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Lactoferrin in breast milk-based powders

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Research Article

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Abstract

This study aimed to determine lactoferrin (LF) in breast milk-based powders and formulas. Lactoferrin is an important whey protein in all mammalian milks and is responsible in large part for the known antimicrobial effects of human milk in particular. As breast feeding is not always possible, formulas based on cows milk have been developed in order to meet the nutritional needs of the newborn, while more recently human breast milk-based powders have been introduced to offer the biological functionality of human milk to pre-term and critically ill babies. In the present work, the amount of LF in commercial breast milk-based powders was tested by a validated RF-HPLC method for the determination of LF in breast milk in order to examine both the applicability of the method but at a second level the amount of LF in these commercial products. The detection of LF was possible but the complexity of the matrix lead us to the use the standard addition methodology in order to achieve quantification. The results indicated that breast milk-based powders had higher amount of LF than cows milk-based formulas, both non-fortified and fortified.

Milk is a complex emulsion or colloid that acts a source of nutrients, but also bioactive components which facilitate postnatal adaptation of the newborn by stimulating cellular growth and digestive maturation, establishing symbiotic microflora and development of gut-associated lymphoid tissues (Petrotos *et al*., 2014). Several short- and long-term health benefits of breast-feeding have been demonstrated by clinical and epidemiological studies (Zhang *et al*., 2022) as breast milk is an excellent source of these bioactive compnents as well as immunological and antimicrobial factors (Chirico *et al*., 2008; Aly *et al*., 2013). The main macronutrient components of human milk are protein (principally caseins), carbohydrate (principally lactose) and fat, which are covering the essential nutritional needs of the infants (Johnston *et al*., 2015). Minor proteins such as secretory IgA, lysozyme, and lactoferrin (LF) have antimicrobial properties (Aly *et al*., 2013), while LF, a-lactalbumin, milk fat globule membrane proteins and osteopontin are considered bioactive with functions on protection from infection and the acquisition of nutrients (Lönnerdal, 2016).

LF, a major whey protein in all mammalian milk, may be the bioactive substance in human milk that is most responsible for its well-known antimicrobial effects (Manzoni, 2016). LF is a non-heme iron binding glycoprotein of the transferrin family (Satué-Gracia *et al*., 2000; Li *et al*., 2012; Huang *et al*., 2018; Tsakali *et al*., 2019; Motoki *et al*., 2020) and is present not only in milk but also in other biological secretions (Ellingson, *et al*., 2019; Zhu, *et al*., 2023). It has a molecular weight of 77–80 DA (Aly *et al*., 2013; Tsakali *et al*., 2014) and it has been extensively studied for its multiple bio-functional properties which potentially include antioxidant, anti-inflammatory, antibacterial, antiviral and antitumor activities as well as activity as a growth factor (Johnston *et al*., 2015; Zhang *et al*., 2021).

Due to the ideal properties of breast milk, exclusive breast feeding for at least the first six months of life is recommended (WHO, 2012). However, there are many factors including insufficient milk production, illnesses, medication or even social reasons such as return to work, that do not allow exclusive breast feeding. Formulas have come to cover this need but as most of them are based on cows milk, whilst they might be similar to breast milk in terms of calories (albeit with more derived from lactose than from fat), vitamins and minerals, they are essentially different regarding their lower protein content (Bezkorovainy, 1977). There are significant differences both in the ratio of casein to whey protein as well as the nature of the individual proteins (Davidsson *et al*., 1994). For example, LF is found at the level of 0.440–4.400 mg/ml in mature human milk but only in concentrations of approximately 0.030–0.485 mg/ml in cow's milk (Johnston *et al*., 2015; Tsakali *et al*., 2019). Formula manufacturers are trying to mimic the biological functionality of human milk by fortification with individual proteins and especially LF. Although this is allowed in some countries including the

European Union, the maximum addition level is set to 1000 mg/l (for both EU and China: European Commission, 2012; Zhang *et al.*, 2017). The approach of using donor milk has attracted interest but there are several practical problems, especially in terms of standardization, storage conditions, shelf life and convenience in daily use. The latest introduction of breast milk-based powders and formulas is moving towards covering these problems as they can have standardized composition, convenience in storage and in use, without losing the essential nutritional and bioactive ingredients of breast milk. Currently, such products are targeted at babies with special needs (pre-term, critically ill) and the availability of donated breast milk is unlikely to satisfy a mass market for such powders.

