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Radosavljević, J.; Stanić-Vučinić, D.; Stojadinović, M.; Radomirović, M.; Simović, A.; Radibratović, M.; Veličković, T. Č. Application of Ion Exchange and Adsorption Techniques for Separation of Whey Proteins from Bovine Milk. *Current Analytical Chemistry* 18 (3), 341–359.



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## REVIEW

# Application of Ion Exchange and Adsorption Techniques for Separation of Whey Proteins from Bovine Milk

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**ABSTRACT:**

**BACKGROUND:** The world production of whey was estimated to be more than 200 million tons per year. Although whey is an important source of proteins with high nutritional value and biotechnological importance, it is still considered as a by-product of the dairy industry with low economic value due to low industrial exploitation. There are several challenges in the separation of whey proteins: low concentration, the complexity of the material and similar properties (pI, molecular mass) of some proteins.

**METHODS:** A narrative review of all the relevant papers on the present methodologies based on ion-exchange and adsorption principles for isolation of whey proteins, known to the authors, was conducted.

**RESULTS:** Traditional ion-exchange techniques are widely used for the separation and purification of the bovine whey proteins. These methodologies, based on the anion or cation chromatographic procedures, as well as combination of aforementioned techniques are still preferential methods for the isolation of the whey proteins on the laboratory scale. However, more recent research on ion exchange membranes for this purpose has been introduced, with promising potential to be applied on the pilot industrial scale. Newly developed methodologies based either on the ion-exchange separation (for example: simulated moving bed chromatography, expanded bed adsorption, magnetic ion exchangers, etc.) or adsorption (for example: adsorption on hydroxyapatite or activated carbon, or molecular imprinting) are promising approaches for scaling up of the whey proteins' purification processes.

**CONCLUSION:** Many procedures based on ion exchange are successfully implemented for separation and purification of whey proteins, providing protein preparations of moderate-to-high yield and satisfactory purity. However, the authors anticipate further development of adsorption-based methodologies for separation of whey proteins by targeting the differences in proteins' structures rather than targeting the differences in molecular masses and pI. The complex composite multilayered matrices, including also inorganic components, are promising materials for simultaneous exploiting of the differences in the masses, pI and structures of whey proteins for the separation.

**Keywords:** whey proteins, ion exchange, alpha-lactalbumin, beta-lactoglobulin, adsorption, membranes

## 1. INTRODUCTION

For a long time, cheese whey has been considered as a by-product of low or no Commercial value, and it was mainly used for animal feed or discarded directly into rivers or public drains without previous treatments, causing serious environmental problems. Fortunately, this has been changed in recent years, making cheese whey a highly valued by-product in the dairy processing industry [1]. However, whey is a difficult medium for fractionation due to the diversity of the ingredients and their low concentrations.

More than 40% of the total milk production is used for cheese, generating cheese whey as a voluminous effluent. On one hand whey components are valuable for food and pharmaceutical industry, especially proteins which are of high nutritional value. On the other hand, whey as effluent of high pollution effect, due to its profound impact on biochemical and chemical oxygen demand. Therefore, in the last few decades various methods have been developed to enable safe discarding of whey and to recycle whey components.

## 2. WHEY PROTEINS OVERVIEW

Whey proteins constitute about 0.7% (w/v) of the total solid content of whey obtained from milk. Separation of whey proteins based on their isoelectric points gives two distinct groups, the major whey proteins: beta-lactoglobulin (BLG), bovine serum albumin (BSA) and alpha-lactalbumin (ALA), which are negatively charged at the pH of rennet whey (pH 6.2 to 6.4); and minor whey proteins: lactoferrin (LF) and lactoperoxidase (LP) that carry a positive net charge at the pH of whey.

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## 2.1. Whey proteins' properties

The main physicochemical properties of main whey proteins are summarized in Table 1 [2-3].

**Table 1** Properties of bovine whey proteins, compiled from references [2-3].

Protein	Isoelectric point (pI)	Molecular weight (kDa)	Concentration (g/L)
Beta-lactoglobulin (BLG)	5.2-5.4	18.3	3-4
Alpha-lactalbumin (ALA)	4.7-5.1	14.2	1.2-1.5
Bovine serum albumin (BSA)	4.9-5.1	66.0	0.3-0.6
Immunoglobulins (Igs)	5.0-8.0	150.0-900.0	0.6-0.9
Lactoperoxidase (LP)	9.6	78.0	0.03
Lactoferrin (LF)	8.0	78.0	0.1
Glycomacropeptide (GMP)	<3.8	8.6	1.5

Beta-lactoglobulin (BLG) is the most represented protein in bovine whey (approximately 50% of total whey proteins), while it is not present in human milk [4]. This protein possesses excellent technological properties due to its ease of gel formation and emulsifying and foaming properties, which justifies its use in various food industries [5]. Moreover, due to its high cysteine content, BLG is considered to be a protein of a high nutritional value [6]. Unfortunately, there are many reports on adverse allergic reactions to infant formulae in children [4, 7-8], so interest in methods for removal of BLG from infant formulae is on the rise.

Alpha-lactalbumin (ALA) is a small protein, which accounts for up to 30% in both bovine and human milk protein content. Similarly to BLG, it is used in food industry to improve techno-functional properties of the food [9], and to fortify the meat and dairy products with essential amino-acid tryptophan [10-11].

Immunoglobulins (Igs) provide passive immunity to various infectious diseases, and they are effective in health protection of newborns [12]. Isolated Igs may be used as therapeutic agents in infected animal neonates [13].

Lactoferrin (LF) is a protein that can chelate iron ions. Its various biological functions (antimicrobial, bone growth stimulating, anticarcinogenic, immuno-modulatory, ion transporting, toxin binding etc.) are, probably, caused by this intrinsic property of lactoferrin [14-15].

Lactoperoxidase (LP) is a whey protein known for its antimicrobial properties. Along with LF, LP can be used for supplementation of infant formulae as a natural antibiotic, hence, extending the shelf-life of the product [16].

Bovine serum albumin (BSA) is an invaluable tool in both industry and research. BSA is used as an additive in various human cell-culture media and stabilizer of various protein/enzyme preparations. Most commonly, BSA is used as a non-specific adhesion blocking agent, preventing the sticking of proteins to reaction vessels and tubes.

Glycomacropeptide (GMP) is a peptide fragment present exclusively in rennet whey. It is liberated by enzymatic cleavage of  $\kappa$ -casein. The most prominent feature of this peptide is lack of aromatic amino acids, so GMP is convenient as a source of amino acids in the nutrition of persons with phenylketonuria [17].

## 2.2. Whey protein formulations

Whey is a multi-component medium, difficult to fractionate due to the diversity of the ingredients and their low concentrations. Whey proteins could be fractionated by various techniques, as membrane filtration, chromatography, precipitation and spray drying leading to various whey protein formulations, p.e. sweet whey powders, whey protein isolate (WPI), acid whey powders, whey protein concentrate (WPC), reduced lactose powders, and demineralized whey.

Sweet whey, which is the most common one, results from the casein precipitation by rennet at pH 6.5 (protein content 11.0 - 14.0%). Acid whey is produced at pH < 5, by the lactic acid precipitation during the manufacture of the fresh and curd cheese (protein content 7.0 - 9.0%), or in casein production when mineral acids are used for precipitation (casein whey, protein content 9.0 - 11.01%).

Whey proteins are available as three different types: concentrates (WPCs), isolates (WPIs) and hydrolysates (WPHs). In contrast to sweet whey powder, containing only 11.0 - 14.0% of protein, WPCs have a protein content ranging from 34% to about 90%. Whey protein concentrates (WPCs) are filtrates of whey obtained by double filtration. Initial filtration removes fat particles, while subsequent membrane filtration concentrates the sample by removal of water and lactose. WPIs are the purest among whey proteins' preparations, containing approximately 90% of whey proteins and only traces of lactose and lipids [18-19]. WPHs are produced by enzymatic hydrolysis of whey proteins in WPC or WPI.

## 3. TRADITIONAL ION-EXCHANGE TECHNIQUES IN WHEY PROTEIN SEPARATION

Ion exchange chromatography separation is based on electrostatic interactions between ionic and polar analytes, ions present in the eluent and ionic functional groups fixed to the chromatographic support. Two distinct mechanisms as follows; ion exchange due to competitive ionic binding (attraction) and ion exclusion due to repulsion between similarly charged analyte ions and the ions fixed on the chromatographic support, play a role in the separation in ion chromatography.

The mixtures of acidic and basic whey proteins could be separated in a single chromatographic run with increasing salt gradient at pH 7.0 using cation and an anion exchange column in series [20].

The traditional ion exchange techniques for isolation of whey proteins are summarized in Table 2.

### 3.1. Anion exchange chromatography

Similar physicochemical properties of whey proteins have made it difficult to selectively separate one protein from another. Major whey proteins BLG, ALA and BSA are negatively charged at the pH value of whey, while minor proteins lactoferrin and lactoperoxidase hold a positive charge at the same pH. Ion exchange column chromatography thus represents a method of choice for separation of whey proteins into two distinct groups based on their differences in isoelectric points. Usage of ion exchange chromatography in separation of whey proteins is further supported by its higher capacity due to bigger surface of beads in comparison to the binding capacity of membranes, as well as its reproducibility, ease of scale-up and low cost.

Isoelectric points of main bovine whey proteins BLG, ALA and BSA fall in the narrow pH range of 4.7 - 5.4 [2-3]. Anion exchange chromatography thus enables adsorption of acidic whey proteins to the positively charged ion exchanger at the pH above their pI, while under the same conditions basic proteins are unadsorbed to the matrix and are subsequently fractionated

using cation exchanger. Elution of acidic proteins is then performed by either changing the pH, increasing ionic strength or using affinity methods.

Both weak and strong ion exchangers are used in the separation of whey proteins from different whey preparations. The greatest challenge in fractionation of whey proteins is separation of its two main proteins – ALA and BLG. For that purpose, weak anion exchangers, such as DEAE-Sepharose, DEAE-Sephadex and DEAE-Cellulose, have been used in various purification protocols based on anion exchange chromatography [21-23]. Depending on the type of matrix and choice of ionic strength and pH of equilibration buffer, ALA can be either unadsorbed or adsorbed on the DEAE column.

Ambient pressure anion exchange chromatography has shown to be highly successful for the purification of BLG in its native form. In the method proposed by de Jongh et al, batch chromatography using diethylaminoethyl (DEAE) Sepharose CL-6B (Pharmacia, Uppsala, Sweden) equilibrated in 0.1 M Tris-HCl pH 7.2 was successfully used for purification of BLG. Under conditions used, most of the whey BLG (85 - 90%) was adsorbed to the matrix, while ionic strength of equilibration buffer stopped other whey proteins from binding to the matrix. Elution of BLG was obtained with 0.25 M NaCl in demineralized water. Gel filtration using Superdex 75 BPG column (Pharmacia, Uppsala, Sweden) was needed for further purification of BLG, as DEAE-eluted material contained traces of ALA and high molecular weight proteins. Obtained BLG was estimated to be >98% pure. Omission of gel filtration step is possible at the expense of the protein yield. Namely, when whey was diluted to conductivity comparable to 0.13 M Tris-HCl (pH 7.2), 60 - 65% of the BLG was bound to DEAE with a purity >98% [21]. Stojadinovic et al. have described another mild method for rapid isolation and purification of native BLG from bovine whey using DEAE Sephadex A-50 (GE Healthcare) equilibrated in 0.02 M Tris-HCl, pH 7.5. Under applied experimental conditions, the majority of whey proteins were adsorbed on DEAE-Sephadex and were eluted from the column using stepwise NaCl gradient (0.04 - 0.28 M) in equilibration buffer [23].

While ALA was predominant in fractions eluted with 0.12 and 0.16 M NaCl, BLG eluted as two peaks with 0.2 and 0.24 M NaCl. In comparison to other methods described in the literature, the method of Stojadinovic et al. involves a single chromatography step for obtaining BLG of high purity of 97 - 99% (depending on protein fraction) with a protein recovery of 80%, comparable to those obtained in the method proposed by de Jongh et al. The method has also proved to be successful in separating BLG isoforms A and B with high purity in some of the fractions (99 and 91% respectively). Apart from being cheap, the procedure requires only one day for the purification of about 300 mg of BLG from a single run using a small column (2.5 cm × 20 cm) [23].

