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Nano-HPLC-HRMS Analysis to Evaluate Leptin Level in Milk Samples: A Pilot Study

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Abstract: Leptin is a 16 kDa lipophilic protein hormone secreted by adipocytes and its most significant function is to inform the brain with negative feedback that regulates food intake. Recently the protein found in human breast milk was related to breast feeding and onset of obesity, and the evidence of a low probability to develop pediatric obesity in children fed with breast milk was also confirmed. Since leptin could have a critical role, its quantitation both in human breast, bovine milk and in infant formula products is interesting. For this reason, we developed an analytical method based on immunoaffinity purification followed by an analysis with nano-High Pressure Liquid Chromatography coupled with High Resolution Mass Spectrometry analyzer (nano-HPLC-HRMS) to identify and quantify leptin in milk samples and performed a pilot study using samples of human breast milk, bovine milk and infant formulas. With an obtained lower limit of quantitation (LLOQ) of 100 ng mL^{-1} we quantified leptin in human breast milk finding an average of 6.70 ng mL^{-1}. Our results show that leptin was under LLOQ both in bovine milk and in infant formula products. In conclusion, the developed analytical method here described was suitable to quantify leptin in milk samples with a good sensitivity and selectivity, and without the use of radioactive reagents.

Keywords: leptin; human breast milk; infant formulas; immunoaffinity; nano-High Pressure Liquid Chromatography; High Resolution Mass Spectrometry

1. Introduction

Leptin is a lipophilic protein of 167 amino acids with a molecular weight of about 16 kDa secreted by adipocytes. It is a protein hormone discovered in 1994 during a study related to obesity in mice [1]. The gene encoding for the protein is expressed on the surface of adipocytes cell membrane; leptin serum concentration levels are related to adipose tissue and food intake. The protein is involved in different organism activities; the most significant is to inform the brain with a negative feedback that regulates food intake. Moreover, the hormone modulates angiogenesis, fertility, and bone formation [2]. Recently, leptin was also related to human breast feeding and obesity since infants that are breastfed showed lower probability to develop pediatric obesity compared with newborn nots fed with human breast milk [3–9]. In light of this consideration, leptin could be considered to be a biomarker of health.

Human milk is characterized by a complex composition which includes nutrients, hormones, and several bioactive components that could influence infant growth, development, and well-being later in life [10]. Leptin is one of the proteins present in mammalian breast milk. These play a fundamental role in infant growing and development and their intake through breast feeding is

important. In particular, human milk contains many other elements, such as lipids, carbohydrates, vitamins, minerals, growing factors, and immunity/stem cells. All of these compounds make human milk a unique vital resource for the newborn. Moreover, human milk changes its composition during lactation: colostrum, produced from the first to the sixth day after birth, has a high concentration of antibodies and nutrients (proteins); transition milk, formed from about six days until 10 to 14 d after birth, shows elevated amount of fat and lactose (concentration of proteins is reduced); and finally the mature milk, composed by high amount of fat and lactose too and low proteins content [11].

A recent systematic review of literature tried to provide evidence on the effect of leptin, ghrelin, Insulin Growth Factor 1, adiponectin and insulin on infants' and children's growth and body composition. The available data reveals conflicting findings concerning the potential role of all these hormones on modeling growth and fat mass apposition and health outcomes later in life [12].

However, in animal models, leptin, when ingested as a component of breast milk, is increasingly recognized as playing a role in the postnatal programming of a healthy phenotype in adulthood age [13,14]. We recently reported that breast milk leptin and maternal BMI could influence infant serum leptin values, and this could be of interest to explain the beneficial effect of breast feeding on preventing obesity in children [15].

Leptin concentration in human whole milk shows different concentration among lactation period: from 0.63 ng·mL^{-1} to 32.7 ng·mL^{-1}, 2–42 and 60–120 d after birth respectively [16,17].

It was reported that breastfed infants have a higher leptin values that formula fed ones [18,19].