Even though a series of methods have been developed for the determination of bovine (and some other) LF there is still no standard/official method apaert from '*Standard Method Performance Requirements (SMPRs®) for Determination of Bovine Lactoferrin in Infant and Adult/Pediatric Nutritional Formula*' (AOAC, 2020). The methods developed for the determination of LF in dairy products include spectroscopic, chromatographic and immunoassay techniques (Huang *et al.*, 2018; Tsakali *et al.*, 2019) but the infant formula matrix can make it difficult to achieve good specificity and accuracy (Ellingson, *et al.*, 2019).

In the present work, breast milk powders and breast milk-based fortified formulas were tested for their LF content, using a validated RF-HPLC method for the determination of LF in breast milk. We wished to examine both the applicability of the method and, at a second level, the amount of LF in these commercial products.

Material and methods

Samples

Breast milk-based commercial products were tested. The first (BMP) was a standardized and pasteurized 100% human milk powder (70P, NeoKare UK), while the second (BMF) was a human milk-derived fortifier made of human milk, calcium glycerophosphate and calcium gluconate (MMF, NeoKare UK). Different batches of them were tested, 5 of BMP and 3 of BMF. Samples were stored at room temperature, in their initial packaging (sealed bags) until the day of analysis. The reconstitution of the powders was performed according to manufacturer's instructions (ready to feed) for BMP (1.55 g with 10 ml water) while for BMF 1 g with 25 ml of water (instead of human milk that was suggested by the manufacturer). Fat was removed by centrifugation at 3000 g for 15 min while separation of serum was performed *via* precipitation using HCl 1 M to pH 4.6 and centrifugation at 3000 g for further 15 min and filtration with 25-mm filters and 0.45-µm Cellulose Acetate Blue Luer Lock filters (Restek, Bellefonte, PA). Lactoferrin from human milk (>95% purity; Sigma-Aldrich, Milwaukee, WI) standards were used. All samples were tested three times and each time in triplicate.

RP-HPLC analysis

The RP-HPLC method tested was initially applied exactly as described by Tsakali *et al.* (2019) on a VWR Hitachi module with a diode-array detector L-2455 Elite La Chrom (VWR International, Radnor, PA). The column was a Zorbax SB 300-C8, 4.6 × 150 mm, 5-µm particle size (Agilent, Santa Clara, CA). After the initial powder testing the method was modified as follows: Mobile phase A consisted of acetonitrile, water, and trifluoroacetic acid in a ratio of 50 : 950 : 1 (vol/vol/vol) and mobile phase B consisted again of acetonitrile, water, and trifluoroacetic acid in a ratio of 950 : 50 : 1 (vol/vol/vol). Linear gradient within a run time of 38 min and combination of flow rates from 1 to 1.5 ml/min were used: 0 to 10 min isocratic to 33% B, 10 to 20 min 33 to 38% B, 20 to 29 min to 38% B, 29 to 38 min to 39–33% B. The column temperature was set to 50°C, whereas the injection volume was 20 µl. The detection was by absorbance at 205 nm. The method was optimized using different concentrations of commercial LF from human milk (Sigma-Aldrich) (25, 50, 100, 200 and 400 µg/ml) corrected by the given purity. All standards were examined twice, on different days and in duplicates each time.

Results and discussion

Elution was monitored at 200–400 nm and detection of LF was achieved at both 205 and 278 nm. At 205 nm, the detection of LF has the advantage that it improves the sensitivity of response, as the LF peak was better baseline resolved. When only LF standards were tested, the elution time of LF was 9.2 min (Fig. 1a).

