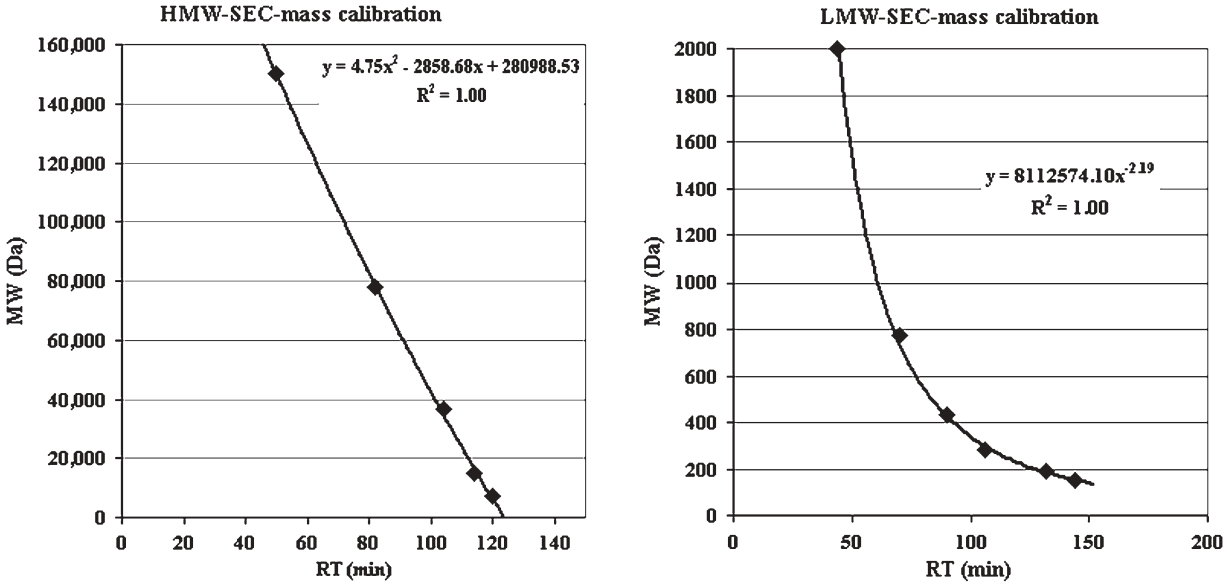

Fig. 1 Mass calibrations of SEC columns (HMW, LMW) are seen (mass/elution time). For HMW-SEC mass calibration γ-globulin (150 kDa), lactoferrin (78 kDa), β-lactoglobulin (36.5 kDa), lysozyme (15 kDa), and metallothionein (7 kDa) were injected and respective retention times were monitored by UV detection. For LMW-SEC mass calibration an analogous procedure (injection of mass-defined compounds and determination of retention time) was performed. The void retention time was determined using lysozyme (>2000 Da). Further mass calibration employed thyroxin (777 Da), *N*,*N*′-bis(*t*-BOC)-L-cystine (440 Da), *N*-CBZ-L-glutamic acid (281 Da) , citric acid (192 Da) and methionine (149 Da).

and Mn determination, followed by SEC separations for size characterisation of binding forms and finally—in a second chromatographic dimension—partial identification of Mn species.

Results and discussion

Manganese analysis in human milk subfractions

In a first step the rough distribution of manganese in human milk to proteins or LMW compounds was elucidated by two centrifugation steps. The first aimed for fat removal, the second distinguished between a protein pellet and a supernatant fraction (predominantly low molecular weight compounds). Manganese determination (number of replicates for human milk as well as of subfractions: $n = 5$) in whole human milk (HM) and these subfractions resulted in 2.83 ± 0.06 μg L^{-1} Mn in HM (100% ± 2%), 2.80 ± 0.05 μg L^{-1} Mn in defatted milk (99% ± 2%), 2.87 ± 0.06 μg L^{-1} Mn in the supernatant fraction (101% ± 2%) and 0.16 ± 0.09 μg L^{-1} Mn in the pellet fraction (5% ± 3%). The manganese concentration determined in human milk corresponds well with data from the literature, *e.g.* references 5–9, which report about 2–5 μg L^{-1}. Because of the identical values for HM and defatted milk no Mn was attributed to the fat fraction. The value from the pellet fraction may be an artifact, due to an incomplete removal of the supernatant, in analogy with reference ref. 16. To keep sample preparation short and simple, defatted milk served for further investigations.

