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# Advanced analytical tools for bovine lactoferrin identification and quantification in raw skim milk to finished lactoferrin powders

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ABSTRACT

As the use of bovine lactoferrin (bLF) has increased, greater regulatory and commercial interest is being placed on bLF identification and quantitation and quality. Several methods can be used to quantitate bLF, but a robust accurate quantification method applicable as a standardised tool for feed milk, process monitoring, and evaluation of end products has been less of a concern. bLF concentrations increase through the bovine lactation cycle, so accurate and timely information is required that can increase both processing plant performance and product quality and output. In this article we evaluate analytical cation exchange, reverse phase high-performance chromatography and size exclusion chromatography methods for the analysis of bLF identification and quantification from whole milk, process intermediates, ingredients, milk and infant formula products. However, the methods described in this article to identify and quantify the bLF may not be directly assimilated to the biological activities of the bLF.



## 1. Introduction

Lactoferrin (LF) is a multifunctional globular glycoprotein which plays a pivotal role in iron metabolism and innate host defense mechanisms of mammalian organisms (Wheeler et al., 2012; Wheeler, Hodgkinson, Prosser, & Davis, 2007). LF has a bi-lobed structure, folded form of a single polypeptide chain, 703 amino acid residues (78 kDa) and an isoelectric point (pI) of 8.7 (Tomita et al., 2009). Globular lobes are represented by N- and C-terminal lobes. Both lobes exhibit high-affinity binding of iron ($Fe^{3+}$), one atom for each lobe coordinated with a bicarbonate anion (Naidu, 2000; Rastogi et al., 2016). Iron binds by coordination chemistry and electrostatic interactions between the negatively charged binding pocket (González-Chávez, Arévalo-Gallegos, & Rascón-Cruz, 2009; Legrand et al., 2008; Nagasako, Saito, Tamura, Shimamura, & Tomita, 1993). Iron is released from LF when the molecule undergoes protonation of aspartic acid and histidine residues at low pH (Baker & Baker, 2004, 2009).

LF is an important component of the immune system and has a range of biological functions including immunomodulation, antimicrobial, serine protease, anti-inflammatory, iron homeostasis, and reduction of osteoporosis (Cornish et al., 2004; Plaut & Geme, 2013; Tomita et al., 2009; Tomita, Takase, Bellamy, & Shimamura, 1994; Van de Looij et al., 2014). As a bacteriostatic agent, LF has been proven to inhibit the growth of bacteria (Teraguchi et al., 1995; Weinberg, 1978) through sequestering essential iron from pathogens (hypoferraemia) through its iron chelating activity (Legrand et al., 2008). The bactericidal activity has also been observed through its ability to disrupt bacterial and fungal membranes (del Olmo, Morales, & Nuñez, 2009). Protection of host cells has been observed by preventing the internalisation of replicating viruses (Caccavo et al., 2002; Legrand et al., 2008). LF is the second most abundant protein in human milk. Lactoferrin concentration in human milk is higher in the early stages of development and plays an important role in developing an infant's immune system. In comparison to human milk, cows' milk has a lower concentration (10–20 fold) of lactoferrin (Abd El Gawad, El-Sayed, Mb, & El-Salam Am, 1996; Billakanti, 2009). These functional properties have led bovine LF (bLF) to become a functional ingredient used by infant formula manufacturers to supplement their formula (James, 2015).

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At present this is predominated by Asian countries that feed lactoferrin fortified infant formula to their babies, with China being the biggest market for LF fortified (premium) infant formula powders. More recently, market conditions for bLF have improved for other nations including the USA with a number of companies including Morinaga Milk Ltd (Kruger, 2012; Kruger & Kobayashi, 2006), and Synlait Milk Ltd, New Zealand, gaining GRAS (Generally Recognized As Safe) notice from the US FDA to use their bLF in infant and toddler milk formula exported to the USA (Drummond, Stein, & Halcrow, 2016). Considering the potential benefits of bLF, many more new products have been developed for different applications (James, 2015). Consequently, there has been an increased global demand for the commercial production of bLF.

A literature review of bLF suggested that there is significant amount of information available for the separation, purification and isolation of LF and lactoperoxidase (LPO) from skim milk, acid whey and sweet whey (Alvarez-Guerra & Irabien, 2012; Andersson & Mattiasson, 2006; Banavara, Alvey, Peters, & Gonzalez, 2017; Billakanti, 2009; Brock, 2012; Fee & Chand, 2006; Levay & Viljoen, 1995; Lu, Xu, Wang, & Yang, 2007; Wolman, Maglio, Grasselli, & Cascone, 2007; Wu & Xu, 2009). Among all the methods, cation exchange resins SP Sepharose Big Beads or SP Sepharose FF or CM Sepharose FF are the three most common resins used (Fee & Chand, 2005, 2006; Lu et al., 2007; Wu & Xu, 2009). The literature focuses on the process optimisation/development for the separation and purification of LF and LPO or fractionation of positively charged proteins (milk basic protein, MBP). However, only limited information is published for the quantification and fundamental characterisation of LF and LPO using analytical cation exchange chromatography. A few papers used Source 30S (Harris et al., 2010) or Mono S (Majka et al., 2013) or similar analytical cation exchange columns (Andrews, Taylor, & Owen, 1985) for secondary purification or characterisation of native LF/LPO. In most cases, the samples used for the analysis are either sweet whey or acid whey and semi-purified/purified LF/LPO. Other than a few recent papers (Yuan et al., 2017; Zhang et al., 2017), none of the published methods discussed a fully optimised/developed analytical cation exchange method for quantifying lactoferrin in skim milk or similar raw materials (milk powders or infant formula powders).

To support the manufacturers of bLF containing products, reliable analytical techniques are required for both the identification and quantitation of LF from raw material to finished products. The importance of accuracy, speed, reliability, robustness, and accessibility of analytical methods is paramount as they need to be able to provide high-quality information quickly that can facilitate manufacturing decisions. At present, a number of platforms exist (ELISA, SPR and chromatography); however, a standardised robust analytical method does not exist. There is limited information available to develop robust, reliable methods for the identification and quantification of bovine lactoferrin. For example, there is no agreed standard available in the dairy industry for marketing bovine LF outside of the CFDA regulations. This gap in the lack of suitable analytical techniques puts pressure on manufacturers and product developers to argue the case for their in-house analytical techniques. This can result in the incorrect grading of products and even loss of business/profit.

Global bLF manufacture has steadily grown there is increased competition for producers around the globe to have rapid and robust analytical techniques for quantification and characterisation of bLF. The importance of accuracy, speed, reliability, robustness and accessibility of analytical methods is paramount especially in applications such as processing of bLF as titer can fluctuate in feed milk and increases over the season. There are several platforms with a wide range of non-validated protocols available in regulatory standards. However, these methods suffer from several limitations. In most cases, they do not allow for the analysis of feed materials and crude intermediates which provides critical information of the desired product quality and yield. This paper focuses on optimising and validating analytical cation exchange chromatography methods (Supplementary material, Table S1) that is novel and useful for an international dairy community. To demonstrate the applicability of this work, the authors have provided bLF analysis of a range of raw materials and products and compared this with selected reversed-phase HPLC methods.

## 2. Materials and methods

### 2.1. Instrumentation and columns

All reversed phase high performance liquid chromatography (RP-HPLC) experiments were carried out on Agilent UPLC (1290 series instrument, Agilent Technologies, USA) equipped with a diode-array detector (DAD) and ChemStation software. An Aeris™ 3.6 μm WIDEPORE XB-C8 200 Å analytical LC column (Part - OOG-4481-EO, 250 × 4.6 mm), a C8 Security Guard Ultra Holder (Part - AJO-9000) and Security Guard Ultra Cartridges (Part - AJO-8771) were purchased from Phenomenex, New Zealand. A Prosphere C4 analytical column (Part - 5126473, 150 × 4.6 mm, 5 μm), All-Guard Cartridge Holder (part – MAISH-80101) and Guard Cartridges Prosphere (part – 5126484) were purchased from Dr Maisch, Germany. All other chromatography experiments were carried out on AKTA pure 150 M LC systems (GE Healthcare Life Sciences, Australia). Tricorn 5/50 (part – 28-4064-09) and Tricon 5/20 (Part – 28-4064-08) empty high-performance columns, Mono S 5/50 GL pre-packed column (Part – 17516801), Superdex 200 Increase10/300 GL column (Part – 28990944) were purchased from GE Healthcare Life Sciences, Australia. Electrophoresis Power Supply, EPS-600 was purchased from GE Healthcare Technologies, Australia. Uvitec Essential (D55 Model) computer based Gel Documentation System was purchased from Total Lab Systems, New Zealand.

### 2.2. Materials and reagents

The reference bovine lactoferrin (WAKO bovine lactoferrin (96.7% purity), (SKU: 129-04121-5G)) was purchased from Novachem, Australia. Trifluoracetic acid (T6508-100 mL) with purity of ≥99% was purchased from Sigma Aldrich, New Zealand, Acetonitrile, Optima® LC-MS grade, was purchased from Fisher Chemicals, New Zealand. Source-15S, 200 mL (Part – 17094405) resin was purchased from GE Healthcare Life Sciences, Australia. Milli-Q® Type-1 water (MQ) was produced in-house using Milli-Q® Direct Water Purification System (John Morris, New Zealand). NuPAGE™ 4–12% bis-Tris precast gels (Cat. No. NP0321BOX), NuPAGE™ MES SDS Running Buffer (20×) (Cat. No. NP0002), See Blue™ Pre-stained Protein Standard (Cat. No. LC5925) were purchased from ThermoFisher Scientific, New Zealand. All other buffer salts and chemical reagents used in this work were analytical grade.

