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Determination of Zn-citrate in human milk by CIM monolithic chromatography with atomic and mass spectrometry detection

Radmila Milačič*, Dejan Ajlec, Tea Zuliani, Dušan Žigon, Janez Ščančar

Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

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ABSTRACT

In human milk zinc (Zn) is bound to proteins and low molecular mass (LMM) ligands. Numerous investigations demonstrated that Zn bioavailability in human milk is for infant much higher than in cow's milk. It was presumed that in the LMM human milk fraction highly bioavailable Zn-citrate prevails. However, literature data are controversial regarding the amount of Zn-citrate in human milk since analytical procedures reported were not quantitative. So, complex investigation was carried out to develop analytical method for quantitative determination of this biologically important molecule. Studies were performed within the pH range 5–7 by the use of synthetic solutions of Zn-citrate prepared in HEPES, MOPS and MES buffers. Zn-citrate was separated on weak anion-exchange convective interaction media (CIM) diethylaminoethyl (DEAE) monolithic chromatographic column using NH_4NO_3 as an eluent. Separated Zn species were determined by flame atomic absorption spectrometry (FAAS) or inductively coupled plasma mass spectrometry (ICP-MS). Quantitative separation of Zn-citrate complexes ($[Zn(Cit)]^-$ and $[Zn(Cit)_2]^{4-}$; column recoveries 94–102%) and good repeatability and reproducibility of results with relative standard deviation (RSD $\pm$ 3.0%) were obtained. In fractions under the chromatographic peaks Zn-binding ligand was identified by electrospray ionization tandem mass spectrometry (ESI-MS-MS). Limits of detection (LOD) for determination of Zn-citrate species by CIM DEAE-FAAS and CIM DEAE-ICP-MS were 0.01 μg Zn mL^{-1} and 0.0005 μg Zn mL^{-1}, respectively. Both techniques were sensitive enough for quantification of Zn-citrate in human milk. Results demonstrated that about 23% of total Zn was present in the LMM milk fraction and that LMM-Zn corresponded to Zn-citrate. The developed speciation method represents a reliable analytical tool for investigation of the percentage and the amount of Zn-citrate in human milk.

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1. Introduction

Human milk is usually the first diet for the infant. It meets all the needs of the new-born and provides essential trace elements like Zn [1]. The importance of Zn for normal growth and development of infant has been well documented. In 1980s Zn deficiency was reported in infants weaned from human to cow's milk [2–5]. These observations initiated the investigations to identify Zn biding ligands in human and cow's milk [2–10]. In cow's milk Zn is predominately bound to milk proteins [2,4,6], while in human milk Zn is distributed between milk proteins and low molecular mass (LMM) ligands [3–10]. The most important LMM ligand in human milk is citrate, present in concentration about 2.5 mmol L^{-1}, while in much lower amounts oxalate (0.08 mmol L^{-1}) and sulfate (0.05 mmol L^{-1}) exist [11]. Phosphorus that is present in concentration about 1 mmol L^{-1} is associated mainly with milk proteins [12]. Based on high citrate content in human milk, it was presumed that in the LMM milk fraction Zn-citrate species prevailed [3]. In first attempts to separate casein fraction from LMM milk constituents ultracentrifugation was applied. Results of these investigations were rather controversial, reporting that the amount of the LMM-Zn fraction in human milk ranged from 35% upto 95% [5–7]. Michalke and coworkers [7] found the highest amount of Zn in the LMM human milk fraction (95%) after separation of proteins by ultracentrifugation. By re-chromatography of the LMM milk fraction using TSK-SEC, they predicted that 95% of Zn in human milk was bound to citrate. These findings significantly differ from reports of other investigators which assumed that only 15–40% of Zn in human milk existed as Zn-citrate [5,6]. In last decade SEC in combination with multielemental detectors was used to separate chemical species of Zn and other elements in human milk [8–10]. Authors reported that among several chromatographic peaks LMM-Zn peak was also observed, but it was not quantified. The presented literature data are controversial regarding the amount of Zn-citrate in human milk. In addition, analytical procedures that were applied in general did not allow quantitative separation of Zn-citrate. Therefore, more powerful speciation procedures were needed for reliable and quantitative determination of this essential Zn species.

* Corresponding author. Tel.: +386 1 477 3560; fax: +386 1 2519 385.
E-mail address: radmila.milacic@ijs.si (R. Milačič).

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In last two decades monolithic chromatography has been progressively used in the field of chromatography as alternative or substitute to conventional liquid chromatography techniques. The advantages of monolithic supports are associated with effective mass transport at low back pressures, good separation efficiencies and possibility to operate the chromatographic runs at flow rates by one order of magnitude higher than usually applied in particles packed columns [13–15]. Methacrylate-based monolithic supports are intensively used in industrial applications and separations of biomolecules [16–22]. Available in disk and column packages, methacrylate-based monoliths bearing cation- and anion-exchange groups are promising chromatographic supports also for efficient separation of charged chemical species of elements [23–29]. The mild chromatographic conditions of weak anion-exchange monolithic supports enable preservation of chemical species during the separation procedure [29] and offers much higher selectivity than SEC columns [27–29]. In our previous research convective interaction media diethylaminoethyl (CIM DEAE) disk was applied for speciation of Zn-citrate and some other Zn complexes [30]. However, the main drawback was that separation of Zn-citrate was not quantitative.