Manganese speciation using SEC-ICP-MS

The DFF sample was introduced to SEC having a high molecular weight separation range. Fig. 3 shows the UV and Mn chromatograms. There are three Mn peaks seen, the first and second one having only low Mn amounts (*ca.* 3% and 10% of injected Mn). They correspond to approximately 107 and 75 kDa (according to mass calibration) and co-elute with high UV signals. This seems to indicate Mn bonding to proteins. When comparing these results with data from a manganese–protein data bank[21] human cytosolic prolidase might match the size 107 kDa. The molecular weight of prolidase is reported at 108 KDa (and from others partly up to 185 kDa) determined by SEC. The MW calculation from DNA sequence (SDS page) is reported to be 2×54.305 Da = 108.610 Da (as it is reported to exist as a dimer).[21] Injection of a prolidase standard also matched the respective retention time. The 75 kDa signal did not meet one of the Mn protein standards. However, refs. 22 and 23 reported Mn binding to transferrin in blood as a usual Mn transport species. The analogue of transferrin in human milk is lactoferrin, having a molecular weight of 78 kDa. Further, ref. 24 found an inhibition of bacterial multiplication by Mn-saturated lactoferrin, which proves that a Mn-lactoferrin was already found. However, Mn-transferrin or Mn-lactoferrin are reported to show a weak metal–protein bonding.

The main Mn peak in HMW-SEC consists of *ca.* 90% of injected Mn and elutes at the terminating volume of the column at around 125 min. Mn-citrate standard or inorganic manganese standards ($MnCl_2$, $Mn(NO_3)_2$) showed the same retention time as the major peak from the sample. These

