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Review

Lactoferrin Production: A Systematic Review of the Latest Analytical Methods

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Abstract: Background: Lactoferrin is a major functional protein involved in maintaining human health, which possesses antioxidant, anti-inflammatory, antibacterial, and antiviral properties. Therefore, it can be used to support the treatment of viral and bacterial diseases, as well as in cancer prevention. Lactoferrin-manufacturing processes may compromise its protein structure and function, so it is necessary to establish reliable analytical methods for production efficiency and quality control purposes. This paper reviews the lactoferrin production processes, summarising the methods using various matrices (milk, milk powder, infant formula, whey, bovine lactoferrin lyophilised powder, yoghurt, colostrum, and human milk), the most popular purification methods, and sample preparation. Material and methods: The Medline and Embase databases were searched using the following phrases: ”lactoferrin” and “purification” or “isolation” or “extraction” or “separation”. The search was limited to recent studies from the last five years published in English up until 12 March 2025. Of the 573 articles identified, 17 were reviewed. Results: Lactoferrin purification and determination methods depend on the matrix used. The latest research focuses on improving parameters of lactoferrin determination, shortening time, improving efficiency or limiting costs, and even reducing toxicity by changing the reagents. The method of separating lactoferrin using magnetic beads or nanoparticles has been developed, as well as the determination parameters using high-performance liquid chromatography (HPLC). Conclusions: The current lactoferrin production techniques are characterised by increased efficiency and quality, but they require standardisation of the purification process depending on the matrix. The latest Lf determination methods are highly precise, and most of them produce high-quality Lf. This allows to introduce on the market a higher quality product, which can significantly improve standard approaches.

Keywords: lactoferrin; instrumental analysis; determination of lactoferrin; purification; dairy products; infant formula; whey

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1. Introduction

1.1. Lactoferrin Structure

Lactoferrin (Lf) is one of the main proteins of mammalian milk whey [1]. Typically, human milk contains approximately 1–2 mg/mL Lf, whereas cow’s milk contains 1–10 µg/mL [2]. It was first reported in 1939 [3] and was subsequently isolated and found to have a similar structure to serum transferrin [4]. It is a glycoprotein consisting of one polypeptide chain with a molecular weight of 80 kDa [1]. Human Lf consists of 691 amino acids, whereas bovine Lf comprises 696 amino acids [5]. The two globular lobes, carboxyl

(C) and amino (N), are joined by an alpha-helix and comprise two domains, C1 and C2, N1 and N2, folded into a beta-sheet [1,5]. The structure visualisation is shown in Figure 1. Each lobe can bind one ferric ion, creating a closed holo-Lf, but when no iron is bound, it creates the open-form apo-Lf [6]. Its positive charge facilitates binding to anionic compounds [5]. Its sensitivity to proteolysis and denaturation is determined by the number of glycosylation sites and their location [7].

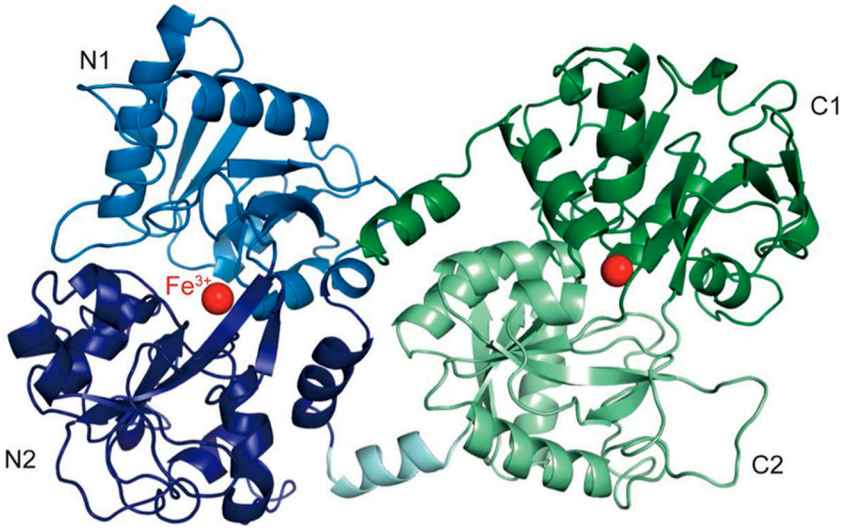

Figure 1. The visualisation of lactoferrin structure [8].

Human and bovine Lf show about 70% structural similarity, suggesting that Lf performs similar bioactive functions in mammals; thus, bovine Lf is a good source of Lf for human use [5].

The Lf concentration in milk depends primarily on the lactation stage [9], with the highest concentration in colostrum (7 mg/mL in human colostrum vs. 2 mg/mL in buffalo colostrum) [2,10]. The average Lf concentrations in milk products are presented in Table 1.

Table 1. Average lactoferrin concentration in milk products.

Product	Lf Concentration	Reference
Human colostrum	7 mg/mL	[11]
Human mature milk	2 mg/mL	[11]
Bovine colostrum	1.5 mg/mL	[10]
Bovine mature milk	0.2 mg/mL	[10]
Goat colostrum	0.39 mg/mL	[12]
Goat mature milk	0.06 mg/mL	[12]
Camel milk	0.229 ± 0.135 mg/mL	[13]
Buffalo milk	0.332 ± 0.165 mg/mL	[14]
Infant formula (cow's milk-based)	0.1 mg/mL	[15]

1.2. The Effects of Bovine Lactoferrin on the Human Body

Lf plays a key role in iron transport and has antiviral, antibacterial, antioxidant, and anti-inflammatory properties [16–18]. It is commonly used to treat iron deficiency anaemia (IDA). Oral bovine Lf supplementation improves haemoglobin concentrations as effectively as standard iron supplementation [19–24], and Lf supplementation of children with IDA is better than iron supplements alone [25–28]. It also has fewer side effects than iron supplements (such as constipation), thus making it a safer solution for children, especially those with different comorbidities [29,30].

Pregnant women are also at higher risk of IDA, but Lf is a safe and effective product to use in this group, increasing the positive effect of ferrous sulphate supplementation and reducing gastrointestinal side effects [31]. It has a similarly positive outcome when used alone and increased haemoglobin levels better than ferrous sulphate [21].

Lf is a safe alternative for high-risk patients; for example, it can be used as a less invasive treatment in patients with cancer, where standard anaemia treatment consists of intravenous iron [32]. Additionally, it reduces hepcidin levels, which rise during the inflammatory response, as hepcidin is an inflammatory mediator. Therefore, it is an effective alternative for patients undergoing regular haemodialysis—a procedure, which, if carried out regularly, increases the inflammatory response [33].

During an infection, iron absorption is restricted due to inflammation to decrease iron availability for pathogens, thus disabling certain metabolic pathways [34]. Besides strong iron absorption properties, Lf also exerts antiviral effects, possibly due to its ability to retain iron in an acidic environment, specific for infection and inflammation. It binds to negatively charged viral fragments and receptors, resulting in viral inhibition [35]. Lf reduces the frequency of common cold events, shortens the duration of infectious diseases, and significantly reduces lower respiratory tract events in infants [36–39]. There is some evidence of its ability to lower the risk of bacterial and fungal sepsis in preterm infants [38,40,41].

Inconsistent findings report Lf's effectiveness in treating chronic hepatitis C virus (HCV). Some studies show a significant increase in virological response in Lf monotherapy, as well as combined with standard HCV therapy [42]. It is an effective treatment for hepatitis B virus (HBV) [43,44]. Similar outcomes were reported on Lf activity against human papillomavirus (HPV) as it inhibits the binding of HPV to the cell surface [45,46]. Recently, Lf nanoparticles have been applied in human immunodeficiency virus (HIV) therapy. Lf can be used as a drug carrier, thereby improving its bioavailability. It can also prevent HIV nuclear translocation and abrogate extracellular entry [47–50].

Studies conducted on participants infected with SARS-CoV-2 and treated with Lf show promising effects. It resulted in a shorter time to test negative and faster clinical recovery since it reduced SARS-CoV-2 seroconversion in patients with mild to moderate symptoms [51–53].