As Zn-citrate is most probably present in the LMM fraction of human milk, the first aim of the present investigation was to develop analytical method for quantitative determination of this biologically important molecule by the use of CIM DEAE monolithic chromatography in combination with mass spectrometry techniques. The second aim was to apply the developed procedure for speciation of Zn in the LMM fraction of human milk.

2. Experimental

2.1. Instrumentations

The separation of Zn species was performed on weak CIM DEAE-1 anion-exchange tube monolithic column (Bia Separations, Ljubljana, Slovenia) (column dimensions 6.7 mm i.d., length 4.2 mm, volume 1 mL, working flow rates upto 16 mL min^{-1}, with matrix supports made of highly porous poly(glycidyl methacrylate-co-ethylene dimethacrylate) bearing a weak diethylaminoethyl (DEAE) anion-exchange functional groups (pH stability 2–14). The column was connected to a Waters (Milford, MA, USA) Model 600E gradient high-pressure pump, equipped with a Rheodyne (Cotati, CA, USA) Model 7725i injector (0.5 mL loop). Total Zn concentrations and separated Zn species were determined by FAAS on a Varian (Mulgrave, Victoria, Australia) SpectrAA 110 atomic absorption spectrometer or by ICP-MS on Agilent 7700x instrument (Agilent Technologies, Tokyo, Japan), equipped with Octopole Reaction System (ORS), featuring helium collision mode. Experimental working conditions for ICP-MS were optimized for plasma robustness and adequate sensitivity using High Matrix Introduction (HMI) system. HMI allows introducing low amounts of salts that were used in the separation procedure. For elimination of potential polyatomic interferences on the analytical mass 66 of Zn ($^{32}S^{34}S$, $^{34}S^{16}O^{16}O$, $^{32}S^{16}O^{18}O$, $^{31}P^{35}Cl$ and $^{54}Fe^{12}C$), He was introduced in ORS [31]. Treatment of data was performed with the Agilent MassHunter software. For identification of Zn-binding ligand negative ion mode mass measurements were run on a hybrid quadruple time of flight mass spectrometer (Q-Tof Premier) provided with an orthogonal Z-spray electrospray (ESI) interface (Waters Micromass, Manchester, UK). Experimental working conditions are summarized in Table 1.

A WTW (Weilheim, Germany) 330 pH meter was employed to determine the pH. Samples were centrifuged on Hettich (Beverly, MA, ZDA) Universal 320 centrifuge. Microultrafiltration was performed on Amicon Ultra-4 Centrifugal Filter Devices (Amicon, Beverly, MA, USA) Ultracel 3 K.

2.2. Reagents and materials

Merck (Darmstadt, Germany) suprapur acids and Ultrapure 18.2 MΩ cm water obtained by Direct-Q 5 Ultrapure water system (Millipore Watertown, MA, USA) were used for the preparation of samples and standard solutions. All other chemicals were of analytical reagent grade. Stock standard solution of Zn (1000 mg L^{-1} in 5% HNO_3) (Merck) was used for preparation of working standard solutions for FAAS and ICP-MS determinations. Working standard solutions were prepared by adequate dilution with water. Citric acid monohydrate (Merck) was used for the preparation of Zn-citrate. In order to adjust pH of Zn species investigated, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-(N-morpholino)propanesulfonic acid (MOPS) and 2-(N-morpholino)ethanesulfonic acid (MES) buffers were used. The selected pH of buffer solutions was adjusted by addition of an appropriate amount of NH_4OH.

2.3. Preparation of synthetic solutions of Zn-citrate

Stock Zn-citrate solution (50 μg Zn mL^{-1}) was made by mixing of an appropriate amount of citric acid monohydrate with 1000 mg L^{-1} Zn^{2+} solution, so that the Zn-to-citric acid molar ratio was 1:100 (similar to that in human milk). Zn-citrate complex was formed within 24 h. Speciation analysis data confirmed that stock Zn-citrate solution was stable for at least four weeks when stored at 4 °C. By speciation analysis it was further established that the identical chromatograms were obtained, regardless the stock solution of Zn-citrate was prepared from Zn-nitrate salt dissolved in water, or from commercially available standard solution of Zn^{2+} in 5% HNO_3. Due to the simplicity, the latter was used for preparation of stock Zn-citrate solution preparation. Working synthetic solutions of Zn-citrate (1 μg Zn mL^{-1}) at pH 5, 6 or 7 were prepared by adding 0.2 mL of stock Zn-citrate solution (50 μg Zn mL^{-1}) to 9.8 mL of 0.2 mol L^{-1} HEPES, MOPS or MES buffers. Before use, these solutions were left

Table 1
Operating conditions for FAAS, ICP-MS and ESI-MS-MS.

FAAS parameters		ICP-MS parameters		ESI-MS parameters	
Wavelength	213.86 nm	Forward power	1500 W	Ionization	Negative mode
Lamp current	3 mA	Outer gas flow	15.0 L min^{-1}	Ion spray voltage	2.7 kV
Flame	Air–acetylene	Carrier gas flow	0.44 L min^{-1}	Nebulizing gas	N_2 10 L min^{-1}
Background correction	Deuterium lamp	Dilution gas flow Ar (HMI)	0.73 L min^{-1}	Source temperature	150 °C
		He gas flow	4.0 mL min^{-1}	Injection	Direct 10 μL min^{-1}
		Isotope monitored	^{66}Zn	Mass range	*m*/*z* 50-1000
		Internal standard	^{72}Ge	Mass resolution	10.000
		Integration time	2.4 s	Scan types	Total ion, MS/MS scan
		Total acquisition time	600 s	External calibration	Leucine-enkephalin

for 16 h to allow equilibration of Zn-citrate species at pH investigated. Working Zn-citrate solutions were stable (stored at 4 °C) for at least five days in HEPES and two days in MOPS and MES buffers. The equilibration time for preparation of working Zn-citrate solutions and their stability were verified by the speciation analysis.