Optimization of the HPLC method was carried out using standard solutions of LF from human milk. The calibration curve was extrapolated in the concentration range of 25–400 µg/ml (R^2 = 0.995). The sensitivity of the method is the slope of the calibration curve of LF. Repeatability was estimated by calculating the relative standard deviation (RSD). A standard LF sample of 200 µg/ml was measured 6 times on three different days. Intraday repeatability was found to be 1.80% RSD, whereas interday repeatability was found to be 5.93% RSD. The limit of detection for 3 S/N (signal: noise ratio) was found to be 35.40 µg/ml while the limit of quantification for 10 S/N was found equal to 51.90 µg/ml. When the aqueous solutions of each powder samples were tested under the same conditions the elution time of LF was altered to approximately 9.0 min. As can be seen in the presented chromatographs the LF peak resolution is different for the aqueous powder solutions samples and LF interferes with other peaks (Figs. 1b and 1c).

Due to its high isoelectric point (pI > 8), LF is largely positively charged at acidic and neutral pH, thus it interacts with anionic molecular and cellular components (Pochet *et al.*, 2018). Also, it has the tendency to form complexes with negatively charged proteins such as soluble β-lactoglobulin, serum albumin and immunoglobulin (Lampreave *et al.*, 1990), soluble β-casein and also with milk fat globule membrane lipopolysaccharides (Fong *et al.*, 2007). Based on this and on the fact that there were deviations between the chromatographs of different batches, the standard addition methodology was applied in order to secure that there is no matrix interference. The aqueous solutions of each powder were spiked (4 : 1 v/v) with LF standard in different concentrations (100, 200 and 400 µg/ml for BMP powders and 25, 50, 100, 200 and 400 µg/ml for BMF powders). The ratio comparison showed that the LF peak increased linearly with increasing LF concentration, indicating the lack of another peak inclusion. Quantification was then performed and the amount of LF in the aqueous (ready to feed) solutions of BMP and BMF powders are given in Table 1.

The average amount of LF in the aqueous BMP was found to be 0.301 ± 0.02 mg/ml of ready to feed product which equates to 1.94 ± 0.13 mg/g LF in powder. This LF content is much higher than in non-fortified cow's milk-based formulas where LF is scare (Johnston *et al.*, 2015). It is also much higher than the

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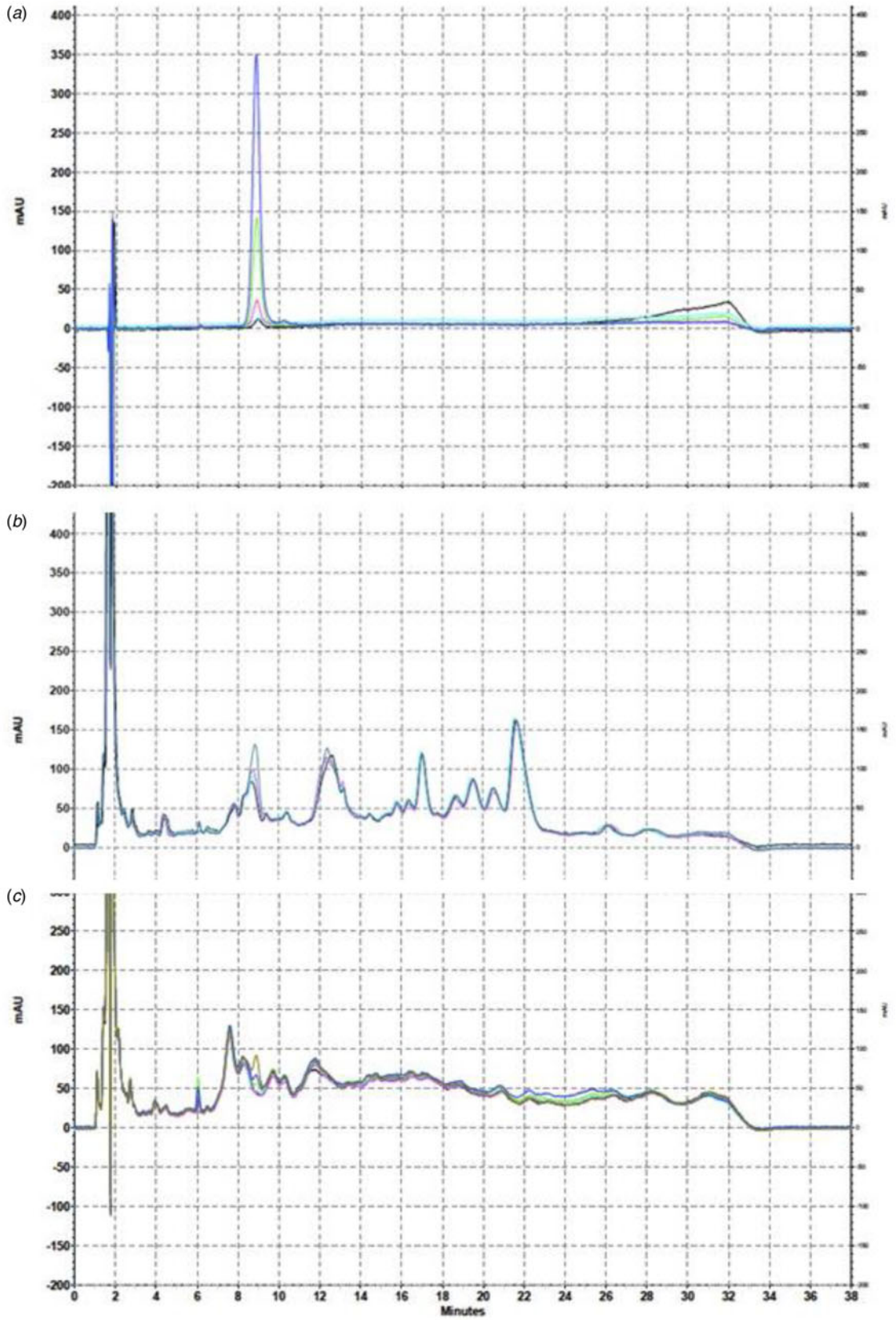