Lf possesses antibacterial properties due to its ability to sequester iron from the environment, making it inaccessible to pathogens. Lf also binds to lipopolysaccharides (LPS) on the bacterial cell wall, decreasing the virulence, entry into human cells, and inducing apoptosis [54,55]. Lf inhibited *Pseudomonas aeruginosa* bacterial biofilm production in cystic fibrosis (CF), with CF patients with lower Lf levels being less resistant to the chronic *P. aeruginosa* lung infection [56]. Lf also significantly reduces the adhesion of *Streptococcus mutans* to abiotic surfaces, the bacterium responsible for most dental caries [57]. The combination of Lf and lysozyme increases the antibacterial properties of both compounds [58]. Lf also displays antibacterial properties towards *Shigella*, *Salmonella*, and *Escherichia coli* [59].

Lf has the potential to improve intestinal health in mice with the combination of Lf and osteopontin used to treat inflammatory bowel disease (IBD) [60]. Lf in combination with the antimicrobial peptide N6 improved the intestinal barrier and mucosal immunity and increased the height of small intestinal mucosal villi in enterotoxigenic *Escherichia coli* (ETEC) infected mice, making Lf a promising therapeutic for treating bacterial enteritis [61]. Lf treatment of rat models of depression resulted in better enterocyte conjunction and lower levels of inflammatory factors, which caused metabolic balance in hippocampal tissue and mitigated depression-like behaviour [62]. Lf prevented *Salmonella* biofilm formation on abiotic surfaces; thus, it is a potent agent for inhibiting drug-resistant *Salmonella* serotypes [63].

Lf is also a neuroprotective agent, regulating iron uptake in neuronal tissue by modifying the activity of iron transporters and reducing oxidative stress [64]. Lf protects neurons from MPP+ mitochondrial toxin that induces damage in Parkinson's disease (PD) [65,66]. Interaction with heparan sulphate proteoglycans (inflammatory mediators) is also being investigated [65]. In a study on patients with Alzheimer's disease (AD), three months of Lf administration significantly improved disease markers such as acetylcholine, serum amyloid beta, and IL-6 [67]. Although researchers still do not understand the specific mechanism of Lf's positive impact on AD, its role in inflammation in the central nervous system is certain [68]. Research is ongoing regarding its effects on prions and hypoxia/ischemia [69,70].

Lf can reduce the inflammatory response in lipopolysaccharide-induced and sepsis-induced acute lung injury models, possibly due to regulation of the PPAR-γ pathway [71,72].

Lf shows renoprotective potential. In studies on glycerol-induced rhabdomyolysis and acute kidney injury, Lf supplementation significantly improved renal function and decreased serum creatinine, blood urea nitrogen, albuminuria, and proteinuria. It decreased kidney injury markers and increased expression of nuclear factor erythroid 2-related factor 2 (NRF2) [73], exhibiting similar properties in gentamicin-induced acute kidney injury. It suppressed ferroptosis, a non-apoptotic cell death process, indicating nephroprotective properties [74].

Lf has been used as an ingredient in gels to promote skin wound healing. Lf/NZ2114/lithium magnesium silicate hydrogel reduced the inflammatory response, promoting wound healing with more hair follicles and healthy glands [75]. The hydrogel was effective when applied to post-operative wounds, showing great potential in wound management [76]. In vitro studies revealed that the mechanism involved fibroblasts and keratinocyte stimulation, re-epithelialisation, and collagen and hyaluronan synthesis [77].

Lf is effective in treating dental issues, and when applied topically, it effectively reduces symptoms of dentin hypersensitivity, such as pain and inflammation [78]. Bovine Lf-derived peptides can be used in treating alveolar bone destruction in periodontitis due to reducing TNF-α expression, osteoprotegerin downregulation, and osteoclastogenesis reduction [79].

1.3. Lactoferrin Production

Due to its unique properties, Lf can be isolated and purified from cheese whey and skimmed milk on an industrial scale [80]. Global Lf production has increased rapidly over the last 20 years, from less than 80 tonnes per year in 2003 to over 300 tonnes per year in 2021 [81,82]. It is estimated that the market size will exceed 265 million in 2027 [82,83]. In Europe, the main producers are Germany (170 tonnes per year) and the Netherlands (70 tonnes) [84–86].

Commercially, Lf is currently isolated from bovine milk or its by-products primarily due to its good availability and relatively low costs. However, Lf can be isolated from any mammalian secretion [81], and recombinant human Lf has been produced in transgenic animals, fungi, cell cultures, or plants [87,88]. However, Lf production from bovine milk is expensive due to the large volume of milk or its products required for the chromatographic and ultrafiltration techniques [89]. To purify Lf to supplement grade, milk or other dairy products must undergo an extraction process. The pulled-out Lf is then eluted with a solution of NaCl, then desalted and condensed by ultrafiltration. The obtained product must then be pasteurised and dried to create a powder (Figure 2).

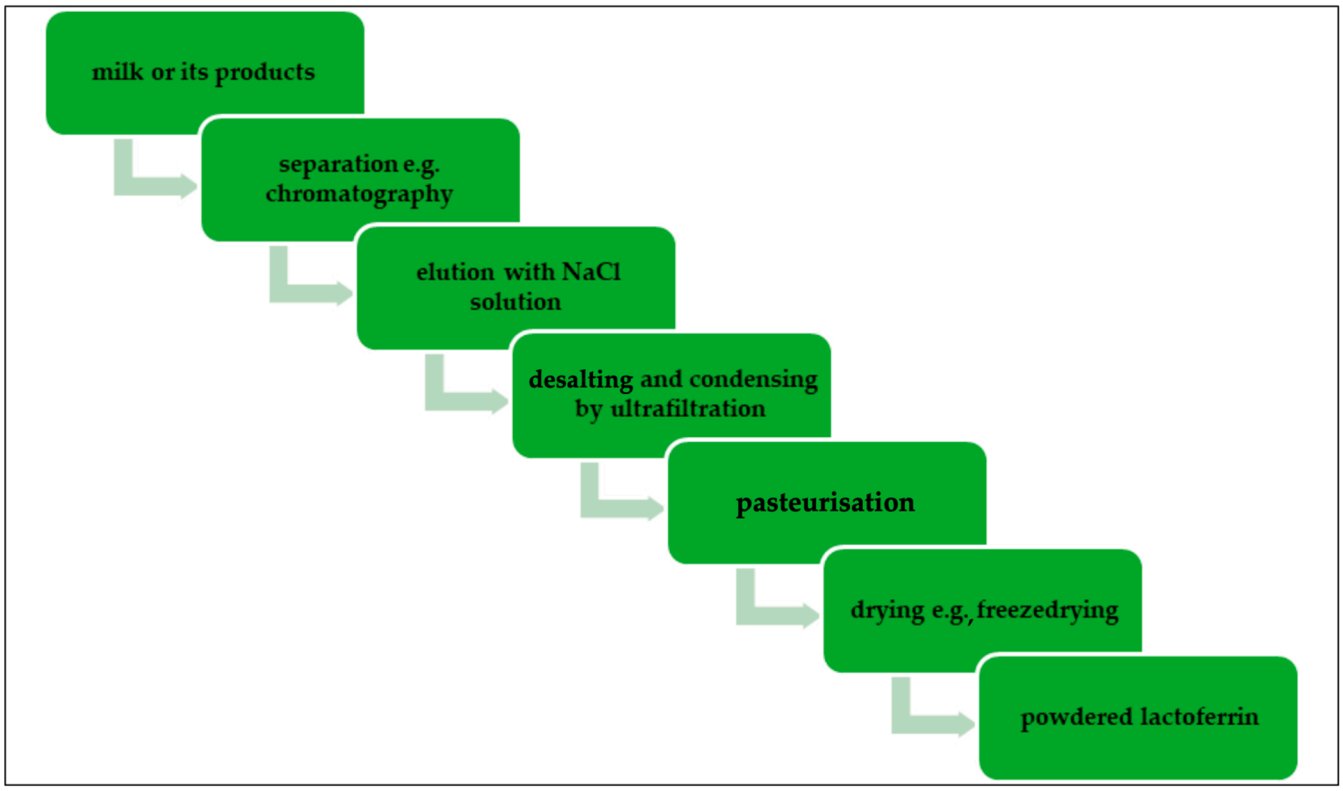

Figure 2. Lactoferrin production process.

