MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society For Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

ISOLATION OF BIOACTIVE MILK PROTEINS: A REVIEW

Mohd Ezuan Khayat [1,3*], Aisamuddin Ardi Zainal Abidin [1] and Fadzlie Wong Rizal Wong [2,3]

[1]*Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.*
[2]*Department of Bioprocess, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.*
[3]*Bioprocessing and Biomanufacturing Research Center, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.*

*Corresponding Author: m_ezuan@upm.edu.my

History
Received: 4th March 2020
Accepted: 19th July 2020

Keywords:

Dairy product; milk; protein; bioactivities; isolation.

Abstract

Proteins are one of the major milk components that are diverse in constructions, structures and functional properties. Based on available evidence, individual milk proteins confer a wide range of potential health benefits, thus, attracting interests from both researchers and manufacturers to develop the most optimal methods for their isolation. The aim is to obtain an optimal yield of proteins with the highest purity in the shortest time. Due to the structural diversity and varying protein stability, a specific method for the isolation of each milk proteins may be needed. Over the past decades, many techniques have been assessed to isolate milk proteins such as filtration, precipitation and chromatographies. Most of the milk proteins require the incorporation of more than one technique to be successfully isolated. The advent of technology has also improved isolation processes. This review aims to present the current knowledge on the development of methods for isolating milk proteins. The progression of the isolation techniques is expected to obviate problems associated with the underutilisation of milk proteins.

1. INTRODUCTION

Milk is a complex and complete food that contains essential nutrients such as proteins, oligosaccharides, lipids and minerals. Conventionally, it provides the nutritional need for the mammalian neonate during its early phases of life [1]. However, milk's role has expanded beyond the nutritional values as it constitutes a broad range of bioactive compounds with a significant role in human metabolism [2]. Bioactive compounds found in foods may exert physiological and biochemical activities which could be consumed by humans through the intake of conventional food, functional foods or dietary supplements [3]. The bioactive properties of milk compounds can be categorised into four main areas which include gastrointestinal development, activity and functions; immunological development and functions; infant development; and microbial activity, including antibiotic and probiotic activities [3]. Hence, each compound may exert multiple bioactive properties. It is also worth noting that some compounds are present naturally in their original forms and are ready for further application following isolation, while others may require pre-treatment (e.g., proteolytic digestion) to liberate the specific compounds (e.g., peptides).

Many studies conducted to date on the health benefit of food components led both food manufacturers and consumers to develop a better understanding of the relationship between food and human health. This relationship has steered the expansion of the global nutraceutical market which is projected to be worth US$ 465,709.8 million by 2027 [4]. The growth of the market is primarily driven by the increase of consumer awareness on

the importance of maintaining health by consuming functional foods and by emphasising on preventive action rather than treatment of chronic diseases. This attitude has led to the emergence of milk fractionation sector in the nutraceutical industries. Many of the compounds from milk have been exploited in the formulation of dairy, non-dairy food and pharmaceuticals [5][6][7]. These compounds are presumed to promote human health by increasing the immune system, reducing elevated blood pressure, combating gastrointestinal infections, controlling body weight and preventing osteoporosis [5][6][7].

On the other hand, the isolation techniques of the milk components are dependent on the type of compounds. Milk compounds are conventionally separated using size-based separation method. For instance, sugars like lactose and oligosaccharides are small biomolecules, hence, their isolation processes are rather straightforward like dialysis, membrane filtration and liquid chromatography [8]. The lipid fraction of milk can be separated via centrifugation. As for vitamins, another small molecule, are usually isolated by liquid-liquid extraction, high-performance liquid chromatography (HPLC) and magnetic solid-phase extraction (MSPE) [9]. Proteins which are the primary components of milk are relatively more challenging to be isolated compared to the previous three compounds. A majority of the milk proteins are made up of caseins and whey proteins. Caseins in milk are found in form of micelle which consists of two types of casein proteins, namely calcium-sensitive (αs1-, αs2- and β- caseins) and calcium-insensitive (κ-casein) [10]. In contrast, whey proteins are biochemically more diverse compared to caseins which include α-lactalbumin, β-lactoglobulin, immunoglobulins, lactoferrin, lactoperoxidase and growth factors [11][12][13] (Figure 1).

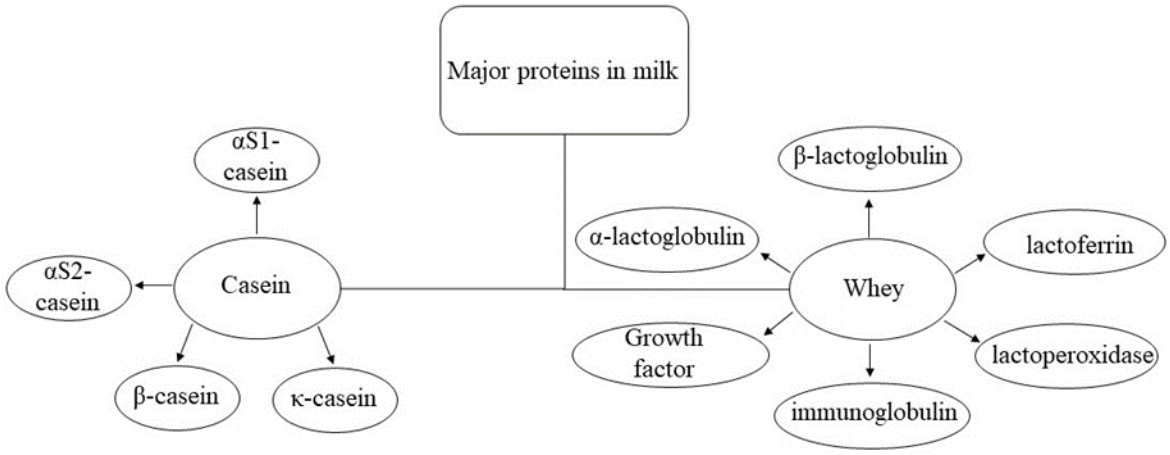

Figure 1: Major bioactive protein from milk.

The recent developments in the isolation and characterization of milk proteins are reviewed in this paper. This review will focus on major milk proteins namely caseins, α-lactalbumin, β-lactoglobulin, lactoferrin, lactoperoxidase, immunoglobulins and growth factors.

2. ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM MILK

2.1 Caseins

Approximately, 80 % of the total milk protein is made up of casein. It is commonly found in the market as sodium casein. Caseins have gained interest as bioactive proteins with functional properties in promoting health such as biodefence mechanism (antibacterial and antiviral properties) [14], opioid receptor ligands inducing naloxone to antagonize antinociception [15], regulating proliferation of lymphocytes [16], production of antibodies and cytokines [17] and close to zero allergenicity to infants compared to human breast milk [18]. Caseins are traditionally extracted from the milk through fermentation which is also known as cheese. Apart from that, casein can also be isolated from whey protein as a liquid by-product of cheese production containing 12 to 20 % of casein glycomacropeptide [19]. Moreover, caseins are purified using chromatography or membrane separation to produce infant formulae. The most developed methods separate caseins in milk using membrane separation such as ultrafiltration (UF) or microfiltration (MF) at low temperatures. It is due to the casein physiochemical properties where αS1-casein, αS2-casein and k-casein coagulate under such conditions [20]. Meanwhile, the traditional method widely used by the milk and dairy industries produce sodium caseinate from the reaction of milk with acid and alkali by heating them together to 31 °C with rennet before the sedimentation process at 4 °C for 48

h [21]. Upon sedimenting, the lower fraction (insoluble fraction) will contain αS1-casein, αS2-casein and k-casein, while the supernatant will contain β-casein. Since the aforementioned steps did not produce highly purified caseins, more efficient separation technologies were developed. A study conducted by Huppertz et al. provided an improvisation to the previous method at an industrial scale [22]. The suggested method consisted of a series of incubation and centrifugation before being filtered from rennet-coagulated pasteurised skim milk. The final product which yielded the total extraction of casein (majorly β-casein) were of good purity based on the urea-PAGE assessment [22].