2.4. Sampling protocol and preparation of milk samples for analysis

Milk samples were obtained from two healthy volunteers (28 years old mother A and 30 years old mother B) in different stage of lactation (8–26 weeks). Mothers collected milk by manual expression before infant feeding. To prevent contamination about 2 mL of milk was first discarded and next 20 mL collected directly into 25 mL Duran glass bottles. Milk sample was immediately stored at −25 °C. Before the analysis, frozen sample was thawed and left at 4 °C for 16 h. The milk fat was gathered above the sample. Defatted milk was carefully collected by a plastic syringe equipped with a stainless steel needle and subjected to 10 min centrifugation at 3000 rpm (958*g*) to additionally remove the remaining fat. To prevent contamination all laboratory ware was soaked with 10% nitric acid for 24 h and then rinsed with Milli Q water. The use of any gloves or creams was omitted during sample collection, handling process and analytical procedure.

2.5. Procedure for the determination of Zn by FAAS and ICP-MS

Total Zn concentration in milk was determined from defatted sample by FAAS (diluted 1:5 with water) or ICP-MS (diluted 1:10 with water). For speciation analysis undiluted milk was used. Prior to speciation procedure proteins were removed by micro-ultrafiltration. For this purpose, 4 mL of defatted milk was transferred into centrifugal filter device (cut off 3000 Da) and centrifuged for 10 min at 8100 rpm (6981*g*). In ultrafiltrate, which represented LMM milk fraction, Zn content was determined by FAAS or ICP-MS. Speciation analysis of the LMM milk fraction were performed by CIM DEAE-FAAS or CIM DEAE-ICP-MS procedures, respectively. For quality control Zn content in reference material Whole milk powder 8435 NIST was analyzed after microwave assisted digestion by FAAS and ICP-MS. In addition, comparative analyses of Zn content in defatted human milk samples and Zn in LMM milk fractions were performed by FAAS and ICP-MS. Results of Zn concentrations in reference material determined by FAAS and ICP-MS were within the range of certified value, indicating the accuracy of analytical procedures applied. Comparable results (agreement within ± 2%) were also obtained for Zn content in human milk samples determined by FAAS and ICP-MS, proving that in milk samples, Zn can be reliably determined by both methods (see Table 1 in Supplementary material).

2.6. CIM DEAE-1 procedure with FAAS or ICP-MS detection

5 mL of sample was injected onto a CIM DEAE-1 monolithic column. An aqueous—1.2 mol L^{-1} NH_4NO_3 linear gradient elution was applied for 10 min at a flow rate of 1.0 mL min^{-1}. The eluate was collected in 1.0 mL fractions in Eppendorf polyethylene cups and the concentration of Zn determined by FAAS immediately after separation by the use of matrix matched standards. Alternatively, the elution profile of Zn during the chromatographic separation was followed on-line by ICP-MS. For quantification of separated Zn species by ICP-MS a sample aliquot was injected on CIM column and chromatographic peaks collected in sample tubes. Zn content in separated peaks was then immediately determined by ICP-MS applying external calibration. After each separation the column was regenerated for 1 min with 4 mol L^{-1} NH_4NO_3 at a flow rate of 10.0 mL min^{-1}, followed by elution with 0.2 mol L^{-1} HEPES (pH 5) for 2 min at a flow rate of 10.0 mL min^{-1}. Finally, the column was equilibrated with water for 11 min at a flow rate of 10.0 mL min^{-1} and for one more min at a flow rate of 1.0 mL min^{-1}. The chromatographic run was completed in 25 min.

If not stated otherwise, all the experiments were done in duplicates.

2.7. Cleaning procedure

20 mL of 1 mol L^{-1} NaOH was pumped through the column at a flow rate of 1 mL min^{-1}. All steps that followed were performed at a flow rate of 10 mL min^{-1}. After application of NaOH the column was rinsed with 50 mL of water. Then 50 mL of 0.2 mol L^{-1} HEPES (pH 5) was applied, followed by rinsing with 20 mL of 2 mol L^{-1} NaCl and 50 mL of 0.2 mol L^{-1} HEPES (pH 5). At the end the column was rinsed with 50 mL of water. It was experimentally proven that the cleaning of column was necessary after approximately 30 successive separations. NH_4NO_3 eluent was cleaned by applying batch Chelex-100 procedure [27,28].

3. Results and discussion

3.1. Development of an analytical procedure for the speciation of Zn-citrate by CIM DEAE-1 column with FAAS or ICP-MS detection

There are several problems related to speciation of Zn. At physiological pH values Zn is readily adsorbed on different chromatographic columns. Zn-citrate complexes are moderately stable and are partially broken on strong anion-exchange chromatographic supports (see Fig. 1. in Supplementary material). This phenomenon was observed also in our previous work [30].

Zn interacts with numerous buffer solutions, which cannot be used in sample preparation and in chromatographic separations. When pH in solutions containing Zn^{2+} ions was adjusted with potassium hydrogen phthalate, piperazine, Tris–HCl, potassium borate or imidazole buffers, broad chromatographic peaks, which were eluted at retention volumes characteristic for negatively charged complexes, were observed [30]. If there was no interaction with buffers, Zn^{2+} ion should be, on anion-exchange columns, eluted with the solvent front. Due to these reasons, separation of Zn species on different chromatographic supports was not quantitative [30]. In order to develop quantitative and reliable procedure for determination of Zn-citrate, the above drawbacks were considered in the present investigation.