### 2.3. Sample preparation protocols

#### 2.3.1. Lactoferrin stock and calibration standards preparation

Commercial (WAKO) bLF stock solution (10 mg $mL^{-1}$) was prepared by accurately weighing 250 mg of powder in a 50 mL Falcon tube. MQ water added to the tube to a final volume of 25 mL or 25 g (assuming the density of water is ≈ 1000 kg $m^{-3}$ at room temperature). The bLF powder was dissolved (typically it dissolves within an hour, care must be taken to avoid foaming while dissolving). The working lactoferrin calibration standards

were prepared from the stock solution in an appropriate buffer system and concentration range. For example, lactoferrin quantification in milk samples requires a calibration curve between 50 and 500 mg $L^{-}$, therefore working LF solutions were prepared in this range by diluting the bLF stock solution (10 mg $mL^{-1}$) in MQ water. Similarly, for RP-HPLC analysis, bLF calibration standards 100–2000 mg $L^{-1}$ for Callaghan Innovation protocol and 4000–14,000 mg $L^{-1}$ for modified GB protocol were prepared in a 0.1% TFA containing MQ water solvent system and MQ water, respectively.

*2.3.2. Sample preparation of liquid milk*

Liquid whole milk samples were purchased from a local super market or dairy farm and were stored at 4 °C upon collection. The milk was transferred into a 40 mL high-speed centrifugation tube (Nalgene™ Oak Ridge high-speed PPCO centrifuge tube, Thermo-Fisher Scientific, New Zealand), centrifuged at 16,000 × *g* for 10 min at 4 °C to remove fat and insoluble compounds. The skimmed milk was collected from the tube and left at 4 °C until further use. If skimmed milk was available, then it was directly used for the analysis.

*2.3.3. Sample preparation of whole milk, skimmed milk powders and infant formula powders*

Twenty grams of milk or infant formula powder was accurately weighed in a 250 mL Duran® bottle or similar container, then 180 mL of MQ water added to the bottle to achieve 10% (w/v) solids and the powder was dissolved using a magnetic stirring bar for 15 min at room temperature. Liquid milk was centrifuged to produce skimmed milk for LF analysis.

*2.3.4. Sample preparation from commercial lactoferrin powders*

All lactoferrin powder samples were prepared at 10 mg $mL^{-1}$ stock solutions in MQ water. Working lactoferrin samples were then prepared in a suitable buffer system depending on the analysis method and protocols.

*2.4. Cation exchange chromatography*

*2.4.1. Source 15S*

A Tricorn (5/50) column was carefully packed with a 0.9 mL of Source 15S resin and the Tricorn (5/20) guard column was packed with a 0.1 mL of Source 15S resin. The guard column was connected to the main column, providing a total column volume of 1 mL. The packed column connected to the AKTA pure 150M system (UV cell path length of 0.2 mm) was washed with 10 column volumes (CV) of each of MQ water and buffer A (50 mM Tris-HCl, pH 7.5 containing 0.5% Tween 20), at 2 mL $min^{-1}$ flow rate for the first time. Generally, the column was equilibrated for 10 CV with buffer A followed by the skim milk sample application (5 mL), then the column washing with buffer A for 15 CV followed by linear gradient elution for 25 CV using buffer B (50 mM Tris-HCl, pH 7.5 containing 0.5% Tween 20 and 1 M NaCl). Finally, the column was regenerated by washing with buffer B (5 CV) and buffer A (5 CV). A chromatogram was recorded at 220 and 280 nm simultaneously; where 280 nm is typically used for proteins (aromatic amino acids) while absorbance maxima at 220 nm is due to peptide bonds. The signal intensity of bLF at 220 nm is approximately 5 times higher than the signal intensity at 280 nm. However, absorbance at 280 nm was used for LF analysis as there are other materials that exhibit absorption at 220 nm. The column was regenerated using a cleaning in place (CIP) protocol using 1.0 M NaOH solution in the upward flow direction; if the column was unused for more than 24 h it was stored in a 20% ethanol solution.

*2.4.2. Mono S*

A Mono S, prepacked column (1 mL), was used for LF analysis. The Mono S column can also be protected using a guard column without compromising the peak resolution (data not shown). The Mono S column connected to the AKTA pure 150M system was washed with MQ water and buffer A (50 mM sodium phosphate pH 7.5), 10 CV (each) at 2 mL $min^{-1}$ flow rate for the first time. Generally, the column was equilibrated for 10 CV with buffer A following the lactoferrin sample application (5 mL), then the column was washed with buffer A for 10 CV followed by linear gradient elution for 25 CV using buffer B (50 mM sodium phosphate, pH 7.5 containing 1 M NaCl). Finally, the column was regenerated by washing with buffer B (5 CV) and buffer A (5 CV). A chromatogram was recorded at 280 and 465 nm. Absorbance at 280 nm was used for LF quantification and absorbance 465 nm (Majka et al., 2013) was used for understanding the difference in the percentage of iron saturation of different lactoferrin samples used in this study. The column was also regenerated using a cleaning in place (CIP) protocol (1 M NaOH/MQ water cycles in downward flow) if keeping the column unused for more than a week it was stored in 20% ethanol solution.

*2.4.3. Size exclusion chromatography*

A Superdex 200 Increase column was connected to the AKTA pure 150 M system and equilibrated and eluted with 50 mM sodium phosphate buffer, pH 7.5 containing 0.25 M NaCl for 1.2 CV. Lactoferrin 0.5 mL at 10 mg $mL^{-1}$ was injected into the column. Similarly, the commercial bLF was injected at the same concentration. The chromatograms at 280 nm were compared for both lactoferrin samples.

*2.4.4. Callaghan Innovation RP-HPLC method*

The RP-HPLC analysis was performed on an Agilent 1290 Infinity LC System equipped with a temperature-controlled column oven and a Diode Array Detector, Agilent Technologies, USA. Detection was carried out at the wavelengths of 220 nm, 280 nm for all whey proteins and 412 nm for LPO only (Morrison, Hamilton, & Stotz, 1957). Separation was performed on the Aeris™ 3.6 μm WIDEPORE XB-C8 200 Å, (250 × 4.6 mm) LC Column (Agilent Technologies, USA). A combination of a linear gradient and isocratic mobile phase solvent profile was employed to elute bound proteins with a flow rate of 1 mL $min^{-1}$ used throughout the analysis. Mobile phase A consisted of MQ water containing 0.1% TFA and mobile phase B was acetonitrile containing 0.1% TFA. The column temperature and the injection volume were set to be 40 °C and 20 μL, respectively. The data were acquired and processed by ChemStation software (Agilent Technologies, USA).

*2.4.5. Modified GB method*

The GB method (GB 1903.17–2016), Chinese national standards issued by the Standards Administration of China (SAC), was implemented in June 2017 for testing bovine lactoferrin powders imported to China. The GB method used Alltech Prosphere C4 column (300 Å, 150 mm × 4.6 mm) and Alltech guard cartridge Prosphere C4 (7.5 mm × 4.6 mm) with all guard cartridge holder. To compare test methods, the work was carried out with necessary amendments of the GB method on the Agilent 1290 Infinity LC System equipped with a temperature controlled column oven and a Diode Array Detector, Agilent Technologies, USA. Detection was performed at 280 nm for all whey proteins and 412 nm for LPO only. Separation was performed using a mixture of linear gradient and isocratic elution at 1 mL $min^{-1}$. Mobile phase A was MQ water containing 1% TFA and mobile phase B acetonitrile containing 1% TFA. The column temperature and the injection volume were set at

35 °C and 50 μL, respectively. The data were acquired and processed by ChemStation software (Agilent Technologies, USA).

### 2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was routinely used for analysis of bovine lactoferrin and other proteins during various stages of development. Each sample (15 μL) was mixed with 5 μL of NuPAGE™ Sample Buffer (4×) (Cat. No. NP0008, ThermoFisher Scientific, New Zealand) and 1 μL 2-mercaptoethanol (Cat. No. M7154, Sigma Aldrich, New Zealand), samples were heated in boiling water to 90 °C for 5 min, and spun-down at 10,000× *g* for 5 min. The supernatant (15 μL) of the above sample preparation was applied to each well of 4–12% NuPAGE Bis-Tris gel. Electrophoresis was performed at a constant 200 V for 35 min. Gels were stained with 0.25% Coomassie in 10% acetic acid, 40% methanol, 5% glycerol for 30 min and de-stained with 10% acetic acid, 40% methanol, 5% glycerol solution for 1 h followed by 8% acetic acid solution for 30 min. De-stained gels were photographed and analysed for protein migration using essential software of Uvitec gel documentation system.

## 3. Results and discussion

### 3.1. LF analysis of whole milk samples by analytical cation exchange chromatography

To optimise the assay protocol, a set of experiments were carried out at different pH values and buffer conditions. Due to the high isoelectric point (pI) of bLF (pI ≈ 8.6), pure bLF demonstrated good binding characteristics over a wide range of conditions when using Source 15S (1 mL). As shown in Table 1, for a commercial reference bLF analysis, no significant difference in bLF peak area was observed for buffer pH values ranging from 6.8 to 8.5; pH 6.8 being the closest to natural milk pH and pH 8.5 being closest to the isoelectric point of bLF. There was a small difference observed for different buffer salts. Sodium phosphate buffer salt demonstrated the greatest bLF peak area compared with HEPES and Tris salt buffer systems. However, it was evident from the literature that the phosphate buffer system is not suitable for bLF analysis in crude milk samples due to the possible formation of insoluble calcium phosphate, which may increase the column back-pressure. Though there is no substantial preference between the remaining two buffer salts, the HEPES buffer salt is 2.7 times more expensive (Sigma Aldrich, New Zealand) than Tris buffer salt, and Tris is commonly used in research laboratories. Therefore, factoring the above considerations, Tris salt was the most appropriate buffer salt for bLF analysis in crude milk samples.

