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Novel method for metalloproteins determination in human breast milk by size exclusion chromatography coupled to inductively coupled plasma mass spectrometry.

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Highlights

- Novel methodology of metalloproteins determination by SEC-ICP-MS in human whey milk protein fractions.
- Two metalloproteins have detectable concentrations of Mn, Co, Cu and Se.
- The levels of Mn, Co, Cu and Se were within the range reported in the literature.
- Samples with long lactation periods still have normal levels of these metals.

Abstract

Levels of essential metals in human breast milk (HBM) have been determined by different analytical techniques, but there is few woks about human whey milk fractions. However, the current trend lies in metalloproteomic and identification of different metalloproteins. In this sense, native separative techniques (N-PAGE and SEC) coupled to ICP-MS provide us with valuable information. Besides it is necessary the development of new methodologies in order to determine with accuracy and precision the profile of such metals and metalloproteins in the different whey protein fractions of HBM. Thus, the aim of this work was to develop a new method for metals and metalloproteins determination by SEC-ICP-MS in whey protein fractions of HBM. Human whey fractions were obtained of HBM samples by ultracentrifugation. Then, protein fractions of whey milk were separated by SEC coupled to ICP-MS for metalloproteins and Mn, Co, Cu and Se quantification. Besides, protein profile of whey milk was determined by N-PAGE and computer assisted image analysis. SEC-ICP-MS results indicated that first and second protein fractions showed detectable levels of the Mn, Co, Cu, and Se. Protein profile determined by N-PAGE and image analysis showed that molecular weight of protein fractions ranged between 68.878-1,228.277 kDa. In this work, metalloproteins were analyzed by SEC coupled to ICP-MS, with adequate sensitivity and accuracy. Our study has shown the presence of Mn, Co, Cu and Se bound to two protein fractions in whey milk of HBM. Metals levels analyzed were within the ranges reported in the literature.

Key words: Metalloproteins; Human Breast Milk; Whey Protein Fractions; Size exclusion chromatography; Inductively coupled plasma mass spectrometry.

1. Introduction

Several studies have shown that breastfeeding offers many benefits for babies, children and mothers [1]. World Health Organization documents recommend the initiation of breastfeeding within the first hour of life of the newborn, its maintenance as the only way of feeding up to six months and, as far as possible; keep breastfeeding supplemented with other safe food to 2 years of life [2]. Thus, human breast milk (HBM) gives babies the right mix of nutrients because contains water, vitamins, carbohydrates, lipids, proteins, antibodies and minerals. The proteins of HBM have different origins: whey proteins are synthesized in the mammary gland, and other proteins such as albumin, various enzymes and protein hormones are transferred to milk from plasma [3]. The HBM proteins can be grouped in: mucins, caseins, and human whey milk proteins [4]. The components of human whey include alpha-lactalbumin, serum albumin, lactoferrin, immunoglobulins, lactoperoxidase enzymes, glycomacropeptides, lactose, and minerals [5]. It has been well recognized that metals bound to proteins play a critical role in biological processes; and several HBM proteins require a metal ion to perform their catalytic activities, stabilize the structure and/or properly perform its functions.

The presence and levels of metal concentrations in HBM have been determined by different techniques [6-10]. Recent studies have determined calcium, iron and zinc levels bound to the IgAs [8]. Other studies have determined the presence of various metals in HBM, without indicating whether they are free or bound to a particular protein fraction [11]. It is necessary to extend this study to other protein fractions of HBM, to elucidate the metals and metalloproteins levels and to find novel roles of them in neonatal nutrition. In this sense it is necessary the development of new methodologies in order to determine with accuracy and precision the profile of such metals and metalloproteins in the different whey protein fractions of HBM. Metalloproteomics allows the study of the proteins-metals and

its structural characteristics. This field of study combines proteomic techniques such as native polyacrylamide gel electrophoresis (N-PAGE) or size exclusion chromatography (SEC) with instrumental techniques for metal analysis, such as inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectrometry (ICP-OES) [8]. Association of these techniques allow the knowledge of biological roles of metals and metalloproteins. In order to avoid loss of non-covalently bound metals and protein structure, nondenaturing techniques such as native-polyacrylamide gel electrophoresis (N-PAGE) and size exclusion chromatography (SEC) has been employed [12].