FAAS was used as an element specific detector, since it is sensitive enough, cheap and fast technique. Column recoveries, calculated as ratio between injected and eluted Zn species, were obtained by summing up Zn concentrations in fractions collected during the chromatographic run. Once the analytical procedure was developed, more sensitive ICP-MS detector was connected to CIM-DEAE-1 column to follow Zn elution profile on-line and for quantification of Zn species under chromatographic peaks.

3.1.1. The choice of an appropriate buffer for speciation of Zn

Due to influence of several buffers on Zn speciation [30], in the present work the choice of an appropriate buffer that would not interact with Zn was further examined. For this purpose, an aqueous synthetic solution of Zn^{2+} (1 μg Zn mL^{-1}, pH 5) was prepared from the stock standard solution (1000 mg L^{-1} of Zn in 5% HNO_3) by dilution with water. The same synthetic solutions of Zn^{2+} at pH 5 were also prepared in 0.2 mol L^{-1} HEPES, MOPS and MES buffers, which are usually used in biochemical analysis [32]. Samples were injected onto a CIM DEAE-1 column and Zn

determined in the separated fractions by FAAS (Fig. 2. in Supplementary material). Results revealed that at pH 5, Zn^{2+} in aqueous solution was quantitatively eluted with a solvent front. The same elution profiles were obtained for Zn^{2+} prepared in 0.2 mol L^{-1} HEPES, MOPS and MES buffers, confirming that these buffers did not interact with Zn^{2+} species. So, they were further used for the adjustment of the pH of Zn species investigated.

3.1.2. Optimization of the parameters for separation of Zn-citrate on CIM DEAE-1 column

For chromatographic separation NH_4NO_3 was used as an eluent since it does not interact with Zn and is compatible with FAAS, ICP-MS and ESI-MS detectors. Different aqueous linear gradient elutions from 0.8 upto 1.4 mol L^{-1} NH_4NO_3 were tested. Zn-citrate was prepared in concentration 1 μg Zn mL^{-1} at pH 7 in 0.2 mol L^{-1} HEPES. At such concentration levels Zn was also found in the LMM human milk fraction. As an element specific detector FAAS was used. The results of the experiment are presented in Fig. 1.

It is evident from Fig. 1 that the column recovery for aqueous—NH_4NO_3 gradient elution is 90.6% for 0.8 mol L^{-1} NH_4NO_3, while elution with 1.0 to 1.4 mol L^{-1} NH_4NO_3 enables quantitative separation of Zn-citrate species. So, aqueous 1.2 mol L^{-1} NH_4NO_3 linear gradient was further applied. It was experimentally proven, that the same elution profiles and quantitative column recoveries were obtained when water or 0.005 mol L^{-1} HEPES (pH 5) were applied in NH_4NO_3 gradient elution. Due to the simplicity, aqueous gradient elution was used. The experimental data also showed that the efficient regeneration of the monolithic column is of crucial importance for repeatable chromatographic separations. Between different combinations examined, application of 4 mol L^{-1} NH_4NO_3, followed by rinsing with 0.2 mol L^{-1} HEPES (pH 5) and equilibration with water, as described under the CIM DEAE-1 procedure, enabled repeatable and reproducible chromatographic separations.

3.1.3. Partitioning of Zn-citrate on CIM DEAE-1 column at different pH values

At pH range 5–7, Zn-citrate exists as a mixture of negatively charged complexes. Powell and Pettit [33] provided a computer program for calculation of metal species over a wide pH range using stability constants from the IUPAC database. On the basis of these calculations, the following Zn-citrate species (Zn to citrate molar ratio 1:100) were predicted: in the pH range 5–7 $[Zn(Cit)]^-$ and $[Zn(Cit)_2]^{4-}$ complexes coexist. At pH 5 $[Zn(Cit)]^-$ is the dominant species, while at pH 7 $[Zn(Cit)_2]^{4-}$ prevails. Citrate forms complexes of moderate stability with Zn. The corresponding log K (at 25 °C and ionic strength 0.16) are 4.98 for $[Zn(Cit)]^-$ and 5.90 for $[Zn(Cit)_2]^{4-}$ complex, respectively [34]. Therefore, to preserve Zn-citrate complexes weak anion-exchange CIM DEAE-1 monolithic column was used. Synthetic Zn-citrate solutions (1 μg Zn mL^{-1}) were prepared in 0.2 mol L^{-1} HEPES, MOPS and MES buffers within a pH range 5–7. Speciation analysis was performed by the CIM DEAE-1—FAAS procedure. The results are presented in Fig. 2.

Data from Fig. 2 indicate that Zn-citrate is quantitatively eluted (recoveries between 96 and 100%) from the column in two peaks with maximal concentrations at 3 min and 6 min. The elution profiles are the same when 0.2 mol L^{-1} HEPES, MOPS or MES were used in preparation of synthetic solutions of Zn-citrate at pH 5, 6 and 7. Based on the theoretical predictions [33] and elution times, it can be deduced that the first peak (eluted with maximal concentration at 3 min) corresponds to $[Zn(Cit)]^-$ complex, while the second peak (eluted with maximal concentration at 6 min) to $[Zn(Cit)_2]^{4-}$ complex. It is also evident that at pH 5 $[Zn(Cit)]^-$ complex prevails, while at pH 7 $[Zn(Cit)_2]^{4-}$ becomes a dominant species. At pH 7, the tailing of the second chromatographic peak is observed due to the appearance of Zn-hydroxo species. These observations are in agreement with the theoretically predicted Zn-citrate speciation in the pH range investigated [33]. Above observations again confirmed that HEPES, MOPS or MES buffers do not form complexes with Zn and are appropriate for their use in Zn speciation analysis.