New techniques of obtaining Lf from sources other than animals are emerging. One of them uses fungi (*Pichia pastoris*) that allows scaling of the production due to the use of bioreactors. In optimal conditions, the yield can reach 2.8 g/L. This method allows for keeping the fungi densely in a limited space, which improves efficiency. However, the initial costs of production infrastructure and equipment are high [90,91].

Another method considers transgenic plants. The yield is lower than that of the fungi systems, but the smaller operational costs make it economically advantageous. A downside of using plant material is the reliance on a longer production cycle and environmental variability [92].

A vital production aspect is to achieve a bioequivalent product, which can be a challenge in transgenic processes, as well as obtaining regulatory approvals. Only then, new methods can be implemented on a mass scale [93].

Global standardisation in the quality of Lf (bovine lactoferin) is progressing through Generally Recognised as Safe (GRAS) and Novel Food. These regulations are relatively high, including more than 95% Lf purity in protein [94]. In addition to lactoferrin content, other quality criteria are also assessed. The most important include low levels of microbiological contamination, minimal content of substances left over from technological processes, and limited levels of heavy metals [95]. Total heavy metal content (cadmium, lead, arsenic, mercury, copper) is max. 1 mg/kg. Microbiological guidelines: standard plate count < 1000 cfu/g; *Enterobacteriaceae* < 10 cfu/g; yeasts < 10 cfu/g; and coagulase-positive *Staphylococcus*: not detected in 1 g [96].

Due to the broad spectrum of Lf's health-promoting effects, with particular emphasis on antiviral and antibacterial effects, it is often a component of dietary supplements that strengthen immunity. The great interest in pharmaceutical products that prevent infectious diseases means a growing interest in commercial Lf production; therefore, this paper reviews the current Lf production methods.

2. Materials and Methods

The literature search, quality assessment, and data extraction were performed independently by two authors using a standardised approach. This review was reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. This study was registered in the Centre for Open Science using the Open Science Framework. The search criteria are presented in Figure 3.

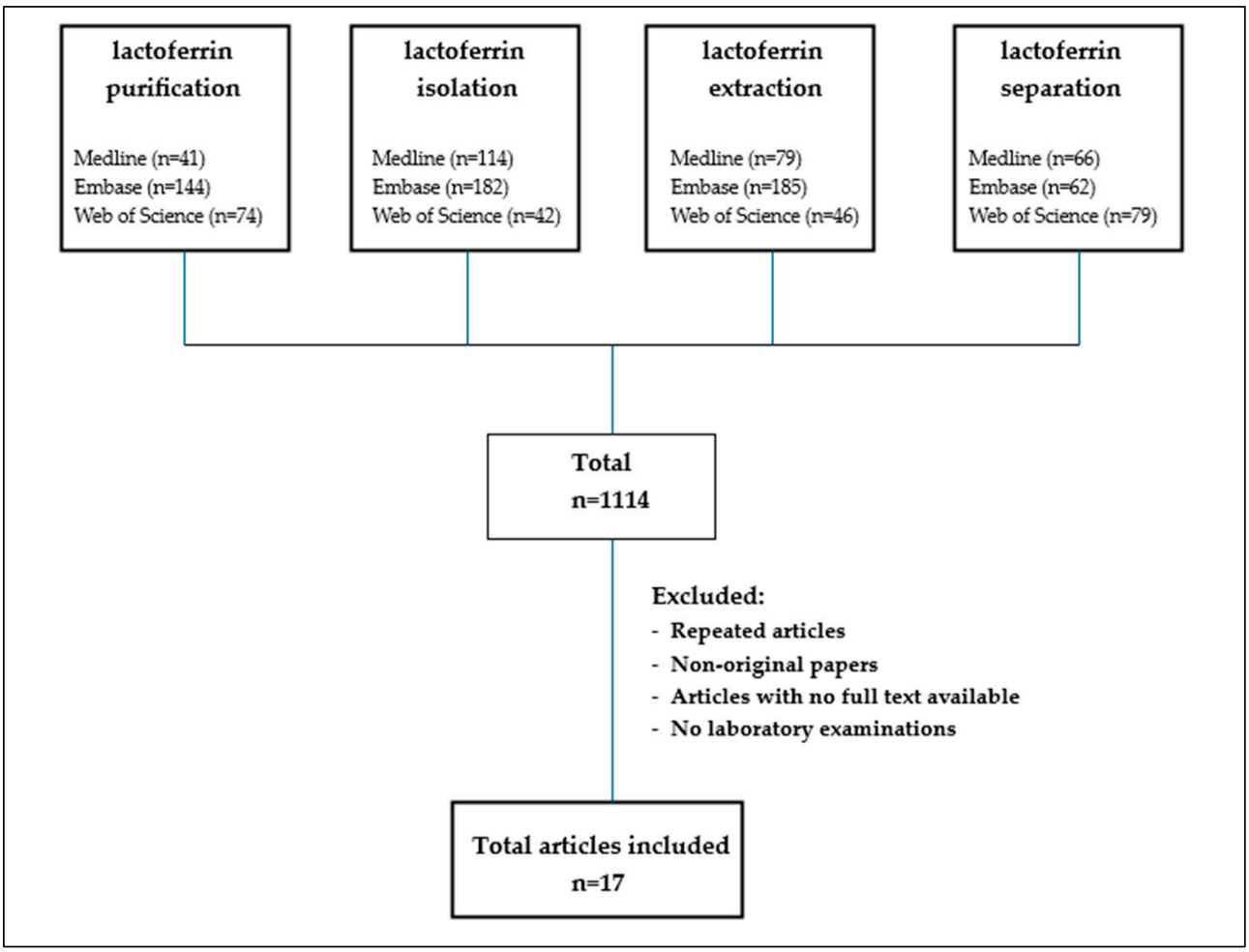

Figure 3. Search strategy.

The databases Medline, Embase, and Web of Science were searched up to the 12th of March 2025 using the following phrases: "lactoferrin" and "purification" or "isolation" or "extraction" or "separation". Inclusion criteria were English language, original research, and publication after 2019. Duplicates were ruled out. The exclusion criteria were no laboratory examination described in the article, absence of sample preparation report or conditions parameters, and using different product other than milk or milk-derived (Figure 3). All authors independently assessed the full-text articles, and agreement was reached among all authors on the articles included for review.

3. Results and Discussion

Obtaining Lf is becoming more popular due to its beneficial health properties. The methods of obtaining and determining Lf differ in their applications; some of them can be used for bovine Lf, others for camel Lf, and human Lf. Typically, bovine Lf is used on the largest scale due to good and cheap access to cow's milk or by-products used to produce dairy based on cow's milk, i.e., whey. Lf from human milk can be used to treat premature

babies, whereas Lf from camel milk is used in areas of camel milk abundance. Nevertheless, obtaining and determining Lf depends on the source material: liquid milk, powdered milk, Lf supplements, and whey. Each of these matrices has its specification, distinct Lf concentration, structural composition, and pH; therefore, the production methods differ.

Over the last five years, new analytical methods have been developed to improve Lf production and determination by reducing production costs and its toxicity, increasing the Lf yield, or improving the accuracy of Lf determination. Table 2 presents the latest purification and determination methods depending on the matrix. Out of 17 studies, 11 covered bovine Lf, but it was not the only source. Lf was mainly prepared from milk, its powdered form [97–103] or milk-based infant formula [97–99,104,105]. Some articles also used whey or whey protein concentrate [106,107], human milk [108,109], colostrum [110], camel milk [111], or lactoferrin powder [112].

Wang et al. used Boron-doped titania (inorganic boric acid and highly hydrophilic titanium dioxide) to detect Lf in dairy products. Parameters like extraction time, pH, desorption time, and solution had to be optimised to achieve an adsorption capacity of 63.9 mg/g. It was confirmed by UHPLC-UV and made a cheap and efficient determination technique [97].