The current methods of casein purification involve membrane separation. The membranes used in casein isolation/purification can be categorised into microfiltration (MF) [23][24][25][26], ultrafiltration (UF) [27][28][29], reverse-osmosis [30] and nanofiltration (NF) [31][32][33] membranes which are usually made up of ceramic or polymers. Furthermore, studies have suggested to not rely on one separation membrane alone but to incorporate more than one type of membrane separation. Among the types of membranes listed above, the reverse-osmosis membrane is one of the unpopular choices of membrane separation as it only removes water to condense the milk protein concentration. Based on the available literature, most studies utilised MF membrane which is made of ceramic [34], polyvinylidine fluoride (PVDF) [23][25] Sephadex [36] and cellulose [36]. The extensive use of MF might be due to its easy availability and cost-effectiveness compared to UF and NF membranes. In 2014, a patent was published on the separation of β-casein from milk using spiral-wound PVDF membrane equipped with reversible thermal-induced aggregation system which demineralises the β-casein to produce β-casein purity of 90% [23]. However, among all the membrane used in isolating/ purifying caseins, the combination of UF and NF demonstrated a better percentage or concentrated caseins (coupled with dehydration) which improved its stability against degradation during storage [31]. As casein is one of the major bioactive proteins in milk and dairy products, MF was shown to be cost-effective in producing a high amount of pure caseins and is deemed as a good protocol for the industry.

On the other hand, chromatography is often used to assess the purity of the separated caseins. Glantz et al. constructed an assessment tool which can isolate, identify and purify caseins based on a library constructed from ultrafiltrated milk [37]. The study utilised asymmetrical flow field-flow fractionation connected to multiangle light scattering (AsF|FFF-MALS) and refractive index (RI) detection to isolate and purify casein micelles via flow cytometry which is then identified by detectors at 658 nm, 632.8 nm and 280 nm [37]. Usually, purity assessment of caseins methods is performed using polyacrylamide gel electrophoresis (PAGE) and reverse phase-high performance liquid chromatography (RP-HPLC). The sodium dodecyl sulfate-PAGE (SDS-PAGE) and Urea-PAGE are the common types of denaturing PAGE analyses used [22][29][38]. The extracted caseins were compared to purified commercial caseins in the polyacrylamide gel to compare its molecular weight (protein ladder) as well as its production. Additionally, another chromatography method known as RP-HPLC provides a more precise quantitative analysis of the extracted caseins. Most HPLC methods are conducted by injecting samples through different types of columns like C18 or C4 columns which utilise polar solvent mobile phase [25][26][35][36][39] and shodex size exclusion column which utilised phosphate buffer as its mobile phase [40]. Similar to PAGE analysis, chromatography analysis identifies caseins at different peaks (based on the detection at a specific wavelength) compared to the standard purified caseins. Furthermore, the integrity of the molecular structure of caseins can be further characterised by the area of the peaks obtained.

2.2 Whey proteins

2.2.1 α-Lactalbumin and β-Lactoglobulin

Karasu et al. identified that most commercial skim milk from the local supermarket contained 1.9 g/L of α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) [24]. Other studies indicated a higher accumulation of α-LA and β-LG in whole milk (no-fat was removed) which can reach approximately 2 to 3 g/L depending on the cattle breed, diet and stage of lactation [41][42]. α-LA plays a major role in lactose synthase and galactosyltransferase (enzyme functions) with a single strong Ca^{2+} binding site [43]. Meanwhile, β-LG possesses ligand-binding functions that bind to a wide range of ligands [44]. Despite the properties, the separation of α-LA and β-LG gained interest because β-LG is considered as an allergen in infant formulas, hence, only purified α-LA was formulated in infant foods [45]. Extensive studies have been carried out since 1957 to separate or fractionate α-LA and β-LG from milk and dairy products with the utilisation of salt concentration [46]. Since then, many methods have been tested including the manipulation of pH with heating [47], precipitation via acid or isoelectric [48][49], membrane separation [50][51][52] and chromatographies [53]. However, most of these techniques were found only proficient at the laboratory scale.

Having said that, many patents have also been published in regards to α-LA and β-LG extraction and purification using the manipulation of heat and pH [54][55], silica-based anion exchanger [56], UF [57] and calcium-binding ionic exchange resin [58]. Years later, a modified manipulation method of pH and heat by Alomirah & Alli with a series of incubation and centrifugation demonstrated promising results for commercial applications [59]. The authors also separated and purified α-LA and β-LG using pH adjustment and dialysation/ lyophilisation. This method yielded 47-69 % of β-LG fractions with 83 % to 90 % purity, while 44-89

% of α-LA fractions with 86 % to 90 % purity from 60g/L dairy product (whey protein).

The chromatography methods for the separation and purification of α-LA and β-LG are only ideal for preparative scale and not on a commercial scale [50][51]. The types of chromatographies include affinity chromatography [60], cation-exchange chromatography [61], anion-exchange chromatography [62][63], size exclusion chromatography [64], high-performance liquid chromatography [56][65], hydrophobic chromatography [66] and ultra-high pressure chromatography [67]. Based on these chromatography studies, most of their yields and purities were not up to par with the modified manipulation method of pH and heat by Alomirah & Alli except for anion-exchange chromatography [59]. Hence, it was postulated that pressure treatment (principle of chromatography) caused denaturation of β-LG [53]. However, the anion-exchange chromatography by De Jongh et al. yielded efficiency up to more than 80 % with more than 98 % of β-LG purity. In the study, affinity chromatography was also utilised prior to the purification of β-LG from fresh bovine milk using anion-exchange chromatography (Superdex 75 BPG)[62].

On the other hand, membrane separation is also one of the most frequently used methods in separating and purifying α-LA and β-LG from milk and dairy products. It treats the milk with heat (thermal aggregation) followed by membrane filtration [68]. Meanwhile, the conventional practices of α-LA and β-LG separation from milk involve the removal of caseins followed by isoelectric precipitation (precipitating α-LA, while β-LG remained soluble) and fractionation via membrane separation or centrifugation. Fractionation could produce purified α-LA and β-LG in the range of 50-80% and 60-99%, respectively [69]. Furthermore, the development of membrane separation methods has also been established to yield a greater outcome. A study indicated that compared to chromatography, utilisation of membrane separation at micro-size pore (MF) can also yield more than 99% and 90% recovery of α-LA and β-LG, respectively [70]. This observation revealed that membrane separation causes lower denaturation or loss of α-LA and β-LG indicating great potential in commercial applications. Novel positively charged membrane UltracelTM PLC regenerated cellulose UF membrane treated with 3-bromopropyl trimethylammonium bromide exhibited promising improvement of α-LA and β-LG at 87 % and 83 % purity, respectively [50][51]. However, this recovery yield was much lower compared to the previously described MF membrane. Hence, it was suggested that the utilisation of a negative polyethersulfone (PES) UF membrane might improve the recovery process [71]. Attempts to utilise membrane separation at a commercial or large-scale operation was achieved by Toro-Sierra et al. [68]. The study utilised a combination of 1) manipulation of heat and pH (to precipitate/ separate between α-LA and β-LG using citric acid), 2) MF and 3) UF. The pilot-scale study analysed 100 L of whey protein to achieve high overall yields of α-LA (60.7 % to 80.4 %) and β-LG (80.2 % to 97.3 %) with 91.3 % and 97.2 % purity respectively.