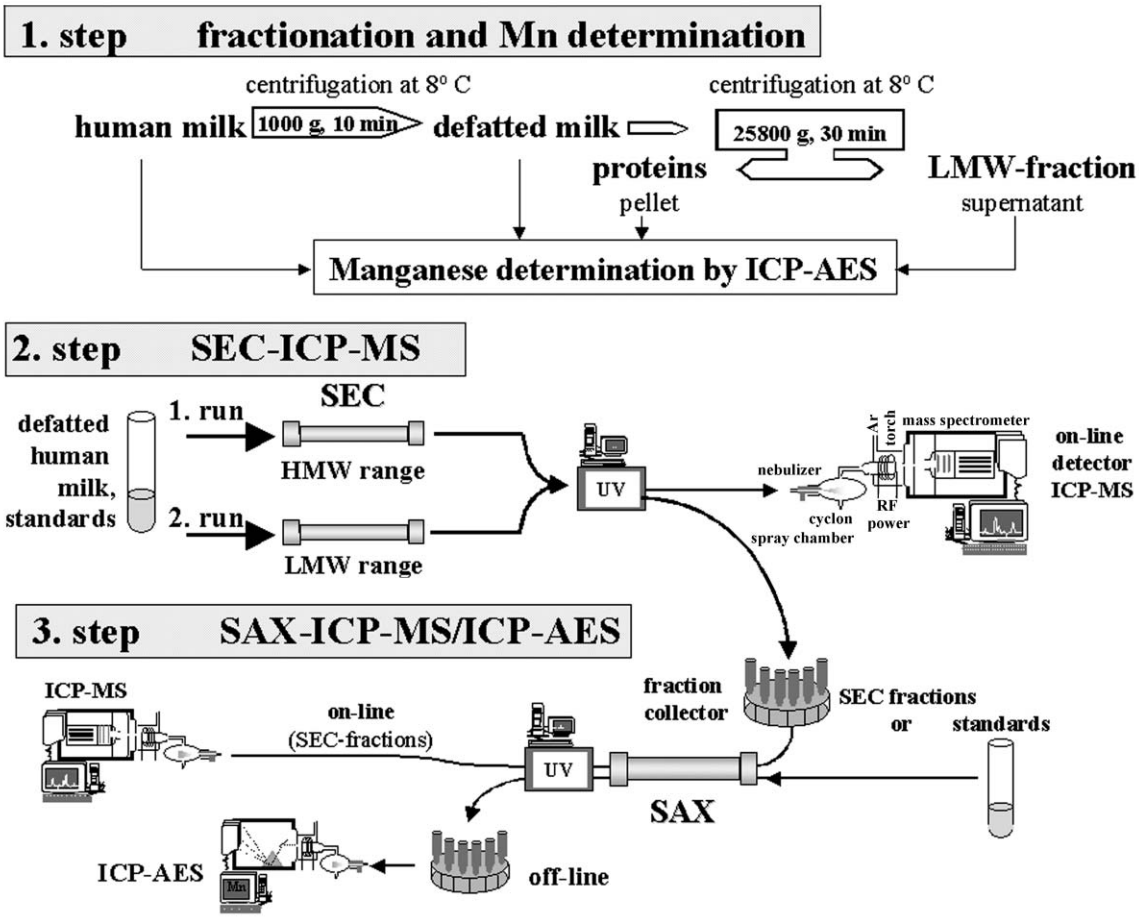

Fig. 2 Analytical concept for manganese speciation in human milk: 1. Step: Attribution of manganese to human milk subfractions using ICP-AES as Mn detector; 2. Step: Size exclusion fractionation with on-line hyphenation to ICP-MS or fraction collection with Mn determination in the fractions by ICP-AES (as a second detector), or for chloride and phosphate determination using IC. 3. Step: Aliquots of SEC fractions were investigated by SAX-ICP-MS. In parallel Mn standard compounds were run on SAX, and monitored by ICP-MS (on-line) and (after fraction collection, 1 min per fraction) by ICP-AES, too.

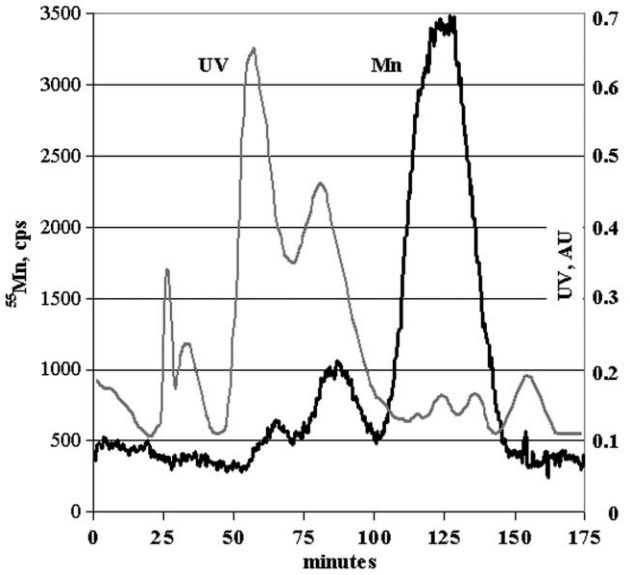

Fig. 3 An UV and Mn chromatogram of a SEC separation from defatted human milk (separation range: 10–150 kDa) is shown. Mn predominantly elutes at the terminating volume at 125 min, indicating a bonding to low molecular weight compounds.

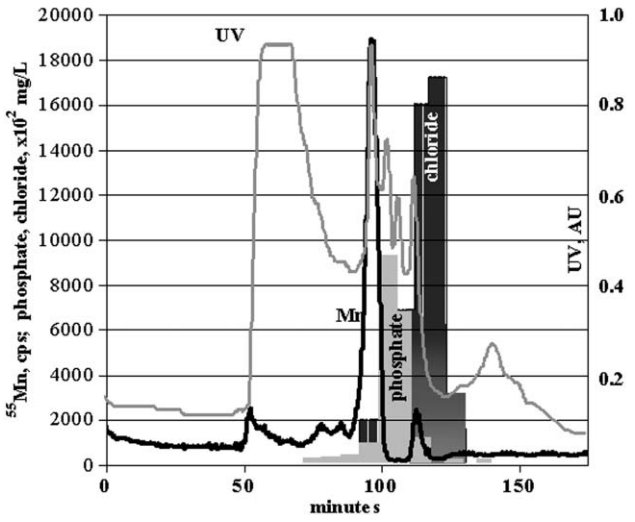

Fig. 4 An UV and Mn chromatogram of a SEC separation from defatted human milk (separation range: 100–2000 Da) is demonstrated. Only a small peak is seen at the void (>2000 Da at 54 min). The major peak elutes at 95 min and a smaller one at 115 min. The IC determination of phosphate and chloride in collected SEC fractions demonstrated a co-elution of only Cl^- with the latest manganese peak. Injection of a Mn-citrate solution resulted in a peak at 95 min (not shown), thus co-eluting with the major Mn peak.