Ellingson et al. developed and validated a method for quantifying Lf in infant formulae using ultra-high performance LC-tandem mass spectrometry (MS/MS). All validation parameters were met with a precision RSDR (mean recovery, repeatability) ranging from 2.1 to 7.1 and an intermediate RSDR ranging from 7.0 to 10.4 across types of infant formulae. Accuracy with certified reference material resulted in mean recoveries of 91.7–96.4%. This study supports manufacturing specifications and nutrient labelling requirements [104].

Gill et al. described and evaluated the analytical performance of an optical biosensor immunoassay to determine Lf in infant formulae and other nutritional powdered formulae. This method is rapid, sensitive, precise, accurate, and simple to implement with an analytical range of 0–200 mg/hg, detection limit of 0.8 mg/hg, recovery of 96.1–109.2%, and repeatability of 1.0–5.3%. Additionally, it provides physiological analytical information because the method is specific for intact Lf, and thermally denatured Lf generates no measurable binding response [98].

Pang et al. described a method using immunoaffinity magnetic purification coupled with high-performance liquid chromatography-fluorescence (HPLC-FL) detection for the determination of Lf in dairy products (pasteurised milk, infant formula powder, whey protein concentrate). This method is more sensitive (curve range 0.8–30 μg/mL) than the HPLC-UV method (10–1200 μg/mL). Additionally, in comparison to LC-MS/MS, where the sensitivity is higher (1–1000 nM), it is possible to distinguish intact Lf from its denatured form. This method is simple, specific, and sensitive, but not suitable for yoghurt because its pH is not conducive to immunoaffinity binding [99]. This method is shown in Figure 4.

Wang et al. used an aptamer affinity column combined with high-performance liquid chromatography HPLC-UVD for the purification and enrichment of LF in milk. This purification method is a good alternative to the heparin affinity column due to reduced cost and improved stability. The detection method is characterised by a wide range and good sensitivity with a detection limit of 3 μg/mL [100].

Table 2. Sample preparation and analytical methods for obtaining lactoferrin from different matrices.

Matrix	Methods	Result	Characteristic of Analytical Method		References
			Sample Preparation	Conditions and Validation Parameters	
Liquid milk, Fermented milk, Infant formula	UHPLC-UV	Developed a cheap and efficient method for detecting Lf in dairy products	4 mL of liquid milk or 4 g of fermented milk and infant formula mixed with 800 µL of dichloromethane and 400 µL of water with 5% acetic acid; centrifuged; supernatant mixed with 8 mL ammonium chloride buffer; added 5 mg of B-doped TiO_2; sonicated for 5 min; shaken for 75 min; 200 µL of water containing 5% formic acid added and shaken to elute the bound Lf; filtered with a 0.22 µm polyethersulfone filter membrane	Detector: UV, column: Waters Acquity TM Protein BEH C4 column (2.1 mm × 100 mm, 1.7 µm). Eluent A: 0.1% formic acid in water, B: acetonitrile/water/ formic acid (71.4:28.6:0.075, *v*/*v*/*v*), gradient elution Flow rate: 0.3 mL/min LOD: 0.0002–0.0012 mg/g LOQ: 0.0006–0.0029 mg/g	Wang M et al. (2022) [97]
Infant formula	LC-MS/MS	Developed and validated a method for quantifying LF in powder infant formulae	1 g powder infant formula or 10 g liquid (ready-to-feed) infant formula + 30 mL extraction buffer and diluted to volume (50 mL) with extraction buffer samples that contained between 1000 and 2000 mg/100 g: diluted by transferring 50 µL to a new well and adding 100 µL of 85 + 15 water–acetonitrile (0.15% formic acid) samples that contained < 1000 mg/100 g: diluted by transferring 50 µL to a new well and adding 50 µL of 80 + 20 water–acetonitrile (0.2% formic acid) final dilutions: added 150 µL internal standard working solution IS-SAMP (for samples containing 1000–2000 mg/100 g) or 100 µL IS-SAMP for samples containing < 1000 mg/100 g)	Column: Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 µm), mobile phase A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile, gradient elution, flow rate 0.6 mL/min. MS/MS: 4000 Q-Trap with Electrospray Ionisation, IonSpray voltage: 4000 V LOQ: 0.25 mg/g LOD: no data	Ellingson DJ et al. (2019) [104]
Infant formula, milk powder, powder reconstituted ready-to-feed basis	Optical Biosensor Immunoassay	Developed and evaluated a rapid, sensitive, precise, and simple method for detecting Lf in nutritional formula powder	Infant formula powder or milk powder diluted 1:2000 *w*/*v*. 0.5 g of sample + 8 mL buffer HPS-EP (2.044 g sodium chloride in 100 mL), stored in the dark ≥ 15 min. 990 µL buffer + 10 µL, cap and vortex Powder reconstituted ready-to-feed basis diluted 1:2000 *w*/*v*. 25 g powder + 200 g water, warm to 25 °C for 10 min and mix. 4.5 g aliquot of sample solution and make up to 10 mL with HPS-EP, cap and vortex. 990 µL solution buffer + 10 µL of the diluted sample, cap and vortex	Biacore T200. HPS-EP flow rate: 10 µL/min, contact time 300 s. LOD: 0.008 mg/g LOQ: 0.025 mg/g	Gill BD et al. (2022) [98]

Table 2. *Cont.*

Matrix	Methods	Result	Characteristic of Analytical Method		References
			Sample Preparation	Conditions and Validation Parameters	
Pasteurisation milk, infant formula, whey protein concentrate	HPLC-FL	Developed a method of Lf purification by immunoaffinity magnetic beads. Determined the Lf by HPLC-FL, which is characterised as more sensitive than HPLC-UV	Liquid samples: 200 µL of milk mixed with 800 µL of PBSN (phosphate buffered saline containing 0.1% Tween 20, pH = 7.4), centrifuged at 10,000 rpm/5 min at 4 °C to remove the fat, 500 µL of supernatant incubated with 5.0 mg of antibody-coated beads at 37 °C/2 h Beads washed (immobilise anti-Lf antibody with magnetic beads) using 1.0 mL of PBSN three times and attracted with a magnet: supernatant removed. The bead-bound target protein was eluted using 1.5 mL of citrate buffer (0.1 M, pH = 3.0) containing 0.1% Tween (CBN) Lf saturated with iron at room temp. with a freshly prepared FeNTA solution [9.9 mM ferric nitrate and 8.5 mM nitrilotriacetic acid (disodium salt) in water, adjusted to pH = 7.0 by adding solid $NaHCO_3$]/1 h Solid samples: 35 mg of samples dissolved in 1.0 mL of PBSN, fat removed by centrifuging, 500 µL of the supernatant incubated with 10 mg of antibody-coated beads at 37 °C/2 h, and treated like a liquid sample	Detector: FL, 280 nm (excitation), 344 nm (emission), Column: Innoval Neo XD C18 column (150 × 4.6 mm, 5 µm), Mobile phase: A—water + 0.1% trifluoroacetic acid, B-acetonitrile + 0.1% trifluoroacetic acid, gradient elution, flow rate 1 mL/min LOD: 0.00025 mg/mL LOQ: 0.0008 mg/mL	Pang J et al. (2020) [99]
Raw milk	HPLC-UVD	Improved Lf purification using an aptamer column combined with HPLC-UVD	10 mL milk + 40 mL phosphate buffer (0.2 mol/L $Na_2HPO_4 \cdot 12H_2O$, pH = 7.5), centrifuged at 4 °C and 10,000 rpm for 15 min. Supernatant was purified using the AAC (activated with 5 mL of binding buffer) 10 mL of the supernatant (10 mL milk + 40 mL buffer) loaded on the column. AAC washed with 10 mL of washing buffer (0.01 mol/L $Na_2HPO_4 \cdot 12H_2O$, pH = 7.5) and flushed with 2–3 mL of air. Lf eluted from the AAC with 2 mL of elution buffer (0.05 mol/L $Na_2HPO_4 \cdot 12H_2O$, 1 mol/L NaCl, pH = 8.0). Eluent filtered through a 0.2 µm syringe filter	Detector: UVD, column: Xbridge Protein BEH C4 300 Å (250 × 4.6 mm, 5 µm), mobile phase: A—0.1% trifluoroacetic acid, B—acetonitrile, gradient elution, flow rate: 0.5 mL/min LOD: 0.000003 mg/mL LOQ: 0.00001 mg/mL	Wang N et al. (2021) [100]