3.1.4. Figures of merits for the developed CIM DEAE-1—FAAS procedure

In order to study the repeatability of the procedure developed, six consecutive determinations of synthetic solutions of Zn-citrate (1 μg Zn mL^{-1}) were prepared in 0.2 mol L^{-1} HEPES and analysis performed by the use of CIM DEAE-1—FAAS procedure. Zn-citrate complexes were quantitatively eluted in two peaks with maximal concentrations at 3 and 6 min. The column recoveries ranged from 94 to 102%. Good repeatability of measurement was obtained with the relative standard deviation (RSD ± 3.0%) (see Table 2 in Supplementary material). The same experiment was made in triplicate determinations after one week by another analyst. The same Zn chromatograms were obtained and the column recoveries ranged from 94 to 100%, confirming that the

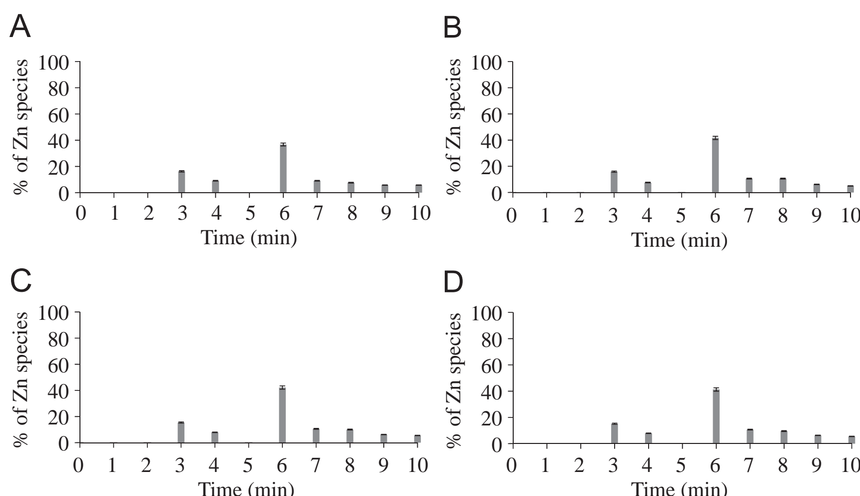

Fig. 1. Distribution of Zn-citrate (1 μg Zn mL^{-1}) at pH 7 prepared in 0.2 mol L^{-1} HEPES, employing CIM DEAE-1—FAAS procedure. Sample volume 0.5 mL, flow rate 1 mL min^{-1}. Linear gradient elution from 100% water to (A) 0.8 mol L^{-1} NH_4NO_3, (B) 1.0 mol L^{-1} NH_4NO_3, (C) 1.2 mol L^{-1} NH_4NO_3 and (D) 1.4 mol L^{-1} NH_4NO_3. Column recoveries: (A) 90.6%, (B) 97.7%, (C) 98.1%, (D) 96.7%.