Strong anion exchangers, such as QAE (quaternary aminoethyl) Toyopearl (QAE-TP), Q Sepharose, Mono Q and Q Hyper D/F, have also been used in fractionation of whey proteins [24-26]. Ye et al. have thus reported a mild, normal-pressure method for rapid and high-yield purification of ALA and BLG genetic variants A and B directly from rennet whey (pH adjusted to 6.5) using strong anion exchanger QAE-TP (Toyosoda, Tokyo, Japan). Following adsorption of basic whey proteins lactoperoxidase and lactoferrin on a strong cation exchange sulfopropyl-Toyopearl (SP-TP) column, fraction of unadsorbed proteins is passed through a QAE-TP column equilibrated with 0.05 M Tris buffer pH 8.5. Both ALA and BLG bind to the matrix under these conditions. Elution of bound ALA is performed using linear concentration gradient of 0 - 0.15 M NaCl in 0.05 M Tris-HCl buffer pH 8.5, while BLG isoforms remain adsorbed under these conditions. For better separation of BLG isoforms, the pH of the QAE-TP column is lowered to pH 6.8. BLG isoforms are eluted with 0.1 - 0.25 M NaCl gradient in 0.05 M Tris-HCl buffer pH 6.8, with BLG isoform B eluting first (0.14 - 0.17 M NaCl), followed by elution of A isoform (0.17 - 0.2 M NaCl) [26].

Fast protein liquid chromatography (FPLC) has also been employed for fractionation of whey proteins [24-25, 27]. Manji et al. investigated fractionation of proteins of sweet and acid wheys using MonoQ HR 5/5 column (Pharmacia, Uppsala, Sweden) coupled to FPLC system equilibrated in various buffers whose pH was adjusted to 6.3. The best separation conditions for fractionation of proteins of acid whey (pH adjusted to 6.8) were achieved using combination of step and inverted linear gradient of water and CH<sub>3</sub>COONa (pH adjusted to 6.3). Electrophoretically pure fractions of BLG isoforms were obtained, while ALA and BSA showed some contamination, probably resulting from immunoglobulins [24]. Simultaneous separation of BLG variants together with fractionation of other whey proteins in the same FPLC run imposes challenges and was not possible under experimental conditions used. Santos et al. managed to develop such a method that involved only anion exchange chromatography. Single-step method for separation of all four major whey proteins, BLG, ALA, BSA and Ig, from a whey protein concentrate having 80% w/w protein content (WPC80) using MonoQ 5/50 GL column (GE Healthcare, Pittsburgh, PA) coupled to a FPLC system was developed. The column was equilibrated with 0.02 M Tris - HCl, pH 6.3 during 5 column volumes and loaded with 500 µl of 5 mg/ml solution of WPC80. Elution buffer was 0.02 M Tris - HCl, pH 6.3 with 1 M NaCl. Inverse salt gradient was used – from 0% to 35% of elution buffer during 40 column volumes, followed by a step gradient from 35% to 50% of elution buffer during 5 column volumes. All immunoglobulins and all ALA of WPC were collected in a single fraction containing some BLG. All the BSA from WPC, although co-eluted with residual BLG, was collected in a single fraction as well. Authors have managed to recover 60.5% of pure BLG from WPC in two fractions containing no other protein, with BLG isoforms B and A eluting as separate fractions [25].

While the resolution of high-pressure methods are of particular importance when it comes to separation of BLG isoforms that are hard to separate in normal-pressure chromatography, these methods are limited by their low binding capacity. For preparative separation, a small decrease in the dynamic binding capacity when the flow rate increases is the most important parameter of the matrix and therefore Couriol et al. separated whey proteins on a preparative scale by using the hyperdiffusive matrix for anion exchange chromatography (Q Hyper D/F, Biosepra) [28]. Nearly pure fractions of the five main acid whey proteins were obtained on the preparative scale at flow of 127 cm/h, whilst separation was focused on ALA, pure ALA was obtained at even 500 cm/h.

### 3.2. Cation Exchange Chromatography

Basic whey proteins, primarily lactoferrin and lactoperoxidase, but also IgGs, are separated from other whey proteins using cation exchange chromatography. Careful selection of experimental conditions allows binding of less abundant basic proteins to the negatively charged resin and their subsequent elution from the matrix. Many reported procedures using cation exchangers report on using a single cation exchange matrix for separation of multiple proteins from whey, not just basic proteins LF and LP. The rationale behind this is the production of different whey protein products, either WPI, ALA, WPI depleted of ALA or purified LA and LP, all using a single system. Doultani et al. have thus developed a method based on the use of inexpensive and food-grade adsorbent and buffers that uses selective elution for whey protein fractionation from cheese whey (pH 6.4 ± 0.1). SP Sepharose Big Beads (Amersham Biosciences, Piscataway, NJ, USA) column was equilibrated in 0.05 M sodium lactate, pH 4.0. Elution of bound ALA to obtain ALA-depleted WPI was performed using 0.1 M sodium acetate (pH 4.9). In the next elution step, 0.05 M phosphate (pH 6.5) was used, eluting BLG, IgG and BSA, while 0.05 M phosphate (pH 6.5) containing either 0.35 M or 1.2 M sodium chloride eluted purified LP and LF, respectively [29]. While the proposed and similar

methods have practical advantages in adjusting a single operating system to multiple demands of the market, purities of single proteins are not of satisfactory grade. Application of cation exchange chromatography has therefore mostly been focused on purification of lactoferrin and lactoperoxidase. There is a growing commercial interest in the purification of lactoperoxidase due to its antimicrobial and nutritional properties [16, 30-31].

Fweja and coauthors compared efficiency of batch and column chromatography in purification of lactoperoxidase from rennet whey using two types of resins, Carboxymethyl-Sephadex C-25 (Kabi Pharmacia, Uppsala, Sweden) and Sylphopropyl-Toyopearl SP-650 (Toyosoda, Tokyo, Japan), both equilibrated in 0.05 M sodium phosphate buffer, pH 6.5. LP was eluted using either 1 M NaCl in equilibration buffer for batch chromatography or linear NaCl gradient (0-1.0 M) in the same buffer for column chromatography. Protein recovery rates on both resins were higher in column chromatography mode than in batch procedures, but the methods were not successful in separating LP from LF [32].

However, Yoshida and Ye reported successful separation of LP and LF by cation exchange chromatography on CM-Toyopearl (Toyosoda, Japan) [33]. They used skimmed milk to produce acid whey and, upon ammonium sulfate precipitation of globulins, used hydrophobic interaction chromatography on Butyl Toyopearl 650M to enrich the preparation with LP and LF. The LP/LF enriched fraction was subjected to cation exchange on The CM-Toyopearl column (1.5 cm x 25 cm) equilibrated with 50 mM phosphate buffer, pH 7.7. The proteins were eluted by linear gradient of sodium chloride (0 – 0.3 M). LP was eluted at lower concentration of sodium chloride in comparison with the LF, that was eluted with higher retention volumes, and, even, separation of different isoforms of LF has been observed. Yoshida and Ye reported a yield of 41 mg of purified LP with preserved enzymatic activity and 88 mg of LF (21 mg of LF isoform a, 67 mg of LF isoform b) per liter of acid whey.

Since the fat and caseins removal from milk prior to protein purification results in protein loss, Fee and coauthors applied chromatography of raw, untreated milk for purification of LP and LF [34]. They used XK16 packed with SP Sepharose Big Beads (GE Healthcare) equilibrated in 0.01 M phosphate buffer (pH 6.7) and eluted proteins either using gradient (0 - 1.0 M NaCl) or step elution (0.4 M NaCl to elute LP and 1.0 M NaCl to elute LF) in the same buffer. Separation of LP and LF is achieved by keeping the temperature around milking temperature to prevent solidification of fats and consequently column blockage.

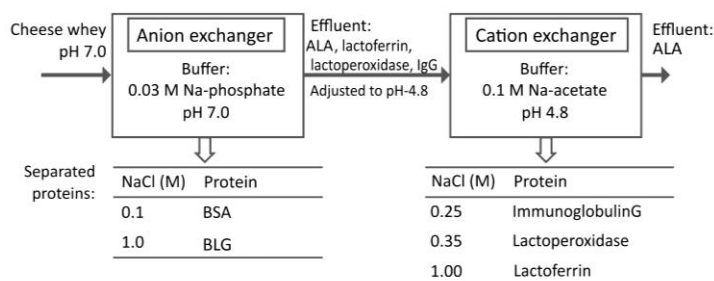
### 3.3. Combined cation-anion exchange chromatography

Isolation and purification of a single protein using ion exchange chromatography has been in the focus of many research papers in the last few decades, but some have proposed simultaneous anion - cation exchange methods for the isolation of more than one protein from whey using membrane adsorption chromatography.

The first report on combination of cation and anion chromatography for purification of whey proteins was published by Kiddy et al. [56]. They precipitated fat, casein and Igs from milk by sodium sulfate and separated whey proteins on CM-cellulose. After that BLG fraction was further chromatographed on DEAE-cellulose to obtain separated BLG A and BLG B.

Voswinkel and Kulozik fractionated whey proteins using Macroporous Sartobind membranes (Sartorius Stedim Biotech, Germany) with strong anionic or cationic ligands (Figure 1) [57]. Pre-treated acid whey of pH 7.0 was injected and negatively charged BSA and BLG were bound, except for ALA which was displaced from the membrane. BSA and BLG were eluted with 0.1 M and 1 M NaCl, respectively. The pH of the effluent was now set to 4.8 and injected onto the anionic membrane, allowing for IgG, LF and LP binding. In this step the excess of protein (3 fold the effluent from the first step) was injected to prevent ALA from binding to the membrane (ALA remains in the effluent). Proteins were eluted from the anion exchanger as follows: IgG at 0.25 M NaCl, LP at 0.35 M NaCl and LF at 1 M NaCl. The recovery and purity of most protein fractions were close to 90% and higher, lactoferrin separation was less successful.

Later in 2014, Voswinkel and Kulozik tested the scalability of the process from lab to pilot scale expecting similar elution patterns due to the same bed height of the membrane although the membrane area was 50 times bigger [58]. Isolated minor proteins were obtained at approximately 90% purity. The yield was between 80 and 97% with LF as the only exception. Depletion of BLG ranged from 96 to 99.8%. The results at lab and pilot scale were consistent in terms of binding capacity and stable membrane performance.