Table 2. *Cont.*

Matrix	Methods	Result	Characteristic of Analytical Method		References
			Sample Preparation	Conditions and Validation Parameters	
Skim milk	IEC RP-HPLC SDS-PAGE	Optimised an industrial chromatographic process for separating Lf from skim milk	Experiments for model development and validation were conducted on an Akta avant 150 (Cytiva®, Sweden). The column was packed with SP Sepharose Big Beads Food Grade strong cation exchanger. The developed model was used for in-silico process optimisation of a two-step elution process, which is typically used in commercial Lf manufacturing	IEC—Column exchange chromatography: SOURCE 15STricorn 5/50 filled with 1 mL of SOURCE 15S resin, buffer A: 50 mM Tris, pH = 7.5, B: 1 M NaCl, 50 mM Tris, pH = 7.5 RP-HPLC—buffer A: MilliQ water, 0.1% TFA, B: Acetonitrile, 0.1% TFA, gradient elution, flow rate 1 mL/min SDS-PAGE—Sodium dodecylsulphate–polyacrylamide gel electrophoresis under reducing conditions on pre-cast BoltTM 12% Bis-Tri Plus 1.00 mm X 12 well gels as per manufacturer recommendations. LOD: no data LOQ: no data	Gerstweiler L et al. (2023) [101]
Milk	Spectrophotometry	Used hybrids constituted of nanomaterials, surface active maghemite nanoparticles, and DNA to bind Lf	Milk samples skimmed by centrifugation at 4000 g for 15 min at 4 °C and then ultra-centrifuged. Bovine whey incubated in the presence of SAMN@DNA (0.5 g/L) and mixed for 2 h at room temperature, leading to the formation of a SAMN@DNA@LF complex. SAMN@DNA@LF hybrid released by incubation in a high ionic strength solution (4 M NaCl) for 1 h at 4 °C. The eluted Lf solutions concentrated using Vivaspin tubes	Spectrophotometry at 280 nm, Lf in the 0.05–0.6 mg/mL concentration range. LOD: no data LOQ: no data	Cecconello A et al. (2024) [102]

Table 2. *Cont.*

Matrix	Methods	Result	Characteristic of Analytical Method		References
			Sample Preparation	Conditions and Validation Parameters	
Camel milk	UHPLC-MS/MS	Determined Lf from camel milk	Camel milk adjusted to pH = 4.6 with acetic acid, centrifuged; aliquots of 100 µL of whey spiked with 100 µL of 1 µM stable isotope-labelled internal standard and mixed with 180 µL of 50 mM NH_4CO_3. reduction by adding 15 µL of 500 mM TCEP solution at 50 °C /30 min; alkylation in the presence of 45 µL of 500 mM IAA solution; 10 µL of 100 mM $CaCl_2$ solution and trypsin added, incubated at 37 °C overnight; terminated by adding 10 µL of formic acid; filtered through a 0.22 mm nylon filter	Column: UPLC BEH C18 (100 mm×2.1 mm, 1.7 µm, 300 A), Mobile phases A: Milli-Q water + 0.1% ammonia, B: acetonitrile, gradient elution, and flow rate of 0.3 mL/min Electrospray ion source of the MS, capillary voltage: 3.50 kV, cone voltage: 15 V, desolvation gas: nitrogen, flow rate of 850 L/h LOD: 0.0038 mg/g LOQ:0.011 mg/g	Li X et al. (2019) [111]
Formula powder, paediatric/adult nutritional powders	UHPLC/UV	Developed and validated a method for detecting Lf in nutritional formula powder.	0.5–1.50 g sample + 11.5 mL warm (40 °C) 0.2 M sodium phosphate (pH = 8.0), shaken for 60 min, centrifuged for at least 20 min at 8000 g at 4 °C	Column: BEH C4 column (4.6 × 150 mm, 3.5 µm), mobile phase A: 0.1% trifluoroacetic acid solution, B: 0.1% trifluoroacetic acid in acetonitrile solution, flow rate: 0.5 mL/min, gradient elution LOD: 0.002 mg/g LOQ: 0.04 mg/g	Frueh JL et al. (2024) [105]
Whey	RP-HPLC-DAD	Developed and validated a method for detecting Lf in whey	Whey samples centrifuged at 15,000× *g* for 10 min (at room temperature) to remove aggregates and micelles	RP-HPLC. Detector: DAD, column: BioResolve RP mAb 450 A Polyphenyl column (2.7 µm, 150 × 4.6 mm), mobile phase A: 99% double distilled water + 1% Acetonitrile + 0.1% of the ion-pairing agent trifluoroacetic acid, B: 99% ACN and 1% AqDD added with 0.072% TFA, gradient elution flow rate: 0.8 mL/min LOD: 0.006 mg/mL LOQ: 0.019 mg/mL	Ostertag F et al. (2021) [106]

Table 2. *Cont.*

Matrix	Methods	Result	Characteristic of Analytical Method		References
			Sample Preparation	Conditions and Validation Parameters	
Whey protein concentrate	Anion exchange chromatography	Improved isolation and purification of Lf (N-glycans) from whey protein concentrate by anion exchange chromatography	SDS-PAGE: the protein concentration was diluted and mixed with 5× loading buffer to reach a concentration of 4 mg/mL, heated at 95 °C for 5 min, and then loaded onto the gel, next run with 5% stacking gel, and 12% separating gel. The running buffer contains 0.05 M Tris, 0.19 M glycine, and 0.1% (*w*/*v*) SDS. Pre-stained protein marker was used for the quantification and molecular weight determination of the protein bands The electrophoresis was run through stacking gels and separating gels at a constant voltage of 80 V and 120 V, respectively. After that, the gels were stained with Coomassie Brilliant Blue R250 for 0.5 h and decoloured overnight	DEAE (diethylaminoethyl) Sepharose Fast Flow column (90 µm particle size) with Advanced Kinetic and Transport Analysis pure system LOD: no data LOQ: no data	Wu X et al. (2024) [107]
Lactoferrin powder	Capillary electrophoresis	Optimised the parameters of uncoated capillary electrophoresis as a perspective for separating Lf	Sample dissolved in buffer and filtered through a 0.22 µm filter	Different tests. Capillary electrophoresis apparatus: uncoated fused capillary, inner diameter of 50 µm, outer diameter of 75 µm, length of 61.2 cm, actual length of 51.2 cm. The best buffer system was determined to be 50 mmol/L phosphates with 6 mol/L urea. The auxiliary pressure of 0.5 psi LOQ: 0.04 mg/mL LOD: 0.01 mg/mL	Chen H. et al. (2021) [112]

Table 2. *Cont.*

Matrix	Methods	Result	Characteristic of Analytical Method		References
			Sample Preparation	Conditions and Validation Parameters	
Capsules, sachets, milk powder	Spectrofluorimetry	Determined Lf by spectrofluorimetry without using toxic reagents	Lacto-Apex® capsules. 10.0 mg of powder. Seventy millilitres of methanol were added, and the flask contents underwent sonication for 10 min, filtered into a 100 mL measuring flask, and further serial dilutions were performed to get the range of study. The steps for constructing the calibration curve. For sachets, Lineal® sachets labelled to contain 100 mg Lf/3 g were used, where a weight of the sachet powder corresponding to 10.0 mg Lf was transferred into a 100 mL measuring flask, and then the procedure mentioned above was followed. 0.5 g of milk powder + methanol to 5.0 mL, sonicated/1 h, followed by 10 min of centrifugation at 8000 rpm. 1 mL of the supernatant transferred to a 5 mL measuring flask + completed to the volume with methanol to obtain the final concentrations of LTF (2.0, 4.0, 6.0, 8.0, and 10.0 µg/mL)	Spectrofluorimeter: Xenon flash lamp operated at 800 V, slit width = 5 nm, smoothing factor of 20, excitation: 230 nm, emission: 337 nm LOD: 0.00002 mg/mL LOQ: 0.000082 mg/mL	Magdy G et al. (2024) [103]