Thus, the aim of this work was to develop a new method for metals and metalloproteins determination by SEC-ICP-MS in whey protein fractions of HBM. Protein profile of samples was determined by N-PAGE and computer assisted image analysis. This method was applied for the analysis of essentials metals (Mn, Co, Cu, and Se) associated to whey protein fractions of HBM samples.

2. Materials and Methods

2.1. Instrumentation

For the electrophoresis separations, a Cooled Wide Dual Mini-Vertical cell, DCX-800C, and a power supply EPS-300 X (CBS-Scientific, Company Inc.) were employed.

SEC was performed coupling the chromatograph (Series 200; Perkin-Elmer. Thornhill, Canada) to an ICP-MS (ELAN DRC-e; Perkin-Elmer SCIEX, Thornhill, Canada). Argon gas with a minimum purity of 99.996% was supplied by Air Liquid (Córdoba, Argentina). Protein markers (IgM hexamer, IgM pentamer, apoferritin band 1, apoferritin band 2, B-phycoerythrin, lactate dehydrogenase, and bovine serum albumin;

which have a molecular mass of 1236, 1048, 720, 480, 242, 146, and 66 kDa, respectively) were used as standards proteins. SEC analysis was applied to determine Mn, Co, Cu, and Se distribution within whey milk fractions. Sulfur, as sulfur oxide (SO), was determined simultaneously in order to investigate the presence of peptides and proteins [13, 14]. The employed SEC column (TSKgel® G3000SW HPLC Column, phase diol, L × I.D. 30 cm × 7.5 mm, 10 μm particle size) separates proteins with molecular weights up to 500 kDa. Extraction procedure was performed with a Tris–HCl solution (pH = 6,8; 50mM). Optimal instrumental conditions for the quantification of the analytes studied have been summarized in table 1.

On the other hand, the total proteins quantification was performed employed a Beckman spectrophotometer DU 520 for the biuret method using the reagents supplied for GT Lab (Rosario, Argentina).

2.2. Reagents

Water distilled and de-ionized, with a resistivity of 18.2 MΩ cm^{-1}, produced by an Easy pure RF system from Barnstead (Dubuque, IA, USA) was employed in further assays. Tris(hydroxymethyl)aminomethane (TRIS) (99.85%, Across Organics, New Jersey, USA), ammonium persulfate, N,N,N′,N′-tetramethylethylenediamine (TEMED), native sample buffer: 62.5×10^{-3} mol L^{-1} TRIS-HCl, pH 6.8; 40% glycerol; 0.01% bromophenol blue, bis-acrylamide 2%, and acrylamide (Bio-Rad, USA), were used. The electrophoretic buffer solution (0.025 mol L^{-1} tris(hydroxymethyl)aminomethane, 0.192 mol L^{-1} glycine, pH 8.3).

For total protein determination, Biuret reagent ($CuSO_4$ 12 mmol L^{-1}, sodium-potassium tartrate 32 mmol L^{-1}, potassium iodide 6 mmol L^{-1}, and sodium hydroxide 250 mmol L^{-1}) was supplied from GT Lab (Rosario, Argentina). Bovine serum albumin (BSA,

powder, lyophilized, defatted and deionized, purity grade >99%) was provided by Fedesa-UNSL.

2.3. Samples

After obtaining a written informed consents, fresh HBM samples (n = 4) were collected in metal-free tubes from adult lactating women (12 month postpartum) in 2016 in San Luis, Argentina. The volunteer subjects were also asked to complete a questionnaire, and subjects that had acute or chronic diseases were excluded. The whole procedure was made anonymous and the study was approved by the Local Ethics Committee. All participants were informed about the purposes and scope of the study, and signed appropriate consent forms. Data assessment was complete for all participants.