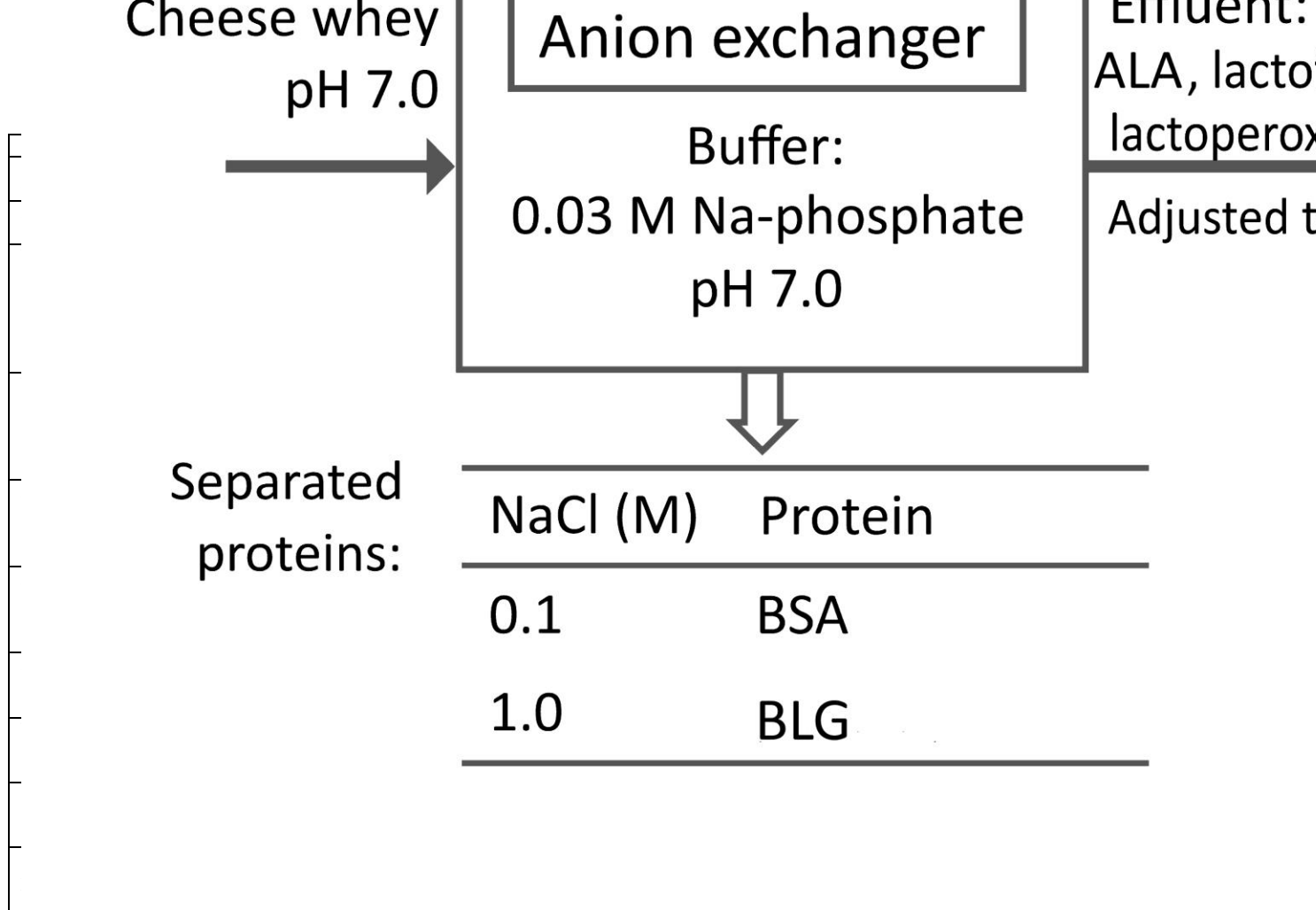


**Figure 1.** Schematic representation of the anion-cation two-step fractionation process of acid whey proteins by Voswinkel and Kulozik [57]. Reproduced with permission from Elsevier.

Although membrane chromatography offers many advantages in protein downstream applications, harsh chemical conditions are applied when membranes are converted from inert to adsorptive, thus possibly damaging the fine membrane structure [59]. To overcome these drawbacks, mixed matrix membranes have been created [60] by incorporating an adsorption resin such as ion exchange into a membrane polymer solution prior to membrane casting. Saufi and Fee managed to produce a single membrane that performs both cation and anion exchange and applied it to whey protein isolation [59]. A mixed mode membrane was synthesized by incorporating 7.5 wt % SP Sepharose cation and 42.5 wt % Lewatit MP500 anion resin into an ethylene vinyl alcohol base polymer casting solution. Ratio of cation to anion was chosen to be relative to the level of acidic and basic proteins present in whey. The authors estimated that a 1000 m<sup>2</sup> spiral-wound membrane module (200 L membrane volume, 1 m<sup>3</sup> module volume) could isolate around 25 kg total of whey protein per hour. Applications for such a membrane in an industry setting are promising, but for research purposes and further protein fractionation additional processes or modifications to the membrane are necessary, but the fact that the membrane can easily be modified to the sample's protein composition offers great versatility.

**Table 2.** Traditional ion exchange chromatographies for isolation of whey proteins

Starting mixture	Protein	Matrix	Mode	Equilibration	Elution	Reference
<b>Anion exchange chromatography</b>						
Rennet whey	BLG	DEAE Toyopearl	Column	50 mM Tris-HCl, pH 8.5	Linear gradient 0 - 0.3 M NaCl in equilibration buffer	[26]
Fraction after gel filtration of buffalo whey	BLG	DEAE- Sepharose	Column	0.02 M phosphate buffer, pH 6.8	Linear gradient 0- 0.5 M NaCl in equilibration buffer	[35]
WPC80 (80% w/w protein)	BLG	Mono Q 5/50 GL	Column	20 mM Tris-HCl pH 6.3	Linear gradient 0- 0.5 M NaCl in equilibration buffer	[25]
Whey	BLG	DEAE-Sephadex A - 50	Column	20 mM Tris-HCl pH 7.5	Step gradient 0.04- 0.28 M NaCl in equilibration buffer	[23]
BLG (A+B)	BLG A and BLG B	Q Sepharose Fast Flow	Column	25 mM Tris-HCl pH 8.8	0.25 M NaCl in equilibration buffer	[36]
Whey	BLG	Nuvia Q	Column	20 mM sodium phosphate, pH 6.4	Linear gradient 0 - 0.3 M NaCl in equilibration buffer	[37]
WPC40	ALA	Mono Q 4.6/ 100 PE	Column	20 mM N - methyl piperazine, and 5 mM NaCl, pH 4	Linear pH gradient from pH 10-4: 20 mM piperazine, 20 mM triethanolamine, 20 mM bis - tris propane, 20 mM N - methyl piperazine, 5 mM NaCl	[38]
Whey precipitated with 50% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	ALA and BLG	DEAE-Sepharose Fast Flow	Column	50 mM Tris - HCl at pH 8.5	Linear gradient 0- 0.5 M NaCl in equilibration buffer	[39]
ALA - enriched WPC	ALA	HiLoad Q Sepharose Fast Flow	Column	200 mM sodium acetate, pH 7.0	Stepwise changes in ionic strength ( 1 M NaCl) and pH (7 - 3) in equilibration buffer	[40]
WPI	ALA	DEAE Sepharose™ Fast Flow	Column	25 mM Tris - HCl, pH 7.5	Linear gradient 0 - 0.35 M NaCl in equilibration buffer	[41]
Whey	ALA and BLG	Mono Q	Column	20 mM Tris - HCl, pH 7.0	Linear gradient 0 - 0.35 M NaCl in equilibration buffer	[42]
Whey	BLG	DEAE Sepharose - CL6B	Batch	100 mM Tris - HCl, pH 7.2	0.25 M NaCl in demineralized water	[21]
Whey	ALA and BLG	Mono Q	Column	20 mM Tris - HCl, pH 7.0	Linear gradient 0 - 0.34 M NaCl in equilibration buffer	[43]
Sweet whey	BLG	Q - Sepharose Big Beads	Column	10 mM sodium acetate, pH 5.8	Two - step simultaneous changes in pH and ionic strength (50 mM NaOAc, pH 5.0 and 100 mM NaOAc, pH 4.0)	[19]
Supernatant of whey precipitated with saturated ammonium sulfate	LF and LP	DEAE Cellulose	Column	5 mM sodium phosphate, pH 8.2	Stepwise elution with 10 and 25 mM sodium phosphate, pH 8.2	[44]
WPI after TCA precipitation, salting - out, thermal precipitation or peptic hydrolysis	BLG A and BLG B	Mono Q HR 5/5	Column	20 mM Tris - HCl bu'ler pH 7.0	Linear gradient 0 - 0.35 M NaCl in equilibration buffer	[45]
Ultrafiltered whey	BLG A and BLG B	Q Sepharose Fast Flow	Column	11.4 mM piperazine, pH 6.20	Linear gradient 0 - 0.5 M NaCl in equilibration buffer	[46]
Acid whey	BLG A and BLG B	Mono Q HR 5/5	Column	70 mM sodium actate, pH 6.3	Stepwise elution with 0 - 0.7 M sodium actate, pH 6.3	[24]
Supernatant of whey precipitated with 50 % ammonium sulfate	BLG	DEAE-cellulose	Column	50 mM Tris, pH 6.5	Stepwise gradient 0 - 0.4 M NaCl in equilibration buffer	[22]
Whey precipitated with 50% ammonium sulfate	BLG A and BLG B	Mono Q HR 5/5	Column	70 mM sodium acetate, pH 6.3	Stepwise elution with 0 - 0.7 M sodium actate, pH 6.3	[22]
Supernatant of acid whey precipitated with 3% TCA	BLG	QAE ZetaPrep 250	Column	50 mM sodium phosphate, pH 6.0	Step elution with 0.3 M NaCl in equilibration buffer	[47]
BLG (A+B+C)	BLGA, BLG B and BLG C	DEAE-Cellulose	Column	50 mM sodium phosphate, pH 5.8	Stepwise elution with 0.01 - 0.14 M NaCl in equilibration buffer	[48]
<b>Cation exchange chromatography</b>						
Supernatant of whey precipitated with 1.8 M ammonium sulfate	LP and LF	CM-Toyopearl	Column	50 mM sodium phosphate, pH 7.7	Linear gradient 0 - 0.3 M NaCl in 50 mM sodium phosphate, pH 7.7	[33]
Lactic acid whey	ALA	S - beads	Column	10 mM sodium lactate, pH 4.0	One - step elution with 100 mM sodium acetate, pH 4.9	[49]



### 3.4. Traditional ion exchange combined with filtration techniques

Allen et al. [61] purified lactoperoxidase by the combination of Amberlite CG 50 cation exchange and gel filtration (Sephadex G 100). Aich et al. [35] isolated BLG from buffalo whey by gel filtration chromatography using Sephacryl S-200 and purified it further by anion-exchange chromatography on DEAE-Sephacryl. Xu et al. [62] have concentrated Igs by selectively removing major whey proteins, ALA, BLG and BSA, using polystyrene anion exchanger IRA93 and Amicon YM100 membrane. Lech et al. [63] described an integrated process of fractionation of whey proteins. After the first step of ultrafiltration, by using cation exchange (CM sepharose), anion exchange (DEAE Sepharose) and gel filtration (Sephadex G-50), they separated BLG, BSA and LF, with BSA and LF of near 100% purity and recovery of 93.2 and 91.7%, respectively. Li et al. [64] isolated BLG from buffalo whey by anion exchange chromatography (DEAE-Sephacryl Fast Flow) followed by gel filtration (Sephadex G-75), obtaining preparation with >90 % purity and preserved antigenicity. Oram et al. [65] have purified LF from cheese whey using cation - exchange Amberlite CG50 resin and Sephadex G-100 gel filtration. Carlstorm et al. [30] purified LF from whey by CM-cellulose in the first step, and by Sephadex G-75 in the second step.

## 4. ION EXCHANGE MEMBRANES FOR WHEY PROTEIN SEPARATION

Conventional chromatographic processes show tremendous disadvantages for a fast and reliable downstreaming of whey, since large volumes and high protein concentrations cause many problems such as fouling of chromatographic material, long cycle times, large pressure drops via chromatographic columns and complicated process control systems. Membrane adsorber technology combines chromatographic separation with filtration, whereas membranes are converted into efficient adsorbers by attaching functional exchanger groups to the inner surface of the micro-porous membranes. In membrane chromatography the rate of association between target proteins and functional groups in ion exchange membranes is very rapid, unlike the slow rate of diffusion through packed columns. The fast convective flow combined with negligible pressure drop enables dramatically reduced processing times compared with packed columns. Also, ion exchange membranes are easy to scale-up, in contrast to the need for lengthy column packing. Commercially available membrane systems for laboratory and process scales are ion - exchange systems with strongly acidic (sulfonic acid), strongly basic (quaternary ammonium), weakly acidic (carboxylic acid) and weakly basic (diethylamine) types.

The performance of ultrafiltration (UF) membranes for the separation of whey proteins relies on two fundamental descriptions of the underlying transport phenomena: the stagnant film model and the sieving coefficient. The stagnant film model explains the build up of protein on the membrane surface (concentration polarization) that results from convective transport of solvent (whey permeate) through the membrane. The sieving coefficient ( $S_0$ ) is a measure of the transmission of protein through the membrane, being a fundamental measure of membrane performance. The sieving coefficient is greatest when the solution pH is near the isoelectric point (pI) of the protein, whereupon the protein carries no net charge, and when the salt concentration is elevated sufficiently to shield the charges on the protein and membrane. When the protein and membrane charges are alike, then electrostatic repulsion causes  $S_0$  to decrease, while when the charges are different, then the charged

proteins adsorb to the oppositely charged membrane and form a dynamic membrane that has the same charge as the protein molecules in solution, causing also electrostatic repulsion and a decrease in  $S_o$ .