Table 2. *Cont.*

Matrix	Methods	Result	Characteristic of Analytical Method		References
			Sample Preparation	Conditions and Validation Parameters	
Human milk (lactoferrin lyophilisate)	HPLC Infrared spectroscopy (FTIR) SDS-PAGE electrophoresis	Obtained a lyophilisate of purified Lf by using a heparin affinity column and determined by the HPLC method	Pre-treatment of the milk: Centrifuged at 4.000 rpm/20 min. Sample extraction: 5 mL of sample + 5 mL of buffer 0.2 M Na_2HPO_4 (pH = 8), mixed for 30 s, centrifuged at 4.000 rpm/20 min at 4 °C Lf purification: 1 mL HiTrap Heparin affinity column (conditioned with 5 mL of 0.2 M Na_2HPO_4 buffer (pH = 8), addition rate of 1 mL/min. The extraction solution: injected into the column, followed by a wash with 10 mL of buffer 0.2 M Na_2HPO_4 (pH = 8). Lf eluted with 3 mL of buffer 0.05 M Na_2HPO_4 and 1.0 M NaCl (pH = 8) Freeze-drying: eluted fractions taken to an ultrafiltration cell, passed through a 30 kDa membrane pressure of 60 psi, performing two washes with 0.05 M NaCl Lyophiliser at 0.147 mBar/6 h	Detector: DAD Column: Kinetex XB C18 (15 × 4.6 mm, 5 µm) Mobile phase: 0.1% trifluoride acetic acid in water, acetonitrile, gradient elution, flow rate 0.6 mL/min Infrared Spectroscopy: Cary 630 IR spectrophotometer analysed with a resolution of 2 cm^{-1}, 32 scans, range of 400 to 4000 cm^{-1}. SDS-PAGE electrophoresis: denaturing and reducing conditions 40 µL of protein in solution in 10% polyacrylamide gel wells. Run at 150 V in a Hoeffer Mighty Small 250 chamber LOQ: 0.004 mg/mL LOD: 0.001 mg/mL	Parra-Saavedra et al. (2022) [108]
Human milk	Latex agglutination assay ELISA	Developed a cheap and fast method for determining Lf in human milk	Milk samples diluted 100-fold (samples with values exceeding 16 µg/mL diluted 200-fold); 4 µL of diluted milk + 100 µL of buffer solution + 100 µL of polystyrene latex particles coated with anti-human LF mouse monoclonal antibody ELISA, milk samples centrifuged 1/50,000 sample dilution preparation: 5 µL of sample + 495 µL of diluent solution; 2 µL of the 1/100 sample + 998 µL of diluent solution	Absorbance measurement: 600 nm, 30 s, and 5 min after adding latex particles The concentration of Lf was measured using a Human Lf ELISA Kit LOD: 0.0002 mg/mL LOQ: 0.02 mg/g	Tanaka M et al. (2024) [109]

Table 2. *Cont.*

Matrix	Methods	Result	Characteristic of Analytical Method		References
			Sample Preparation	Conditions and Validation Parameters	
Colostrum	Electroanalysis electrochemical quartz crystal microbalance (EQCM) Single radial immunodiffusion (SRID)	Determined Lf in colostrum	- Samples centrifuged (3000 rpm/5 min) - Sample diluted (1:100) in 0.1 M PBS (phosphate buffer solution) containing 3.0 mM $[Fe(CN)_6]^{3-/4-}$ electroanalysis - Samples diluted	Electroanalysis: Autolab electrochemical analyser. LBP as a working electrode, Ag/AgCl reference electrode, and platinum rod counter electrode are used for all electrochemical investigations. 0.1 M PBS (pH = 7.0) solution containing 3.0 mM [Fe(CN)6]3-/4-redox probe. CV: range of −0.2 to +0.6 V; scan rate of 100 mVs−1. DPV: range of −0.1 to +0.4 V; scan rate of 10 mV s^{-1}. The impedance spectra were recorded at an open-circuit voltage in the frequency range of 100 kHz to 1 Hz and an alternating voltage amplitude of 10 mV Electrochemical quartz crystal microbalance: The module is connected to a PGSTAT 302N potentiostat/galvanostat, and the working electrode is a gold-coated AT-cut quartz crystal sensor with a fundamental resonant frequency of 6 MHz. A gold wire counter electrode and a KCl-saturated gel electrolyte Ag/AgCl reference electrode were used to complete the electrochemical circuit Single radial immunodiffusion: Specific antibody and 1.2% agar in 0.005 M barbital buffer (pH = 7.3), incubation: 37 °C for 15–20 h LOD: 65.2 nM LOQ: no data	Ebrahimi F et al. (2022) [110]

AAC—aptamer affinity column; DAD—diode array detector; IAA—iodoacetamide; IEC—ion-exchange chromatography; FL—fluorescence; RP-HPLC—reversed-phase high-performance liquid chromatography; TCEP—Tris (2-carboxyethyl) phosphine hydrochloride; UHPLC—ultra high-pressure liquid chromatography; UV—ultraviolet; LC-MS/MS—liquid chromatography tandem spectrometry mass; UVD—ultra-variable-pressure detector.

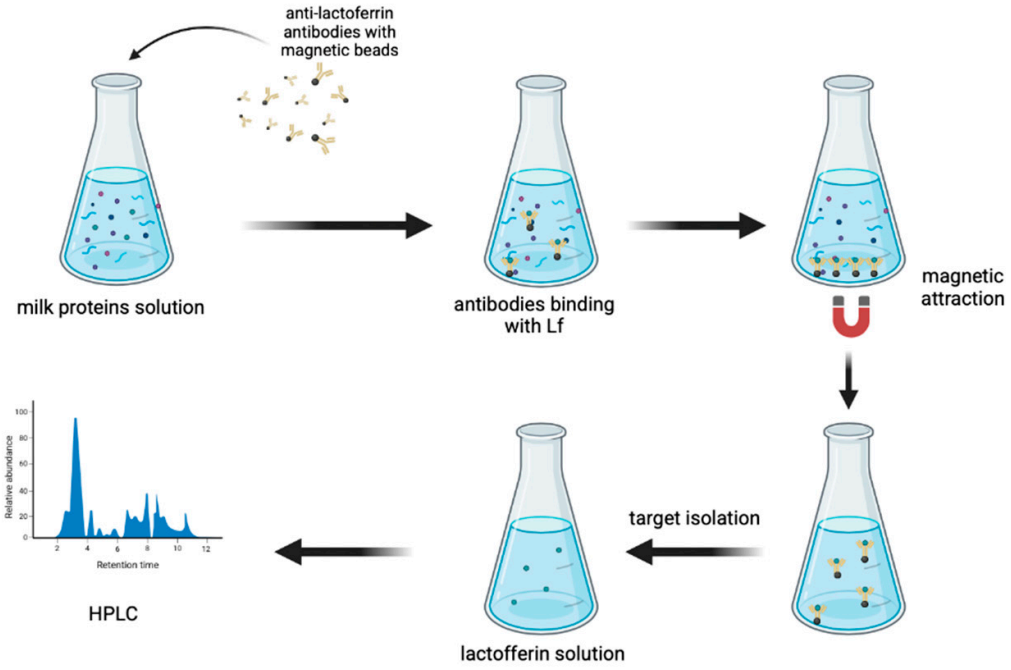

Figure 4. Lf isolation with magnetic beads.