However, since breast feeding is not always possible or practical, in this case the newborn is fed with infant formulas. These are defined by Commission Directive 2006/141/EC of 22 December 2006 as "the only processed foodstuff which wholly satisfies the nutritional requirements of infants during the first months of life until the introduction of appropriate complementary feeding [...]. The essential composition of infant formulas must satisfy the nutritional requirements of infants in good health as established by generally accepted scientific data" [20]. The European Commission Directive 2006/141/EC lists minimum and maximum concentrations of the compounds contained in infant formulas, such as proteins, lipids, vitamins, mineral salts, and so on.

Most of the infant formula products are produced with dairy products; the most important is certainly bovine milk, which contains bovine leptin, appropriately processed. Infant formula products are classified in two categories: powdered and liquid formulas. These latter are offered as three formulations: ready to use, concentrated liquid, and powder. The processing techniques of raw materials (i.e., bovine milk) used to obtain infant formulas are largely described in the literature [11,21]. Briefly, for liquid infant formula, five steps are required to process raw materials and gain a final product perfectly proper for infant fed, growing and health. These steps are recombination and standardization, emulsification, second standardization, sterilization, and finally aseptic storage and filling. This process leads to a complete isolation and adaption of raw materials ingredients (proteins, carbohydrates, and lipids as the main classes) taking as reference the human milk. Since leptin is secreted by adipocytes and expressed mainly on their surface, when bovine milk is defatted to obtain infant formula preparation, it can be assumed leptin is removed with the lipids (adipocytes) because of its lipophilicity [22].

The aim of the study was the development of an analytical method suitable for the trace analysis and determination of lipophilic protein leptin in different milk samples. At the present time, because of great attention to the environment, it is significant to reduce the use of organic solvents and of radioactive assays. The developed method was based on the use of the technique nano-High Pressure Liquid Chromatography coupled through a nano-ESI source to High Resolution Mass Spectrometry (nano-HPLC-HRMS) which consumed for the chromatographic separation a reduced amount of organic solvents since the flow of the nano system was 300 nL/min.

High Resolution Mass Spectrometry is demonstrated to be a powerful tool to analyze protein contents in milk [23–26]. Proteomics research and studies in milk (and in general in food) are essential to control safety, quality, and transformation of the food. The proteome profile of milk is characterized by

a huge number of caseins (80%) and as other protein components as lactoglobulins, lactalbumins, serum albumin, and immunoglobulins (20%) for a total concentration of 35 g/L of proteins [27]. Furthermore, others minor compounds are present in milk, such as free amino acids, urea, creatinine, mineral salts, and hormones, such as leptin during lactation period. Among these massive quantity of compound, as previously reported, leptin has low concentration in whole milk [16,17]. Therefore, an enrichment of this molecule in milk samples is mandatory. Besides many protocols, we selected an immunoaffinity process [28,29] to purify milk samples and isolate leptin with the use of specific antibodies.

To our knowledge, this is the first study that provides a quantification of leptin in milk samples, both maternal, bovine, and liquid infant formulas, using an analytical method based on immunoaffinity purification and nano-HPLC-HRMS analysis.

2. Materials and Methods

Human leptin analytical standard (assay ≥ 97%), iodoacetamide, DL-dithiothreitol, trypsin, monoclonal anti-LEP antibody produced in mouse (species reactivity: human), water for ultratrace analysis and acetonitrile hypergrade for LC-MS were purchased from Merck (Merck, Milan, Italy).

Polyclonal anti-LEP (anti-leptin) antibody produced in rabbit (species reactivity: Bos taurus, bovine (cattle)) was acquired from S.I.A.L. (Rome, Italy).

Glycolink™ micro immobilization kit was purchased from Thermo Scientific (Thermo Scientific, Milan, Italy).

Human breast milk from the first three months of life and liquid infant formula samples were kindly offered by the Department of Public Health and Pediatrics, University of Turin, Turin, TO, Italy. Bovine whole milk used to build calibration curves was purchased in a local supermarket. Bovine milk sampled in a farm in the Northern Italy from a cow that gave birth to a calf 6 d old (called bovine colostrum) was used to identify bovine leptin, since no analytical standard was available.