2.2.2 Lactoferrin and Lactoperoxidase

Apart from caseins, lactoferrin (LF) and lactoperoxidase (LPO) are the other bioactive proteins present in milk (often in whey), which are known for their antimicrobial properties [72]. LF or formerly known as lactotransferrin is an iron-binding glycoprotein protein which is structured in 2 globular lobes with iron-binding sites in each lobe [73][74]. LPO, on the other hand, is a haemoprotein consisting of a single polypeptide chain [75][76]. It was estimated that the contents of LF and LPO in whey are approximately 0.003% and 0.002% [77], and may vary depending on the cattle breeds [78]. Previous studies revealed a slight weight gain, lower counts of Escherichia coli in faeces, colon and jejunum, together with lower incidence of diarrhoea and higher blood immunoglobulin level in calves fed with milk containing 0.1 % LF and 0.2 % LPO than that of the calves fed with commercial cow milk replacer (LF and LPO content was approximately 0%) [79][80]. Besides that, LF and LPO in milk were also extensively studied in humans and model organisms whereby antimicrobial, antiviral activities, as well as immunomodulatory effects, were identified [76][81]. Apart from their nutraceutical and pharmaceutical potentials, the demand LF and LPO extraction and purification were also high due to their usage in food preservatives. The traditional thermal treatment of foods to kill pathogens and spoilage microorganism can alter the taste or nutritional value due to denaturation of vitamins and volatile compounds [41]. Several studies have assessed the application of LPO in food preservation including dairy products, beverages, desserts and salad dressings [75][82]. Recently, several studies highlighted the potential of LF and LPO as food preservatives with the combination of high-pressure treatments [83][84][85].

The most effective methods to extract and purify LF and LPO are chromatography with some modifications [86]. Many studies employed affinity chromatography [87][88][89], ion-exchange chromatography [77][90][91][92][93], size exclusion chromatography [94][95][96] and HPLC [97][98][99]. Of these, ion exchange and HPLC are widely used method at the moment. Numerous patents have been filed on the isolation and purification of LF and LPO from milk and other dairy products but most of them expired before 2014. Affinity chromatography was one of the earliest methods in LF and LPO separation, whereby a company called Snow Brand Milk Products Co Ltd patented a method using a monoclonal antibody to trap LF and LPO from raw milk [100]. In 1987, the same company filed a different patent for separating LF and LPO from raw milk using membrane separation comprises polysaccharide cross-linked with sulfuric ester, that yields up to more than 95 to 98 % purity [101]. Since then, most studies or patents shifted to ion-exchange

chromatography where greater yield and purity was obtained. Patents published varied based on the types of polymer used, pore diameter, and diameter and length of the column [102][103][104][105]. Although isolation and purification methods of LF and LPO have been well established [86], current studies involved the utilisation of new types of polymers in chromatography [77][87][94]. However, most of the findings were not significantly different from that of the previous studies which could enhance the commercial production of LF and LPO cost-effectively.

2.2.3 Immunoglobulins

According to Dubuisson et al. (2002), cow's milk was identified as one of the top six listed food allergens [106]. Apart from caseins and β-LG which contribute to allergic reactions, milk also contains immunoglobulins. There are five types of immunoglobulins found in mammals include IgA, IgD, IgE, IgG and IgM. It was identified that immunoglobulins in milk contain approximately 1 to 2 % of total milk protein [53], whereby, IgG is one of the major immunoglobulins found in milk [107]. While IgA and IgM are present at low levels [108]. IgG which comprises of two subclasses namely IgG1 and IgG2 constitutes 0.72 mg/ml in milk and 32 to 212 mg/ml in colostrum [108]. Having said that, bovine colostrum and milk have been traditionally used to prevent infection and has been a major research interest for decades [53]. Currently, with the advancement of separation and purification technologies, large-scale production or isolation of IgG have caused a remarkable growth of the dairy industries for nutraceutical food market [109].

Unlike the other bioactive proteins, immunoglobulins from dairy products are sensitive towards processing treatments [110]. A study by Elfstrand et al. revealed a 25 % reduction in immunoglobulins content in whey proteins that were treated with heat and freeze-drying [110]. During the treatment process, IgM was identified to be the most sensitive immunoglobulin since it did not survive (0%) the pasteurisation and freeze-drying treatment. Therefore, separation and purification of immunoglobulins were achieved using high-performance liquid chromatography [99][111], size exclusion chromatography [112] [113], affinity chromatography [107][112][114], ion-exchange chromatography [115][116][117], membrane filtration [110][118], electrophoresis [119][120][121] and immuno-based technique [122][123][124][125][126][127]. Similar to the other aforementioned bioactive proteins in milk, chromatography seems to be the most common method of choice as it has been demonstrated to be practical and accurate for high specific protein purification. However, there is no single method that has been demonstrated to accurately quantify all bioactive proteins in milk simultaneously. Specific chromatography such as size exclusion chromatography, ion-exchange chromatography and affinity chromatography can increase the yield and purity of the immunoglobulins by approximately 94% and more than 95% respectively [53][116].

Additionally, membrane separation is also an effective method in isolating and purifying immunoglobulins. Some methods combined membrane separation with chromatography for a better outcome [53]. For instance, optimisation of immunoglobulins extraction and purification from transgenic goat's milk using MF yielded more than 95% with 15 to 20 % purity [128]. However, further attempts in separating and purifying immunoglobulins from transgenic goat's milk using UF reduced the yield to 80% but increased the purity up to 80% [129]. Nevertheless, a study which utilised both MF and UF successfully extracted immunoglobulins from bovine colostrum at different time scales of milking (80 hours per post after the previous milking) during three different milkings. The IgG1 concentration was estimated at 48 to 120 mg/ml on the first day of milking, 26 to 42 mg/ml (second milking) and 11 to 40 mg/ml (third milking) [110].

On the other hand, electrophoresis is one other common method used to analyse protein structure, expression (quantification) and integrity. Studies have utilised electrophoresis to isolate and purify immunoglobulins at a laboratory scale. One such electrophoresis method is the SDS-PAGE, where IgG, β-lg and α-la were successfully isolated and identified from whey protein through ion-exchange chromatography [99]. Although chromatography has been the method of choice in separating and purifying immunoglobulins, it was not suitable for large scale production [119][130]. Therefore, a novel extraction method which involves electrophoresis was employed using reverse micelles extraction [119]. This method involved the pooling of the immunoglobulins and other soluble proteins into small droplets using electrostatic interaction by creating micelles using surfactant ranging nanometers in diameter [131]. Moreover, Su & Chiang successfully extracted IgG1, IgG2, β-LA and α-LG using anionic surfactant bis(2-ethylhexyl) sodium sulfosuccinate (AOT) as a surfactant and purified them using SDS-PAGE [119].

Lastly, the immuno-based technique is also a preferable analytical technique to assess the purity of immunoglobulins. Immuno-based techniques include radial immunodiffusion (RID), nephelometry, enzyme-linked immunosorbent assay (ELISA), lateral flow immunoassay and surface plasmon resonance (SPR). Due to the diversity of these techniques, a collaborative study was conducted to analyse immunoglobulins. The AOAC published an immunoassay method to determine IgG in bovine colostrum powders, bovine milk powders and dietary supplements containing bovine colostrum products at concentrations of 0.4 to 15% in powder, excluding skim milk powder and milk protein isolate using SPR [122].

2.2.4 Growth Factors

The presence of hormones and growth factors in milk and dairy products are also recognised as important or high-value bioactive proteins. Among the growth factors are the insulin-like growth factor - 1 (IGF-I), IGF-II, platelet-derived growth factor, acidic and basic fibroblast growth factors (FGF) and transforming growth factor-β (TGF-β) [132][133]. Hence, it was suggested that dairy industries could replace the traditional source growth factors in mammalian cell cultures [134]. Moreover, Belford et al. identified that whey-derived extracts (containing the growth factors) may support the growth of distinct cells such as human epithelial cells and myoblasts cells, while the addition of bovine milk may provide other mitogenic factors [132]. On the other hand, growth factor has also been used to treat gastrointestinal disorder, wounds and skin diseases [135]. This review will discuss the isolation and purification of hormones which are considered as one of the high-value products from milk and other dairy products.

Hormones from milk and dairy products can be successfully extracted and purified using chromatography and membrane filtration ([53][110][135][136]. Gauthier et al. indicated that MF poorly separates the growth factor hormones compared to UF [135]. However, Elfstrand et al. utilised both MF and UF to successfully obtain 289 ± 10 ng/ml of TGF-β2 and 590-870 ng/ml of IGF-1 hormone [110]. Furthermore, an attempt for large scale isolation of IGF-1 and IGF-II was found to be promising via alkaline diafiltration with double UF [137]. The diafiltration process utilises a recycling mode that recycles the alkaline buffer as well as the permeate that is collected from the first filter.