findings confirm the results of centrifugation experiments with the supernatant (LMW-) fraction carrying nearly the total manganese in human milk.

Mass balances were calculated and found to be between 93–103% ($n = 5$). It should be noted that at the terminating volume (at 125 min) no further differentiation between Mn species is possible. Therefore, the defatted human milk sample was injected onto a SEC column having a separation range between *ca*. 100 Da and 2000 Da . This is shown in Fig. 4. The UV chromatogram shows a broad overflow peak at the exclusion volume paralleled by a small Mn peak (*ca*. 8% of total eluted Mn). Four more Mn peaks were detected at 65, 80, 95 and 115 min, attributed to *ca*. 600 Da, 500 Da, 300 Da and 260 Da by mass calibration of the column and showing 3%, 2%, 81% and 7% of eluted Mn. Again mass balances were calculated and found to range from 95–106% ($n = 5$). Mn-citrate was run on the system, too. Its retention time turned out to be at 95 min in parallel to the major Mn peak seen from human milk. As manganese phosphate was thought to be a relevant Mn species in human milk from ref. 7, fraction collection was carried out and phosphate was determined by ion chromatography. The elution profile is also seen in Fig. 4, having a maximum at 110 min. The elution pattern of phosphate and Mn did not show co-elution. The maximum of phosphate comes up between two Mn peaks at 95 min and 115 min. This indicates that the assumption of ref. 7 was not confirmed in these experiments. On the other hand, the elution pattern found for chloride (determined by ion chromatography in respective SEC fractions from DFF, Fig. 4) as well as the elution time of a $MnCl_2$ standard in LMW-SEC demonstrated a co-elution of the latest Mn peak with chloride, indicating that inorganic Mn (shown with $MnCl_2$) is present too.

Manganese speciation in defatted human milk using SAX-ICP-MS

However, a definite attribution of Mn to specific ligands is not possible with SEC alone. Therefore a SAX method was developed for separation of up to 10 Mn compounds. Fig. 5 shows respective chromatograms and gives the retention times determined by SAX-(UV)-ICP-MS or fraction numbers containing the Mn compounds determined by ICP-AES.

Defatted human milk was also analysed by the SAX method, shown in Fig. 6. The UV chromatogram demonstrates an effective separation of the sample. However, the Mn trace showed only two Mn main peaks at 1.41 min and 13.28 min as well as very minute traces at 11.87 min, 14.58 min, 16.92 min and 18.41 min. The first Mn peak may be attributed to inorganic Mn, such as $MnCl_2$, and the very small peak at 11.87 min may correspond to Mn-citrate, whilst the second major peak at 13.28 min elutes near the retention time of galactosyl transferase. But the other Mn signals did not clearly fit any of the investigated standards and thus were not attributed to specific Mn compounds. The total Mn detected in this samples was 3.2 μg L^{-1}, which corresponds to 113% recovery compared to Mn determination in HM. This increased value was attributed to the problem of the significantly increased noise on chromatograms during the second half of run time. This was obviously due to stronger interference on the ^{55}Mn isotope coming from the eluent "C" as proven by several blank runs (without any sample).

Manganese speciation using SAX-ICP-MS after SEC separation

As peak identification gave no satisfying results in the whole human milk sample, the Mn containing SEC fractions from HMW and LMW separations were investigated additionally with SAX-ICP-MS. This provided that only Mn species of the fractionated size were analysed by SAX-ICP-MS at one time.