Lucas et al. [66] investigated UF fractionation of negatively charged proteins at pH 7, ALA (target protein) and BLG (contaminant protein) from whey protein concentrate. Positively charged membranes, obtained by chemical modification of inorganic membranes with polyethyleneimine coating, have shown a high selectivity of ALA in the permeate owing to the strong interactions between the positive charges and BLG. Cowan et al. [67] prepared negatively charged polyethersulfone ultrafiltration membranes (100 kDa) by the formation of sulfonated polystyrene grafted chains within the membrane pores. The five times better selectivity of charged membrane for BLG, resulting in its retention, was due to a reduced pore size combined with electrostatic repulsion between negatively charged BLG and the negatively charged membrane.

Plate et al. [68] developed downstream procedure for the isolation of LF, LP and lactoferrin from sweet cheese whey based on strong cation - exchange membrane adsorber technology (Sartobind S, Sartorius, Gottingen, Germany). The procedure was upscaled to an industrial scale with two 1m<sup>2</sup> modules (type S-10k - 15–25) in series, obtaining LF with recovery of 88% (7 g lactoferrin per cycle). Valino et al. [69] separated a binary protein mixture containing BSA and LF using charged ultrafiltration membranes, obtaining a total selectivity in the most suitable conditions. BSA separation was obtained by applying positively charged membranes at pH 5.0, while the negatively charged membranes at pH 9.0 permitted the direct recovery of the permeated LF.

Freitag et al. [70] have separated whey proteins (BLG, ALA, BSA and IgG) using a mixed - mode cation and anion ion exchange system (cellulose-based Q15 and S15, Sartorius). At pH 6.0 the Igs were retained on the cation exchanger, while the other whey proteins are retained on the anion exchanger. Prior to elution, Q and the S units were disconnected and eluted separately in a NaCl gradient to prevent the Igs from coeluting at the same ionic strength with BLG. Ulber et al. [71] developed a very fast isolation method for direct removal of LF from sweet whey using strong cation membrane (Sartobind®, SARTORIUS). The permeate obtained within the crossflow filtration was directly used for downstreaming of LF ion exchange membrane in a continuous process. Bhattacharjee et al. [72] separated BLG from WPC by two - stage ultrafiltration (30 and 10 kDa) followed by ion - exchange membrane chromatography (Vivapure™ Q Mini - H column). They obtained 87.6% purity of BLG, although lactose and minerals still remained. Bhushan et al. [2] prepared anion exchange ultrafiltration membranes by covalent binding of quaternary amine to the surface of the membrane to fractionate glycomacropptide (GMP) from cheese whey. For the binary mixture of GMP and BLG adding a charge to the membrane increased selectivity by about 600%. Hossain et al. [73] separated mixture of pure ALA, BLG and BSA, as a simulation of whey sample, using anion exchange membrane (Sartobind R Anion Exchanger-D75), with capacity of 1.71 mg/cm<sup>2</sup>, 4.3 mg/cm<sup>2</sup> and 0.43 mg/cm<sup>2</sup>, respectively. Goodall et al. [74] applied Sartobind MA ion exchange membranes (weak anion exchanger with diethylamine groups and a strong anion exchanger with quaternary ammonium groups) to selectively separate BLG, BSA, and ALA from rennet whey. Both membranes were selective primarily for BLG, obtaining relatively pure BLG elution fraction and a permeate fraction enriched in ALA and BSA. Using a highly charged membrane, low ionic strength solutions, and a pH that causes one protein to be highly charged compared to another protein enhances protein fractionation [2]. Column membrane chromatographic system, described by Gerstner et al. [75], consist of stacked disks of macroporous cross-linked regenerated cellulose membranes functionalized with ion exchange moieties. Fluid flow through the macropores of these membranes results in rapid mass transport to and from the adsorbent surface.

The adsorptive microporous membranes can overcome the major limitations of conventional packed bed chromatography for protein separation, as they are not compressible eliminating diffusion limitations, hence enabling higher throughput and shorter processing times. However, application of membranes with very fine pores requires the use of equally fine microfilters on the raw whey as a pretreatment. Using a membrane with a much larger pore size (3–5 μm) can eliminate retention of lipids and proteins, and are able to reduce transmembrane pressure drop. Different functionalized polymer brushes could be attached to microporous membranes and permeating a protein solution through the pores rimmed by the polymer brushes, an ideal capturing rate of the proteins with a negligible diffusional mass - transfer resistance could be achieved [76]. Chiu et al. [16] used a microporous membrane, containing immobilized sulfonic acid moieties, for the rapid fractionation of the LP and LF from whey resulting in their recovery of 73.56% and 50.55 %, respectively. This membrane system was more rapid, smaller in size, and used a higher flow rate than traditional bead-based systems. Voswinkel et al. [57] developed selective purification of ALA, BLG, BSA, Ig, LF and LP from acid whey under non-denaturing conditions using coupled anion and cation exchange membranes. The recovery and purity of most protein fractions were close to 90% and higher. In the following study Voswinkel et al. [58] scaled - up chromatographic separation 50 - fold and improved fluid distribution, thereby avoiding high back pressures, by applying a radial flow membrane adsorber system. In the first anion exchanger step BLG and BSA were removed, and in the second cation exchanger step LF, LP and Ig were isolated, while ALA remained in the serum phase. The radial flow was applied to allow smaller bed heights, e.g. to maintain path length through the membrane pores, and thus to obtain elution profile as in lab scale. High-performance tangential flow filtration (HPTFF) is a technology recently developed to improve the separation based on size and charge difference of biomolecules. Incorporation of charge on the surface of UF permits the use of membrane with wider pore size that provides higher fluxes with no inhibition on the protein recovery. Arunkumar et al. [77] separated mixture of ALA and BLG by HPTFF by covalently attaching a positive charge on a 300 kDa membrane and using a two-stage flow configuration, obtaining 87% pure ALA. In a three-stage membrane system with recycle was calculated to give 87% pure ALA and 83% pure BLG [78], demonstrating that proteins 15–20 times smaller than the membrane cut off can be fractionated from a natural dairy fluid at its innate conductivity using charged ultrafiltration membranes.

#### 4.1. Ion exchange mixed matrix membrane

Ion exchange mixed matrix membrane (MMM) is prepared by physical incorporation of an ion exchange resin into a membrane polymer solution, prior to membrane casting. This procedure eliminates the need for chemical modification to induce a charged group in the membrane. MMM combine the advantages of membrane technology, such as easy scale-up, low pressure drop and high throughputs, with column chromatography, such as selectivity and high binding capacity. MMM can be reused in multiple cycles without significant loss of performance. Avramescu et al. [79] prepared MMM by incorporation of Lewatit cation exchange resins CNP 80 into an EVAL (copolymer of ethylene and vinyl alcohol) porous structure as base membrane, to prepare heterogeneous membranes with high protein adsorption capacity, in various geometries (flat sheet membranes and solid or hollow fiber). The obtained MMM, investigated using BSA as a model protein, has shown high protein binding capacity (40–45 mg/mL of MMM) at low backpressure leading to lower denaturation, as well as high protein recovery (90%). Saufi et al. [5] reported separation of BLG from whey using an anion exchange MMM, prepared by incorporation of Lewatit ion exchange resin (MonoPlus™ MP500) into EVAL. MMM showed selective binding towards BLG compared to other proteins, and dynamic binding capacity of BLG in whey solution was about 80 mg/g membrane (24 mg /mL of



membrane). The maximum BLG binding capacity was achieved around pH 5–6, which is near to its pI. In their further study these authors [59] incorporated Lewatit MP500 anionic resin and SP Sepharose cationic resin into EVAL, obtaining MMM able to bind both basic and acidic proteins simultaneously from whey. Binding capacities of ALA, LF, BLG and Ig were 7.16, 11.40, 59.21 and 1.11 mg/g membrane, respectively. They also incorporated Lewatit CNP105 cation exchanger and Lewatit MP500 anion exchanger resin into cellulose acetate and EVAL, obtaining almost pure protein mixture of BLG and LF after the batch elution of LF - spiked whey [80]. Saufi et al. [81] successfully recovered LF from whey by MMM made of embedded ground SP Sepharose cation exchange resin into an EVAL. The chromatography system was operated in cross - flow mode to minimize fouling and enhance LF binding, resulting in an LF recovery of 91%, with high purity.

## 5. RECENTLY DEVELOPED APPROACHES FOR ION EXCHANGE - BASED SEPARATION OF WHEY PROTEINS

### 5.1. Simulated moving bed chromatography

In comparison to traditional column chromatography, simulated moving bed (SMB) technology enables higher productivity, higher product concentration, reduced buffer consumption, more efficient use of raw material and higher target purity. is a way of making column processes more efficient and thus more economical. The SMB approach is based on the simulation of a true countercurrent operation between the solid and the liquid phase, by valve switching over a series of columns or column movement in a carousel. Andersson et al. [82] have developed 20 column SMB process for the separation of LP and LF from WPC on cation exchanger SP-Streamline (GE Healthcare, Sweden). SMB demonstrated better process data than the non moving bed process (48% rise in productivity, 6.5 times lower buffer consumption and 4.8 times higher target protein concentration with a better raw material utilization), although productivity would be lower when using whey instead of WPC due to lower protein concentration.

### 5.2. Expanded bed adsorption

Expanded bed adsorption (EBA) allows the adsorption of target proteins directly from an unclarified feedstock, such as whey, as it makes possible processing of viscous and particulate liquids. EBA is robust technology very favorable in industry as it integrates the clarification, concentration, and primary purification into a unit operation. It is able to process a large volume of feedstock with a high operation flow, increasing the overall yield, reducing the operational time and the cost for capital investment and consumables. Although EBA technology is widely used in rough separation of proteins, Du et al. [83] purified LF from whey in one step using EBA with a strong cation exchanger Fastline SP (Upfront Chromatography A/S, Denmark). LF with a high purity (88.5%) and a reasonable recovery (77.1%) was obtained, where the purification factor reached even 553. In their further study Du et al. [84] used two sequential expanded beds where LF was isolated from sweet whey in the first expanded bed packed with Fastline SP, and the flow through was loaded into the second expanded bed packed with mixed-mode hydrophobic and cation exchanger Streamline Direct CST-1 (GE Healthcare, Sweden) to separate IgG. Moreover, the stream flowed out of the integrated process was collected and separated by ultrafiltration to produce whey protein concentrate providing, fully utilized sweet whey.

### 5.3. High-porosity chromatographic materials

The whey protein separation methods which utilize monolithic columns are still in development. In packed particle columns, due to the discontinuous structure, mobile phase flows preferentially between the particles, and formed eddies create dispersion and deteriorate the resolution of the separation on account of the dilution of the analytes. The eddies also create flow dependant shear forces which can damage large labile biomolecules. Monolithic columns overcome these problems as a consequence of the continuous structure of monolith media. In contrast to packed particle columns where both convective (laminar and turbulent) and diffusive mass transport are present, only laminar convective mass transport is characteristic for monoliths. Hence, monoliths provide faster separation times at no loss of resolution [1]. Albrecht et al. [1] developed a ion exchange HPLC-MS method for separation of the main proteins from WPI using short anion exchanger convective interaction media (CIM) monolithic columns (CIM DEAE, BIA Separations, Slovenia). The separation of ALA and BLG variants A and B was achieved by gradually lowering the pH of the mobile phase, although BSA and BLG B partially overlapped. The authors underlined monoliths at least as an alternative to the packed particle columns as they obtained rapid separation, very good pH gradient linearity and MS compatibility. Teepakorn et al. [85] compared strong cation exchange membrane (Sartobind S, Sartorius) and monolith (CIM, BIA Separations) chromatography for LF and BSA separation, using membranes stacks and monolith discs with identical bed heights placed into the same housing of an axial flow column. Although LF was bound, whereas BSA passed in the effluent in both cases, the monolith showed lower LF binding capacity and broadened and nonsymmetrical elution peaks.