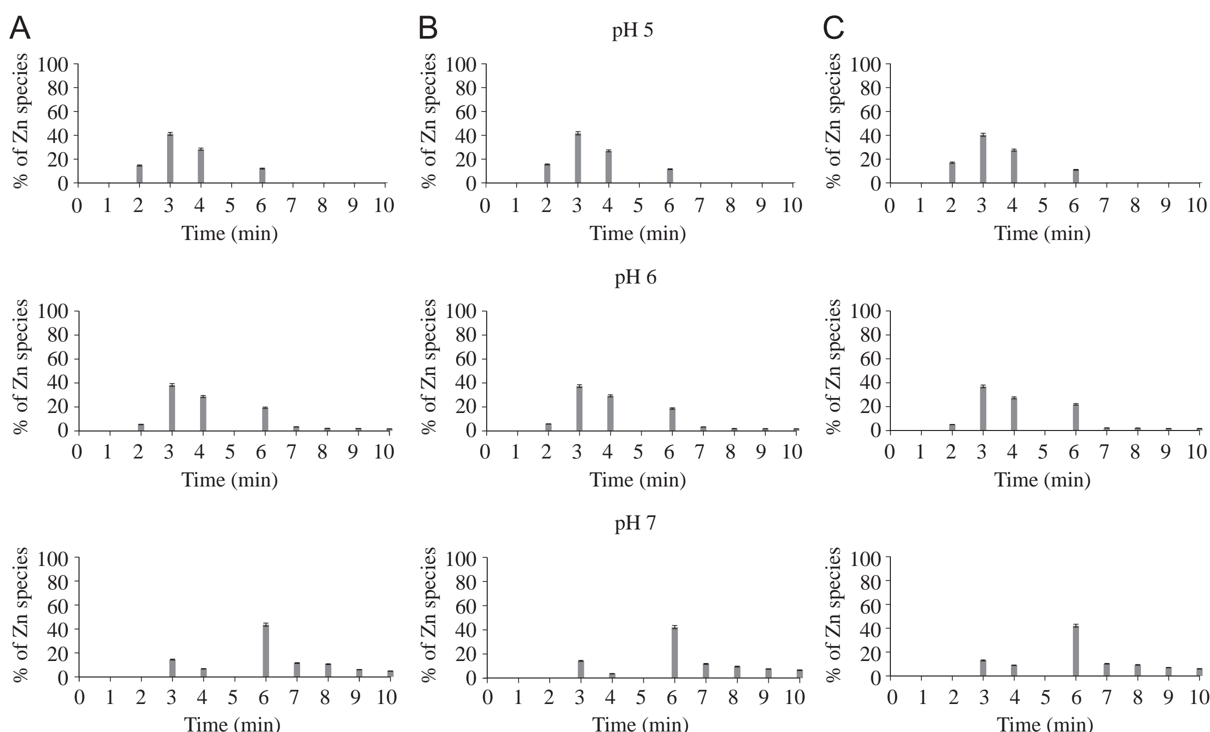

Fig. 2. Distribution of Zn-citrate (1 μg Zn mL^{-1}) prepared in (A) 0.2 mol L^{-1} HEPES, (B) 0.2 mol L^{-1} MOPS and (C) 0.2 mol L^{-1} MES at different pH, employing CIM DEAE-1—FAAS procedure. Sample volume 0.5 mL, aqueous—1.2 mol L^{-1} NH_4NO_3 linear gradient elution, flow rate 1 mL min^{-1}. Column recoveries: pH 5: (A) 96.2%, (B) 95.7%, (C) 96.0%, pH 6: (A) 101.5%, (B) 100.4%, (C) 99.2%, pH 7: (A) 97.9%, (B) 96.4%, (C) 96.5%.

procedure developed was also reproducible (see Table 3 in Supplementary material).

Linearity of measurement of synthetic solutions of Zn-citrate for CIM DEAE-1—FAAS procedure was obtained in the concentration range 0.02 to 2.0 μg mL^{-1} for separated Zn species with correlation coefficient better than 0.998. The limit of detection (LOD), calculated as the concentration equal to 3 times standard deviation (3*s*) of the blank sample, was 0.01 μg mL^{-1} for the determination of separated Zn species.

3.1.5. Partitioning of Zn-citrate on CIM DEAE-1 column with ICP-MS detection and figures of merits

Once the analytical procedure was developed, ICP-MS was connected to CIM-DEAE column to follow Zn elution profile on-line. The representative chromatogram of the synthetic solution of Zn-citrate (1 μg Zn mL^{-1}) prepared in 0.2 mol L^{-1} HEPES at pH 7, together with the elution profile of blank sample is presented in Fig. 3.

The elution profile of separated $[Zn(Cit)]^-$ and $[Zn(Cit)_2]^{4-}$ complexes is the same, as in CIM DEAE-1—FAAS procedure, while continuous elution followed on-line by ICP-MS allows more precise observation of the separated Zn complexes. Blank sample did not contribute to the measured signal of ICP-MS determinations. It was experimentally proven that the linearity of measurement of Zn content in separated Zn-citrate peaks by ICP-MS was obtained in the concentration range from 0.001 to 1.0 μg mL^{-1} with correlation coefficient better than 0.999. The LOD of the separated Zn species determined, based on 3 times standard deviation (3s) of the blank sample, was 0.0005 μg mL^{-1}.

3.2. Speciation of Zn in the LMM fraction of human milk

The developed CIM DEAE-1 procedure enables quantitative and reliable determinations of Zn-citrate within biologically and environmentally important pH ranges and was applied in

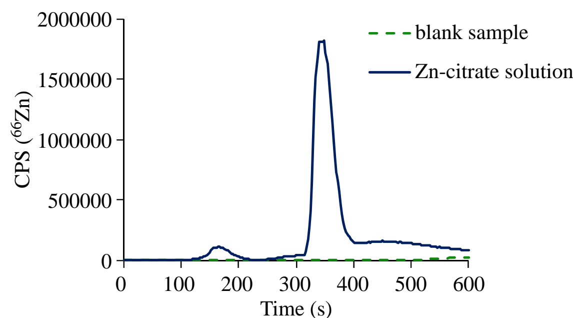

Fig. 3. Distribution of Zn-citrate (1 μg Zn mL^{-1}) at pH 7 prepared in 0.2 mol L^{-1} HEPES and blank sample, employing CIM DEAE-1—ICP-MS procedure. Sample volume 0.5 mL, aqueous—1.2 mol L^{-1} NH_4NO_3 linear gradient elution, flow rate 1 mL min^{-1}. Column recovery for Zn-citrate: 103%.

speciation of Zn in the LMM fraction of human milk. Four milk samples of two different volunteers (28 years old mother A and 30 years old mother B) in different stage of lactation were analyzed. Sampling and sample preparation followed the protocol described under section 2.4. *Sampling protocol and preparation of milk samples for analysis*. Concentrations of Zn in defatted milk, Zn in the LMM milk fraction (Zn in ultrafiltrate, cut off 3000 Da) and the amount of Zn-citrate complexes (eluted from the chromatographic columns) were determined by FAAS or ICP-MS. Ultrafiltrate was further used for speciation of Zn in the LMM milk fraction by CIM DEAE-1 procedure. Regarding the Zn concentration, FAAS or more sensitive ICP-MS detectors were used to follow the elution profiles and quantification of separated Zn species. These results are presented in Fig. 4, while the corresponding Zn concentrations in Table 2.