2.1. nanoHPLC Set Up and Parameters

An Ultimate 3000 nano-HPLC (Thermo Scientific, Milan, MI, Italy) equipped with an NCS-3500-RS nanopro flow pump and a WPS-3000 TPL RS autosampler was used.

The chromatographic separation was achieved with a PepMap™ RSLC C18 column (2 µm, 100 Å, 75 µm × 50 cm; Thermo Scientific, Milan, Italy) preceded by a nano-pre-concentration column (C18 PepMap 100 Å, 5 µm, 300 µm × 5 mm; Thermo Scientific, Milano, Italy). Eluents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile/water 8/2 (B) for the separation column, and trifluoroacetic acid 0.05% in water/acetonitrile 98/2 (P) for the pre-concentration one. The run gradient started from 5% of B, isocratic for 5 min (to pre-concentrate), increased to 99% of B in 95 min and recondition. Flow was set to 300 nL min^{-1}, injection volume was 1 µL.

The pre-concentration step was attained with 100% of P at flow rate of 5 µL $min^{-1.}$

2.2. HRMS Set Up and Parameters

An Orbitrap Fusion (Thermo Scientific, Milan, Italy) with a nano-ESI source was used. Source parameters were set as follows: spray positive voltage, 2400 V; ion transfer tube temperature, 325 °C. Full scan spectra were acquired in the range of *m/z* 200–2000 with a resolution of 50,000. Data dependent analysis (DDA) spectra were acquired in the range between the ion trap cut-off and precursor ion *m/z* values with 60,000 of resolution. CID (collision induced dissociation) energy was maintained at 30%. Once selected the unique peptides, one as qualifier and one other as quantifier ions, after tryptic digestion of leptin (see above, section "Data processing") dedicated CID MS/MS spectra of ions were performed. The mass accuracy of recorded ions (vs. calculated) was ±2 milli mass units (without internal calibration).

2.3. Sample Preparation and Purification

Samples of human breast milk, of bovine milk, of bovine colostrum and of infant formulas were prepared and purified using the immunoaffinity process. Samples were processed using two different antibodies: monoclonal anti-LEP antibody produced in mouse with specific reactivity for human, and polyclonal anti-LEP antibody produced in rabbit specific for bovine. Therefore, the antibodies bound human and bovine leptin, respectively.

To link the antibodies anti-LEP at the affinity columns of immobilization link we followed the procedure described in the manuals. We used both the antibodies anti-LEP at concentration of 4 µg/mL, suitable for the amount of leptin in breast milk [16,17].

When the affinity columns were ready and the antibody was linked, 300 µL of milk sample(s) were loaded and the incubation lasted over night at 4 °C under mechanical rotation. The columns were then washed and leptin was eluted in triplicate with 200 µL of glycine 0.2 M in hydrochloric acid, pH 2.5–3. 10 µL of Tris buffer 1 M in hydrochloric acid, pH 8.5–9, were added to neutralize the solution.

Each sample was loaded, for each antibody, in triplicate using three different affinity columns, for a total of 900 µL of sample and 1.8 mL of elution solution, which were evaporated using a CentriVap Cold Trap with CentriVap Concentrator (Labconco Co., Kansas City, Missouri, MO, United State of America). Samples were reconstituted in 50 µL 0.1% formic acid in water, vortexed and enzymatically digested with trypsin.

To ensure an appropriate pH range for tryptic activity (pH 7–9), the reconstituted samples were diluted in ammonium bicarbonate 50 mM. Then DL-dithiothreitol, iodoacetamide, and trypsin (1/20 ratio) were added to the solution which was digested for 18 h at 37 °C. Samples were placed into low binding vial and analyzed with nano-HPLC-HRMS.