Similarly, bLF analysis was optimised at different buffer pH values by performing experiments at pH 6.8, 7.5 and 8.0 containing 50 mM Tris-HCl. The pH 8.5 buffer was close to the pI of bLF therefore it was not included in this set of experiments. The buffer at pH 6.8 and pH 7.5 demonstrated similar levels of lactoferrin in the applied skim milk sample while at pH 8.0 it had an estimated 10% lower binding (Table 2). The skimmed milk pH was always kept as received, and usually it is around pH 6.6. To confirm the results, skimmed milk samples applied to the Source 15S column were fractionated into unbound, wash, and eluted fractions. The eluted fractions for all buffer systems demonstrated good peak resolution for bLF from the majority of other positively charged proteins present in milk. However, all the fractions showed some faint minor protein impurities (6–14 kDa) that were co-eluted with the bLF fraction (Supplementary material Fig. S1, highlighted region of Lanes A7–A8, B8–B10, and C8–C10). Among all pH conditions, pH 7.5 showed a better resolution for bLF from other positively charged proteins of skimmed milk (Supplementary material Fig. S1, Lanes B8–B10). To improve the bLF peak resolution further, a buffer system containing 50 mM Tris-HCl, pH 7.5 containing 0.5% Tween 20, a nonionic detergent, was employed. This buffer system showed the best bLF resolution from other protein impurities as shown in Fig. 1 (Fig. 1A at 39.12 mL peak and Lanes B8–B9 corresponds to

**Table 2**
Bovine lactoferrin content of skimmed milk determined using various combinations of parameters.[a]

| Buffer/Sample | Detected LF content (mg $L^{-1}$) |
|---|---|
| Set 1 – pH optimisation | |
| pH 6.8 | 83.34 ± 3.45 |
| pH 7.5 | 84.66 ± 1.77 |
| pH 8.0 | 76.61 ± 0.68 |
| Set 2 – skimmed milk, injection volume | |
| 5 mL | 100.72 ± 3.35 |
| 10 mL | 204.21 ± 2.40 |
| 15 mL | 303.05 ± 11.80 |
| 20 mL | 412.43 ± 7.10 |
| Set 3 – skimmed milk, pre-incubation temperature | |
| 10 °C | 91.77 ± 0.64 |
| 25 °C | 98.21 ± 1.13 |
| 50 °C | 101.90 ± 2.31 |
| Set 4 – skimmed milk, spiking level | |
| 0 | 98.21 ± 1.13 |
| 51.3 mg $L^{-1}$ | 151.93 ± 0.07 |
| 99.0 mg $L^{-1}$ | 207.53 ± 8.04 |
| 189.1 mg $L^{-1}$ | 298.58 ± 5.57 |

[a] Combinations were buffer pH, sample injection volume, sample temperature, and spike sample recovery. The column used in this study was a Source 15S (1 mL) custom packed and contained a guard column. The buffer system used for buffer pH optimisation (Set 1) was 50 mM Tris-HCl containing 0.5% Tween 20; all other sets used 50 mM Tris-HCl containing 0.5% Tween 20 at pH 7.5. Sample pre-incubation for Set 3 was for 15 min. Analysis was performed at room temperature (≈25 °C). The commercial pasteurised skim milk samples used for each of set were different (but same manufacturers) and the bLF content of milk is seasonal; therefore, different bLF content was observed for different skim samples used in the above list of experiments.

**Table 1**
Summary of bovine lactoferrin peak area measured for three different buffer salts at four buffer pH conditions.[a]

| pH | Sodium phosphate | HEPES | Tris-HCl | Average | STDEV | RSD (%) |
|---|---|---|---|---|---|---|
| 6.8 | 1209.35 ± 4.34 | 1190.55 ± 2.45 | 1187.37 ± 2.74 | 1195.76 | 11.88 | 0.99 |
| 7.5 | 1209.95 ± 0.67 | 1191.08 ± 1.00 | 1186.72 ± 1.05 | 1195.92 | 12.35 | 1.03 |
| 8.0 | 1210.43 ± 3.61 | 1197.01 ± 3.36 | 1188.49 ± 7.75 | 1198.58 | 10.96 | 0.91 |
| 8.5 | 1212.43 ± 1.89 | 1195.93 ± 2.31 | 1186.48 ± 1.45 | 1198.28 | 13.13 | 1.10 |
| Average | 1210.49 | 1193.64 | 1187.27 | | | |
| STDEV | 1.34 | 3.30 | 0.90 | | | |
| RSD (%) | 0.11 | 0.28 | 0.08 | | | |

[a] Buffer concentration was 50 mM in all cases. The bLF concentration (1 mg $mL^{-1}$), sample injection volume and source of the bLF were kept consistent during this study. The column used in this study was a Source 15S (1 mL) custom packed and contained a guard column.

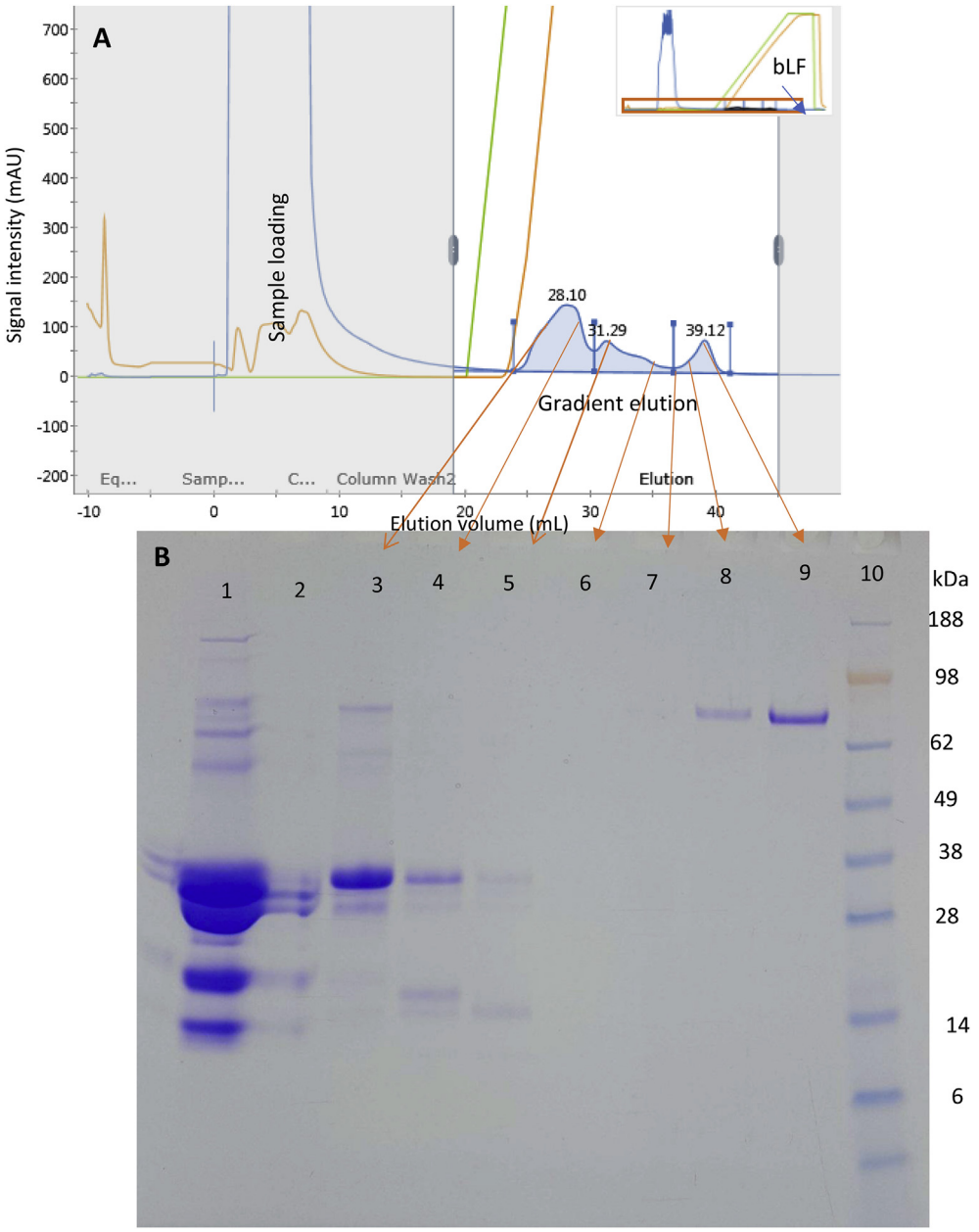


**Fig. 1.** A typical chromatogram (A) of skimmed milk loaded onto to the Source15S (1 mL) for quantification of bLF. The chromatogram was monitored at UV 280 nm and fractions were labelled with unique names that were used later in the SDS-PAGE (reduced, 4–12%) analysis (B): lane 1, skimmed milk; lane 2, empty (overflow of L1); lanes 3–9 elution fractions (lanes 8 and 9 correspond to bLF); lane 10, molecular mass marker.

bLF). This could be due to the fact that the nonionic detergent, Tween 20, eliminates undesirable hydrophobic interactions that have a negative impact on bLF peak resolution. Therefore, 50 mM Tris-HCl, pH 7.5 containing 0.5% Tween 20 was employed as the optimum buffer system for bLF quantification in bovine skimmed milk using a column packed with Source 15S resin. The above conditions were used for all subsequent optimisation experiments.

To understand the minimum amount of skimmed milk sample required for accurate LF quantification, a series of skimmed milk sample injections were trialled at 5, 10, 15 and 20 mL volumes, while keeping the same buffer system, flow rate, temperature and elution gradient. The results in Table 2, set 2, indicated that there was no significant difference in terms of bLF concentration (mg $L^{-1}$) determined for varying injection volume when normalised. For example, for a selected skimmed milk sample, 5 mL injection estimated a bLF concentration of 100.72 mg $L^{-1}$ [RSD (n = 3) of 3.4%], while 20 mL injection estimated 412.43 mg $L^{-1}$ [RSD (n = 3) of 1.7%] bLF, respectively. The normalised (with respect to 5 mL injection volume) bLF content of selected skimmed milk was approximately 103.43 mg $L^{-1}$ (i.e., 412.43/4 = 103.43). Though all milk sample injections produced a similar bLF content (101.74 ± 1.1 mg $L^{-1}$), column compression for >10 mL skimmed milk loading was slightly higher than smaller volume injections (smaller sample injections allow a higher number of sample analysis per column), and the overall analysis time was longer for larger sample injections. Considering the bLF concentration in bovine milk (50–500 mg $L^{-1}$) along with the above advantages of smaller sample injection volumes it was concluded that skimmed milk injection volume of 5 mL was optimal and was used in all subsequent experiments.