In development of new chromatographic adsorbents in bioseparation, cryogels are emerging as particularly interesting materials, which can be prepared from appropriate monomers by copolymerization in semi - frozen liquid media. Advantages of cryogels are macroporosity (with pore sizes from several microns to several hundreds of microns), favorable biocompatibility, elastic tissue, physical/chemical stability, ease of preparation and variety of possibilities for functionalization. Cryogels can be prepared as beads, monoliths, sheets, disks, as well as composite cryogels, such as with embedded beads within monolithic cryogels. Billakanti and Fee [86] applied cation exchange cryogel for separation of LF and LP from whey, obtaining LF with a yield of 92% and a purity of 94%. Although the binding capacity was low in comparison with commercial packed bed chromatographic resins (SP Sepharose, GE Healthcare), high flow rates (550 cm/h) and large load volumes (50 mL) were achieved for 5 mL of cryogel. Dong et al. [87] efficiently isolated IgG from whey using monolithic anion exchange cryogel. High purity IgG (>95%) was obtained by employing suitable elution conditions and maximum IgG recovery of about 94% by choosing a suitable loading volume. Pan et al. [88] prepared cation exchange composite hydroxyethyl methacrylate-based (HEMA) cryogel embedded with cellulose beads for separation of LP from whey with purity of 98.0 - 99.8% and recovery of 92%. This matrix, having favorable porosity and permeability, also combines the supermacroporous properties of HEMA for the permission of passage of crude feedstocks and the macroporous cellulose beads for the diffusion of biomolecules. Machado et al. [89] produced supermacroporous cryogel poly(acrylamide)-based monolithic column functionalized with sulfo groups, having large pores, high porosity, weak axial dispersion and low HETP. Determination of ALA adsorption behavior on this cation exchanger demonstrated that it was spontaneous, being the process entropically driven, producing endothermic enthalpy changes of adsorption that increased in magnitude with the addition of salt.

Templated mesoporous silica materials have a high surface area and porosity, and a variety of interconnected pore structures, making them useful for separation. Sarvi et al. [90] synthesized amine functionalized mesoporous silica (FDU-12),

with different pore sizes, for selective separation of ALA and BLG. By increasing the entrance size of pores and amine functionalization the amount of both proteins adsorbed increased significantly, and separation of ALA and BLG from a mixture was achieved for short contact times (under non-equilibrium conditions) as ALA was adsorbed much faster than BLG.

#### 5.4. Selective adsorption

The proteins become adsorbed on the chromatographic matrix by interacting at several sites on the surface of the protein molecule and support. This fact is used for the design of selective adsorption of proteins using supports with a very low activation degree, resulting in selective adsorption of the proteins that are large enough to establish several interactions with support. On the other hand, large proteins may be strongly adsorbed onto conventional matrices since their large external surface permits a great area of interaction with the support, resulting in difficulties in their desorption requiring very high ionic strength, drastic pH values, etc. Therefore, the use of poorly activated supports might avoid these problems. Itoyama et al. [91] prepared porous chitosan beads with a specific capacity to adsorb lactoferrin directly from bovine milk by the partial sulfation reactions, where adsorption of lactoferrin was efficiently regulated by the amount of sulfate groups, apparent density, and mechanical properties. Pessela et al. [92-93] developed two - steps method for purification of Igs from a WPC. In the first step BSA has been eliminated by adsorption on highly activated DEAE-agarose matrix, and after that IgGs, contaminated only by very small proteins (ALA nad BLG), were purified by selective adsorption on lowly activated monoaminoethyl-N-aminoethyl (MANAE)-agarose matrix. Bolivar et al. [94] achieved selectivity by limiting accessibility of proteins to matrix active groups. They have designed an anion exchanger support for the selective adsorption of small proteins by activating an aminated support with glutaraldehyde and further coating the support surface with bovine serum albumin (BSA). In this support, "wells" are generated by two neighborhoods BSA molecules, and on the bottom of those "wells" glutaraldehyde groups are exposed out ready to react with small molecules that have a size small enough to be accommodated between two BSA molecules on the pre-existing support. As BSA surface was not inert enough adsorbing many proteins, immobilized BSA was coated with dextran. Selective adsorption of low molecular mass proteins, ALA and BLG, from dairy whey was achieved. This approach may be exploited for designing tailor-made supports using other kind of adsorbing groups (chelating agents, boronic acid, etc.), or using proteins with different sizes to coat the support.

El-Sayed et al. [95] exploited cation exchange selective adsorption on SP Sepharose FF to separate a pure binary mixture of ALA and BLG, with the purpose of establishing a process for isolating them from whey. They observed competitive adsorption between the two proteins, obtaining complete separation of both proteins in a pure form. However, when separated WPC separation was incomplete resulting in BLG with 95% purity and 80% of recovery of, while ALA showed an 84% recovery. El-Sayed et al. [96] showed evidence of competitive adsorption as ALA displaced and eluted all BLG from the column in a pure form, and the remaining ALA was eluted thereafter at high purity and with 91% recovery. In fact, Kd for ALA was almost four times larger than that of BLG, while maximum protein binding capacity for ALA was 1.3 times higher. Moreover, by using confocal laser scanning microscopy (CLSM) to visualize the adsorption of ALA and BLG to SP Sepharose FF (pH 3.7) at the level of an individual bead, El-Sayed et al. [97] observed that BLG filled the bead more rapidly than ALA in a shell - wise fashion until the whole bead was filled, while ALA binding took place only at the outermost region of the bead, implying their different transport mechanisms. The confocal results also showed evidence that the weaker-affinity protein ALA was able to displace the more strongly adsorbed one BLG. In their further study [98] they investigated adsorption of proteins from whey at pH 3.7 using SP Sepharose FF, in order to improve separation process. They observed that ALA was able to displace and drive BLG off the column and therefore BLG was collected in a pure form. Based on this they established a consecutive two-stage process for separating ALA and BLG from WPC, with BLG and ALA recoveries of 98% and 67%, respectively, showing increases of 49% and 33% over the one - stage process. The purity of BLG was 95% in the first stage and 86% in the second one. These authors also simulated the breakthrough curves for the adsorption of ALA and BLG SP Sepharose FF [99] and, for adsorption of WPC, the simple kinetic model accurately predicted the individual breakthrough curves for ALA and BLG after correcting the Langmuir isotherm parameters for BLG using the same factors as proved necessary for pure mixtures.

#### 5.5. Magnetic ion exchangers and high gradient magnetic separation (HGMS)

The magnetic separation technology an alternative to other separation techniques currently used for the isolation of proteins from crude feedstocks due to the fast processing rates and the no longer required pretreatment of the feedstock, such as whey. In the batch mode magnetic decantation is mediated by an external magnetic field. Low - cost starting materials and simple preparation of magnetic particles, as well as their chemical and mechanic resistance enabling long-term reusability, are additional advantages [100]. Meyer et al. [101] prepared magnetic micro-ion exchangers (MMIEX) by functionalization of nano-sized superparamagnetic magnetite crystals, and in combination to HGMS succeeded to directly capture of LF from whey, with adsorption capacity of 12.6 mg/g. At MMIEX concentration of 5 g/l 70% recovery and purification factor of 11 was observed. Brown et al. [102] converted co-metal magnetite-PVA composite particles into ultra-high capacity weak cation exchange adsorbents featuring dense brush of polyacrylic acid (pAAc) chains. Binding capacities of LF and LP were 585 and 685 mg/g, respectively. Heeboll-Nielsen et al. [103] constructed high-gradient magnetic fishing (HGMF) process for the fractionation of whey, where LP was purified 36 fold and concentrated 4.7 fold. Superparamagnetic iron oxide crystals were coated with an amino-silane layer, followed by coating with a thin layer of polyglutaraldehyde (PG), and finally the cation exchanger was prepared by reacting PG-coated supports with bromoethane sulfonic acid. In their further study [104] these authors prepared superparamagnetic anion and cation exchangers, using them sequentially in bovine whey fractionation. From the cation exchangers Igs could be resolved from LP and LF, whereas adsorption to the anion exchangers was surprisingly selective for BLG resulting in high purity was obtained, although low resolution was obtained during elution.

#### 5.6. Displacement chromatography

In displacement chromatography there is a competition of the feed components for a limited amount of binding sites on the stationary phase surface, and it is enforced by the introduction (step-function) of the highly concentrated displacer solution, a displacer being a substance with even higher affinity for the stationary phase than any of the feed components. Liao et al. [105] separated BLG A and BLG B by high performance displacement chromatography on an anion exchange column (DEAE functionalized Vydac macroporous silica, The Separation Group, USA) and with chondroitin sulfate as the displacer. Not only that high resolving power was demonstrated, but also the amount of sample that can be separated is much larger than that by elution, enabling preparative separations. Vogt et al. [106] have tested several polyanions as displacer for separation of proteins from technical dairy whey on anion - exchange column (Bio-Scale Q2, BioRad). Polyacrylic acid as displacer has shown to be the most effective enabling ALA and BLG yield of 79% and 93%, respectively.

## 6. ADSORPTION - BASED TECHNIQUES IN WHEY PROTEIN SEPARATION

Generally, proteins tend to adsorb both on hydrophobic and hydrophilic surfaces due to the variety of forces involved in the protein-surface interaction (e.g., solvation effects, hydrophobic effect, electrostatic interactions, etc.). Adsorption is often used in biomolecule separation processes, through various adsorbent - adsorbate interactions, including ionic, affinity, and hydrophobic bonding. Adsorption is lately highlighted as a strategy for whey protein recovery mainly by development of activated carbons produced from agricultural waste as a promising alternative to reduce the cost of the adsorption process.

### 6.1. Ion affinity

Immobilized metal ion affinity chromatography (IMAC) is an effective method for isolating proteins from non-clarified mixtures, employing chelated metal ions, such as  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ , as a coordination agent for protein links. The adsorption of proteins by IMAC is mainly determined by the presence of the histidine residues exposed on the protein surface and LF and Igs have shown high affinity to copper ion loaded matrices.

In an attempt to isolate Igs and LF from cheese whey Al - Mashikhi et al. [107] applied several adsorption solid supports (silica, controlled pore glass, and alumina), where controlled pore glass showed the highest adsorption of immunoglobulins but with low capacity. Therefore, they have also used 1,4 - butanediol diglycidyl etheriminodiacetic acid modified Sepharose 6B with loaded copper ion. Of the two peaks eluted using pH gradient, they obtained the first yellowish peak rich in lactoferrin and the second rich in Ig. They demonstrated that the modification of histidine residues in Ig with diethyl pyrocarbonate almost completely eradicated the adsorption, implicating that adsorption mechanism was based on the coordination with histidine in Ig and Cu on the chelating column. Blomkalns et al. [108] isolated ALA from bovine WPC by IMAC using  $\text{Cu}^{2+}$  - Chelating Sepharose Fast Flow, obtaining a purity of 90% and recovery of 80%. Carvalho et al. [109] reported isolation LF by its direct capture on supermacroporous column of polyacrylamide cryogel loaded with copper ions (through the bond with iminodiacetic acid). The cryogel demonstrated good hydraulic permeability, porosity and low coefficient of liquid axial dispersion in a wide speed range.

### 6.2. Hydroxyapatite (HA)

Hydroxyapatite is an adsorbent with the formula  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  having a high affinity for proteins and has been used for the separation and purification processes of several proteins. Rossano et al. [110] separated whey proteins on HA followed by Superdex 75 gel filtration for final purification. By elution with 0.4 M phosphate ALA and IgG were mainly eluted at pH 5.0, whereas BLG and BSA were eluted at pH 6.0. Schlatterer et al. [111] applied ceramic HA chromatography with a fluoride ion gradient in phosphate buffer as displacement agent. BLG was completely eluted in one peak at a fluoride concentration of about 0.6 mol/L with purity of at least 96% and yield of 50 - 55%. Following size exclusion chromatography on Superdex 75 effectively removed contaminants resulting in BLG purity of 99%. Ng and Yoshitake [112] have used a mixed - mode ceramic HA chromatography method for one - column fractionation of LF from whey. They succeeded in removing LP by initial desorption from the matrix under isocratic conditions. Sousa et al. [113] evaluated the adsorption of ALA on HA using solid - liquid phase equilibrium data reported as adsorption isotherms and their Van't Hoff's thermodynamics analysis showed that the adsorption process is entropically driven. Vyas et al. [114] used all-trans retinal covalently immobilized on calcium bio-silicate to purify BLG. Adsorption and desorption of BLG were carried out at pH 5.1 and 7.0, using 0.01 and 0.1 M phosphate buffers, respectively. The stirred tank and fluidized bed column modes gave higher BLG purity (>95%) and recovery (2.88 g BLG/kg of matrix) then packed column mode (80% and 0.65 g/kg), attributed to insufficient contact between the passing fluids and matrix during adsorption, desorption, and intermittent washing. Tercinier et al. [115] characterized the adsorption behavior of whey proteins showing that the amount of whey proteins from WPI adsorbing on to HA particles varied depending on ionic strength, pH and mineral composition due to alteration in the zeta - potential of the HA. Vogt and Freitag [108] compared of strong anion exchange and HA displacement chromatography for separation of technical dairy whey. HA displacement chromatography has shown to be less successful in whey separation than displacement on strong ion exchanger due to the pH sensitivity of the BLG.