To quantify leptin in human breast milk and infant formula samples, a calibration curve of the protein in bovine milk was prepared. Fresh whole cow milk was fortified with increasing amount of human leptin analytical standard (0-100-250-500-1000-2500-3000 µg mL^{-1}). Calibration standards were processed as previously described.

2.4. Data Processing

Using Mascot software (Matrix Science, London, UK; version 2.3.02), all CID MS/MS spectra of breast milk and calibration samples were analyzed for database searching. Mascot was used to search the SwissProt human database (20,417 entries) by assuming the digestion enzyme trypsin. Carbamidomethylation of cysteine was specified as a fixed modification and parent and fragment ion mass tolerance was 0.05 Da. Using BLAST (Basic Local Alignment Search Tool, registered as trademark of the National Library of Medicine), we selected two typical peptides belonged to human leptin (UniProt KB code P41159, Homo sapiens (Human)). The amino acids sequences of peptides are proposed in Table 1. Ions with *m/z* 708.3599, z = 2, and *m/z* 764.4099, z = 2, were selected as quantifier and qualifier respectively.

Table 1. Amino acids sequence, *m/z* ratio and retention time of selected peptides from human leptin (UniProt KB code P41159, Homo sapiens (Human)).

Amino Acids Sequence	m/z (z = 2)	Retention Time (min)
INDISHTQSVSSK	708.3599	15.24
NVIQISNDLENLR	764.4099	35.30

Amino acids sequence of human and bovine leptin is quite similar: only 26 amino acids change among the two species. As Table 2 shows, the quantifier peptide m/z 708.4, z = 2, with the sequence INDISHTQSVSSK, is in common between the species. The qualifier ion, m/z 764.4, z = 2, with the sequence NVIQISNDLENLR changed between the species: in bovine leptin there is a valine instead an isoleucine.

Table 2. Comparison between sequence of human (UniProt KB code P41159, Homo sapiens (Human)) and bovine (UniProt KB code P50595, Bos taurus (Bovine)) leptin. Bold letters denote the different amino acids between human and bovine leptin sequence. Italic and underline letters indicate the selected peptides to qualify and quantify human leptin. The first peptide is in common between species.

Human Leptin (Homo Sapiens)
MHWGTLCGFLWLWPYLFYVQAVPIQKVQDDTKTLIKTIVTR*INDISHTQSVSSK*QKVTGLDFIP GLHPILTLSKMDQTLAVYQQILTSMPSR*NVIQISNDLENLR*DLLHVLAFSKSCHLPWASGLETLD SLGGVLEASGYSTEVVALSRLQGSLQDMLWQLDLSPGC
Bovine leptin (Bos taurus)
M**R**C**G**PL**YR**FLWLWPYL**S**YV**E**AVPI**R**KVQDDTKTLIKTIVTRINDISHTQSVSSKQ**R**VTGLDFIPG LHP**LL****S**LSKMDQTLA**I**YQQILTS**L**PSRNV**V**QISNDLENLRDLLHLLA**A**SKSC**P**LP**QVRA**LE**S**L**ES LG**V**VLEAS**L**YSTEVVALSRLQGSLQDML**R**QLDLSPGC

The same process was used to identify bovine leptin in bovine breast milk and infant formula samples. We used Mascot software (Matrix Science, London, UK; version 2.3.02) to analyze raw CID MS/MS spectra of cow milk. Mascot was used to search the SwissProt Bos taurus database (37,513 entries) by assuming the digestion enzyme trypsin. Carbamidomethylation of cysteine was specified as a fixed modification and parent and fragment ion mass tolerance was 0.05.

3. Results

To quantify leptin in milk samples (human, bovine, and infant food) we used the selected peptide with *m/z* ratio of 708.3599, z = 2. The calibration curves in bovine milk were in the ranges between 0 and 3000 ng mL^{-1}. No leptin was quantified in bovine whole milk without fortification used for the calibration curves. Curves were repeated three times and the coefficients of correlation R^2 were very close to the unit (Figure 1). The obtained lower limit of quantitation (LLOQ) was 100 ng mL^{-1}, and the LOD (limit of detection) was 27 ng mL^{-1}.