To understand the impact of sample temperature during the analysis of skimmed milk, a set of experiments were carried out at different sample pre-incubation temperatures (10–50 °C). Experiments were performed using 50 mM Tris-HCl, pH 7.5 containing 0.5% Tween 20, 5 mL sample injection, 2 mL $min^{-1}$ flow rate during sample injection and 4 mL $min^{-1}$ flow rate for washing and elution were employed. Skimmed milk (pH 6.6) samples used in this experiment were pre-incubated for 15 min at 10 °C, 25 °C and 50 °C before injecting on to the column. The bLF analysis (Table 2) for skimmed milk pre-incubated at 10 °C was 91.77 ± 0.64 mg $L^{-1}$, while pre-incubation at 25 °C and 50 °C were 98.21 ± 1.13 and 101.90 ± 2.31, respectively. The results indicated that the amount of bLF quantified in cold skimmed milk (10 °C) samples showed approximately 10% lower recovery than at 50 °C and 6.5% lower recovery than for the 25 °C sample. However, when frozen skimmed milk samples were analysed after freshly thawing without any pre-heating a 40% lower bLF concentration was obtained previously (data not shown). However, the difference between the 25 °C and 50 °C samples was 3.6%, which was not very significant and considered as within the acceptable limit (<5%). Considering the advantages, generally, higher temperature helps to avoid fat precipitation of bovine milk (melting point of dairy fats ≤40 °C) and lowers the column back pressure, while temperatures higher than 55 °C may cause bLF denaturation. Therefore, it is recommended to pre-heat skimmed milk samples to 25–50 °C before injecting on to the column for accurate quantification of bLF in milk samples.

Finally, the performance of optimised conditions were verified by bLF spiking of skimmed milk samples. Experiments were performed using 50 mM Tris-HCl, pH 7.5, containing 0.5% Tween 20 buffer system, 5 mL sample injection, 2 mL $min^{-1}$ flow rate during sample injection and 4 mL $min^{-1}$ flow rate for washing and elution, skimmed milk pre-incubation at 25 °C was employed. The sample pre-incubation temperature was kept at 25 °C, which is close to room temperature. The skimmed samples were spiked with three different known concentrations of bLF as indicated in Table 2 and set 4, and the samples were incubated for 15 min with continuous mixing. Each sample was tested in triplicate and the bLF content was estimated against a bLF calibration curve (Supplementary material Fig. S2) and results summarised in Table 2. The percentage recoveries achieved for the three spiking experiments were 104.6%, 110.4% and 105.9% with an average of 107.0% ± 3.0%. The results indicate that optimised conditions were produced resulting in an acceptable error range (±10%) for such crude sample analysis. To show the applicability of the test method, a wide range of raw materials were tested for LF content (Table 3). The optimised method was successfully employed for the quantification of LF content from skimmed milk, acid whey, whole milk, skimmed and whole milk powders, infant formula powders, and whey protein concentrates (WPC). The spike recovery of skimmed milk samples was between 101% and 106%, skimmed milk and whole milk powders was around 93%, infant formula powders were between 95% and 118%. Most results were within 7% error range while infant formula powders were within 23% error range. Knowing the complexity of infant formula powders, the percentage of bLF recovery is still within an acceptable error range compared with other available methods (up to 25%). Therefore, the optimised method is suitable for LF quantification in a wide variety of samples.

### 3.2. LF analysis of purified to semi-purified samples by analytical cation exchange chromatography

High resolution pre-packed Mono S 5/10 GL column (1 mL) was selected over Source 15S column for the quantification of bLF in purified to semi-purified samples because it provides superior peak resolution. The Mono S column was pre-packed with 10 μm size beads while Source 15S was packed in-house with 15 μm beads otherwise they were produced using the same base matrix, polystyrene divinylbenzene. Mono S column produces much higher resolution than Source 15S but may not be suitable for crude samples like skim milk. Based on the preliminary findings (Table 1),

**Table 3**
Content of bovine lactoferrin detected in different types of dairy products with and without spiking.[a]

| Sample type | Spiking (mg $L^{-1}$ or mg 100 $g^{-1}$) | Calculated LF (mg $L^{-1}$ or mg 100 $g^{-1}$) | LF spike recovery (%) |
|---|---|---|---|
| Commercial Skimmed Milk 1 (organic) – liquid | Nil | 82.25 ± 2.28 | NA |
| | 109.2 | 197.40 ± 2.11 | 105% |
| Commercial Skimmed Milk 2 (regular) – liquid | Nil | 107.47 ± 1.74 | NA |
| | 107.7 | 222.13 ± 6.87 | 106% |
| Skimmed Milk prepared from commercial non-homogenised whole milk – liquid | Nil | 78.07 ± 0.51 | NA |
| | 160.6 | 240.96 ± 4.02 | 101% |
| Commercial Skimmed Milk 3 (regular) – liquid | Nil | 102.58 ± 1.83 | NA |
| | 109.6 | 217.14 ± 4.79 | 105% |
| Acid whey (prepared from a commercial skimmed milk) – liquid | Nil | 115.96 ± 0.85 | NA |
| | 100 | 219.83 ± 1.39 | 95% |
| Skimmed prepared from commercial whole milk powder | Nil | Not detected | NA |
| | 107.2 | 100.10 ± 12.04 | 93% |
| Commercial Skimmed Milk Powder | Nil | Not detected | NA |
| | 124.7 | 115.54 ± 0.52 | 93% |
| Commercial infant formula powder – Stage 1 (Birth+) | Nil | Not detected (below MOD) | NA |
| | 125.5 | 148.44 ± 5.52 | 118% |
| Commercial infant formula powder – Stage 2 (6 Months+) | Nil | Not detected (below MOD) | NA |
| | 125.5 | 131.00 ± 13.55 | 107% |
| Commercial infant formula powder – Stage 3 (12 Months+) | Nil | Not detected (below MOD) | NA |
| | 125.5 | 134.22 ± 3.57 | 111% |
| Commercial infant formula powder – Stage 2 | Nil | 60.55 ± 0.40 | NA |
| | 28.73 | 88.66 ± 0.45 | 98% |
| | 41.05 | 99.28 ± 0.75 | 95% |
| WPC35 powder (commercial powder) | Nil | 9.82 ± 0.27 | NA |
| WPC 80 powder 1 (commercial powder) | Nil | 157.48 ± 0.13 | NA |
| WPC 80 powder 2 (commercial powder) | Nil | 186.84 ± 0.50 | NA |
| Lactoperoxidase fraction (LPO) – concentrate - Liquid | Nil | 577.59 ± 1.41 | NA |

[a] Spiking experiments were used to evaluate the robustness of the test method; spiking levels are in mg $L^{-1}$ for liquid products and mg 100 $g^{-1}$ for powder products. Products were skimmed milk, whole milk (non-homogenised), acid whey, milk powders, whey powders, and infant formula powders. The column used in this study was a Source 15S (1 mL) custom packed and contained a guard column; the binding buffer system used was 50 mM Tris-HCl, pH 7.5 containing 0.5% Tween20.

buffer conditions were further optimised using 50 mM sodium phosphate and 50 mM Tris-HCl buffer systems at pH 7.5 and 8.0. A commercially purified bovine lactoferrin (1.0 mg $mL^{-1}$) was used. Both sodium phosphate and Tris buffer systems (Table 4) showed that pH 7.5 was superior than pH 8.0 in terms of LF peak areas (1162 mAU vs 1129 mAU) and little difference in terms of LF purity was obtained (96.0% versus 95.8%; Table 4). These results were in-line with Source 15S findings, therefore, pH 7.5 was selected for future optimisation.

Having a closer observation of the chromatograms indicated that sodium phosphate buffer, pH 7.5 produced a better peak resolution for impurity proteins present in the sample compared with Tris buffer, pH 7.5 (Fig. 2; blue trace). However, analysis with sodium phosphate buffer pH 7.5 also produced an additional unbound protein peak during the sample loading (Fig. 3A). Analysis of this fraction by SDS-PAGE (Fig. 3B) revealed that this fraction contains bLF [Lane 2 (FT); Fig. 3B]. In contrast, this peak was not observed for Tris-HCl, pH 7.5 buffer system for the same sample (Fig. 2; blue trace – no unbound protein peak). Other than this difference, there was no significant difference observed for bLF elution for either buffer system. To accurately quantify bLF it should bind and elute off the column. Considering the presence of unbound bLF peak in the sodium phosphate buffer system, it was concluded that 50 mM Tris-buffer system was preferred over the phosphate buffer system. However, sodium phosphate buffer system may still be useful if the better separation of impurities required for certain applications. To showcase this further, a LPO (80 kDa, pI of 9.2–9.9) fraction, which contains the majority of positively charged proteins (Harris et al., 2010) that bind to cation exchange resin, a by-product of bLF manufacturing, was injected through Mono S column using 50 mM Tris-HCl pH 7.5 buffer system and the same gradient elution was employed as above. The chromatogram was recorded at 280 nm (Fig. 4a); fractions collected for every 0.5 mL and all major peaks at 280 nm were analysed using 4–12% SDS-PAGE analysis (Fig. 4b). The results clearly indicated that bLF was well separated under the optimised conditions from all other positively charged proteins that routinely bind to cation ion exchange chromatography resin during bLF production, though it was noticed that all other protein peaks were not well resolved. For example, lanes 5 to 8, represent multiple protein bands corresponding to peaks eluted on Mono S column and the proteins ranged between 8 and 80 kDa. The protein band at ≈80 kDa for lanes 6 and 7 corresponds to LPO (confirmed by RP HPLC) and the protein bands that appeared ≈14–17 kDa range for lanes 7 and 8 correspond to RNase4 (~17 kDa, pI ~9.2) and angiogenin (~15 kDa, pI ~ 9.5) and similar results were reported in the literature (Gupta, Haigh, & Wheeler, 2016; Harris et al., 2010; Maes et al., 1988; Ye, Cheng, & Ng, 1999).