Various crystal planes of HA have been reported to exhibit selective adsorption providing the development of methods for selective protein adsorption by controlling HA morphology, chemical compositions and crystallinities [116]. Kawachi et al. [117] achieved highly selective adsorption of BSA on synthesized needle-shaped HA crystals at low phosphate buffer concentration. The selectivity is consequence of needle-shaped HA morphology, with large surface area, in comparison to irregularly shaped HA. Cetinkaya et al. [118] modified HA microbeads by embedding in ethylene glycol dimethacrylate (EGDMA) in the presence of pore makerwre, and used them to isolate ALA from cow's milk by a single step. ALA separation was performed without fat and casein removal steps. HA microbeads were synthesized by dispersing pure HA agglomerates in a polymer matrix where only the target protein ALA was adsorbed on to the HA microbeads. The authors demonstrated that all whey proteins adsorb on pure HA with none selectivity, whereas the small proteins (ALA and BLG) adsorb on HA microbeads with a preferential adsorption of ALA. The pores of newly synthesized HA microbeads reduce diffusional mass transfer resistance and facilitate convective transport ALA compared with other milk proteins.

### 6.3. Activated carbon

As synthetic adsorbents have a high cost, this aroused interest for the application of alternative adsorbents such as the activated carbon. The active carbons are materials well known for their complex pore structure, high internal surface area and good chemical stability. They also may have various functional groups containing oxygen on the surface. The textural characteristics of the adsorbent are of paramount importance in the adsorption process, since the pore structure limits the size of the molecules that can be adsorbed, while the available surface area limits the amount of material absorbed by the matrix. On the other hand, the surface chemistry of the carbonaceous materials depends essentially on their content of heteroatoms, mainly in superficial oxygen complexes. These functional groups may affect the adsorption capacity and can be modified by thermal and chemical treatments [119].

In the last few years there are several studies investigating potential of activated carbon prepared from agro - industrial byproducts (such as babassu nut shell, cocoa shells, siriguella seeds, coconut endocarp), as low - cost matrices from renewable resources, for separation of whey proteins. These studies investigated adsorptive capacity, mechanisms of adsorption, kinetic and adsorption isotherms of model whey proteins. Alves et al. [120] demonstrated that active babassu coal has the potential for isolation of BSA from whey, although it has inferior chemisorption characteristics compared to HA and synthetic HA. Adsorption properties of BSA for activated carbon prepared from *Elaeagnus* stone with chemical activation with  $\text{ZnCl}_2$  was

also investigated [121]. Pereira et al. [122] prepared activated carbons from cocoa shells and siriguela seeds, and evaluated the whey protein adsorption on produced activated carbon. Impregnation of residues with  $H_3PO_4$  was more effective in modifying the structure of and carbon textural properties and was favorable for the adsorption of ALA, while impregnation with  $ZnCl_2$  resulted in activated carbons which were more effective in the adsorption of BSA. Andrade et al. [123] investigated the potential of coconut endocarp as the carbon precursor material for the synthesis of the activated carbon as an adsorbent for whey protein purification, activated by  $ZnCl_2$ . Batista et al. [124] developed a mesoporous activated carbon from defective coffee beans for the adsorption of fresh whey protein, using activation by phosphoric acid. Adsorption properties of BSA for different activated carbons, such as thermally expanded graphite and graphene nanoplatelets were also investigated [125].

#### 6.4. Amphiprotic polymer nanomaterials

The amphiprotic polymers, consisting of hydrophobic and hydrophilic blocks, endowing it with various nanoarchitectures and desired specific functions, seems to be promising matrices for selective adsorption of proteins. Guo et al. [126], inspired by the effective grafting technique and strong adsorption capacity of CM-cellulose for heavy metal ions, fabricated an amphiprotic polymer-Zn complex magnetic nanospheres merited with multifunctionality for selective adsorption of LF from human colostrum whey. Nanospheres were synthesized by coating of hydrophobic polycaprolactone (HPCL) onto the magnetic  $Fe_3O_4$  nanospheres, followed by hydrophilic CM-cellulose grafting onto the HPCL blocks via esterification reaction and finally chelating with  $Zn^{2+}$  ion with the polyhydroxy structure of CM-cellulose. This approach, immobilizing CM-cellulose onto the HPCL blocks, enabled the regeneration of the nanomaterials and offered excellent performance on reducing the non-specific protein adsorption. The authors used apo-LF to improve the adsorption performance based on the coordination between the Fe vacancy domain of LF and  $Zn^{2+}$  on the surface of magnetic nanospheres achieving the adsorption capacity of apoLF up to 615.3 mg/g. In their further study Guo et al. [127] prepared 2D boron-titanate monolayer nanosheets for highly selective adsorption of Igs, exhibiting a superior adsorption capacity of 1669.7 mg/g and favorable selectivity for the adsorption of glycoproteins. The adsorbed IgG is readily eluted by using 0.1% (m/v) cetane trimethyl ammonium bromide (CTAB). The adsorption behavior was attributed to the binding sites from boric acid and the intercalation capacity from layered titanate nanosheets. For selective isolation of BLG from whey Chen et al. [128] prepared novel organic-inorganic hybrid polyoxometalate (TPPA- $PMo_{12}$ ). BLG selective adsorption was based on the pyridyl and phenyl groups of the TPPA- $PMo_{12}$  hybrid occupying the BLG's hydrophobic cavity and are directed to the calyx entrance, leading to a high adsorption efficiency of 99.2% at pH 5.0. It was demonstrated that hydrogen bonding interactions, rather than the electrostatic interactions facilitate the adsorption of BLG with a favorable adsorption capacity of 1428 mg/g.

#### 6.5. Molecular imprinting

Molecular imprinting technology is a method for making molecularly imprinted polymers with tailor-made binding sites corresponding to the template molecules in shape, size and functional groups. The functional monomers are self-assembled around the template molecule by covalent or noncovalent interactions between the functional groups residing on both the template and the functional monomer. This is followed by the copolymerization of the functional monomer with a bifunctional or trifunctional cross-linker and subsequent removal of the imprint molecules. However, this method is still in its infancy using proteins as templates as, in contrast to small organic molecules, large molecular sizes, fragility and complexity of proteins make difficulties. Soleimani et al. [129] synthesized molecularly imprinted polymers (MIPs) using BSA as a template, 2-VP (2-vinylpyridine) as a functional monomer and EGDMA (ethylene glycol dimethacrylate) as a cross-linker. They achieved maximum adsorption of 24 mg of BSA per g of fabricated MIPs, with recovery of ~80% and the extraction of BSA from whey had a selectivity and enrichment property. Jiménez-Guzmán et al. [130] developed MIP for the recovery of lactoferrin using vinylpyridin as functional and monomerethylene-glycol dimethacrylate as a linker, demonstrating retaining of 34.5% of the total LF content. Recently, Wang et al. [131] fabricated BSA-imprinted monodisperse poly(glycidyl methacrylate)/polystyrene (PGMA/PS) microspheres via dopamine auto-polymerization in alkaline solution. Because dopamine does not affect the validity of the protein structure, the imprinted cavity structure on the surface of the MIPs can match the template protein molecules completely. The obtained micron-sized BSA-imprinted PGMA/PS microspheres demonstrated an excellent saturation adsorption capacity ( $Q = 72.7$  mg/g) and good selective adsorption for BSA (IF (imprinting factor) = 4.6). In their further study, Wang et al. [132] successfully fabricated BSA-imprinted magnetic microspheres by a precipitation copolymerization, in which 2-hydroxyethyl acrylate (HEA) and water-soluble chitosan separately work as functional monomers and cross-linking agents. In order to reduce the nonspecific adsorption and improve the selectivity, sulfobetaine methacrylate (SBMA) is introduced into the imprinted polymer as an anti-protein segment. The optimized MIP ultimately resulted in excellent adsorption capacity (121.7 mg/g) and excellent selective adsorption of BSA (IF = 5.0).

## 7. CONCLUSION

Future approaches for separation of whey proteins by their adsorption should be mainly directed to differences in their structure rather than simple differences in molecular masses and pI. The complex composite multilayered matrices, including also inorganic components, have great potential for simultaneous exploiting of differences in mass, pI and structure for whey protein separation. Moreover, the tailoring of these matrices could be finely tuned to better exploit specific structure patterns of whey proteins approaching affinity-based adsorption.

### CONSENT FOR PUBLICATION

Not applicable.

### FUNDING

This work was funded the Ministry of Education, Science and Technological Development of Republic of Serbia, through Contract number: 451-03-68/2020-14/200168 (project 172024); Belgian Special Research Fund BOF StG No. 01N01718; Serbian Academy of Sciences and Arts GA No. F-26, and the European Commission, under the Horizon2020, FoodEnTwin project, GA No.810752. The EC does not share responsibility for the content of this works.

### CONFLICT OF INTEREST

The authors declare no conflict of interest. All authors contributed to the writing of the manuscript. JR, DSV and TCV analyzed the data and proposed the concept.

## ACKNOWLEDGEMENTS

Declared none.