Gerstweiler et al. described model-based process optimisation of an industrial chromatographic process for separating Lf from skimmed milk. The authors point out that besides a high purity, achieving high Lf productivity and recovery is equally important. Several parameters determine achieving good quality Lf, such as the Lf concentration in the source material, residence time, resin dynamic binding capacity, and elution conditions, e.g., conductivity, pH, and contact time. The General Rate Model, including film diffusion, pore diffusion, and surface diffusion, was combined with the Steric Mass Action model to describe the protein binding to improve production parameters. The disadvantage of using this model is the high costs, but they may be offset by the improved production output [101].

Cecconello et al. used hybrid nanomaterials, surface-active maghemite nanoparticles, and DNA to bind Lf. The purity of the Lf was estimated by SDS-PAGE to be well above the commercially available Lf. This magnetic purification method preserved the Lf structure and specifically recognised Lf in a short time (1 min incubation). An automatic modular pilot system was applied for the continuous magnetic separation and Lf purification, which are suitable for industrialisation [102].

Lf obtained from camel milk also has similar beneficial health properties to that isolated from cows, but not all methods are equally suitable for obtaining Lf from camel and buffalo milk due to differences in the amino acid sequences. Therefore, Li et al. developed and validated a method for determining Lf in camel milk by ultrahigh-performance liquid chromatography–tandem mass spectrometry using an isotope-labelled winged peptide as an internal standard. Camel Lf had to be first purified on a heparin affinity column and confirmed by SDS-PAGE. Then an internal standard was chosen for a specific sequence, ionisation, and a lack of cysteine/methionine. This allowed the winged peptide internal standard to be synthesised and used to determine camel Lf after trypsin digestion. The method had reasonable specificity, sensitivity, repeatability, and precision [111].

Frueh et al. developed and validated an Lf production method using heparin affinity extraction and reverse-phase HPLC/UV capable of meeting the AOAC INTERNATIONAL standard method performance requirements, which had good repeatability (RSD: 2.0–4.8%), recovery (92.1–97.7%), and analytical range (4–193 mg/100 g). This method enables the

determination of only bovine Lf in powdered food products intended for feeding both infants and adults [105].

Most Lf is obtained from buffalo milk, but some is extracted from whey, a by-product of cheese production; thus, interest is growing in the use of this potential abundant source. Ostertag et al. developed a validated RP-HPLC DAD method to simultaneously quantify minor and major whey proteins. This method had an accuracy of 95–103%, precision >96%, recovery of 96–102%, peak homogeneity and linearity R2 $\geq$ 0.990, and limit of quantification (LOQ) < 24 mg/L [106].

Wu et al. also described the isolation and purification of Lf (N-glycans) from whey protein concentrate by ultrafiltration. They improved the ultrafiltration separation by changing the feed flow rate (10 rpm) and molecular weight cut-off (100 kDa membrane) [107].

Chen et al. optimised uncoated capillary electrophoresis to detect Lf, reporting that the optimal parameters were pH 4.0, phosphate concentration of 50 mM with 6 M urea, auxiliary air pressure of 0.5 psi, limit of quantification of 0.04 mg/mL, and detection limit of 0.01 mg/mL. This study provides a future perspective for separating LF by capillary electrophoresis [112].

Magdy et al. validated a spectrofluorimetric method for Lf determination. The advantage of this method is Lf determination in powdered products, such as infant milk and supplements, without using toxic reagents. This method had a concentration range of 0.1–10.0 µg/mL with quantitation and detection limits of 0.082 and 0.027 µg/mL, respectively [103].

Human milk contains more Lf than cow's milk and is used for premature babies due to its highly beneficial bactericidal effect. Parra-Saavedra et al. developed a strategy to obtain Lf from breast milk using a heparin affinity column by HPLC. They achieved a coefficient of determination over 0.99. This method also enables maintenance of the molecules' integrity intact, allowing human Lf to be used in pharmacological applications [108].

Preterm infants are also fed whole human breast milk, so Lf concentrations are routinely measured by ELISA, a rather expensive and laborious method. Therefore, Tanaka et al. developed and validated a new latex reagent for measuring Lf in human milk using the latex agglutination method, a cheaper and faster method for quantifying Lf [109].

Ebrahimi et al. used impedimetric and single-frequency capacitance spectroscopy for label-free rapid Lf screening. This system was characterised by the good stability of the Lf biosensing platform, suitable sensor reproducibility, and long Lf life sensor compared to antigen enzyme-based Lf sensor; thus, it is a good method for determining Lf in colostrum [110].

3.1. Conclusion Remarks

Modern research related to Lf focuses not only on its health-promoting properties on the human body, but also on its production, mainly purification from raw materials, e.g., milk, and the analysis of its content. During the analysis and experiments of the purification process, the authors pay attention mainly to the efficiency, ergonomics, and purity of the obtained Lf, including residues of post-production substances. During purification, raw materials contain many components, e.g., proteins, lipids, polysaccharides, and others, which have an unfavourable effect on the process itself [113]. In the case of methods for determining the presence/content of Lf, the costs of reagents and equipment, time, and validation parameters such as limit of detection and limit of quantification become an important aspect [88]. The efficacy of novel techniques is shown in Table 3.

Table 3. Efficacy of novel techniques of obtaining Lf.

Matrix	Method	Yield/Recovery	Study
Liquid milk, fermented milk, infant formula	UHPLC-UV	Recovery: 83.6–90.8%	Wang M et al. (2022) [97]
Infant formula, milk powder, powder reconstituted ready-to-feed basis	Optical Biosensor Immunoassay	Recovery: 96.1–109.2%	Gill BD et al. (2022) [98]
Milk, infant formula, whey protein concentrates (WPC)	HPLC-FL	Recovery: 96.3% for milk, 95.8% for infant formula, 93.3% for WPC	Pang J et al. (2020) [99]
Raw milk	HPLC-UVD	Recovery: 103.44%	Wang N et al. (2021) [100]
Skim milk	IEC, RP-HPLC, SDS-PAGE	Recovery: up to 100%	Gerstweiler L et al. (2023) [101]
Milk	Spectrophotometry	Yield: ≥98%	Cecconello A et al. (2024) [102]
Capsules, sachets, milk powder	Spectrofluorimetry	Recovery: 96.45–104.92%	Magdy G et al. (2024) [103]
Infant formula	LC-MS/MS	Recovery: 91.7–96.4%	Ellingson DJ et al. (2019) [104]
Formula powder, paediatric/adult nutritional powders	UHPLC/UV	Recovery: 92.1–97.7%	Frueh JL et al. (2024) [105]
Whey	RP-HPLC-DAD	Recovery: 96–102%	Ostertag F et al. (2021) [106]
Human milk	HPLC, Infrared Spectroscopy (FTIR), SDS-PAGE electrophoresis	Recovery: 70%	Parra-Saavedra et al. (2022) [108]
Human milk	Latex agglutination assay, ELISA	Recovery: 90–120%	Tanaka M et al. (2024) [109]
Camel milk	UHPLC-MS/MS	Recovery: 74.5–103.6%	Li X et al. (2019) [111]

For comparison, the patented cation-exchange chromatography, that is, used for mass scale production, and the yield is 80–96% (Patent No. US5596082A) [114].

The use of every matrix requires a standardisation protocol to optimise the production. The most efficient methods of determining Lf from liquid milk turned out to be the Optical Biosensor Immunoassay and HPLC-UVD. The best results based on whey as a matrix were obtained with ultrafiltration, and for human milk, heparin-affinity columns. Nevertheless, there are some challenges to implementing those methods on a mass-scale production. We covered this topic in Section 3.1.3 of this paper.

3.1.1. Conclusion Remarks

Most of the reviewed methods used bovine milk as a source material. This is due to the fact that cow lactoferrin can reach the widest spectrum of recipients. Supplements containing lactoferrin are based on bovine Lf and can be used by most people. Other

sources of Lf are less frequently studied, due to the limited group of recipients of the final product. Camel lactoferrin, although having very similar characteristics to cow lactoferrin, will not be as widely distributed due to the limited areas where camels are bred. It may therefore be a good source of Lf in narrow areas of camel farms, but cow farms are more widespread. Lactoferrin obtained from human milk is used to supplement newborns and infants. It is the safest source of Lf for such young children, which is why some studies focus on human milk. Nevertheless, human milk is a highly valued product with very limited resources. These features make it a less frequently used source and, furthermore, not used in studies as often as cow's milk.