To determine bLF concentration a calibration curve was generated as indicated in Supplementary material Fig. S3, the reference bLF has produced a good linear calibration curve for the selected concentration range (0.27–1.5 mg $mL^{-1}$, triplicate analysis) with $R^2$ value of 0.9999. Following this, a range of commercially produced bovine lactoferrin powders, tablets and LPO concentrate (liquid) samples were tested for quantification of bLF content and purity using the optimised assay conditions on the Mono S column (Table 5, columns 2 and 3).

The bLF results for the Mono S column were compared with two other RP-HPLC methods. Interestingly, commercial lactoferrin powders demonstrated a different level of LF content and purities. Overall, the variation in bLF purity was approximately 5% (between 94% and 99%). In contrast, the bLF content was significantly different (up to 18%) from each other, ranging from 77 g per 100 g–95 g per 100 g. All the commercial lactoferrin powders look very similar in physical appearance (light pink), but had different particle sizes and were produced by different manufacturing technologies, which may produce LF of different qualities.

Two common variations that bLF manufacturers adapt are sterilisation (microfiltration versus pasteurisation) and drying/

**Table 4**
Summary of bovine lactoferrin peak area and purity assessed in two different buffer systems.[a]

| Buffer pH | Sodium phosphate | | Tris-HCl | |
|---|---|---|---|---|
| | LF peak area (mAU × mL) | LF purity (%) | LF peak area (mAU × mL) | LF purity (%) |
| 7.5 | 1165.10 ± 59.60 | 95.8 ± 0.3 | 1157.82 ± 10.61 | 96.3 ± 0.3 |
| 8.0 | 1114.10 ± 9.40 | 95.8 ± 0.2 | 1144.64 ± 8.50 | 95.7 ± 0.2 |

[a] Buffer concentration was 50 mM; bLF concentration (1 mg $mL^{-1}$), sample injection volume and source of the bLF were kept consistent. The column used was an analytical Mono S (1 mL, prepacked) without a guard column.

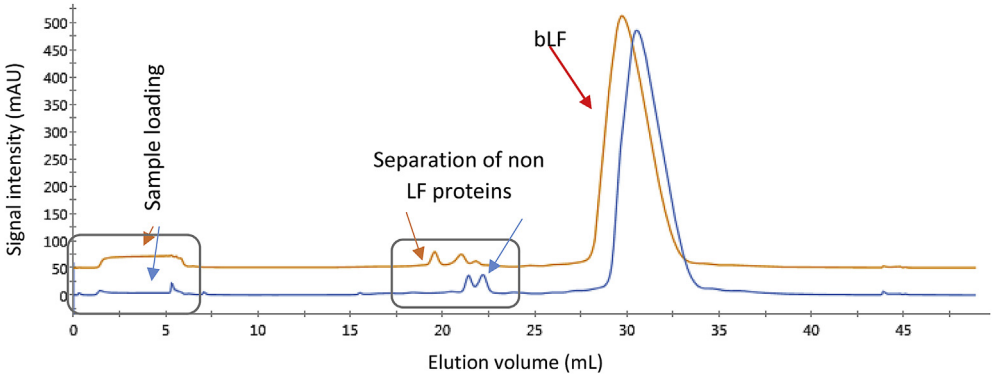


**Fig. 2.** Overlay of chromatograms monitored at UV 280 nm for a reference bLF (1.0 mg $mL^{-1}$) analysis by Mono S (1 mL) pre-packed column. The blue chromatogram (lower trace) corresponds to bLF analysis under 50 mM Tris-HCl, pH 7.5 buffer system while the orange chromatogram (upper trace) corresponds to the same bLF analysis under 50 mM sodium phosphate, pH 7.5 buffer system. Under sodium phosphate buffer system, some of the bLF was present in unbound fraction but provided a superior peak resolution (3 peaks) for other impurities present in the reference bLF sample compared with Tris-HCl buffer system. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

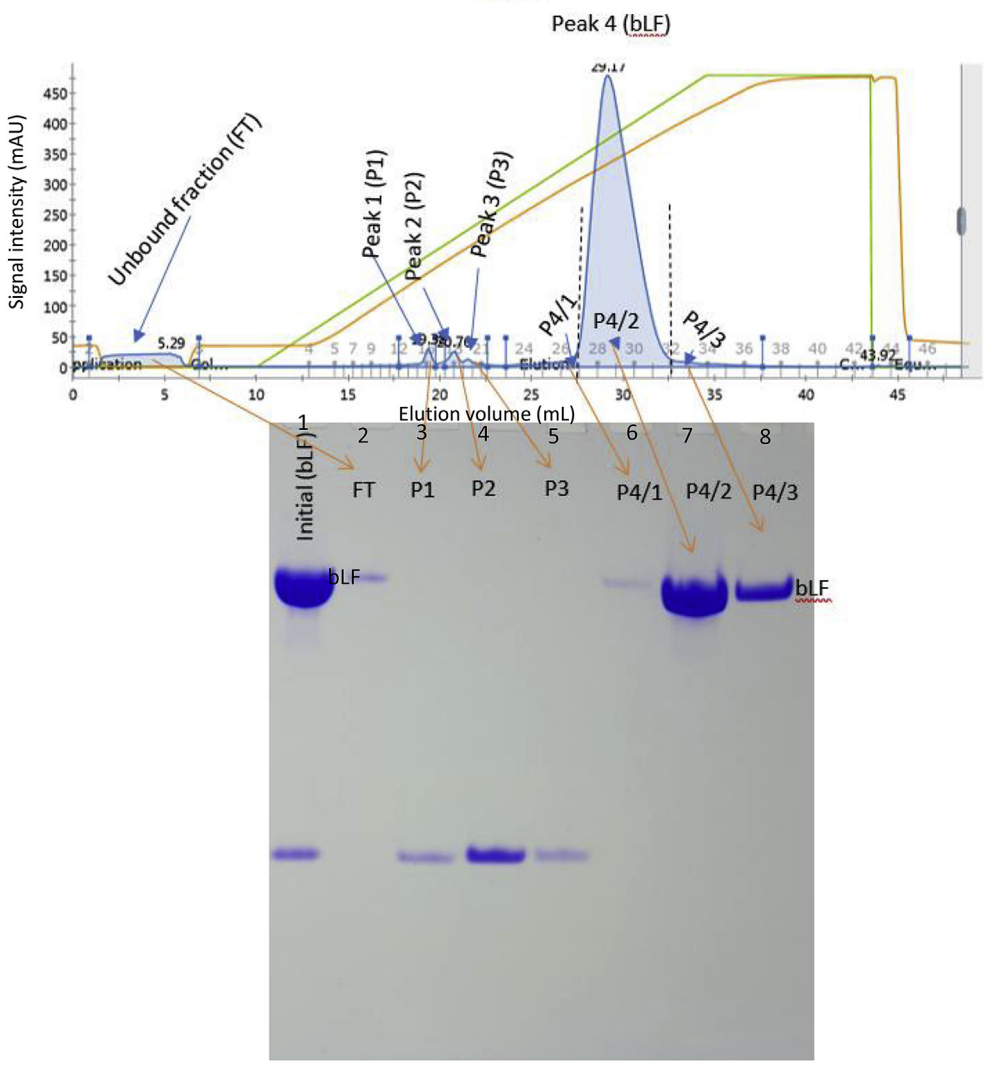


**Fig. 3.** A typical chromatogram (A) of reference bLF analysis by Mono S (1 mL) pre-packed column using 50 mM sodium phosphate buffer, pH 7.5; all the fractions obtained from this analysis were analysed by SDS-PAGE (reduced, 4–12%; B) to confirm protein bands: lane 1, bLF initial sample; lane 2, unbound fraction (FT); lanes 3–8, elution fractions (lanes 6–8 correspond to bLF).

dewatering technologies (freeze-drying versus spray-drying) applied for bLF production. To show the impact of temperature on bLF denaturation, an experiment was conducted. For this experiment, freshly prepared bLF from fresh skimmed milk (unpasteurised skimmed milk) was used. Approximately 0.5 mL of 100 mg $mL^{-1}$ bLF samples (duplicate) were exposed to selected temperature and time combinations (Fig. 5). After heat treatment, samples were immediately cooled in the refrigerator. Samples were centrifuged (1000× *g*, 10 min) to remove any insoluble LF (denatured aggregates) produced during the heat treatment, then the supernatants were diluted (100 × ) with MQ water. All the dilutions were performed by weight basis (wt %) to minimise the human pipetting errors with viscous solutions. As expected, no loss of bLF was observed when heated at 50 °C for 15 min because bLF denaturation begins at 55 °C (data not shown). Samples treated at 75 °C for 2 min produced a recovery of 99% of applied bLF, while samples treated at 75 °C for 5 min and 90 °C for 2 min produced only a recovery of 95% and 96%, respectively. These data clearly show that the heat-treatment or exposure to heat during the pasteurisation or drying conditions should be carefully selected to minimise the percentage of bLF denaturation. Though it was not detailed in this paper, the level of denaturation of bLF varies greatly with sample pH, buffer salt concentration and percentage of impurities present in the product. The Mono S method described in this paper could be used as a tool for assessing the bLF denaturation level in their production processes. This was also visible in the chromatogram (Fig. 5) as partially denatured LF co-elutes as a shoulder peak and similar results were previously demonstrated by Valck and Perraudin (2016). If bLF badly denatured, it may end up resulting in the polymerised or precipitate material. Considering the effect of heat-treatment of bLF observed in this work, it is therefore concluded that different commercial bLF samples might have been exposed to different processing conditions during the manufacturing of bLF powders. Hence the different amount of LF quantities were observed (Table 5).