In order to skim the raw milk samples, each sample was centrifuged at 4000*g*, at 4ºC, during 20 min and the solidified fat was removed using a glass spatula. Whey proteins were obtained from HBM samples by isoelectric precipitation at pH 4.2 with hydrochloric acid (4 mol L^{-1}). The caseins were removed by centrifugation and the resulting supernatant contains whey proteins. The samples were stored at 4ºC until analysis.

2.4. Native-PAGE

Whey samples of HBM previously treated were diluted (1/4) with native sample buffer. The electrophoresis was performed using separating gels with a 7.5% (w/v) of acrylamide/bis-acrylamide, and a pH value of 8.8; the stacking gels were operated with 4.5% (w/v) of acrylamide/bis-acrylamide in a pH value of 6.8. Also, the aliquots of 10 μL of milk samples were loaded per well; a solution of 0.025 mol L^{-1} tris(hydroxymethyl) aminomethane (TRIS) and 0.192 mol L^{-1} glycine (pH 8.3) was employed as buffer running

system. The electrophoretic separation was carried out at constant voltage of 45V per gel (10 ×15 cm) during 3.5 h at laboratory temperature (21ºC). After the electrophoretic running, the slab gel was stained with a solution of 0.1% (w/v) Coomassie brilliant blue G250, 5% (w/v) acetic acid and 25% (v/v) methanol. Then, two distaining steps of 1 h and overnight, with 500 mL of 40% (v/v) methanol and 10% (v/v) acetic acid as distaining solution were performed. The gels distaining steps were performed under continuous agitation. Finally, the distained gels were scanned at 600 dpi resolution in TIFF format and then were analyzed by Image J (version 1.45; NIH) software program.

2.5. Quantitative assessment of PAGE profiles and molecular weight estimation

The gel images were processed through Adobe Photoshop CS3 (version 10.0) to obtain the best contrast for densitometric analysis through software. Best photo scans were taken and their relative optical density (ROD) and percentage amount of protein fraction in HBM (%PF) was done using Image J (version 1.45) software program. The results were expressed as mean ± SD for all data sets (Table 2).

The molecular mass of the protein fractions in HBM was determined through comparison of its electrophoretic relative mobility (Rf) with those corresponding to proteins contained in the molecular weight marker (Native Mark Unstained Protein Standard, Life Technologies, Table 2).

2.6. Protein Identification by Mass Spectrometry Analysis

Protein digestion and Mass Spectrometry analysis were performed at the Proteomics Core Facility CEQUIBIEM, at the University of Buenos Aires/CONICET (National

Research Council) as follows: Coomassie-stained N-PAGE gel excised protein bands were sequentially washed and destained with 50 mM ammonium bicarbonate, 25 mM ammonium bicarbonate 50% acetonitrile, and 100% acetonitrile; reduced and alkylated with 10 mM DTT and 20 mM iodoacetamide and in-gel digested with 100 ng Trypsin (Promega V5111) in 25 mM ammonium bicarbonate overnight at 37°C.

Peptides were recovered by elution with 50% acetonitrile-0.5% trifluoroacetic acid, including brief sonication, and further concentrated by speed-vacuum drying. Samples were resuspended in 15 uL of water containing 0.1% Formic Acid. After that, the peptides were purified and desalted with ZipTip C18 columns (Millipore).