## REFERENCES

1. Albrecht, A.; Vovk, I. Applicability of analytical and preparative monolithic columns to the separation and isolation of major whey proteins. *J. Chromatogr. A.*, **2012**, *1227*, 210-218.
2. Bhushan, S.; Etzel, M.R. Charged ultrafiltration membranes increase the selectivity of whey protein separations. *J. Food Sci.*, **2009**, *74*(3), E131-E139.
3. Stanic, D.; Radosavljevic, J.; Stojadinovic, M.; Cirkovic Velickovic, T. Application of ion exchanger in the separation of whey proteins and lactin from milk whey. In *Ion exchange technology ii: Applications*, Inamuddin, D.; Luqman, M., Eds.; Springer Netherlands: Dordrecht, **2012**; pp 35-63.
4. Monaci, L.; Tregoat, V.; van Hengel, A.J.; Anklam, E. Milk allergens, their characteristics and their detection in food: A review. *Eur. Food Res. Technol.*, **2006**, *223*(2), 149-179.
5. Saufi, S.A.; Fee, C.J. Fractionation of beta-lactoglobulin from whey by mixed matrix membrane ion exchange chromatography. *Biotechnol. Bioeng.*, **2009**, *103*(1), 138-147.
6. de Wit, J.N. Nutritional and functional characteristics of whey proteins in food products. *J. Dairy Sci.*, **1998**, *81*(3), 597-608.
7. El-Agamy, E.I. The challenge of cow milk protein allergy. *Small Rumin. Res.*, **2007**, *68*(1), 64-72.
8. Suutari, T.J.; Valkonen, K.H.; Karttunen, T.J.; Ehn, B.M.; Ekstrand, B.; Bengtsson, U.; Virtanen, V.; Nieminen, M.; Kokkonen, J. Ige cross reactivity between reindeer and bovine milk beta-lactoglobulins in cow's milk allergic patients. *J. Investig. Allergol. Clin. Immunol.*, **2006**, *16*(5), 296-302.
9. Pearce, R.J.; Marshall, S.C. New ways with whey components. *Aust. J. Dairy. Technol.*, **1991**, *46*(2), 105-107.
10. Jost, R.; Maire, J.C.; Maynard, F.; Secretin, M.C. Aspects of whey protein usage in infant nutrition, a brief review. [erratum: Apr 2000, v. 35 (2), p. 255.] *Int. J. Food Sci. Technol.*, **1999**, *34*(5/6), 533-542.
11. Muller, A.; Chaufer, B.; Merin, U.; Daufin, G. Purification of alpha-lactalbumin from a prepurified acid whey: Ultrafiltration or precipitation. *Lait*, **2003**, *83*(6), 439-451.
12. Hutchens, T.W.; Magnuson, J.S.; Yip, T.T. Secretory iga, igg, and igm immunoglobulins isolated simultaneously from colostrum whey by selective thiophilic adsorption. *J. Immunol. Methods.*, **1990**, *128*(1), 89-99.
13. Zydney, A.L. Protein separations using membrane filtration: New opportunities for whey fractionation. *Int. Dairy J.*, **1998**, *8*(3), 243-250.
14. Arnold, D.; Di Biase, A.M.; Marchetti, M.; Pietrantoni, A.; Valenti, P.; Seganti, L.; Superti, F. Antiadenovirus activity of milk proteins: Lactoferrin prevents viral infection. *Antivir. Res.*, **2002**, *53*(2), 153-158.
15. Baveye, S.; Ellass, E.; Mazurier, J.; Spik, G.; Legrand, D. Lactoferrin: A multifunctional glycoprotein involved in the modulation of the inflammatory process. *Clin. Chem. Lab. Med.*, **1999**, *37*(3), 281-286.
16. Chiu, C.K.; Etzel, M.R. Fractionation of lactoperoxidase and lactoferrin from bovine whey using a cation exchange membrane. *J. Food Sci.*, **1997**, *62*(5), 996-1000.
17. Van Calcar, S.C.; MacLeod, E.L.; Gleason, S.T.; Etzel, M.R.; Clayton, M.K.; Wolff, J.A.; Ney, D.M. Improved nutritional management of phenylketonuria by using a diet containing glycomacropeptide compared with amino acids. *Am. J. Clin. Nutr.*, **2009**, *89*(4), 1068-1077.
18. 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.; Pearce, R.J.; Regester, G.O. New opportunities from the isolation and utilization of whey proteins. *J. Dairy Sci.*, **1996**, *79*(8), 1454-1459.
19. Gerberding, S.J.; Byers, C.H. Preparative ion-exchange chromatography of proteins from dairy whey. *J. Chromatogr. A.*, **1998**, *808*(1-2), 141-151.
20. El Rassi, Z.; Horvath, C. Tandem columns and mixed-bed columns in high-performance liquid chromatography of proteins. *J. Chromatogr.*, **1986**, *359*, 255-264.
21. de Jongh, H.H.J.; Groneweld, T.; de Groot, J. Mild isolation procedure discloses new protein structural properties of beta-lactoglobulin. *J. Dairy Sci.*, **2001**, *84*(3), 562-571.
22. Neyestani, T.R.; Djalali, M.; Pezeshki, M. Isolation of alpha-lactalbumin, beta-lactoglobulin, and bovine serum albumin from cow's milk using gel filtration and anion-exchange chromatography including evaluation of their antigenicity. *Protein Express. Purif.*, **2003**, *29*(2), 202-208.
23. Stojadinovic, M.; Burazer, L.; Ercili-Cura, D.; Sancho, A.; Buchert, J.; Cirkovic Velickovic, T.; Stanic-Vucinic, D. One-step method for isolation and purification of native  $\beta$ -lactoglobulin from bovine whey. *J. Sci. Food Agric.*, **2012**, *92*(7), 1432-1440.
24. Manji, B.; Hill, A.; Kakuda, Y.; Irvine, D.M. Rapid separation of milk whey proteins by anion exchange chromatography. *J. Dairy Sci.*, **1985**, *68*(12), 3176-3179.
25. Santos, M.J.; Teixeira, J.A.; Rodrigues, L.R. Fractionation of the major whey proteins and isolation of beta-lactoglobulin variants by anion exchange chromatography. *Sep. Purif. Technol.*, **2012**, *90*, 133-139.
26. Ye, X.Y.; Yoshida, S.; Ng, T.B. Isolation of lactoperoxidase, lactoferrin, alpha-lactalbumin, beta-lactoglobulin b and beta-lactoglobulin a from bovine rennet whey using ion exchange chromatography. *Int. J. Biochem. Cell B.*, **2000**, *32*(11-12), 1143-1150.
27. Kunz, C.; Lönnerdal, B. Human milk proteins: Separation of whey proteins and their analysis by polyacrylamide gel electrophoresis, fast protein liquid chromatography (fplc) gel filtration, and anion-exchange chromatography. *Am. J. Clin. Nutr.*, **1989**, *49*(3), 464-470.
28. Couriol, C.; Le Quellec, S.; Guihard, L.; Molle, D.; Chaufer, B.; Prigent, Y. Separation of acid whey proteins on the preparative scale by hyperdiffusive anion exchange chromatography. *Chromatographia*, **2000**, *52*(7-8), 465-472.
29. Doulhani, S.; Turhan, K.N.; Etzel, M.R. Fractionation of proteins from whey using cation exchange chromatography. *Process. Biochem.*, **2004**, *39*(11), 1737-1743.
30. Carlström, A. Lactoperoxidase. Identification of multiple molecular forms and their interrelationships. *Acta Chem. Scand.*, **1969**, *23*, 171-184.

31. Rombauts, W.A.; Schroeder, W.A.; Morrison, M. Bovine lactoperoxidase. Partial characterization of the further purified protein. *Biochemistry*, **1967**, *6*(10), 2965-2977.
32. 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), 357-367.
33. Yoshida, S.; 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.
34. 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.
35. Aich R; Batabyal S; SN, J. Simple purification method for beta-lactoglobulin from buffalo milk. *Adv. Anim. Vet. Sci.*, **2014**, *2*(2), 78-80.
36. Pavlov, G.; Hsu, J.T. The pH, temperature, and protein structure effect on -lactoglobulin a and b separation in anion-exchange chromatography. *Aiche. J.*, **2018**, *64*(6), 1928-1937.
37. Ng, P.K.; Snyder, M.A. Purification of -lactoglobulin with a high-capacity anion exchanger: High-throughput process development and scale-up considerations. *J. Sci. Food Agr.*, **2013**, *93*(13), 3231-3236.
38. Nfor, B.K.; Ripic, J.; van der Padt, A.; Jacobs, M.; Ottens, M. Model-based high-throughput process development for chromatographic whey proteins separation. *Biotechnol. J.*, **2012**, *7*(10), 1221-1232.
39. Mao, X.; Zhang, G.F.; Li, C.; Zhao, Y.C.; Liu, Y.; Wang, T.T.; Duan, C.Y.; Wang, J.Y.; Liu, L.B. One-step method for the isolation of -lactalbumin and -lactoglobulin from cow's milk while preserving their antigenicity. *Int. J. Food Prop.*, **2017**, *20*(4), 792-800.
40. Geng, X.L.; Tolkach, A.; Otte, J.; Ipsen, R. Pilot-scale purification of alpha-lactalbumin from enriched whey protein concentrate by anion-exchange chromatography and ultrafiltration. *Dairy Sci. Technol.*, **2015**, *95*(3), 353-368.
41. Fucinos, C.; Fucinos, P.; Estevez, N.; Pastrana, L.M.; Vicente, A.A.; Rua, M.L. One-step chromatographic method to purify alpha-lactalbumin from whey for nanotube synthesis purposes. *Food Chem.*, **2019**, *275*, 480-488.
42. Andrews, A.T.; Taylor, M.D.; Owen, A.J. Rapid analysis of bovine milk proteins by fast protein liquid chromatography. *J. Chromatogr. A*, **1985**, *348*, 177-185.
43. Felipe, X.; Law, A.J.R. Preparative-scale fractionation of bovine, caprine and ovine whey proteins by gel permeation chromatography. *J. Dairy Res.*, **1997**, *64*(3), 459-464.
44. Groves, M.L. Preparation of some iron-binding proteins and alpha-lactalbumin from bovine milk. *Biochim. Biophys. Acta*, **1965**, *100*, 154-162.
45. Konrad, G.; Lieske, B.; Faber, W. A large-scale isolation of native beta-lactoglobulin: Characterization of physicochemical properties and comparison with other methods. *Int. Dairy J.*, **2000**, *10*(10), 713-721.
46. Kristiansen, K.R.; Otte, J.; Ipsen, R.; Qvist, K.B. Large-scale preparation of beta-lactoglobulin a and b by ultrafiltration and ion-exchange chromatography. *Int. Dairy J.*, **1998**, *8*(2), 113-118.
47. Imafidon, G.I.; Ng-Kwai-Hang, K.F. Isolation and purification of beta-lactoglobulin by mass ion-exchange chromatography. *J. Dairy Res.*, **1992**, *59*(1), 101-104.
48. Kalan, E.B.; Greenberg, R.; Walter, M.; Gordon, W.G. Chemical properties of beta-lactoglobulins a, b and c. *Biochem. Biophys. Res. Commun.*, **1964**, *16*(3), 199-203.
49. Turhan, K.N.; Etsel, M.R. Whey protein isolate and alpha-lactalbumin recovery from lactic acid whey using cation-exchange chromatography. *J. Food Sci.*, **2004**, *69*(2), E66-E70.
50. Liang, Y.F.; Wang, X.W.; Wu, M.B.; Zhu, W.P. Simultaneous isolation of lactoferrin and lactoperoxidase from bovine colostrum by spec 70 sls cation exchange resin. *Int. J. Env. Res. Pub. He.*, **2011**, *8*(9), 3764-3776.
51. Ingle, U.; Lali, A. Development and optimization of a single-step cation chromatographic whey protein fractionation process: Evaluation and comparison of scale-up strategies. *Braz. J. Chem. Eng.*, **2018**, *35*(2), 805-818.
52. Hahn, R.; Schulz, P.M.; Schaupp, C.; Jungbauer, A. Bovine whey fractionation based on cation-exchange chromatography. *J. Chromatogr. A*, **1998**, *795*(2), 277-287.
53. Faraji, N.; Zhang, Y.; Ray, A.K. Optimization of lactoperoxidase and lactoferrin separation on an ion-exchange chromatography step. *Separations*, **2017**, *4*(2).
54. Faraji, N.; Zhang, Y.; Ray, A.K. Impact of operating conditions on chromatographic column performance: Experimental studies on adsorption of high-value minor whey proteins. *Aims Bioeng.*, **2017**, *4*(2), 223-238.
55. Faraji, N.; Zhang, Y.; Ray, A.K. Determination of adsorption isotherm parameters for minor whey proteins by gradient elution preparative liquid chromatography. *J. Chromatogr. A*, **2015**, *1412*, 67-74.
56. Kiddy, C.A.; Townend, R.E.; Thatcher, W.W.; Timasheff, S.N. B-lactoglobulin variation in milk from individual cows. *J. Dairy Res.*, **1965**, *32*(2), 209-217.
57. Voswinkel, L.; Kulozik, U. Fractionation of whey proteins by means of membrane adsorption chromatography. *Procedia Food Sci.*, **2011**, *1*, 900-907.
58. Voswinkel, L.; Kulozik, U. Fractionation of all major and minor whey proteins with radial flow membrane adsorption chromatography at lab and pilot scale. *Int. Dairy J.*, **2014**, *39*(1), 209-214.
59. Saufi, S.M.; Fee, C.J. Simultaneous anion and cation exchange chromatography of whey proteins using a customizable mixed matrix membrane. *J. Chromatogr. A*, **2011**, *1218*(50), 9003-9009.
60. Avramescu, M.E.; Borneman, Z.; Wessling, M. Mixed-matrix membrane adsorbers for protein separation. *J. Chromatogr. A*, **2003**, *1006*(1-2), 171-183.
61. Allen, P.Z.; Morrison, M. Lactoperoxidase. Iv. Immunological analysis of bovine lactoperoxidase preparations obtained by a simplified fractionation procedure. *Arch. Biochem. Biophys.*, **1963**, *102*(1), 106-113.
62. Xu, Y.; Sleight, R.; Hourigan, J.; Johnson, R. Separation of bovine immunoglobulin g and glycomacropptide from dairy whey. *Process Biochem.*, **2000**, *36*(5), 393-399.
63. Lech, M.; Niesobska, A.; Trusek-Holownia, A. Dairy wastewater utilization: Separation of whey proteins in membrane and chromatographic processes. *Desalination Water Treat.*, **2016**, *57*(48-49), 23326-23334.
64. Li, X.; Luo, Z.L.; Chen, H.B.; Cao, Y.S. Isolation and antigenicity evaluation of beta-lactoglobulin from buffalo milk. *Afr. J. Biotechnol.*, **2008**, *7*(13), 2258-2264.
65. Oram, J.D.; Reiter, B. Inhibition of bacteria by lactoferrin and other iron-chelating agents. *Biochim. Biophys. Acta*, **1968**, *170*(2), 351-365.
66. Lucas, D.; Rabiller-Baudry, M.; Millesime, L.; Chaufer, B.; Daufin, G. Extraction of alpha-lactalbumin from whey protein concentrate with modified inorganic membranes. *J. Membrane Sci.*, **1998**, *148*(1), 1-12.
67. Cowan, S.; Ritchie, S. Modified polyethersulfone (pes) ultrafiltration membranes for enhanced filtration of whey proteins. *Sep. Sci. Technol.*, **2007**, *42*(11), 2405-2418.