Fig. 1. Chromatographs of LF from human milk standards (a) and of spiked BMP (b) and spiked BMF (c). (a) LF at 25 (black), 50 (turqouise), 100 (magenta), 200 (lime) and 400 (blue) μg/ml. In (b), spikes are all at 4 : 1 v/v and comprise BMP with H_2O (black) and BMP with LF at 100 (turquoise), 200 (magenta) and 400 (lime) μg/ml. In (c), spikes are all at 4 : 1 v/v and comprise BMF with H_2O (black) and BMF with LF at 25 (turquoise), 50 (magenta), 100 (lime), 200 (blue) and 400 (green) μg/ml. LF is lactoferrin, BMP is a commercial breast-milk derived powder (70P, NeoKare UK) and BMF is a second commercial breast-milk derived powder (MMF, NeoKare UK).

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Table 1. Concentration of LF in aqueous powder solutions of BMP and BMF samples

Sample ID	LF (mg/ml)	Average value ± stdev
BMP s1	0.300	0.301 ± 0.019
BMP s2	0.280	
BMP s3	0.320	
BMP s4	0.284	
BMP s5	0.320	
BMF s1	0.166	0.174 ± 0.69
BMF s1	0.247	
BMF s1	0.109	

limit set by Chinese government for LF in fortified infant formula (0.30–1 mg/g) and almost three times higher than the average level of LF usually set as quality parameter (0.50 mg/g) in other countries such as U.S.A., Japan and Korea (Li *et al.*, 2012; Jie and Clever, 2023). As might be expected, it is lower than the average amount in actual breast milk (average 2 mg/ml) since pasteurization at 62.5°C for 30 s (Holder methods) can cause a decrease in LF up to 75% (Manzoni, 2016).

The BMF powders showed different sensory characteristics to BMP and differences in the chromatographs were also observed, most probably due to the use of water instead of breast milk during the reconstitution of the powders. However, the chosen alteration in the reconstitution of the powders assists in studying the LF content in the powders without the complication of the LF of the breast milk. Still, the detected amount of LF of 0.174 ± 0.07 mg/ml in aqueous samples ready for breast milk fortification can be useful since they would add almost 20% to the already existing LF of the breast milk.

In conclusion, an already validated RP-HPLC for the determination of LF in breast milk was tested for its applicability for the determination of LF in breast milk-based powder and fortifier. The method had to be slightly altered and with the application of standard addition method, gave satisfying results for the determination of LF in breast milk-based powder products. The amount of LF found in standardized and pasteurized 100% human milk powder was found to be lower than in breast milk but much higher than in both non-fortified and fortified cows milk-based powders. The human milk-derived fortifier could add up to 20% additional LF when reconstituted with breast milk.

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