Apart from the abovementioned chromatography methods, other types can also be used to separate and purify hormones from milk and dairy products. Most studies hydrolysed raw milk with β-glucuronidase before the clean-up preparation using UF membrane to be injected into the chromatography [138][139][140]. Further optimisation and modification of chromatography to identify more hormones from milk was achieved by utilising the high-pressure liquid chromatography-electrospray ionisation tandem mass spectrometry (LC-ESI-MS) [140]. The study prefiltered enzyme-treated milk using UF before injecting the samples into a C18 ultra-performance liquid chromatography (UPLC) column. The system successfully identified up to 50 different types of anabolic hormones from the milk with repetitive analysis yielding a marginal error of 7.9 to 23.2 % relative standard deviation (RSD). Furthermore, an in-depth study was conducted by Altomare et al. successfully identified 634 proteins (not only hormones) using nano-chromatography system (C18 column) followed by mass spectrometry. Among total protein identified using functional proteomic analyses by STRING software (a database of known and predicted protein-protein interactions), 93 proteins were found to have similar growth factors characteristics in wound healing process [141]. The study possesses tremendous application and permits wider approaches in identifying new potential growth factors hormone or novel hormones for study.

3. CONCLUSION

The isolation of new milk proteins can unravel their health potentials as each protein can fortify and enhance the functional properties of food products. Hence, it has attracted researchers and manufacturers worldwide to develop the best isolation techniques to archive optimal productivity. Most of the isolation procedures involve more than a single technique (Figure 2). Precipitation and filtration are usually employed as a preliminary method to separate and recover a bulk of proteins. The advancement in membrane technology has also yielded better separation of proteins, which can reduce the number of processes to obtain their purest forms. In terms of chromatographic methods, which are the most established methods for high-resolution purification, some innovations have also been reported to reduce the overall cost and improve productivity. These continuous efforts in developing effective isolation techniques for milk proteins can be incorporated into human health.

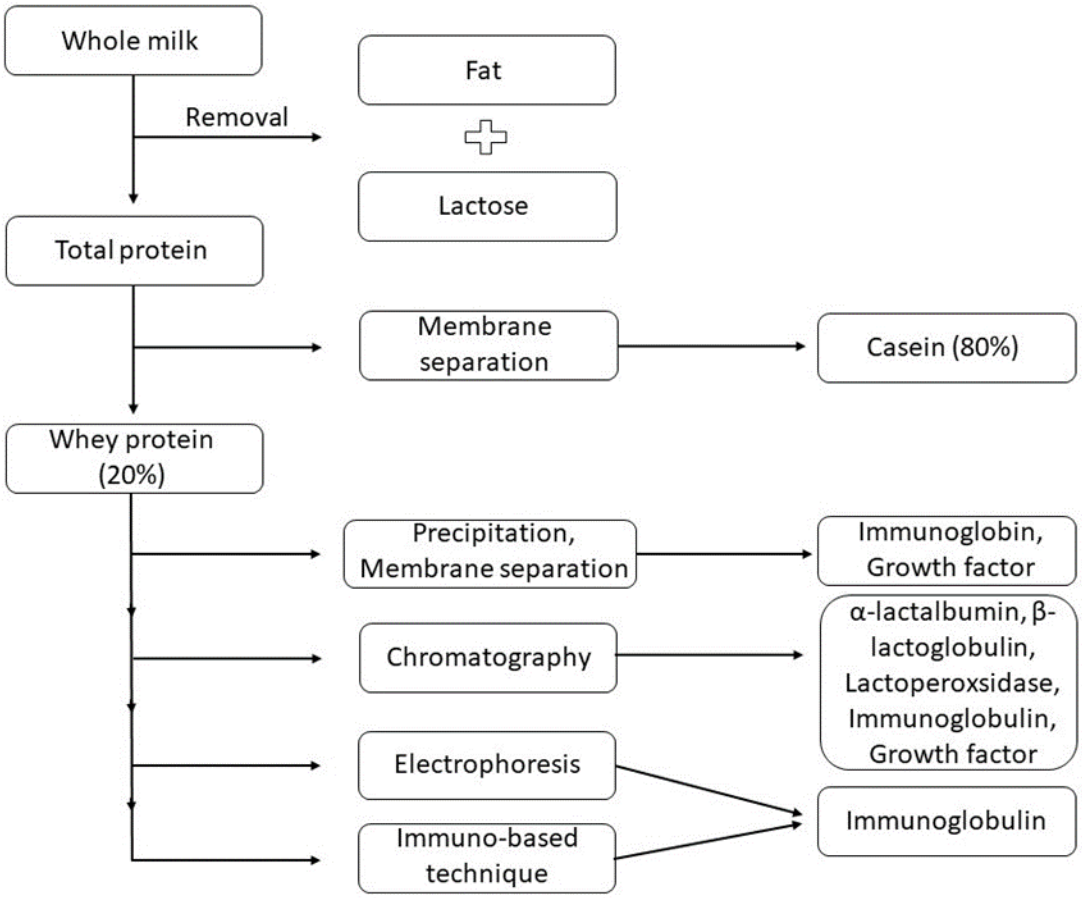

Figure 2: Summary of isolation techniques for bioactive proteins from milk.

REFERENCES

1. Raikos, V.; Dassios, T. Health-promoting properties of bioactive peptides derived from milk proteins. *Dariry Sci. Technol*. 2014, 94(2), 91–101.

2. Park, Y; Nam, M. Bioactive peptides in milk and dairy products: A review. *Korean J. Food Sci*. *An*. 2015, 35(6), 831–840.

3. Gobbetti, M.; Minervini, F.; Rizzello, C. G. Bioactive peptides in dairy products. In *Handbook of Food Products Manufacturing*. Hui, Y. H. Eds.; John Wiley & Sons Inc., New Jersey, U.S.A., 2007; pp. 489–517.

4. Nutraceuticals Market Set for 7.1% Explosive Growth to 2027 to Reach $465,709.8 Million During (2019-2027). Available online: https://3wnews.org/uncategorised/139967/nutraceuticals-market-set-for-7-1-explosive-growth-to-2027-to-reach-465709-8-million-during-2019-2027/ (9 march 2020).

5. FitzGerald, R. J.; Murray, B. A.; Walsh, D. J. Hypotensive peptides from milk proteins. *J. Nutr*. 2004, 134(4), 980S–988S.

6. Korhonen, H.; Marnila, P. Bovine milk antibodies for protection against microbial human diseases. In *Nutraceutical Proteins and Peptides in Health and Disease*. Mine, Y., Shahidi, S., Eds.; Taylor & Francis Group, Florida, U.S.A., 2006; pp. 137–159.

7. Hartmann, R.; Meisel, H. Food-derived peptides with biological activity: from research to food applications. Curr. Opin. Biotech. 2007, 18(2), 1–7.

8. Martinez-Ferez, A.; Rudloff, S.; Guadix, A.; Henkel, C. A.; Pohlentz, G.; Boza, J. J.; Guadix, E. M.; Kunz, C. Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology. *Int. Dairy J*. 2006, 16(2), 173–181.

9. Jiao, Z.; Zhang, Y.; Fan, H. Ultrasonic-microwave method in preparation of polypyyrole-coated magnetic particles for vitamin D extraction in milk. *J. Chromatogr. A*. 2016, 1457, 7–13.

10. Müller-Buschbaum, P.; Gebhardt, R.; Roth, S.V.; Metwalli, E.; Doster, W. Effect of calcium concentration on the structure of casein micelles in thin films. *Biophys. j*. 2007, 93(3), 960–968.

11. Pihlanto, A.; Korhonen, H. Bioactive peptides and proteins. In *Advances in food and nutrition research*. Taylor, S.L., Eds.; Elsevier Inc., San Diego, U.S.A., 2007; Volume 47, pp. 175–276.