The investigation of HMW-SEC-fractions by SAX-ICP-MS only partly confirmed the initial indication by SEC retention times. The 107 kDa fraction did not match any of the standard compounds in SAX. A single peak was seen at 7.1 minutes, which is far from prolidase (17.7 min in SAX). Therefore prolidase was not confirmed to be in this sample. An analogous result was found for the 75 kDa HMW-SEC fraction. No Mn compounds matched in SAX-ICP-MS. However, a Mn-lactoferrin standard was not available for comparison. This compound, suggested as possible by HMW-SEC, thus could not be confirmed.

However, the situation was different for the major peak at the HMW-SEC terminating volume. Here Mn peaks in SAX were seen, fitting to known Mn-compounds. Fig. 7 shows a typical SAX chromatogram of this SEC fraction with two peaks at 1.41 min and 11.8 min. The first one fits inorganic Mn,

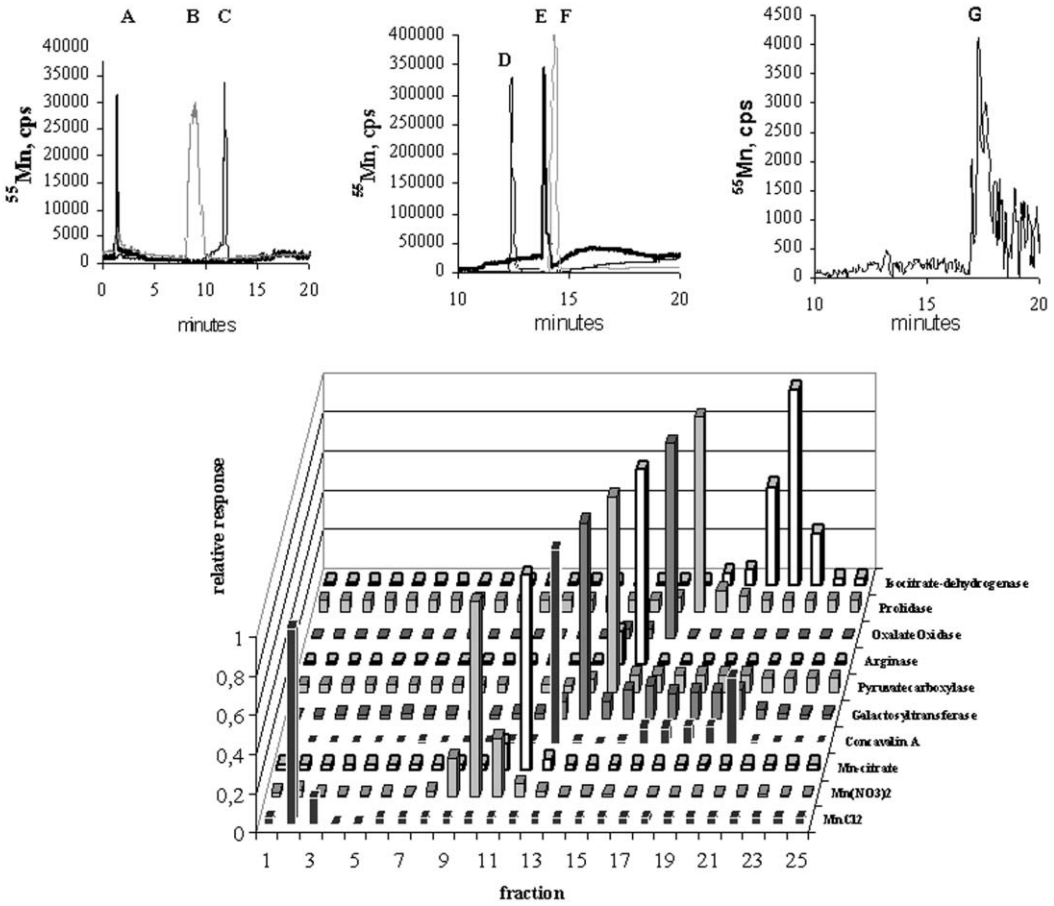

Fig. 5 Upper: Chromatograms of Mn compound standards are shown for SAX-ICP-MS (on-line). As some of the standard compounds showed increased interference on the ^{55}Mn isotope (*e.g.* oxalate oxidase), additional 1 minute fractions were taken after SAX separation and the fractions were analyzed off-line by ICP-AES. A = $MnCl_2$, B = $MnNO_3)_2$, C = Mn-citrate, D = concanavalin A, E = galactosyl transferase, F = pyruvate carboxylase, G = prolidase. Lower: Mn determination in fractions after SAX separation of each Mn compound by ICP-AES. Good agreement is achieved between retention time (monitored by ICP-MS) and fraction number (determined by ICP-AES), *e.g.* Mn-citrate: RT = 11.8 min by ICP-MS, fraction number 12, collected from 11–12 min, by ICP-AES. Retention times determined by UV and ICP-MS and respective fractions are listed below: Table 2