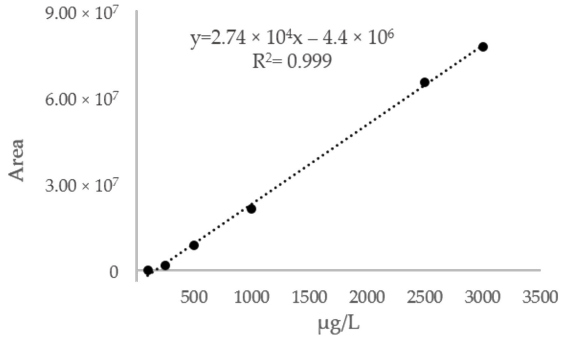

Figure 1. Calibration curve in breast milk sample processed with immunoaffinity purification. LLOQ and LOD were 100 and 27 ng mL^{-1} respectively.

With the developed calibration curve leptin was detected and quantified in milk samples. Each sample was repeated three times with the use of one immunoaffinity column for each. Human breast milk samples showed an average concentration of leptin of 6.70 ng mL^{-1}. Bovine milk, bovine colostrum and infant formula products showed an amount of protein under lower limit of quantitation. However, bovine leptin was identify using Mascot software in bovine colostrum, as expected. In Table 3, the results of milk samples are listed.

Table 3. Concentration value of leptin in human breast milk, cow milk and liquid infant formula samples. The amount is expressed in ng·mL^{-1}. Nd, not detectable.

Milk Sample	Leptin Concentration (ng mL^{-1})
Human breast milk sample 1	7.1 ± 1.06
Human breast milk sample 2	6.4 ± 1.28
Human breast milk sample 3	7.5 ± 1.12
Human breast milk sample 4	5.8 ± 1.16
Cow milk sample 1	Nd
Cow milk sample 2	Nd
Cow milk sample 3	Nd
Cow milk sample 4	Nd
Liquid infant formula sample 1	Nd
Liquid infant formula sample 2	Nd
Liquid infant formula sample 3	Nd
Liquid infant formula sample 4	Nd

4. Discussion

Before the quantitation of leptin in milk samples, a deep investigation about MS/MS fragmentation of selected peptides was conducted. With the orbitrap fusion high resolution mass spectrometry analyzer we performed the precursor ion fragmentation both with CID, HCD (high collision induced dissociation) and ETD (electron transfer dissociation). The precursor ions *m/z* ratio were 708.4 (quantifier) and 764.4 (qualifier). With CID we obtained the best sensitivity. Figure 2 shows the liquid chromatographic separation and tandem mass spectra of the protonated peptides. Product ions are listed in Table 4; as expected, the y and b ions were the main species formed by CID fragmentation.