12. Korhonen, H. Milk-derived bioactive peptides: From science to applications. *J. Funct. Foods*. 2009, 1(2), 177–187.

13. Pihlanto, A. Whey proteins and peptides. *Nutrafoods*. 2011, 10, 29–42.

14. Floris, R.; Recio, I.; Berkhout, B.; Visser, S. Antibacterial and antiviral effects of milk proteins and derivatives thereof. *Curr. Pharm Des*. 2003, 9(16), 1257–1275.

15. Teschemacher, H. Opioid receptor ligands derived from food proteins. *Curr. Pharm. Des*. 2003, 9(16):1331–44.

16. Sütas, Y.; Soppi, E.; Korhonen, H.; Syväoja, E. L.; Saxelin, M.; Rokka, T.; Isolauri, E. Suppression of lymphocyte proliferation in vitro by bovine caseins hydrolyzed with Lactobacillus casei GG-derived enzymes. *J. Allergy and Clin. Immunol*. 1996, 98(1), 216–224.

17. Bennett, L. E.; Crittenden, R.; Khoo, E.; Forsyth, S. Evaluation of immune-modulatory properties of selected dairy peptide fractions. *Aust. J. Dairy Technol*. 2005, 60(2), 106–109.

18. Walker-Smith, J. Hypoallergenic formulas: Are they really hypoallergenic? *Ann. Allergy Asthma Immunol*. 2003, 90(6), 112–114.

19. Bonnaillie, L. M.; Tomasula, P. M. Whey Protein Fractionation. In *Whey processing, functionality and health benefits*. Onwulata, C.I., Huth, P.J., Eds.; John Wiley & Sons Inc., New Jersey, U.S.A., 2008; pp. 15–38.

20. Jovanović, S.; Barać, M.; & Maćej, O. Whey proteins-properties and possibility of application. Mljekarstvo. 2005, 55(3), 215–233.

21. Ward, L. S.; Bastian, E. D. A method for isolating beta-casein. *J. Dairy Sci*. 1996, 79(8),1332–1339.

22. Huppertz, T.; Hennebel, J. B.; Considine, T.; Shakeel-Ur-Rehman; Kelly, A. L.; Fox, P. F. A method for the large-scale isolation of β-casein. *Food Chem*. 2006, 99(1), 45–50.

23. O'Mahony, J. A.; Smith, K. E.; Lucey, J. A. Purification of beta casein from milk. 2014. U.S. patent, US8889208B2.

24. Karasu, K.; Glennon, N.; Lawrence, N. D.; Stevens, G. W.; O'Connor, A. J.; Barber, A. R.; Yoshikawa, S.; Kentish, S. E. A comparison between ceramic and polymeric membrane systems for casein concentrate manufacture. *Int. J. Dairy Technol*. 2010, 63(2), 284–289.

25. Lawrence, N. D.; Kentish, S. E.; O'Connor, A. J.; Barber, A. R.; Stevens, G. W. Microfiltration of skim milk using polymeric membranes for casein concentrate manufacture. *Sep. Purif. Technol*. 2008, 60(3), 237–244.

26. Tolkach, A.; Kulozik, U. Fractionation of whey proteins and caseinomacropeptide by means of enzymatic crosslinking and membrane separation techniques. *J. Food Eng*. 2005, 67(1–2), 13–20.

27. Mikhaylin, S.; Nikonenko, V.; Pourcelly, G.; Bazinet, L. Hybrid bipolar membrane electrodialysis/ultrafiltration technology assisted by a pulsed electric field for casein production. *Green Chem*. 2015, 18(1), 307–314.

28. Holder, A.; Birke, A.; Eisele, T.; Klaiber, I.; Fischer, L.; Hinrichs, J. Selective isolation of angiotensin-I-converting enzyme-inhibitory peptides from micellar casein and β-casein hydrolysates via ultrafiltration. *Int. Dairy J*. 2013, 31(1), 34–40.

29. Donato, L.; Guyomarc'h, F.; Amiot, S.; Dalgleish, D. G. Formation of whey protein/κ-casein complexes in heated milk: Preferential reaction of whey protein with κ-casein in the casein micelles. *Int. Dairy J*. 2007, 17(10), 1161–1167.

30. Hurt, E.; Zulewska, J.; Newbold, M.; Barbano, D. M. Micellar casein concentrate production with a 3X, 3-stage, uniform transmembrane pressure ceramic membrane process at 50°C[1]. *J. Dairy Sci*. 2010, 93(12), 5588–5600.

31. Cao, J.; Wang, G.; Wu, S.; Zhang, W.; Liu, C.; Li, H.; Li, Y.; Zhang, L. Comparison of nanofiltration and evaporation technologies on the storage stability of milk protein concentrates. *Dairy Sci. Technol*. 2016, 96, 107–121.

32. Cao, J.; Zhang, W.; Wu, S.; Liu, C.; Li, Y.; Li, H.; Zhang, L. Short communication: Effects of nanofiltration and evaporation on the physiochemical properties of milk protein during processing of milk protein concentrate. *J. Dairy Sci*. 2015, 98(1), 100–105.

33. Román, A.; Wang, J.; Csanádi, J.; Hodúr, C.; Vatai, G. Partial demineralization and concentration of acid whey by nanofiltration combined with diafiltration. *Desalination*. 2009, 241(1-3), 288–295.

34. Neocleous, M.; Barbano, D. M.; Rudan; M. A. Impact of low concentration factor microfiltration on milk component recovery and cheddar cheese yield. *J. Dairy Sci*. 2002, 85(10):2415–24.

35. Blades, M. Stability and antioxidative activities of casein peptide fractions during simulated gastrointestinal digestion in vitro: Charge properties of peptides affect digestive stability. *Nutr. Food Sci*. 2000, 52(1), 334–341.

36. Bonizzi, I.; Buffoni, J. N.; Feligini, M. Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context. *J. Chromatogr. A*. 2009, 1216(1), 165–168.

37. Glantz, M.; Håkansson, A.; Lindmark Månsson, H.; Paulsson, M.; Nilsson, L. Revealing the size, conformation, and shape of casein micelles and aggregates with asymmetrical flow field-flow fractionation and multiangle light scattering. *Langmuir*. 2010, 26(15), 12585–12591.

38. Bonnaillie, L.; Qi, P.; Wickham, E.; Tomasula, P. Enrichment and Purification of Casein Glycomacropeptide from Whey Protein Isolate Using Supercritical Carbon Dioxide Processing and Membrane Ultrafiltration. *Foods.* 2014, 3(1), 94–109.

39. Moreno, F. J.; Recio, I.; Olano, A.; Lopez-Fandino, R. Chromatographic characterization of ovine κ-casein macropeptide. *J. Dairy Res*. 2000, 67(3), 349–59.

40. Bhattacharjee, S.; Bhattacharjee, C.; Datta, S. Studies on the fractionation of β-lactoglobulin from casein whey using ultrafiltration and ion-exchange membrane chromatography. *J. Membr. Sci*. 2006, 275(1–2), 141–150.

41. Ye, A.; Singh, H.; Oldfield, D. J.; Anema, S. Kinetics of heat-induced association of β-lactoglobulin and α-lactalbumin with milk fat globule membrane in whole milk. *Int. Dairy J*. 2004, 14(5), 389–398.

42. Havea, P.; Singh, H.; Creamer, L. K. Characterization of heat-induced aggregates of β-lactoglobulin, α-lactalbumin and bovine serum albumin in a whey protein concentrate environment. *J. Dairy Res*. 2001, 68(3), 483–97.

43. Permyakov, E. A.; Berliner, L. J. α-Lactalbumin: structure and function. *FEBS Lett*. 2000, 473(3), 269–274.

44. Kontopidis, G.; Holt, C.; Sawyer, L. Invited review: β-lactoglobulin: Binding properties, structure, and function. *J. Dairy Sci*. 2004, 87(4), 785–796.