### *3.3. LF analysis of purified to semi-purified samples by analytical RP-HPLC columns*

RP-HPLC is one of the most common methods used by industry to assess the quality of finish products. In this paper, we briefly described two RP-HPLC methods for testing bLF content and purity

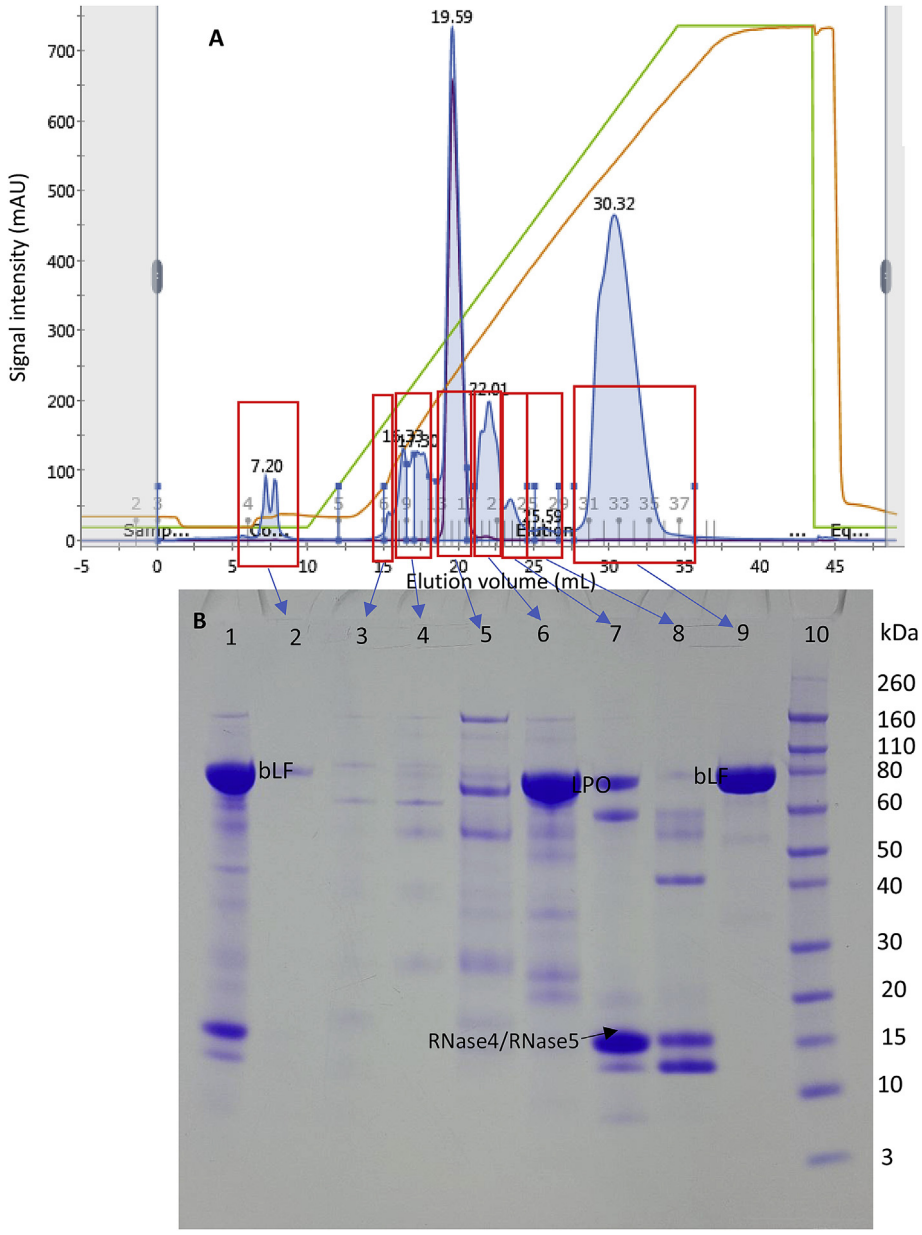


**Fig. 4.** A typical chromatogram (A) of milk basic proteins mixture (also known as LPO fraction or bLF by-product) analysis by Mono S (1 mL) pre-packed column using 50 mM Tris-HCl buffer, pH 7.5; all the fractions obtained from this analysis were analysed by SDS-PAGE (reduced, 4–12%; B): lane 1, bLF by-product initial sample; lane 2, wash fraction; lanes 3–8, elution fractions (lane 9 corresponds to bLF); lane 10, molecular mass marker.

in various LF products. The first RP-HPLC method was developed at Callaghan Innovation for determining bLF content and purity in a range of lactoferrin products (unpublished). The second RP-HPLC method used in this paper was a modified GB method, where the GB method was recently introduced by the Chinese Food and Drug Administration, China for testing bLF quality of lactoferrin products imported into China. The existing version of the GB method uses a single point calibration and applies a high concentration of protein load [50 μL of 10 mg $mL^{-1}$ sample (0.5 mg per injection)], which was close to the upper end of the column binding capacity. Due to this, some significant differences in bLF determination were observed. Therefore, amendments were deemed to improve the robustness of the GB method.

The major improvements of the method included are (i) a 4-point calibration instead of a single point calibration, (ii) 20 μL sample injection per assay instead of 50 μL and (iii) addition of 1% TFA directly to the solvent A and solvent B instead of using a third pump to deliver and mix TFA (10%) into the solvent system. Using the modified protocol, a linear calibration (4–11 mg $mL^{-1}$ or 0.085–0.23 mg of bLF per injection) curve was produced with a $R^2$ value of 0.9996 (Supplementary material Fig. S4). Similarly, using the modified GB method, the bLF content and purity of a range of lactoferrin products were determined and are summarised in Table 5 (columns 6 and 7).

Before the GB method became available in the public domain, Callaghan Innovation had developed a RP-UPLC method for quantification of LF content and purity of lactoferrin products to support various local and international customers. This method was routinely used for testing various lactoferrin products over the past 4 years for several customers. The Callaghan Innovation

**Table 5**
Content of bovine lactoferrin detected in different types of lactoferrin products using three different novel analytical methods/protocols.[a]

| Sample | LF quantification by Mono S column (CIEC) | | LF quantification by C8 column (RP-HPLC) | | LF quantification by C4 column (RP-HPLC) | |
|---|---|---|---|---|---|---|
| | LF (g 100 $g^{-1}$) | LF Purity (%) | LF (g 100 $g^{-1}$) | LF Purity (%) | LF (g 100 $g^{-1}$) | LF Purity (%) |
| S1 | 95.11 ± 0.12 | 97.8 ± 0.2 | 94.33 ± 0.08 | 96.2 ± 0.2 | 95.78 ± 2.57 | 95.9 ± 0.0 |
| S2 | 84.34 ± 0.62 | 95.0 ± 0.0 | 85.27 ± 0.09 | 94.5 ± 0.1 | 84.98 ± 0.66 | 93.9 ± 0.3 |
| S3 | 91.13 ± 1.04 | 96.9 ± 0.1 | 89.59 ± 0.01 | 95.1 ± 0.1 | 90.72 ± 1.35 | 95.0 ± 0.1 |
| S4 | 93.22 ± 0.18 | 96.8 ± 0.1 | 94.40 ± 0.22 | 96.4 ± 0.1 | 94.20 ± 1.81 | 95.7 ± 0.0 |
| S5 | 89.27 ± 0.41 | 94.6 ± 0.2 | 90.32 ± 0.08 | 93.9 ± 0.0 | 89.88 ± 0.96 | 93.4 ± 0.0 |
| S6 | 82.77 ± 2.50 | 98.6 ± 0.5 | 84.72 ± 0.43 | 95.0 ± 0.3 | 85.77 ± 1.25 | 95.5 ± 0.0 |
| S7 | 86.28 ± 0.71 | 99.0 ± 0.1 | 85.30 ± 0.12 | 95.0 ± 0.2 | 86.81 ± 2.75 | 95.5 ± 0.0 |
| S8 | 87.17 ± 0.63 | 98.9 ± 0.0 | 88.24 ± 0.97 | 95.6 ± 1.2 | 90.34 ± 1.34 | 96.9 ± 0.1 |
| S9 | 92.59 ± 0.24 | 97.6 ± 0.0 | 94.13 ± 0.09 | 95.8 ± 0.2 | 95.12 ± 2.51 | 96.0 ± 0.0 |
| S10 | 79.05 ± 0.64 | 96.6 ± 0.2 | 82.51 ± 0.07 | 95.8 ± 1.7 | 82.23 ± 3.00 | 95.8 ± 0.0 |
| S11 | 77.18 ± 0.13 | 96.5 ± 0.6 | 80.89 ± 0.08 | 94.4 ± 0.1 | 80.66 ± 3.48 | 95.7 ± 0.1 |
| S12 | 84.02 ± 0.83 | 96.8 ± 0.4 | 85.11 ± 0.06 | 94.5 ± 0.0 | 83.20 ± 3.65 | 96.0 ± 0.1 |
| S13 | 95.20 ± 1.46 | 98.2 ± 0.0 | 97.32 ± 0.27 | 97.1 ± 0.1 | 97.44 ± 2.62 | 96.9 ± 0.1 |
| LF tablet 1 | 38.84 ± 0.15 | 98.7 ± 0.1 | 39.28 ± 0.24 | 98.5 ± 0.6 | 41.40 ± 1.43 | 97.2 ± 0.2 |
| LF tablet 2 | nd | nd | 29.06 ± 0.47 | 97.3 ± 0.1 | 29.60 ± 0.88 | 96.7 ± 0.1 |
| LPO (concentrate) – liquid | 4.50 ± 0.61 | 47.0 ± 0.1 | 4.76 ± 0.03 | 54.7 ± 0.6 | 4.42 ± 6.34 | 58.6 ± 0.3 |

[a] Abbreviations are: S1–S13, Bovine lactoferrin (LF) powders S1–S13, respectively; LPO, lactoperoxidase; CIEC, cation exchange chromatography, RP-HPLC, reverse pahse high performance liquid chromatography. Expected lactoferrin contents of LF tablets 1 and 2 were 40 g 100 $g^{-1}$ and 28.13 g 100 $g^{-1}$, repectively; nd, not determined.