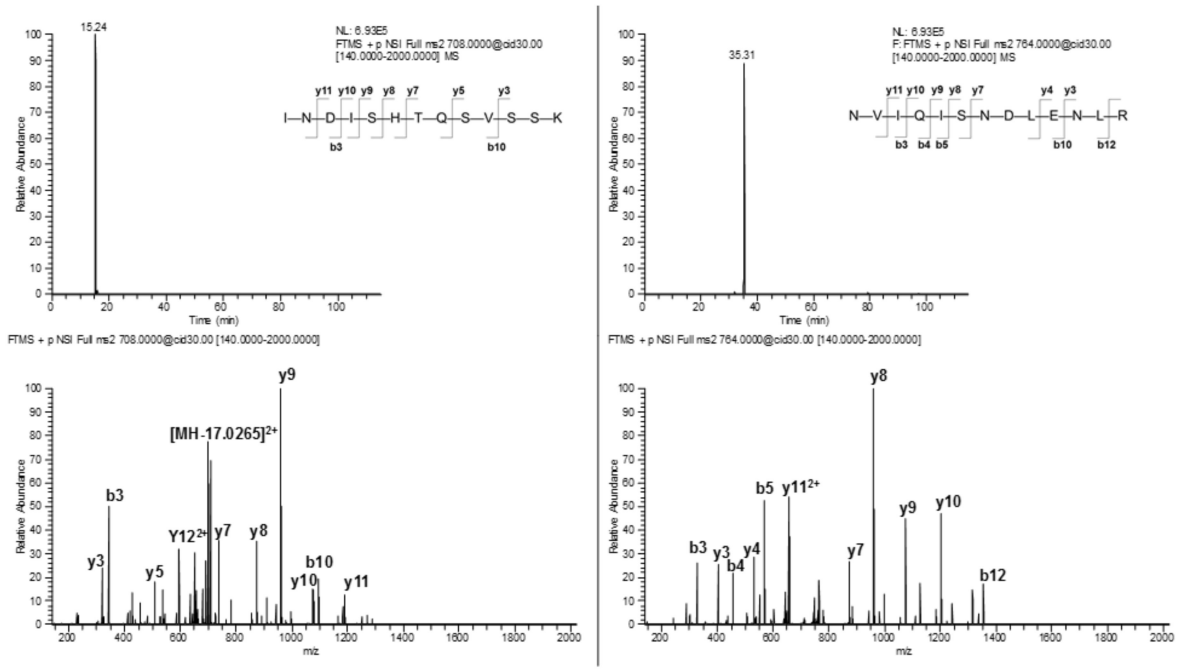

Figure 2. Chromatographic separation of human leptin analytical standard at concentration of 250 ng mL^{-1} digested peptides and MS/MS fragmentation products of quantifier (*m/z*= 708.3599, z = 2) and qualifier (*m/z* = 764.4099, z = 2) ions.

Table 4. Precursor protonated ions *m/z* of selected peptides and monoisotopic mass of the product CID ions. As expected, y and b ions were the only formed with CID fragmentation.

Precursor Ion m/z $[MH]^{2+}$	Product Ion m/z	ppm Error	Ions Type
708.3599	960.4745	+0.425	$y9^{+}$
	699.8466	+0.385	$[MH\text{-}NH_3]^{2+}$
	343.1612	+0.265	$b3^{+}$
	736.3836	+0.475	$y7^{+}$
	873.4452	+0.397	$y8^{+}$
	651.8179	+0.463	$y12^{2+}$
	321.1769	+0.245	$y3^{+}$
	1095.5429	+0.423	$b10^{+}$
	507.2773	+0.368	$y5^{+}$
	1073.5586	+0.478	$y10^{+}$
	1188.5855	+0.386	$y11^{+}$
764.4099	960.4745	+0.398	$y8^{+}$
	568.3453	+0.465	$b5^{+}$
	657.8542	+0.381	$y11^{2+}$
	1201.6171	+0.451	$y10^{+}$
	1073.5586	+0.421	$y9^{+}$
	873.4425	+0.369	$y7^{+}$
	531.2885	+0.436	$y4^{+}$
	327.2027	+0.298	$b3^{+}$
	402.2459	+0.236	$y3^{+}$
	455.2613	+0.312	$b4^{+}$
	1353.7009	+0.481	$b12^{+}$

Once obtained the mass spectrum information, bovine milk samples were used for calibration curve. As previously reported, we gained a LLOQ and LOD of 100 and 27 mg mL^{-1} respectively.

The LLOQ was quite higher if compared with other analytical methodologies developed for leptin quantitation. These methods were based on chemiluminescent or enzyme immunoassay (CLEIA and EIA), radioimmunoassay (RIA), immunofluorimetric assay (IFMA), enzyme-linked immunosorbent assay (ELISA) and HPLC-MS and reported very low limit of quantitation [30–40].

CLEIA and EIA leptin analysis attained by the research groups of He [30] and Sekiguchi [31] detected the protein in serum. He and co-workers with their developed chemiluminescent immunosensor gained a LOD of 1.9 pg mL^{-1} [30]. Using the anti-leptin polyclonal antibody with a chemiluminescent enzyme immunoassay Sekiguchi team measured leptin in human serum with a LOD of 0.1 pg mL^{-1} [31].