45. Jost, R.; Maire, J. C.; Maynard, F.; Secretin, M. C. Aspects of whey protein usage in infant nutrition, a brief review. *Int. J. Food Sci. Technol*. 1999, 34(5–6), 533–542.

46. Aschaffenburg, R.; Drewry, J. Improved method for the preparation of crystalline β-lactoglobulin and α-lactalbumin from cow's milk. *Biochem J*. 1957, 65(2), 273–277.

47. Pearce, R.J. Thermal separation of beta-lactoglobulin and alpha-lactalbumin in bovine Cheddar cheese whey. *Aust. J. Dairy Technol*. 1983, 38(4), 144–8.

48. Bramaud, C.; Aimar, P.; Daufin, G. Whey protein fractionation: Isoelectric precipitation of α-lactalbumin under gentle heat treatment. *Biotechnol. Bioeng*. 1997, 56(4), 391–397.

49. Fox, K. K.; Holsinger, V. H.; Posati, L. P.; Pallansch, M. J. Separation of β-lactoglobulin from other milk serum proteins by trichloroacetic acid. *J. Dairy Sci*. 1967, 50(9), 1363–1367.

50. Arunkumar, A.; Etzel, M. R. Fractionation of α-lactalbumin and β-lactoglobulin from bovine milk serum using staged, positively charged, tangential flow ultrafiltration membranes. *J. Membr. Sci*. 2014, 454, 488–495.

51. Arunkumar, A.; Etzel, M. R. Fractionation of α-lactalbumin from β-lactoglobulin using positively charged tangential flow ultrafiltration membranes. *Sep. Purif. Technol*. 2013, 105, 121–128.

52. Sannier, F.; Bordenave, S.; Piot, J. M. Purification of goat β-lactoglobulin from whey by an ultrafiltration membrane enzymic reactor. *J. Dairy Res*. 2000, 67(1), 43–51.

53. Korhonen, H.; Pihlanto, A. Technological options for the production of health-promoting proteins and peptides derived from milk and colostrum. *Curr. Pharm. Des*. 2007, 13(8), 829–43.

54. Pearce, Robert J. Enriched whey protein fractions and method for the production thereof. 1995. U.S. patent, US5455331A.

55. Stack, F. M.; Hennessy, M.; Mulvihil, D.; O'kennedy, B. T. Process for the fractionation of whey constituents. 1998. U.S. patent, US5747647A.

56. Ayers, J. S.; Elgar, D. F.; Palmano, K. P.; Pritchard, M.; Bhaskar, G. B. Process for separation of whey proteins using a novel anion exchanger. 2006. U.S. patent, US7018665B2.

57. Bottomley, R. C. Process for obtaining concentrates having a high α-lactalbumin content from whey. 1991. U.S. patent, 5008376.

58. De wit, J. N.; Bronts, H. Process for the recovery of α-lactalbumin and β-lactoglobulin from a whey protein product. 1995. U.S. patent, US5420249A.

59. Alomirah, H. F.; Alli, I. Separation and characterization of β-lactoglobulin and α-lactalbumin from whey and whey protein preparations. *Int. Dairy J*. 2004, 14(5), 411–419.

60. Bläckberg, L.; Hernell, O. Isolation of lactoferrin from human whey by a single chromatographic step. *FEBS Lett*. 1980, 109(2), 180–3.

61. Uchida, T.; Dosako, S.; Sato, K.; Kawakami, H. Sequential separation of lactoferrin, lactoperoxidase, and secretory component by sulfate-linked ion-exchange chromatography. *Milchwissenschaft*. 2003, 58(9):482–486.

62. De Jongh, H. H. J.; Gröneveld, T.; De Groot, J. Mild isolation procedure discloses new protein structural properties of β-lactoglobulin. *J. Dairy Sci*. 2001, 84(3), 562–571.

63. Outinen, M.; Tossavainen, O.; Tupasela, T.; Koskela, P.; Koskinen, H.; Rantamäki, P.; Syväoja, E. L.; Antila, P.; Kankare, V. Fractionation of proteins from whey with different pilot scale processes. *LWT - Food Sci. Technol*. 1996, 29(5–6), 411–417.

64. Kang, D. Y.; Moon, J. M.; Lee, S. Comparison of size-exclusion chromatography and flow field-flow fractionation for separation of whey proteins. *B. Korean Chem. Soc*. 2011, 32(4), 1315–1320.

65. Bobe, G.; Beitz, D. C.; Freeman, A. E.; Lindberg, G. L. Separation and quantification of bovine milk proteins by reversed-phase high-performance liquid chromatography. *J. Agric. Food Chem*. 1998, 46(2), 458–463.

66. Santos, M. J.; Teixeira, J. A.; Rodrigues, L. R. Fractionation and recovery of whey proteins by hydrophobic interaction chromatography. *J. Chromatogr. B*. 2011, 879(7–8), 475–9.

67. Kleber, N.; Maier, S.; Hinrichs, J. Antigenic response of bovine β-lactoglobulin influenced by ultra-high pressure treatment and temperature. *Innov. Food Sci. Emerg. Technol*. 2007, 8(1), 39–45.

68. Toro-Sierra, J.; Tolkach, A.; Kulozik, U. Fractionation of α-lactalbumin and β-lactoglobulin from whey protein isolate using selective thermal aggregation, an optimized membrane separation procedure and resolubilization techniques at pilot plant scale. *Food Bioprocess Tech*. 2013, 6, 1032–1043.

69. Timmer, J. M. K.; Van der Horst, H. C. Whey processing and separation technology: State-of-the-art and new developments. *Whey: Bulletin 9804*. International Dairy Federation, Brussels, Belgium., 1998; pp. 40–65.

70. Cheang, B.; Zydney, A. L. A two-stage ultrafiltration process for fractionation of whey protein isolate. *J. Membr. Sci*. 2004, 231(1–2), 159–167.

71. Kim, J. T.; Shin, G. H. Adsorption behavior of β-lactoglobulin onto polyethersulfone membrane surface. *J. Adhes. Sci. Technol*. 2015, 29(20), 2245–2255.

72. Séverin, S.; Wenshui, X. Milk biologically active components as nutraceuticals: Review. *Crit. Rev. Food Sci. Nutr*. 2005, 45(7-8), 645–656.

73. Wakabayashi, H.; Yamauchi, K.; Takase, M. Lactoferrin research, technology and applications. Int. *Dairy J*. 2006, 16(11), 1241–1251.

74. Ward, P. P.; Paz, E.; Conneely, O. M. Multifunctional roles of lactoferrin: A critical overview. *Cell. Mol. Life Sci*. 2005, 62(22), 2540–2548.

75. Seifu, E.; Buys, E. M.; Donkin, E. F. Significance of the lactoperoxidase system in the dairy industry and its potential applications: A review. *Trends Food Sci. Technol*. 2005, 16(4), 137–154.

76. Shin, K.; Wakabayashi, H.; Yamauchi, K.; Teraguchi, S.; Tamura, Y.; Kurokawa, M.; Shiraki, K. Effects of orally administered bovine lactoferrin and lactoperoxidase on influenza virus infection in mice. *J. Med. Microbiol*. 2005, 54(8):717–723.

77. Fee, C. J.; Chand, A. Capture of lactoferrin and lactoperoxidase from raw whole milk by cation exchange chromatography. *Sep. Purif. Technol*. 2006, 48(2), 143–149.

78. Turner, S. A.; Thomson, N. A.; Auldist, M. J. Variation of lactoferrin and lactoperoxidase in bovine milk and the impact of level of pasture intake. *New Zeal. J. Agr. Res*. 2007, 50(1), 33–40.

79. van Leeuwen, P.; Huisman, J.; Kerkhof, H.; Kussendrager, K. Small intestinal microbiological and morphological observations in young calves fed milk replacers enriched with a combination of lactoperoxidase system and lactoferrin. *J. Anim. Feed Sci*. 1998, 7(1), 223–228.