In Table 2 the percentage of Zn in LMM milk fraction, pH of samples and stage of lactation are also given. Results of these experiments revealed that total Zn concentrations in milk of both mothers declined with time of lactation (Table 2), what is in

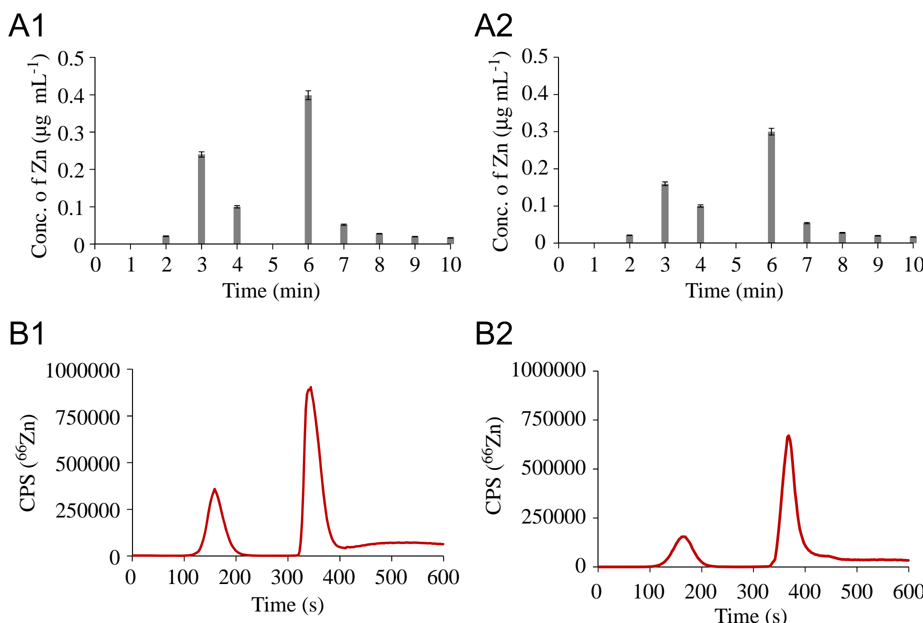

Fig. 4. Distribution of Zn in human milk employing CIM DEAE-1 column with FAAS detection (samples A1 and A2) and ICP-MS detection (samples B1 and B2). Sample volume 0.5 mL, aqueous—1.2 mol L^{-1} NH_4NO_3 linear gradient elution, flow rate 1 mL min^{-1}. Column recoveries: (A1) 97.8%, (A2) 98.6%, (B1) 102%, (B2) 97.7%.

Table 2
Concentrations of Zn in defatted human milk in LMM milk fraction (cut off 3000 Da), Zn-citrate in LMM milk fraction, percentage of Zn in LMM fraction, pH of samples and time of lactation. Zn concentrations were determined by FAAS and ICP-MS#. Samples were taken from of two different healthy volunteers (A and B).

Human milk sample	Concentration of Zn in defatted milk (μg Zn mL^{-1})	Concentration of Zn in LMM milk fraction (μg Zn mL^{-1})	Concentration of Zn-citrate in LMM milk fraction (μg Zn mL^{-1})	Percentage of Zn in LMM milk fraction (%)	pH of milk	Stage of lactation (weeks)
A1	3.93 ± 0.11	0.90 ± 0.02	0.88 ± 0.03	22.9	6.5 ± 0.1	16
A2	3.06 ± 0.08	0.71 ± 0.02	0.70 ± 0.02	23.2	6.5 ± 0.1	26
B1#	1.82 ± 0.02	0.409 ± 0.004	0.417 ± 0.012	22.5	6.6 ± 0.1	8
B2#	1.12 ± 0.02	0.259 ± 0.003	0.253 ± 0.007	23.1	6.5 ± 0.1	26

accordance with the reported literature data [35,36], while the percentage of Zn in the LMM milk fraction remained constant (about 23%), regardless the stage of lactation (8–26 weeks). Chromatograms of speciation analysis of the LMM milk fractions (Fig. 4) demonstrate similar elution profiles of separated Zn species for four samples investigated. On the basis of the elution time and the pH of samples (pH about 6.5), it may be presumed that the first peak eluted at maximal Zn concentration of about 3 min corresponded to $[Zn(Cit)]^-$ and the second peak eluted at maximal Zn concentration at 6 min, corresponded to $[Zn(Cit)_2]^{4-}$ complex. In order to identify the Zn-binding ligands, the chromatographic peaks that contained Zn were collected and subjected to ESI-MS-MS analysis. First, the mass spectrum of standard solution of Zn-citrate was recorded. The most intensive peak in the mass spectrum at m/z 191 correspond to deprotonated molecular ion $[M–H]^-$ of citric acid. This peak was selected as a parent ion for the collision-induced dissociation experiment (CID) and the resulting daughter ion mass spectra were recorded under optimum conditions. During dissociation daughter ions at m/z 129, m/z 111, m/z 87 and m/z 85 were formed and observed in MS-MS spectrum. After optimization of mass spectrometric parameters on standard solution, MS analyses were also performed on separated fractions of the first and the second chromatographic peaks of four milk samples. Mass spectra and the corresponding MS-MS scans for first and second chromatographic peaks of the LMM fraction of milk sample A1 are shown in Fig. 5.