The digests were analysed by nanoLC-MS/MS in a Thermo Scientific QExactive Mass Spectrometer coupled to a nanoHPLC EASY-nLC 1000 (Thermo Scientific). For the LC-MS/MS analysis, approximately 1 µg of peptides was loaded onto the column and eluted for 120 minutes using a reverse phase column (C18, 2 µm, 100A, 50 µm x 150 mm) Easy-Spray Column PepMap RSLC (P/N ES801)) suitable for separating protein complexes with a high degree of resolution. The flow rate used for the nano column was 300 nL min^{-1} and the solvent range from 7% B (5 min) to 35% (120 min). Solvent A was 0.1% formic acid in water whereas B was 0.1% formic acid in acetonitrile. The injection volume was 2µL. The MS equipment has a high collision dissociation cell (HCD) for fragmentation and an Orbitrap analyzer (Q-Exactive-Thermo Scientific Germany). A voltage of 3,5 kV was used for Electro Spray Ionization (Thermo Scientific, EASY-SPRAY).

2.6.1. MALDI-TOF/TOF MS, and Data Analysis

Samples were mixed 1:1 with a matrix a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 0.1% TFA 30:70 water: acetonitrile and spotted on a stainless steel MALDI target plate.

Samples were analyzed using an Ultraflex II Bruker Daltonics UV-MALDI-TOF-TOF mass spectrometer, equipped with a Nd:YAG laser (λ_{em} 355 nm). Spectra were obtained in positive reflectron mode, within a mass range of 800-4000 m/z. The positive-ion mass spectra were calibrated externally using the Bruker Protein Calibration Standard I.

The generated spectra were visualized and compared with FlexAnalysis 3.3 software. The generated peak list was based on signal-to-noise filtering and an exclusion list. The resulting file was then searched by Mascot (Matrix Science, Boston, MA) with database search parameters including a mass tolerance of 20-100 ppm, one missed cleavage, oxidation of methionines, and carbamidomethylation of cysteines. Only matched proteins with significant scores ($P < 0.05$) were considered. The obtained hits were validated by MS/MS fragmentation of one or two high S/N peaks per sample.

3. Results and Discussion

3.1. Protein profiles, MALDI TOF/TOF MS and Data Analysis

The protein concentration mean in the HBM samples analyzed was 7.83 ± 0.05 g L^{-1}. These values were found in the lower extreme of total protein concentrations range (7.60-20.40 g L^{-1}) reported in the literature [15, 16].

Parameters optimized during the separation procedure were: percentage of separating gel (5.25-20% w/v) of acrylamide/bis-acrylamide, applied voltage (45-55 V gel), run time (200-480 min) and dilution of samples (1/2, 1/4, and 1/6 in sample buffer). Optimal conditions were: percentage of separating gels with a 7.5% of acrylamide/bis-acrylamide, constant voltage 45V per gel, running time 210 min (3.5 h), sample dilution 1/4.

Also, whey protein fractions profile obtained by N-PAGE showed 8 fractions. The first and sixth fractions constituted the highest protein percentage and ROD, while the third fraction had the lowest values for both parameters (Table 2). Molecular weight estimation showed that whey proteins of HBM ranged between 68.878-1,228.277 kDa (Table 2).

MALDI TOF/TOF MS and data analyzes indicated that protein fraction 1 is constituted by α-lactalbumin and lactoferrin. This fact is consistent with the gradual decrease in α-lactalbumin concentrations relative to lactoferrin [17], since concentrations of 2.6 mg/mL of α-lactalbumin can be found in one-year lactation samples, while lactoferrin concentrations same samples were 1.44 mg/mL. The main constituent identified in protein fraction 2 was serum albumin. This is in agreement with previous studies that reported concentrations of 0.44 mg/mL of this protein in samples of one year of lactation [17].

3.2. SEC-ICP-MS Analysis

Several authors have reported metal concentrations in milk samples. Reported levels of Mn, Co, Cu and Se range from 0.07-10.70 mg L^{-1} [6, 9, 18, 20], 0.19-74 μg L^{-1} [6, 10, 19], 2.9-2195 μg L^{-1} [6, 7, 9, 19, 20], and 10.5-32.1 μg L^{-1} (7, 9, 10) respectively. Some studies have indicate the binding of manganese with various protein fractions of HBM. Chan et al. [21] have demonstrated the presence of two proteins in HBM that bind manganese, with a molecular weight of 407 KDa and 128 KDa, respectively. Subsequently,

Lönnerdal et al. [22] showed that the major part (71%) of manganese was found in whey in human milk.