RIA was used to quantify leptin concentration in serum and breast milk [15,32–34]. Savino research group measured with RIA leptin concentrations in 36 serum of healthy terms infant exclusively breast feeding and in 24 samples of breast milk obtaining a median concentration of 3.42 ng mL^{-1} in infant serum and 0.51 ng mL^{-1} in breast milk [32]. In a similar experiment performed in 2016 with RIA detection of leptin, the group confirmed the obtained results testing 58 samples of infant and mother serum, and mother milk [15]. Quite comparable results were found by Schuster et al. [33] with RIA detection of leptin in 23 healthy, lactating women and their newborn infants.

RIA and ELISA assays were also used by Shin and co-workers to quantify leptin in mice serum exposed to high fat diet [34]. Leptin was quantified both with RIA and ELISA with a sensitivity of 25 and 46.7 pg mL^{-1} respectively.

All these studies were conducted with a commercially available radioimmunoassay with a declared sensitivity of 0.04 ng mL^{-1} [15,32] or 0.25 pg mL^{-1} [15].

However, RIA presents some important weaknesses, such as the presence of radioisotope and rather high cost of reagents and assay [35].

Immunofluorimetric assay with species-specific antibody was used by Schmidt group [36] to quantify leptin in pig saliva with limit of detection of 1.047 ng mL^{-1}.

Although by using ELISA, Yiman et al. [37] obtained a good sensitivity of 0.05 ng mL^{-1}, and other studies used this technique to detect leptin [38,39], it is well known that ELISA normally suffers from cross-reactivity [40].

Wang et al. [41] performed the quantitation of human leptin in human serum using a HPLC-tandem mass instrument and declared 15.63 ng mL^{-1} as LOQ; the authors did not mention any LOD value. However, serum is a simpler matrix compared with milk, whose content is not only a significant amount of proteins, but above all a massive quantity of lipids.

Nevertheless, leptin is primarily secreted from adipose tissue and here is present in cytoplasm in the interface between nuclei and rim surrounding lipid droplets [42,43]. Consequently, it was not possible to precipitate the lipids with, for example, heptane solution, before the immunoaffinity extraction to avoid the precipitation of leptin too.

Methods based on immunoaffinity approach retaining highly selectivity due to the use of the selected antibody; but the immobilization columns, since the loaded antibody cannot cover quantitatively the total surface of the resin, may bond to other molecules, such as lipids or sugars. We thought that this was the reason of our quite higher LLOQ vs. Wang limit (100 vs. 15.63 ng mL^{-1} respectively [41]).

However, the LLOQ (100 ng mL^{-1}) obtained with this method based on immunoaffinity sample purification and nano-HPLC-HRMS was suitable to quantify the protein hormone leptin in milk samples.

Quality control (QC) samples, consisting of bovine whole milk without leptin fortification, were analyzed and we did not detect endogenous bovine leptin. Using the immunoaffinity columns bound with polyclonal antibody anti-LEP (from rabbit, specific for Bos taurus species), the Mascot software did not recognize any bovine leptin in whole bovine milk. On the contrary, when bovine colostrum that did not undergo any treatment was analyzed, the software identified bovine leptin with a coverage percentage of 85%. Moreover, as mentioned above in "Material and methods", whole bovine milk samples were purchased in a local supermarket; they were commercially available products. After milking, bovine milk is subjected to industrial processes comprising pasteurization, homogenization, centrifugation and eventually skimming. During the centrifugation step fat globules are separated from aqueous milk component leaving three categories of milk: whole (fat content 3.5–4%), semi-skimmed (fat content between 1.5 and 1.8%), skimmed (fat content less than 0.5%). During lactation leptin amount in bovine mature milk was 6.14 μg L^{-1}, but the concentration diminished over time [44–46] and in addition the milk is processed with a final step of centrifugation (lipid separation). Therefore, leptin, because of its lipophilicity, was not detectable.