80. Still, J.; Delahaut, P.; Coppe, P.; Kaeckenbeeck, A.; Perraudin, J. P. Treatment of induced enterotoxigenic colibacillosis (scours) in calves by the lactoperoxidase system and lactoferrin. *Ann. Rech. Vet*. 1990, 21(2), 143–52.

81. van Hooijdonk, A. C. M.; Kussendrager, K. D.; Steijns, J. M. In vivo antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. *Br. J. Nutr*. 2000, 84(1), 127–134.

82. Naidu, A. Lactoperoxidase. In *Natural Food Antimicrobial Systems*. Naidu, A., Eds.; CRC Press, Boca Raton, U.S.A., 2000; pp. 17–102.

83. Cissé, M.; Polidori, J.; Montet, D.; Loiseau, G.; Ducamp-Collin, M. N. Preservation of mango quality by using functional chitosan-lactoperoxidase systems coatings. *Postharvest Biol. Tec*. 2015, 101, 10–14.

84. Montiel, R.; Martín-Cabrejas, I.; Medina, M. Reuterin, lactoperoxidase, lactoferrin and high hydrostatic pressure on the inactivation of food-borne pathogens in cooked ham. *Food Control*. 2015, 51, 122–128.

85. Mazri, C.; Sánchez, L.; Ramos, S. J.; Calvo, M.; Pérez, M. D. Effect of high-pressure treatment on denaturation of bovine lactoferrin and lactoperoxidase. *J. Dairy Sci*. 2012, 95(2), 549–557.

86. Mulvihill, D. M.; Ennis, M. P. Functional Milk Proteins: Production and Utilization. In *Advanced Dairy Chemistry—1 Proteins*. Fox, P.F., McSweeney, P.L.H., Eds.; Springer, Boston, U.S.A., 2003; Volume 1, pp. 1175–1228.

87. Baieli, M. F.; Urtasun, N.; Miranda, M. V.; Cascone, O.; Wolman, F. J. Isolation of lactoferrin from whey by dye-affinity chromatography with Yellow HE-4R attached to chitosan mini-spheres. *Int. Dairy J*. 2014. 39(1), 53–59.

88. Al-Mashikhi, S. A.; Li-Chan, E.; Nakai, S. Separation of immunoglobulins and lactoferrin from cheese Whey by chelating chromatography. *J. Dairy Sci*. 1988, 71(7):1747–55.

89. Lönnerdal, B.; Carlsson, J.; Porath, J. Isolation of lactoferrin from human milk by metal-chelate affinity chromatography. *FEBS Lett*. 1977, 75(1):89–92.

90. Faraji, N.; Zhang, Y.; Ray, A. K. Optimization of lactoperoxidase and lactoferrin separation on an ion-exchange chromatography step. *Separations*. 2017, 4, 10.

91. Fweja, L. W. T.; Lewis, M. J.; Grandison, A. S. Isolation of lactoperoxidase using different cation exchange resins by batch and column procedures. *J. Dairy Res*. 2010, 77(3):357–67.

92. Doultani, S.; Turhan, K. N.; Etzel, M. R. Fractionation of proteins from whey using cation exchange chromatography. *Process Biochem*. 2004, 39(11), 1737–1743.

93. Shigeru, Y.; Ye, X. Isolation of lactoperoxidase and lactoferrins from bovine milk acid whey by carboxymethyl cation exchange chromatography. *J. Dairy Sci*. 1991, 74(5), 1439–1444.

94. Carvalho, B. M. A.; Carvalho, L. M.; Silva, W. F.; Minim, L. A.; Soares, A. M.; Carvalho, G. G. P.; Da Silva, S. L. Direct capture of lactoferrin from cheese whey on supermacroporous column of polyacrylamide cryogel with copper ions. *Food Chem*. 2014, 154, 308–314.

95. Di Nino, G.; Turacchio, M.; D'Archivio, A. A.; Lora, S.; Corain, B.; Antonini, G. Catalytic activity of bovine lactoperoxidase supported on macroporous poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate). *React. Funct. Polym*. 2004, 61(3), 411–419.

96. Yoshida, S. Isolation of some minor milk proteins, distributed in acid whey from approximately 100,000 to 250,000 daltons of particle size. *J. Dairy Sci*. 1988, 71(1), 1–9.

97. Tsakali, E.; Petrotos, K.; Chatzilazarou, A.; Stamatopoulos, K.; D'Alessandro, A. G.; Goulas, P.; Massouras, T.; Van Impe, J. F. M. Short communication: Determination of lactoferrin in Feta cheese whey with reversed-phase high-performance liquid chromatography. *J. Dairy Sci*. 2014, 97(8), 4832–4837.

98. Palmano, K. P.; Elgar, D. F. Detection and quantitation of lactoferrin in bovine whey samples by reversed-phase high-performance liquid chromatography on polystyrene-divinylbenzene. *J. Chromatogr. A*. 2002, 947(2), 307–311.

99. Elgar, D. F.; Norris, C. S.; Ayers, J. S.; Pritchard, M.; Otter, D. E.; Palmano, K. P. Simultaneous separation and quantitation of the major bovine whey proteins including proteose peptone and caseinomacropeptide by reversed-phase high-performance liquid chromatography on polystyrene-divinylbenzene. *J. Chromatogr. A*. 2000, 878(2), 183–196.

100. Kawakami, H.; Shinmoto, H.; Dosako, S.; Ahiko, K. Method for separating bovine lactoferrin from cow's milk and purifying same. 1984. U.S. patent, US4668771A.

101. Kawakami, H.; Tanimoto, M.; Dousako, S. Method for separating and purifying lactoferrin from milk by use of sulfuric ester. 1987. U.S. patent, US4997914A.

102. Kussendrager, K. D.; Kivits, M. G. C.; Verver, A. B. Process for isolating lactoferrin and lactoperoxidase from milk and milk products, and products obtained by such process. 1997. U.S. patent, US5596082A.

103. Uchida, T.; Sato, K.; Kawasaki, Y.; Dosako, S. Separation of lactoperoxidase, secretory component and lactoferrin from milk or whey with a cation exchange resin. 1996. U.S. patent, US5516675A.

104. Burling, H. Process for extracting pure fractions of lactoperoxidase and lactoferrin from milk serum. 1992. U.S. patent US5149647A.

105. Okonogi, S.; Tomita, M.; Tomimura, T.; Tamura, Y., Mizota, T. Process for producing bovine lactoferrin in high purity. 1987. U.S. patent, US4791193A.

106. Dubuisson, C.; La Vieille, S.; Martin, A. Allergies alimentaires : Etat des lieux et propositions d'orientations. *Rapport AFFSA*. 2002.

107. Kabir, S. Immunoglobulin purification by affinity chromatography using protein A mimetic ligands prepared by combinatorial chemical synthesis. *Immunol. Invest*. 2002, 31(3–14):263–178.

108. Blakeslee, D.; Rapacz, J.; Butler, J. E. Bovine immunoglobulin allotypes. *J. Dairy Sci*. 1971, 54, 1319–1320.

109. Gapper, L. W.; Copestake, D. E. J.; Otter, D. E.; Indyk, H. E. (2007). Analysis of bovine immunoglobulin G in milk, colostrum and dietary supplements: A review. *Anal. Bioanal. Chem*. 2007, 389(1):93–109.

110. Elfstrand, L.; Lindmark-Månsson, H.; Paulsson, M.; Nyberg, L.; Åkesson, B. Immunoglobulins, growth factors and growth hormone in

bovine colostrum and the effects of processing. *Int. Dairy J*. 2002, 12(11), 879–887.

111. Léonil, J.; Mollé, D.; Gaucheron, F.; Arpino, P.; Guénot, P.; Maubois, J. L. Analysis of major bovine milk proteins by on-line high-performance liquid chromatography and electrospray ionization-mass spectrometry. *Le Lait*. 1995, 75 (3), 193–210.