Ours results indicated that first and second protein fractions showed detectable levels of the analyzed metals (Table 3, Figure 1). First protein fraction could be serum albumin according to molecular weight estimation. This fraction had the highest metals concentrations, except for Mn mainly bound to fraction 2, and the greatest diversity of metals. Finally, the levels of the metals analyzed were within the range of values reported in the literature [6, 7, 9, 10, 18-20].

3.3. Analytical performance

The limits of detection (LOD) and quantification (LOQ) were calculated in accordance to the formulas given by the official compendia methods [23], using the relation k(SD)/m where k = 3.3 for LOD and 10 for LOQ. SD is the standard deviation from 15 responses of blank replicates and m is the slope of the calibration curve (Table 4).

4. Conclusions

Levels of essential metals in HBM have been determined by different analytical techniques, but there is few woks about human whey milk fractions. In this work, metalloproteins (α-lactalbumin, lactoferrin, and serum albumin) and bound metals have been analyzed and quantified by the proposed methodology in whey protein fractions of HBM with adequate sensitivity and accuracy. Our study has shown the presence of different metals bound to whey protein fractions in HBM, which opens the door to future research that could determine metalloproteins role in nutrition of infants in a long breastfeeding stage (after 12 months). Determined levels of Mn, Co, Cu and Se were

accordance with the WHO guidelines regarding the quality and quantity of nutrients that breast milk provides to infants.

Future research in this area is needed that may include metalloprotein characterization and association with potential biochemical functions.

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Figure Caption

Figure 1: Chromatogram of SEC-ICP-MS corresponding to whey protein fractions of HBM.