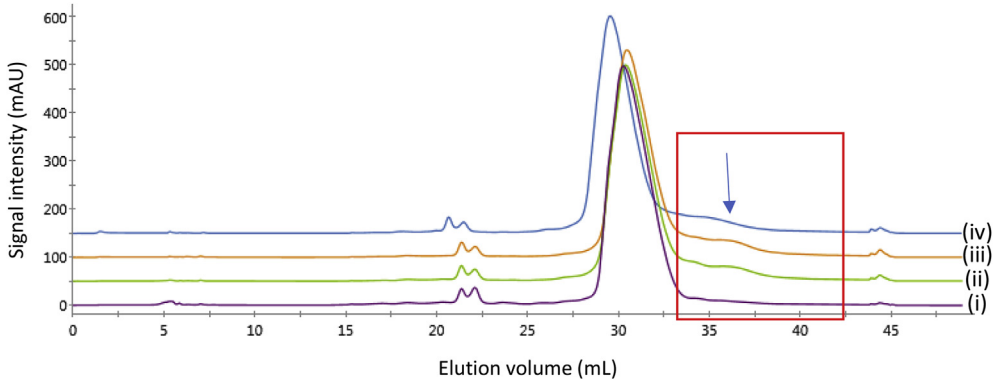


**Fig. 5.** Overlay chromatograms (UV 280 nm) of a reference bLF exposed to different heat treatment and time course studies analysed by Mono S (1 mL) pre-packed column: traces are (i) no heat treatment, (ii) 75 °C, 2 min, (iii) 75 °C, 5 min, (iv) 90 °C, 2 min. 50 mM Tris-HCl, pH 7.5 buffer system was used. The shoulder peak was more prominent for bLF samples which underwent higher temperature treatments and for longer time periods.

protocol used a different column from the GB method hence they demonstrate a different level of assay sensitivities. The method was thoroughly validated (unpublished) following the standard industry protocols of validating chromatography methods. A linear calibration curve (0.5–2.0 mg $mL^{-1}$ or 0.011–0.045 mg of bLF per injection) was produced with $R^2$ value of 1 using an optimised protocol (Supplementary material Fig. S5). Similarly, using this optimised Callaghan Innovation protocol; the bLF content and purity of a variety of LF products were determined and are summarised in Table 5 (columns 4 and 5). A typical chromatogram profile of selected bLF using various analytical methods investigated in this paper is presented in Fig. 6. Each method demonstrated a different type of impurities. For example, RP-HPLC methods (Fig. 6A,B) bLF as main big peak while impurities eluted at different retention times. For the same bLF, analytical cation exchange analysis demonstrated the main peak being LF (Fig. 6C) along with a shoulder denatured LF (broad shoulder peak). Similarly, size exclusion chromatography (SEC) analysis (Fig. 6D) of bLF demonstrated monomer, dimer and other higher molecular structures of lactoferrin. All these analytical techniques indicate that each analytical technique was unique and illustrates different characteristics to identify bLF quantification.

### *3.4. bLF structural integrity analysis by size exclusion chromatography*

SEC using a S200 Increase column (GE Healthcare Technologies) was employed to study the bLF integrity of various commercial lactoferrin powders used in this publication. The commercial bLF powders used in this study demonstrated a different level of LF content using various analytical methods described elsewhere in this paper (Table 5). The 50 mM sodium phosphate, pH 7.5, 0.25 M NaCl buffer showed good peak resolution for various forms of bLF when compared with other buffer systems (50 mM Tris-HCl, pH 7.5, 0.25 M NaCl and 50 mM HEPES, pH 7.5, 0.25 M NaCl). Lower salt concentration (<0.25 M NaCl) in the mobile phase provided poor peak resolution of bLF oligomers (broad over-lapping peaks). A typical chromatogram profile of different bLF on a SEC column is demonstrated in Fig. 7, where the monomer of bLF (78 kDa) was eluted at 14.3 min, the dimer (156 kDa) was eluted at 12.3 min [this may also correspond to IgG monomer (150 kDa)], the oligomer form (>468 kDa) was eluted at 8.7 min [this may also correspond to an IgG trimer (450 kDa)]. However, when the same samples were analysed by SDS-PAGE under reducing conditions, predominantly bLF was present in the monomer form (corresponding SDS-PAGE in

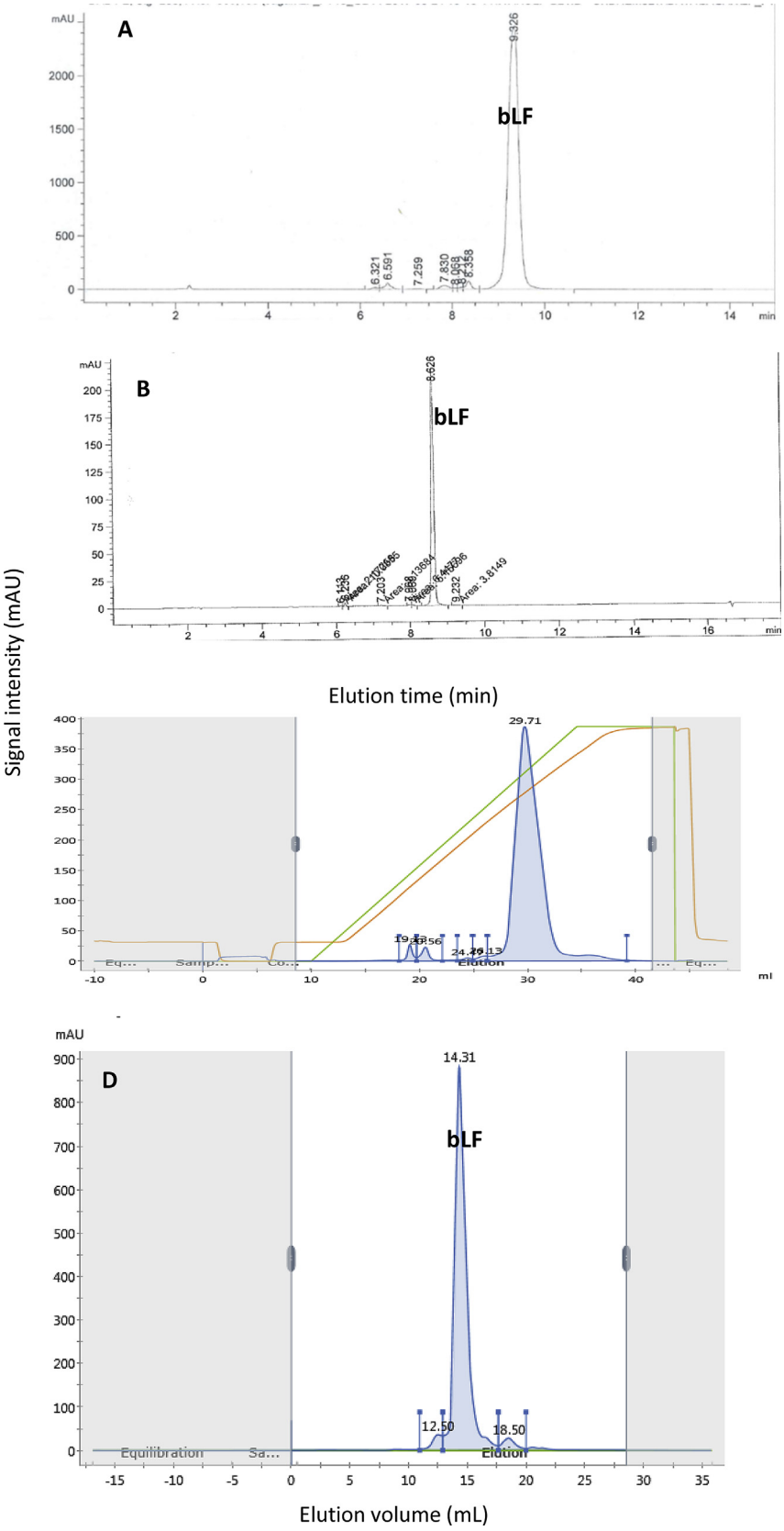


**Fig. 6.** Typical chromatograms of a bLF analysed using (A) the modified GB method, (B) the Callaghan Innovation method, (C) a Mono S (1 mL) column, and (D) a S200 Increase (10/300 GL) column.