112. Andrew, S. M.; Titus, J. A. Purification of immunoglobulin G. *Curr. Protoc. Immunol*. 2001, 5(1), 16.3.1–16.3.12.

113. Merin, U.; Bernstein, S.; Van Creveld, C.; Yagil, R.; Gollop, N. Camel (Camelus dromedarius) colostrum and milk composition during the lactation. *Milchwissenschaft*. 2011,

114. Copestake, D. E. J.; Indyk, H. E.; Otter, D. E. Affinity liquid chromatography method for the quantification of immunoglobulin G in bovine colostrum powders. *J. AOAC Int*. 2006, 56(2):70–74.

115. Daoud-Attieh, M.; Chaib, H.; Armutcu, C.; Uzun, L.; Elkak, A.; Denizli, A. Immunoglobulin G purification from bovine serum with pseudo-specific supermacroporous cryogels. *Sep. Purif. Technol*. 2013, 118(30), 816–822.

116. Dong, S.; Chen, L.; Dai, B.; Johnson, W.; Ye, J.; Shen, S.; Yun, J.; Yao, K.; Lin, D. Q.; Yao, S. J. Isolation of immunoglobulin G from bovine milk whey by poly(hydroxyethyl methacrylate)-based anion-exchange cryogel. *J. Sep. Sci.* 2013, 36(15), 2387–2393.

117. Wu, M. B.; Xu, Y. J. Isolation and purification of lactoferrin and immunoglobulin G from bovine colostrum with serial cation-anion exchange chromatography. *Biotechnol Bioproc E*. 2009, 14, 155–160.

118. Du, Q. Y.; Lin, D. Q.; Zhang, Q. L.; Yao, S. J. An integrated expanded bed adsorption process for lactoferrin and immunoglobulin G purification from crude sweet whey. *J. Chromatogr. B*. 2014, 947–948, 201–207.

119. Su, C. K.; Chiang, B. H. Extraction of immunoglobulin-G from colostral whey by reverse micelles. J. Dairy Sci. 2003, 86(5), 1639–1645.

120. Curadi, M. C.; Orlandi, M.; Greppi, G. F.; Toppino, P. M.; Barzaghi, S.; Cattaneo, T. M. P. Identification of protein fractions in mare's colostrum and milk. *Milchwissenschaft*. 2000, 55(8), 446–449.

121. Kinghorn, N. M.; Paterson, G. R.; Otter, D. E. Quantification of the major bovine whey proteins using capillary zone electrophoresis. *J. Chromatogr. A*. 1996, 723(2), 371–379.

122. Gapper, L. Determination of bovine immunoglobulin G in bovine colostrum powders, bovine milk powders, and dietary supplements containing bovine colostrum products by an automated direct immunoassay with optical biosensor: Collaborative study. *J. AOAC Int*. 2013, 96(5), 1026–1032.

123. Trujillo, A. J.; Castro, N.; Quevedo, J. M.; Argüello, A.; Capote, J.; Guamis, B. Effect of heat and high-pressure treatments on microbiological quality and immunoglobulin G stability of caprine Colostrum. *J. Dairy Sci*. 2007, 90(2), 833–839.

124. Chinowsky, T. M.; Quinn, J. G.; Bartholomew, D. U.; Kaiser, R.; Elkind, J. L. Performance of the Spreeta 2000 integrated surface plasmon resonance affinity sensor. *Sensor Actuat. B–Chem*. 2003, 91(1–3), 266–274.

125. Li, S. Q.; Zhang, Q. H.; Lee, Y. Z.; Pham, T. V. Effects of pulsed electric fields and thermal processing on the stability of bovine immunoglobulin G (IgG) in enriched soymilk. *J. Food Sci*. 2003, 68(4), 1201–1207.

126. Collin, R.; Prosser, C.; McLaren, R. O. B.; Thomson, M.; Malcolm, D. Development and validation of a nephelometric immunoassay for IgG1 in milk. *J. Dairy Res*. 2002, 69(1):27–35.

127. Newgard, J. R.; Rouse, G. C.; McVicker, J. K. Novel method for detecting bovine immunoglobulin G in dried porcine plasma as an indicator of bovine plasma contamination. *J. Agric. Food Chemi*. 2002, 50(11), 3094–3097.

128. Baruah, G. L.; Belfort, G. Optimized recovery of monoclonal antibodies from transgenic goat milk by microfiltration. *Biotechnol Bioeng*. 2004, 87(3), 274–285.

129. Baruah, G. L.; Nayak, A.; Winkelman, E.; Belfort, G. Purification of monoclonal antibodies derived from transgenic goat milk by ultrafiltration. *Biotechnol Bioeng*. 2006, 93(4), 747–754.

130. Luong, J. H. T.; Male, K. B.; Nguyen, A. L. A continuous affinity ultrafiltration process for trypsin purification. *Biotechnol Bioeng*. 1988, 31(6), 516–20.

131. Krei, G. A.; Hustedt, H. Extraction of enzymes by reverse micelles. *Chem. Eng. Sci*. 1992, 47(1), 99–111.

132. Belford, D. A.; Rogers, M. L.; Francis, G. L.; Payne, C.; Ballard, F. J.; Goddard, C. Platelet-derived growth factor, insulin-like growth factors, fibroblast growth factors and transforming growth factor β do not account for the cell growth activity present in bovine milk. *J. Endocrinol*. 1997, 154(1), 45–55.

133. Rogers, M. L.; Goddard, C.; Regester, G. O.; Ballard, F. J.; Belford, D. A. Transforming growth factor β in bovine milk: Concentration, stability and molecular mass forms. *J. Endocrinol*. 1996, 151(1), 77–86.

134. Smithers, G. W.; Ballard, F. J.; Copeland, A. D.; De Silva, K. J.; Dionysius, D. A.; Francis, G. L.; Goddard, C.; Grieve, P. A.; Mcintosh, G. H.; Mitchell, I. R.; John Pearce, R.; Regester, G. O. New Opportunities from the Isolation and Utilization of Whey Proteins. *J. Dairy Sci*. 1996, 79(8), 1454–1459.

135. Gauthier, S. F.; Pouliot, Y.; Maubois, J. L. Growth factors from bovine milk and colostrum: Composition, extraction and biological activities. *Lait*. 2006, 86(2), 99–125.

136. Jouan, P. N.; Pouliot, Y.; Gauthier, S. F.; Laforest, J. P. Hormones in bovine milk and milk products: A survey. *Int. Dairy J*. 2006, 16(11), 1408–1414.

137. Hossner, K. L.; Yemm, R. S. Improved recovery of insulin-like growth factors (IGFs) from bovine colostrum using alkaline diafiltration. *Biotechnol. Appl. Bioc*. 2000, 32(3), 161–166.

138. Tölgyesi, Á.; Tölgyesi, L.; Sharma, V. K.; Sohn, M.; Fekete, J. Quantitative determination of corticosteroids in bovine milk using mixed-mode polymeric strong cation exchange solid-phase extraction and liquid chromatography-tandem mass spectrometry. *J. Pharmaceut. Biomed*. 2010, 53(4), 919–28.

139. Yan, W.; Li, Y.; Zhao, L.; Lin, J. M. Determination of estrogens and bisphenol A in bovine milk by automated on-line C30 solid-phase extraction coupled with high-performance liquid chromatography-mass spectrometry. *J. Chromatogr. A*. 2009, 1216(44), 7539–45.

140. Yang, Y., Shao, B., Zhang, J., Wu, Y., & Duan, H. Determination of the residues of 50 anabolic hormones in muscle, milk and liver by very-high-pressure liquid chromatography-electrospray ionization tandem mass spectrometry. *J. Chromatogr. B*. 2009, 877(5–6), 489–96.

141. Altomare, A.; Fasoli, E.; Colzani, M.; Parra, X.M.P.; Ferrari, M.; Cilurzo, F.; Rumio, C.; Cannizzaro, L.; Carini, M.; Righetti, P.G.; Aldini, G. An in depth proteomic analysis based on ProteoMiner, affinity chromatography and nano-HPLC–MS/MS to explain the potential health benefits of bovine colostrum. *J. Pharmaceut. Biomed*. 2016, 121, 297-306.