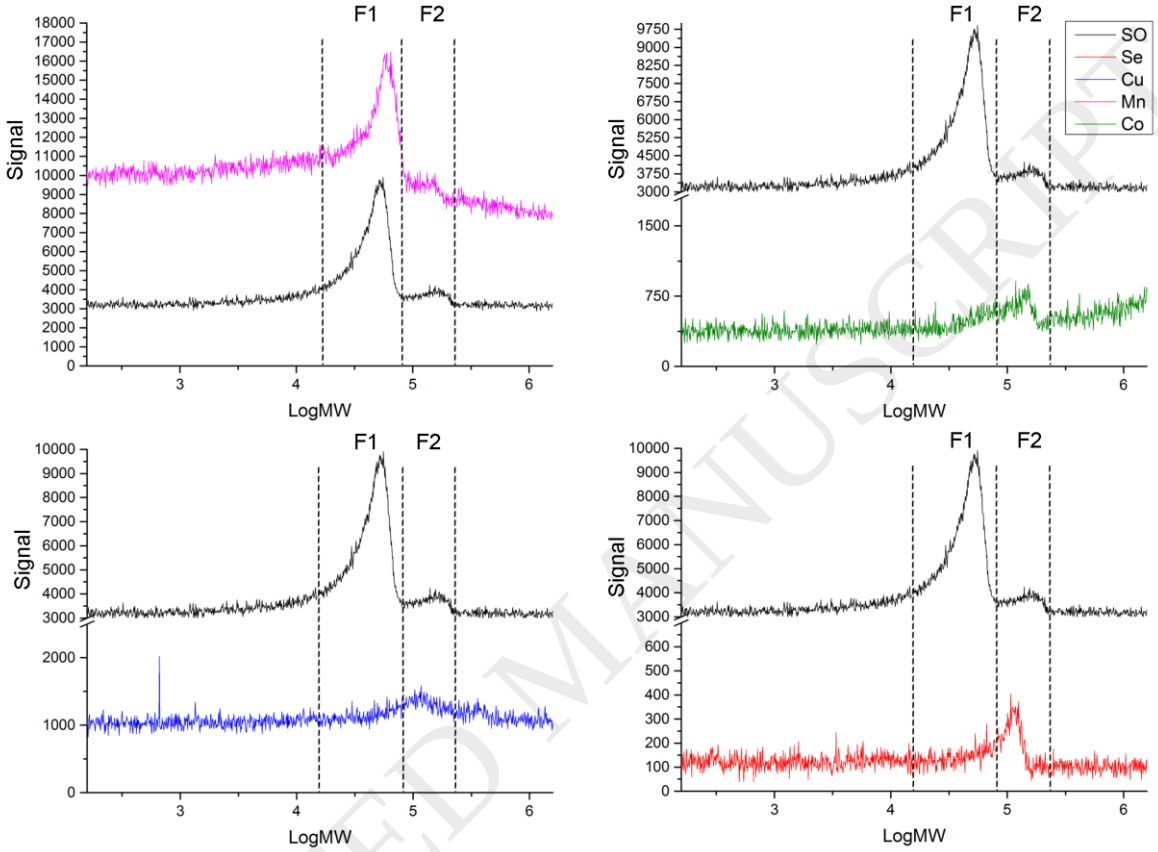

Table 1. Chromatographic conditions for SEC-ICP-MS or Mn, Co, Cu and Se bound to HBM proteins.

Size Exclusion Chromatography	**Chromatograph Series 200 (Perkin-Elmer)**
Mobile phase	TRIS-HCl buffer, 50mM, pH = 6.8
Elution mode	Isocratic
Flow rate	1 mL min^{-1}
Column	TSK gel G3000SW (300 mm × 7.5 mm× 10 µm)
Sample loop	100 µL
Sample dilution	1/10
ICP-MS	**Elan DRC-e (Perkin-Elmer)**
RF power (W)	1050
Gas flow rates (mL min^{-1})	
Plasma	13
Auxiliary	1.35
Nebulizer	0.75
Resolution	Normal
Isotope monitored	^{55}Mn, ^{59}Co, ^{63}Cu, ^{82}Se, $^{32}S^{16}O$.
Scanning mode	Peak hopping
Dwell time	500 ms

Table 2: Results of quantitative assessment of PAGE profiles and estimated molecular weight of whey protein fractions of HBM.

Protein Fraction	Protein Percentage	Relative Optical Density	Rf	Molecular weight (kDa)
1	17.82 ± 2.56	1.00	1.00	68.878
2	10.36 ± 1.16	0.57	0.49	240.776
3	7.07 ± 0.51	0.39	0.42	355.320
4	8.56 ± 0.74	0.47	0.37	415.853
5	8.29 ± 0.92	0.46	0.19	674.186
6	17.99 ± 1.93	0.99	0.17	721.207
7	16.55 ± 3.29	0.93	0.04	1,136.841
8	13.38 ± 1.59	0.74	0.01	1,228.277

Protein percentage values have been expressed as mean ± SEM.

Table 3: Results of SEC-ICP-MS analysis of whey protein fractions of HBM (μg L^{-1}).

Analyte	*Mass*	*Protein Fractions*	
		Fraction 1	**Fraction 2**
Mn	55	592.90 ± 53.12	253.85 ± 7.62
Co	59	<LOD	21.93 ± 0.16
Cu	63	<LOD	121.90 ± 8.26
Se	79	<LOD	163.63 ± 0.03

The results have been expressed as mean ± SEM.

Table 4. Analytical performance: figures of merit.

Analyte	LOD (μg L^{-1})	LOQ (μg L^{-1})	Range of linearity (μg L^{-1})	R^2
Mn	2.81	8.51	8.51-100	0.996
Co	3.39	10.27	10.27-100	0.997
Cu	1.46	4.43	4.43-100	0.991
Se	5.86	17.75	17.75-100	0.993