checked by $MnCl_2$, the second peak elutes at the retention time of Mn-citrate.

Further investigations were focused on the SEC fractions from the LMW range column.

The SEC void fraction containing only minute amounts of Mn resulted in a SAX chromatogram with four very small peak signals at 3.95, 6.99, 9.47 and 11.15 min, each of them close to baseline. None of them fitted one of the retention times of investigated Mn compounds. The 75–85 min fraction (*ca.* 500–600 Da) showed a small peak in the void at 1.41 min and a clear peak at 10.21 minutes, the latter again not paralleling any standard retention time. Most interesting, however, was the result from the 95–105 min fraction, which contained *ca.* 80–85% of Mn in LMW-SEC fractionation. The UV as well as the Mn chromatogram showed a clear separation of several peaks, demonstrated in Fig. 8. Three manganese species are monitored at 1.42 min, 11.84 min and 12.82 min. Standard addition confirmed Mn-citrate at 11.84 min and $MnCl_2$ addition increased the peak at 1.41 min. The latest Mn peak at 12.82 min was not identified by standard addition.

The finding that there is Mn-citrate in human milk is not surprising. The presence of citrate in human milk was discussed during the 1980s[25–28] and was quantified *e.g.* from ref. 14 in 1991 at 525 mg L^{-1} for a lactation state between the 2nd–7th day. It is likely that inorganic Mn^{2+} present in human milk gets complexed by citrate. Ref. 22 found a transferrin independent Mn uptake and transport system in the LMW range.

The investigation of the SEC-Mn peak at 115 min by SAX-ICP-MS showed only a Mn signal at 1.41 min, which also was seen for a $MnCl_2$ standard. Mn-citrate was not now seen. On the other hand, the elution pattern found for chloride (determined by ion chromatography in respective SEC-fractions from DFF) as well as the elution time of a $MnCl_2$ standard in LMW-SEC and SAX suggests at least an inorganic Mn species which is probably $MnCl_2$.

Table 2 Retention times of Mn standard compounds and respective fraction where they were determined by ICP-AES

Mn compound	RT (min)	Fraction number	Mn-compound	RT (min)	Fraction number
$MnCl_2$	1.4	2	Pyruvate carboxylase	14.4	15
$Mn(NO_3)_2$	9.1	10	Arginase	15.6	16
Mn-citrate	11.8	12	Oxalate oxidase	16.3	17
Concanavalin A	12.5	13	Prolidase	17.7	18
Galactosyl transferase	13.8	14	Isocitrate dehydrogenase	21.6	22

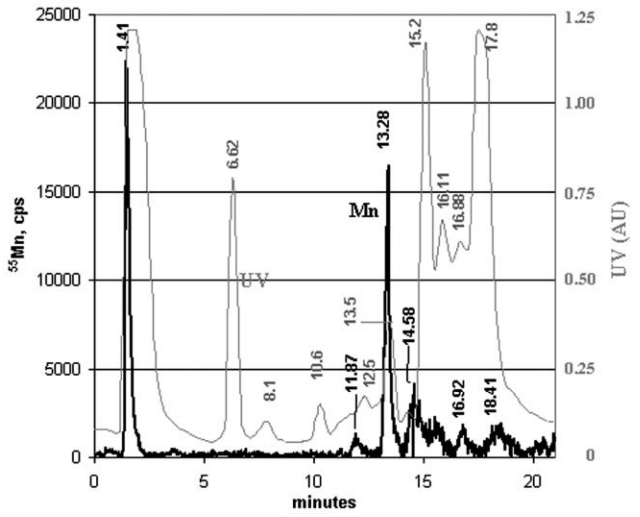

Fig. 6 UV and Mn chromatograms of defatted human milk analysed by SAX-ICP-MS are shown. Although an acceptable separation is seen, an identification of Mn species is not clearly possible.