The most popular preparation and purification methods used buffers, inorganic reagents, centrifugation, magnetic separation, and filtration through membranes or filter columns, e.g., HiTrap Heparin or strong cation exchanger (Sepharose Big Beads). The most common solution to dilute source products was sodium phosphate, with the pH of the dilutions adjusted to 7–8 [99,100,105,109]. Centrifugation was an essential step to remove fat from the samples heated, vortexed, incubated, or further diluted with designated buffers depending on the method. Commonly used solutions included ultrapure water, acetonitrile, and ammonium chloride [97,104,111]. Typically, the solutions were filtered to remove any impurities using nylon or polyethersulfone 0.22–0.2 µm thick filters [97,103,111].

Magnetic microbeads coated with anti-Lf antibodies were also used to elute pure Lf [99]. This method involves using magnetic beads coated with anti-Lf antibodies. The protein bound to its antibody, beads were attracted with a magnet, and the rest of the solution could be discarded, leaving pure Lf to be eluted with fresh buffer [99]. Some methods also involved sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) to produce Lf [107]. This method requires a specific type of sample preparation. In SDS-PAGE electrophoresis, the migration of proteins is independent of their electric load, so the samples have to be treated with SDS (sodium dodecyl sulphate) and denatured at high temperatures in order to enable the process [107].

3.1.2. Determination and Identification of Lactoferrin

The most common currently used methods to identify and determine the content of Lf were high-performance liquid chromatography (HPLC) with an ultra-vis (UV) [97,105], fluorescent (FL) [99], ultraviolet (UVD) [100], or diode array detector (DAD) [109], reversed phase high-pressure liquid chromatography (RP-HPLC) [101] with a DAD [106] and HPLC coupled with dual mass spectrometry (MS/MS) [104,111]. Only two types of chromatographic columns were used for HPLC: C4 or C18 with the mobile phase water and acetonitrile, with formic acid or trifluoroacetic acid with gradient flow. Most authors using HPLC reported method validation parameters, including LOD and LOQ. The lowest LOD and LOQ were reported using UHPLC-UV, 0.0002 g/1 g and 0.0006 g/1 g, respectively [100]. In contrast, the highest values were obtained by HPLC-FL, 0.25 mg/1 mL and 0.8 mg/1 mL, respectively [99]. However, this method can distinguish intact Lf from its denatured form compared to high-sensitivity LC-MS/MS, which does not have such capabilities [99,104]. Despite various instrument limitations (e.g., price), liquid chromatography methods are the most popular for detecting Lf due to their high specificity and accuracy. Currently, the less popular but still used method, ion exchange chromatography, is being replaced by newer methods [101] because of the high running costs resulting in a variable and relatively low Lf yield [115]. Impurities can also be introduced to the final product, requiring more purification steps [116].

Despite the great popularity of Lf determination by chromatographic methods, many authors used other methods:

- optical biosensor immunoassay [98] with an immobilised anti-Lf antibody, which offers the advantages of simpler sample preparation and estimation of intact physiologically active protein. It has been implemented in the New Zealand dairy industry for 18 years.
- Image J software to determine the intensity of the protein bands after SD-PAGE [107].
- capillary electrophoresis, which is a good alternative for determining the content of obtained Lf, but is affected by the system pH, surfactant, organic solvent, buffer system, and auxiliary air pressure [103].
- Fourier transform infrared spectroscopy (FTIR) with an attenuated total reflectance (ATR) attachment [109]. Infrared spectroscopy measures the absorption of infrared radiation by each bond in a molecule and results in a spectrum that is commonly defined as % transmittance per wavenumber (cm). The ATR mode is simple compared to the conventional transmission model [117].
- spectrofluorimetry to determine the Lf content of pharmaceutical preparations (capsules, sachets) and milk powder, obtaining a low LOD: 0.00002 mg/mL and LOQ: 0.000082 mg/mL. The authors indicate that the availability, sensitivity, durability, and affordability of this method make it better than routine Lf analysis [108].
- the latex agglutination assay based on the agglutination that occurs when shiny latex beads come into contact with antigens or filters. It is a very quick and simple method for determining Lf with very good validation parameters [110].
- electroanalysis, electrochemical quartz crystal microbalance (EQCM), and single radial immunodiffusion (SRID). The EIS results are within a linear range with an LOD of 125 nM to 3250 μM and 65.2 nM, respectively [118]. The application of impedance and single-frequency capacitance spectroscopy was successfully demonstrated for Lf determination and shortened analysis time.

The remaining methods for determining Lf were relatively simple, such as spectrophotometry at a wavelength of 280 nm, but they required the use of more precise purification methods [102].

3.1.3. Industrial-Scale Challenges

Although the latest described methods can significantly improve Lf production, there are some limitations to implementing them on an industrial scale. Firstly, not all methods accommodate different matrices. Immunoaffinity magnetic purification used by Pang et al. is limited by the pH level of the source material, thus, cannot use yogurt, which is a product with a low pH [99]. In methods using antibodies, these particles are specific to one protein. Both the Optical Biosensor Immunoassay and Isotope-Labelled Winged Peptide method require matrix-specific antibodies, which make them applicable only to one source material [98,111]. Another issue is the need for frequent changes of either a part of the apparatus or the materials used. Wu et al. used ultrafiltration and anion exchange chromatography on the DEAE Sepharose Fast Flow column to isolate and purify n-glycans (including Lf) from whey protein concentrate with effective results. Nevertheless, this process caused clogging of the membrane after long running, as a layer of gel formed, due to the effect of concentration polarisation and worsened the filtration efficiency [107]. Similarly, an aptamer affinity column used by Wang et al. could be used 10 times before losing the efficiency of the process, then had to be changed [100]. In the boron-doped titania method, the sorbent lasted for 5 cycles and needed to be replaced [115], and the colloidal ɤ-Fe2O3-DNA hybrids proposed by Cecconello et al. for a large-scale Lf purification could only run for two cycles before replacement [102]. Some of the presented methods are still too time-consuming to be applicable for mass production. Optical Biosensor Immunoassay conducted by Gill et al. required calibration before running each experiment [98]. The

HPLC performed by Li et al. involved a time-consuming sample preparation, together with the Boron-doped titania method, which needed a long process of extraction and desorption of samples [111,114]. Another example was a validation process that required multiple gradient elution experiments to implement the optimisation proposed by Gerstweiler et al. [101]. Due to being a novelty, some methods need very specific conditions to run effectively, which can be difficult to achieve in a large-scale production site. Chen et al. proposed a very efficient method. Still, it required a simultaneous match of many variables to perform it [112]. Similarly, Gerstweiler et al. had very high recovery rates, but fitting all parameters at once turned out to be impossible [101]. Mass-scale production calls for a relatively low-cost process to meet the demand of buyers, which means that the elements required have to be largely available. Tanaka et al. came up with an innovative technique that gave effective results but needed very specific, costly elements (polystyrene latex particles coated with anti-human LF mouse monoclonal antibody) [109]. Ebrahmi et al.'s impedimetric technique was also efficient, but the cost of pure gold electrodes might exceed a rational budget [110]. In the uncoated capillary electrophoresis performed by Chen et al., the capillaries had to be changed, which is a high cost, considering an industrial-scale process [112]. Lastly, producers have to take the safety of the production site into account. Methods like the Heparin Affinity Extraction with HPLC/UV proposed by Frueh et al. can be problematic due to toxic and potentially dangerous chemicals used in the process [105].

These are the method-specific obstacles that need to be addressed before implementing on a mass scale.

Current Lf determination methods are characterised by high precision, and most methods produce high-quality Lf. It is necessary to develop highly effective techniques for sample preparation, not only in the laboratory but also on an industrial scale, which may be a bigger challenge than Lf determination in samples. There is a need for further research on Lf, especially the production techniques and analysis of Lf content, as well as their validation methods.

4. Conclusions

The current lactoferrin production techniques are characterised by increased efficiency and quality, but they require standardisation of the purification process depending on the matrix.

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