Mass spectrum in Fig. 5(A) and (C) of the first and the second chromatographic peaks contain basic peak at m/z 191, which correspond to deprotonated molecular ion of citric acid. Additionally, a characteristic fragment ion of deprotonated citric acid at m/z 111 is also present in mass spectrum. Based on the analysis of blank sample, separated on the CIM DEAE-1 column and collection of fractions at retention volumes of the first and the second chromatographic peaks, all other ions recorded, correspond to the signals from the eluent used in ESI-MS procedure and eluent of the chromatographic run applied in the CIM DEAE-1 procedure. To confirm the structure of ion at m/z 191, MS-MS analysis of this parent ion was made and the daughter ion mass spectra recorded. As it is evident from Fig. 5(C) and (D), after dissociation, masses m/z 129, m/z 111, m/z 87 and m/z 85 of the daughter ions were present in the MS-MS spectra of the first and the second chromatographic peaks of milk sample. Additional confirmation of the structure of $[M–H]^-$ was done by accurate mass measurements of ions at approximate mass resolution 10,000 with Q-Tof mass spectrometer. Measured accurate mass 191.0192 of $[M–H]^-$ corresponds to elemental composition: $C_6H_7O_7$ and confirmed the structure of deprotonated citric acid. The same ESI-MS-MS spectra were obtained for the LMM fraction of other human milk samples analyzed. These findings revealed that Zn in the LMM fraction of human milk is bound to citrate. Concentration of Zn eluted under Zn-citrate peaks was summed and is presented in Table 2. It may be seen that Zn-citrate

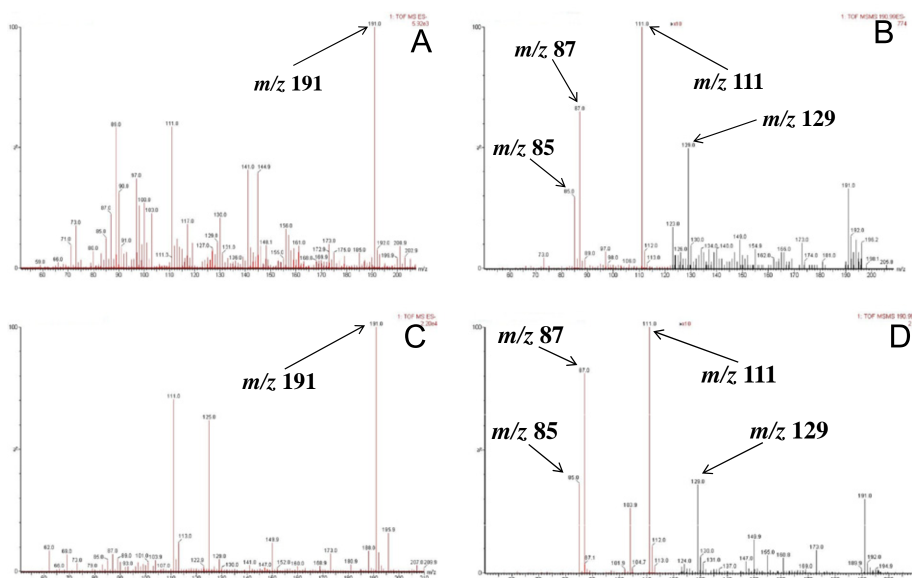

Fig. 5. MS spectra and MS-MS spectra of the chromatographic peaks after separation of LMM milk fraction (sample A1) on CIM DEAE-1 column. (A) MS spectrum and (B) MS-MS spectrum of daughter ions (m/z 191) of the first chromatographic peak. (C) MS spectrum and (D) MS-MS spectrum of daughter ions (m/z 191) of the second chromatographic peak.

represents the majority of the LMM milk fraction. However, more human milk samples should be analyzed for statistical evaluation. To the best of our knowledge this is the first report on reliable and quantitative determination of Zn-citrate in human milk. The information on the amount and composition of the LMM Zn species in human milk is, with respect to Zn bioavailability, very important and may be valuable for producers of infant formulas. Similarly, Pabón and Lönnerdal [37], who observed an enhancing effect on Zn bioavailability when citrate was added to fat or pellet from infant formula, assumed that supplementation of infant formulas with citrate may be advantageous. Our study provides complementary information to findings of Pabón and Lönnerdal [37].

4. Conclusions

Analytical method was developed for quantitative determination of biologically important molecule Zn-citrate. Study was performed with synthetic solutions of Zn-citrate complexes within the pH range 5–7. Zn species were prepared in 0.2 mol L^{-1} HEPES, MOPS and MES buffers, which do not interact with Zn ions. Speciation analysis was performed on a weak anion exchange CIM DEAE-1 monolithic column with FAAS or ICP-MS detection. Aqueous—1.2 mol L^{-1} NH_4NO_3 linear gradient elution allowed fast separation of $[Zn(Cit)]^-$ and $[Zn(Cit)_2]^{4-}$ complexes. For the identification of the Zn binding ligands ESI-MS-MS analysis of separated chromatographic peaks was performed. The analytical procedure developed enabled quantitative, reliable and reproducible determinations of Zn-citrate complexes and was applied in speciation of Zn in four human milk samples of two mothers in different stage of lactation. Results revealed that Zn content in milk declined with time of lactation and that in samples investigated the percentage of LMM-Zn fraction remained approximately the same (about 23% of total Zn in milk). The prevailing LMM-Zn species was found to be Zn-citrate. This information may be valuable for producers of infant formulas and is complementary to findings of other researchers who demonstrated the enhancing effect on Zn bioavailability when citrate was added to fat or pellet from infant formula. The developed speciation method provides a reliable analytical tool for further investigation of the percentage and the amount of Zn-citrate in human milk during the course of lactation. It also represents a potential for determination of Zn-citrate in different biological and environmental samples.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.09.002.

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