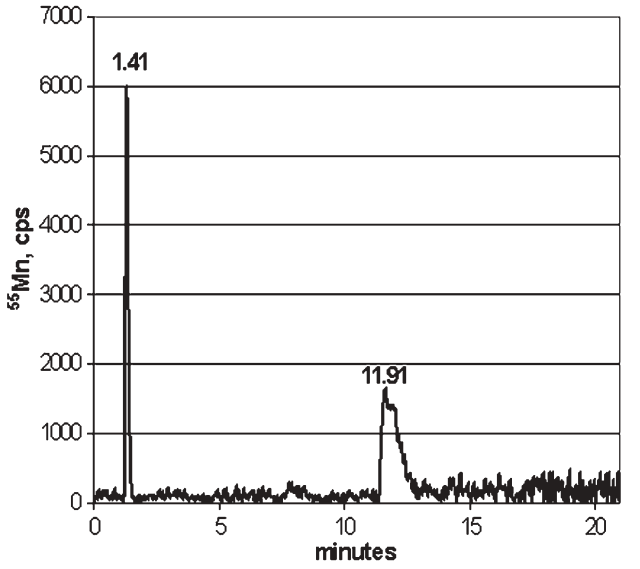

Fig. 7 SAX-ICP-MS analysis of the predominant Mn peak collected from HMW-SEC separation at 125 min is plotted. Standard addition of $MnCl_2$ and Mn-citrate, applied to this fraction increases the peaks and thus may identify the two peaks by standard addition (not shown).

Conclusion

The speciation of manganese in human milk is still analytically demanding due to the very low total Mn concentration with the single species being of even lower concentration and the difficulties in sensitive ICP-MS detection (interference on ^{55}Mn). Even so, several Mn species were monitored and some were identified. Mn-citrate and inorganic manganese, such as $MnCl_2$, were finally found by HMW- and LMW-SEC-ICP-MS as well as by the combination of SEC *and* SAX-ICP-MS (two-dimensional). Their presence in human milk appears likely and they seem to provide the predominant Mn species in human milk of the lactation state investigated. The Mn citrate complex promises a well controlled availability of Mn for a normal development of the newborn in analogy to *e.g.* the citrate complex with Zn known already in human milk.[14] The other Mn compounds of human milk were seen at low amounts, but were not identified as they did not match retention times of investigated standard compounds. This emphasises the need for ESI-MS investigation with sufficient detection sensitivity in future.

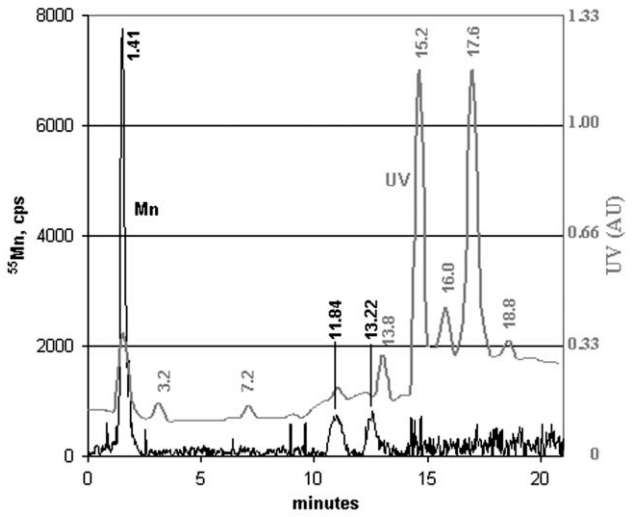

Fig. 8 SAX-ICP-MS analysis (UV and Mn chromatogram) of the predominant Mn peak collected from LMW-SEC separation at 95 min is seen. Standard addition of $MnCl_2$ and Mn-citrate increases the peaks at 1.4 min ($MnCl_2$) and the one at 11.84 min (Mn-citrate). The third Mn peak at 13.22 does not match a standard compound and thus is not identified.

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