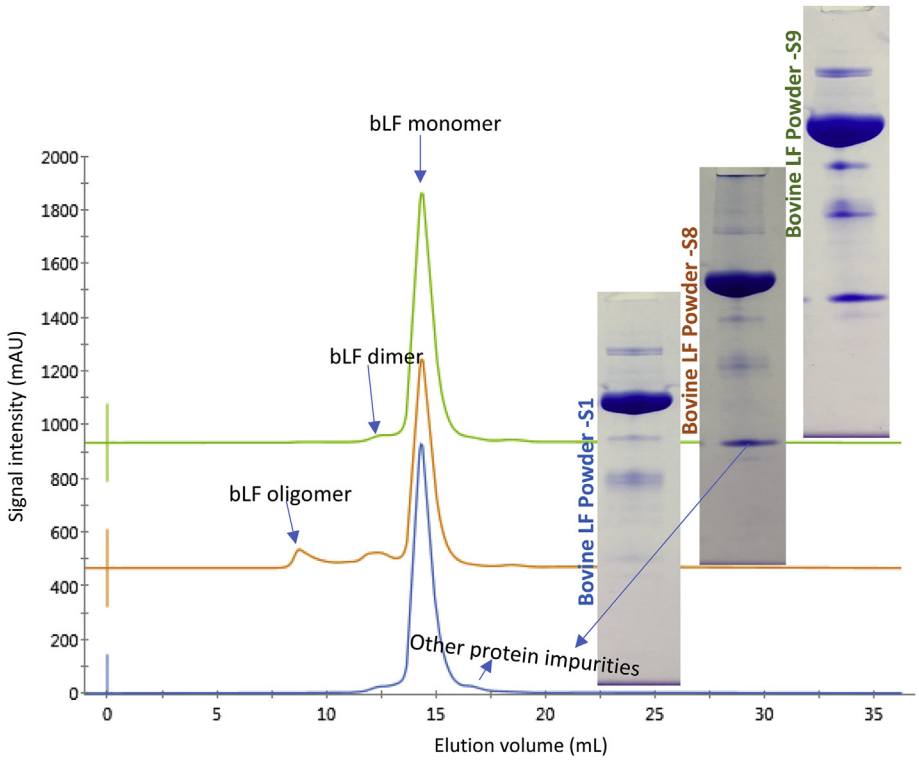


**Fig. 7.** Overlay of chromatograms monitored at UV 280 nm for thee selected commercial bLF powder samples injected onto the SEC column. The peaks at 14.35, 12.51 and 8.69 mL correspond to a monomer, dimer and oligomer form of bLF, respectively. The inserts show corresponding analysed using 4–12% SDS-PAGE.

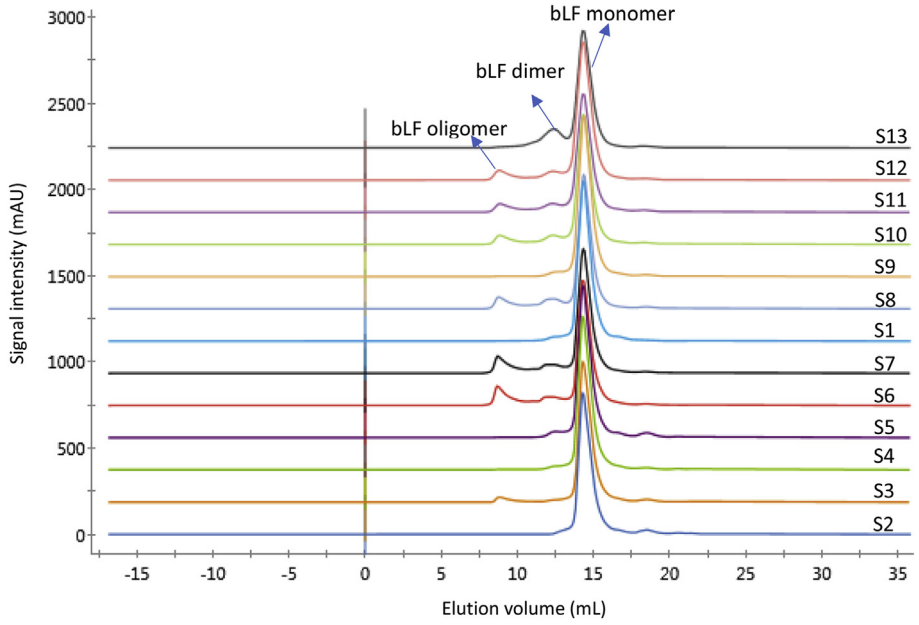


**Fig. 8.** Overlay of chromatograms monitored at 280 nm for 13 commercial bLF powder samples (S1–S13) injected onto the SEC column. The peaks at 14.35, 12.51 and 8.69 mL correspond to a monomer, dimer and oligomer form of bLF, respectively.

Fig. 7). This could possibly be explained by the reduction of the various IgG forms into 50 kDa and 25 kDa domains. These results indicate that bLF exists in different forms under native buffer conditions or that there are contaminating proteins present in the commercial bLF samples. Similar observations have been published elsewhere (Valck & Perraudin, 2016). Interestingly, commercial bLF sourced from various sources demonstrated different percentages of monomer, dimer and oligomer forms of bLF (Fig. 8). The monomer form was the predominant form found in the commercial samples of bLF (Table 6). SEC analysis data provide evidence that these variations could be due to the different processing conditions employed during the bLF manufacture as discussed in earlier sections.

**Table 6**
Proportion of monomer, dimer and oligomer forms of bovine lactoferrin detected in thirteen commercial bLF powders (S1–S13).

| Sample | Bovine lactoferrin form (%) | | |
|---|---|---|---|
| | Monomer | Dimer | Oligomer |
| S1 | 98.52 | 1.23 | 0.00 |
| S2 | 93.35 | 1.50 | 0.00 |
| S3 | 93.05 | 1.05 | 3.68 |
| S4 | 95.56 | 2.10 | 0.00 |
| S5 | 92.82 | 3.53 | 0.00 |
| S6 | 77.82 | 7.91 | 14.23 |
| S7 | 78.10 | 8.69 | 13.21 |
| S8 | 79.48 | 10.30 | 8.42 |
| S9 | 95.65 | 2.68 | 0.00 |
| S10 | 83.60 | 7.68 | 7.00 |
| S11 | 82.29 | 7.90 | 7.17 |
| S12 | 84.43 | 6.84 | 7.08 |
| S13 | 78.49 | 19.47 | 0.31 |

## 4. Conclusions

The protocol validated in this paper for quantification of bLF in various types of crude samples using Source 15S (1 mL) is accurate (linearity for 60–450 mg $L^{-1}$ with an $R^2 = 0.9992$), reliable (RSD of crude milk sample testing 2.2%, n = 21), robust (RSD of spike recovery 8%, n = 11), and fast (18 min). The Source 15S protocol offers a huge benefit for the bLF manufacturing dairy industry to provide critical information, thereby increasing processing efficiencies and product quantity. Having this test method in-house assists the manufacturer to assess the bLF content in the feed material therefore they can calculate the amount of milk to be loaded onto the column to get maximum yield and good product quality.

Column overloading with bLF due to seasonal variation (50–500 mg $L^{-1}$) on the purification cycle can reduce the overall bLF yield due to significant losses in the unbound fraction. On the other hand, under-loading may result in low purity product, (reduced economic value), so the product may not meet the market requirements and therefore will attract a low market price.

Similarly, the same method was also useful for assessing the performance of bLF fortified infant formula. This would allow blending technologies at different stages for formula manufacturing that includes optimising the infant formula blending plant to produce a consistent product that gives the desired level of bLF content. The current methods available for bLF quantification in crude and infant formula powders are either expensive or have limited utility or do not produce reliable test results due to several inherent problems.

The Mono S protocol optimised in this paper was shown to be accurate (linearity 0.27–1.5 mg $mL^{-1}$ with $R^2 = 0.9999$), reliable (RSD of triplicate analysis 0.84%, n = 13), fast (15 min), and provides some insights of bLF surface modifications during the manufacturing practices. The Mono S protocol validated in this paper could serve as an assessment tool for determining the level of bLF denaturation (intensity of a shoulder peak by the main bLF peak) or surface modifications (an unbound portion of bLF) that occurs during the manufacturing process or the presence of bacterial lipopolysaccharides attached to peptide 1–52 of the bLF, induces the processes of inflammation. Such information is critical for assessing the biological functions of bLF, which is not detected by RP-HPLC methods. Therefore, the RP-HPLC methods do not provide a detailed understanding of denaturation and surface modifications of bLF as obtained by the Mono S protocol.

The lactoferrin products tested in this paper indicated that the Mono S results were similar to RP-HPLC data (Bovine LF powder S1 to S5, LPO concentrate, LF Table 5) but some other products (Bovine LF powder S8 to S13) showed significant differences from that of the RP-HPLC data. These differences could be attributed to the separation of intact bLF, surface modified bLF and denatured bLF on the Mono S column whilst co-elution as a single peak on the RP-HPLC column. The RP-HPLC methods are routinely used as a quality analysis tool in terms of purity for assessing product quality.

In this paper, two independent protocols are described (modified GB protocol with linearity between 4 and 11 mg $mL^{-1}$, $R^2 = 0.9999$ and the Callaghan Innovation protocol with linearity between 0.5 and 2.0 mg $mL^{-1}$, $R^2 = 1.0$) with necessary amendments to assess the purity of various lactoferrin powders. Both protocols showed similar results for most of the lactoferrin products tested in this paper, and this indicated that either method has the potential for being implemented as a purity tool for the release of finished products, though the column chemistry was different for the two selected methods.

The other protocol established in this study was SEC analysis of bLF products. Interestingly, SEC analysis provided some additional insights into various commercial lactoferrin products. All of the commercial lactoferrin powders predominantly contained the monomer form of bLF under native conditions but some products demonstrated an increased percentage of dimer and oligomer forms or the presence of contaminating proteins. Bovine LF powder – S1 demonstrated >98% monomer form while Bovine LF powders – S6/S7 demonstrated approximately 78% monomer, 8.3% dimer and 13.7% oligomer form and Bovine LF powder – 13 demonstrated approximately 79% monomer, 20% dimer and <1% oligomer forms. These results together indicate that though all manufacturers use cation exchange chromatography for capturing and purifying bLF from milk or whey they are unable to produce the same quality final product and this could be attributed to their proprietary technologies employed during post-purification or formulation stages. The implications of such modifications towards bLF biological functions were not investigated in this paper but the protocols developed and validated in this paper could be used to increase the purity and yield of bLF products. It is also to be noted that the producers obtain a high degree of purity does not mean to demonstrate a high degree of biological activity. Therefore the methods described in this article to identify and quantify the bLF may not be directly assimilated to the biological activities of the bLF.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.104546.

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