To quantify leptin in samples of breast human milk, bovine milk, and infant food we used the peptides with *m/z* ratio of 708.3599, z = 2. As declared in "Results" section, we quantified human leptin only in human breast milk samples. The results were in accordance with those found by Resto et al. [47] who quantified a concentration of 5.18 ± 4.96 ng mL^{-1}, by Uçar and co-workers [48] who detected with RIA 3.36 ± 1.0 ng mL^{-1} in whole milk samples and by Kugananthan research group [49] which compared whole and skimmed human milk. Figure 3 shows the detected peptides of leptin in human breast milk sample 1.

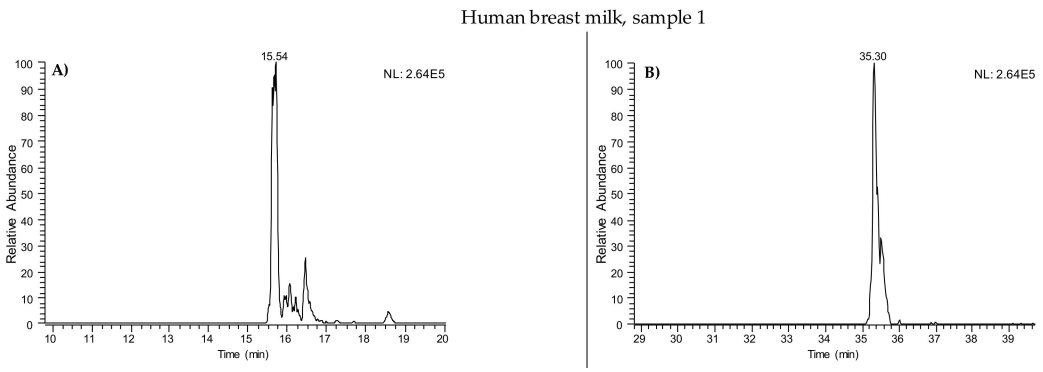

Figure 3. Chromatographic separation of quantifier ion *m/z* = 708.4, z = 2 (panel **A**) and qualifier ion *m/z* = 764.4, z = 2 (panel **B**) oh human leptin detected in human breast milk sample 1.

In liquid infant formula products samples, no leptin was detected (under LLOQ). Once more as for QC samples, we processed the samples also with the immunoaffinity columns bound with polyclonal antibody anti-LEP from rabbit with specificity for bovine. Then we run the raw files with Mascot software and none of bovine leptin digested peptides were found in samples. This finding confirmed the alleged loss of leptin during the defatting process of bovine milk used as principal raw material.

5. Conclusions

The developed analytical method based on immunoaffinity purification and analysis with nano-HPLC-HRMS instrumentation of milk samples shows a good sensitivity and selectivity suitable to identify and quantify leptin in human breast and bovine milk and in infant formula products. For this purpose, we selected two typical peptides, one as qualifier and one other as quantifier, derived from tryptic digestion of the human protein. Peptides were characterized and a deep study of their tandem mass fragmentation pathway was led.

Commercially available bovine milk samples were used as quality control samples and, after leptin fortification, as a matrix for calibration curve development.

After the immunoaffinity process of purification, obtained using specific and selective antibodies anti-leptin, samples were analyzed with nano-HPLC-HRMS developed method. In human breast milk samples leptin were detected and quantified (average of 6.70 ng mL^{-1}); bovine leptin was identified by Mascot software in bovine colostrum. No human, nor bovine leptin (searching for the specific peptides formed by tryptic digestion) was quantified both in bovine milk and in infant formula products (under obtained LLOQ for human leptin). This finding confirmed the high probability to loss leptin during milk processing, because of its lipophilicity.

Last but not least, the developed immunoaffinity/nano-HPLC-HRMS method used a reduced amount of organic solvents (approximately 10 µL for each analysis) and avoid the use of radioactive assays.

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