68. Plate, K.; Beutel, S.; Buccholz, H.; Demmer, W.; Fischer-Fruhholz, S.; Reif, O.; Ulber, R.; Scheper, T. Isolation of bovine lactoferrin, lactoperoxidase and enzymatically prepared lactoferricin from proteolytic digestion of bovine lactoferrin using adsorptive membrane chromatography. *J. Chromatogr. A*, **2006**, *1117*(1), 81-86.
69. Valino, V.; San Roman, M.F.; Ibanez, R.; Ortiz, I. Improved separation of bovine serum albumin and lactoferrin mixtures using charged ultrafiltration membranes. *Sep. Purif. Technol.*, **2014**, *125*, 163-169.
70. Freitag, R.; Splitt, H.; Reif, O.W. Controlled mixed-mode interaction chromatography on membrane adsorbers. *J. Chromatogr. A*, **1996**, *728*(1-2), 129-137.
71. Ulber, R.; Plate, K.; Weiss, T.; Demmer, W.; Buchholz, H.; Scheper, T. Downstream processing of bovine lactoferrin from sweet whey. *Acta Biotechnol.*, **2001**, *21*(1), 27-34.
72. Bhattacharjee, S.; Bhattacharjee, C.; Datta, S. Studies on the fractionation of beta-lactoglobulin from casein whey using ultrafiltration and ion-exchange membrane chromatography. *J. Membrane Sci.*, **2006**, *275*(1-2), 141-150.
73. Hossain, M.; Mohammad, A.; Collins, P.; Evaluation of a commercially available ion exchange membrane for separation of proteins from a whey protein mixture: Modeling and experimental results. *Adv. Food Process. Technol.*, **2018**, AFPT-118.
74. Goodall, S.; Grandison, A.S.; Jauregi, P.J.; Price, J. Selective separation of the major whey proteins using ion exchange membranes. *J. Dairy Sci.*, **2008**, *91*(1), 1-10.
75. Gerstner, J.A.; Hamilton, R.; Cramer, S.M. Membrane chromatographic systems for high-throughput protein separations. *J. Chromatogr. A*, **1992**, *596*(2), 173-180.
76. Kawai, T.; Saito, K.; Lee, W. Protein binding to polymer brush, based on ion-exchange, hydrophobic, and affinity interactions. *J. Chromatogr. B*, **2003**, *790*(1-2), 131-142.
77. Arunkumar, A.; Etzel, M.R. Fractionation of alpha-lactalbumin from beta-lactoglobulin using positively charged tangential flow ultrafiltration membranes. *Sep. Purif. Technol.*, **2013**, *105*, 121-128.
78. Arunkumar, A.; Etzel, M.R. Fractionation of alpha-lactalbumin and beta-lactoglobulin from bovine milk serum using staged, positively charged, tangential flow ultrafiltration membranes. *J. Membrane Sci.*, **2014**, *454*, 488-495.
79. Avramescu, M.E.; Girones, M.; Borneman, Z.; Wessling, M. Preparation of mixed matrix adsorber membranes for protein recovery. *J. Membrane Sci.*, **2003**, *218*(1-2), 219-233.
80. Shiew, C.F.; Shamsinar, N.; Rasli, N.I.; Saufi, S.M. Multiple interactions mixed matrix membrane chromatography using anion and cation exchanger resin for whey protein fractionation. *J. Teknol.*, **2015**, *75*(1), 259-263.
81. Saufi, S.M.; Fee, C.J. Recovery of lactoferrin from whey using cross-flow cation exchange mixed matrix membrane chromatography. *Sep. Purif. Technol.*, **2011**, *77*(1), 68-75.
82. Andersson, J.; Mattiasson, B. Simulated moving bed technology with a simplified approach for protein purification - separation of lactoperoxidase and lactoferrin from whey protein concentrate. *J. Chromatogr. A*, **2006**, *1107*(1-2), 88-95.
83. Du, Q.Y.; Lin, D.Q.; Xiong, Z.S.; Yao, S.J. One-step purification of lactoferrin from crude sweet whey using cation-exchange expanded bed adsorption. *Ind. Eng. Chem. Res.*, **2013**, *52*(7), 2693-2699.
84. 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*, 201-207.
85. Teepakorn, C.; Fiaty, K.; Charcosset, C. Comparison of membrane chromatography and monolith chromatography for lactoferrin and bovine serum albumin separation. *Processes*, **2016**, *4*(3).
86. Billakanti, J.M.; Fee, C.J. Characterization of cryogel monoliths for extraction of minor proteins from milk by cation exchange. *Biotechnol. Bioeng.*, **2009**, *103*(6), 1155-1163.
87. Dong, S.S.; Chen, L.; Dai, B.; Johnson, W.; Ye, J.L.; Shen, S.C.; Yun, J.X.; Yao, K.J.; 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.
88. Pan, M.M.; Shen, S.C.; Chen, L.; Dai, B.; Xu, L.H.; Yun, J.X.; Yao, K.J.; Lin, D.Q.; Yao, S.J. Separation of lactoperoxidase from bovine whey milk by cation exchange composite cryogel embedded macroporous cellulose beads. *Sep. Purif. Technol.*, **2015**, *147*, 132-138.
89. Machado, A.P.D.; Minim, L.A.; Fontan, R.D.I.; Minim, V.P.R.; Goncalves, G.R.F.; Mol, P.C.G. Adsorptive behavior of alpha-lactalbumin on cation-exchange supermacroporous monolithic column. *Fluid Phase Equilib.*, **2015**, *401*, 64-69.
90. Sarvi, M.N.; Bee, T.B.; Gooi, C.K.; Woonton, B.W.; Gee, M.L.; O'Connor, A.J. Development of functionalized mesoporous silica for adsorption and separation of dairy proteins. *Chem. Eng. J.*, **2014**, *235*, 244-251.
91. Itoyama, K.; Nishimura, S.-i.; Kawamura, Y.; Seo, H.; Sato, K.; Dosako, S.-i.; Nishi, N.; Tokura, S. Selective adsorption of bovine lactoferrin onto partially sulfated chitosan beads. *Sen'i Gakkaishi*, **1993**, *49*(11), 580-585.
92. Pessela, B.C.C.; Munilla, R.; Betancor, L.; Fuentes, M.; Carrascosa, A.V.; Vian, A.; Fernandez-Lafuente, R.; Guisan, J.M. Ion exchange using poorly activated supports, an easy way for purification of large proteins. *J. Chromatogr. A*, **2004**, *1034*(1-2), 155-159.
93. Pessela, B.C.; Torres, R.; Batalla, P.; Fuentes, M.; Mateo, C.; Fernandez-Lafuente, R.; Guisan, J.M. Simple purification of immunoglobulins from whey proteins concentrate. *Biotechnol. Prog.*, **2006**, *22*(2), 590-594.
94. Bolivar, J.M.; Batalla, P.; Mateo, C.; Carrascosa, A.V.; Pessela, B.C.; Guisan, J.M. Selective adsorption of small proteins on large-pore anion exchangers coated with medium size proteins. *Colloids Surf. B*, **2010**, *78*(1), 140-145.
95. El-Sayed, M.M.H.; Chase, H.A. Separation of the two major whey proteins using cation-exchange adsorption. *Lect. Notes Eng. Comp.*, **2008**, 39-43.
96. El-Sayed, M.M.H.; Chase, H.A. Single and two-component cation-exchange adsorption of the two pure major whey proteins. *J. Chromatogr. A*, **2009**, *1216*(50), 8705-8711.
97. El-Sayed, M.M.H.; Chase, H.A. Confocal microscopy study of uptake kinetics of alpha-lactalbumin and beta-lactoglobulin onto the cation-exchanger sp sepharose ff. *J. Sep. Sci.*, **2009**, *32*(18), 3246-3256.
98. El-Sayed, M.M.H.; Chase, H.A. Purification of the two major proteins from whey concentrate using a cation-exchange selective adsorption process. *Biotechnol. Prog.*, **2010**, *26*(1), 192-199.
99. El-Sayed, M.M.H.; Chase, H.A. Simulation of the breakthrough curves for the adsorption of alpha-lactalbumin and beta-lactoglobulin to sp sepharose ff cation-exchanger. *Biochem. Eng. J.*, **2010**, *49*(2), 221-228.
100. Nicolas, P.; Ferreira, M.L.; Lassalle, V. A review of magnetic separation of whey proteins and potential application to whey proteins recovery, isolation and utilization. *J. Food. Eng.*, **2019**, *246*, 7-15.
101. Meyer, A.; Berensmeier, S.; Franzreb, M. Direct capture of lactoferrin from whey using magnetic micro-ion exchangers in combination with high-gradient magnetic separation. *React. Funct. Polym.*, **2007**, *67*(12), 1577-1588.
102. Brown, G.N.; Muller, C.; Theodosiou, E.; Franzreb, M.; Thomas, O.R.T. Multi-cycle recovery of lactoferrin and lactoperoxidase from crude whey using fimbriated high-capacity magnetic cation exchangers and a novel "rotorstator" high-gradient magnetic separator. *Biotechnol. Bioeng.*, **2013**, *110*(6), 1714-1725.

103. Heeboll-Nielsen, A.; Justesen, S.F.L.; Hobley, T.J.; Thomas, O.R.T. Superparamagnetic cation-exchange adsorbents for bioproduct recovery from crude process liquors by high-gradient magnetic fishing. *Sep. Sci. Technol.*, **2004**, *39*(12), 2891-2914.
104. Heeboll-Nielsen, A.; Justesen, S.F.L.; Thomas, O.R.T. Fractionation of whey proteins with high-capacity superparamagnetic ion-exchangers. *J. Biotechnol.*, **2004**, *113*(1-3), 247-262.
105. Liao, A.W.; El Rassi, Z.; LeMaster, D.M.; Horváth, C. High performance displacement chromatography of proteins: Separation of  $\beta$ -lactoglobulins a and b. *Chromatographia*, **1987**, *24*(1), 881-885.
106. Vogt, S.; Freitag, R. Comparison of anion-exchange and hydroxyapatite displacement chromatography for the isolation of whey proteins. *J. Chromatogr. A*, **1997**, *760*(1), 125-137.
107. 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), 1747-1755.
108. Blomkalns, A.L.; Gomez, M.R. Purification of bovine alpha-lactalbumin by immobilized metal ion affinity chromatography. *Prep. Biochem. Biotechnol.*, **1997**, *27*(4), 219-226.
109. Carvalho, B.M.; Carvalho, L.M.; Silva, W.F., Jr.; Minim, L.A.; Soares, A.M.; Carvalho, G.G.; 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.
110. Rossano, R.; D'Elia, A.; Riccio, P. One-step separation from lactose: Recovery and purification of major cheese-whey proteins by hydroxyapatite--a flexible procedure suitable for small- and medium-scale preparations. *Protein Expr. Purif.*, **2001**, *21*(1), 165-169.
111. Schlatterer, B.; Baeker, R.; Schlatterer, K. Improved purification of beta-lactoglobulin from acid whey by means of ceramic hydroxyapatite chromatography with sodium fluoride as a displacer. *J. Chromatogr. B, Analyt. Technol. Biomed. Life Sci.*, **2004**, *807*(2), 223-228.
112. Ng, P.K.; Yoshitake, T. Purification of lactoferrin using hydroxyapatite. *J. Chromatogr. B*, **2010**, *878*(13-14), 976-980.
113. Sousa, R.d.C.S.d.; Coimbra, J.S.d.R.; Augusto, L.d.L.X.; Reis, L.S.T. Adsorção de alfa-lactalbumina do soro de leite em hidroxiapatita: Efeito do pH e da temperatura e análise termodinâmica. *Quim. Nova*, **2014**, *37*, 950-955.
114. Vyas, H.K.; Izco, J.M.; Jimenez-Flores, R. Scale-up of native beta-lactoglobulin affinity separation process. *J. Dairy Sci.*, **2002**, *85*(7), 1639-1645.
115. Tercinier, L.; Ye, A.Q.; Singh, A.; Anema, S.G.; Singh, H. Effects of ionic strength, pH and milk serum composition on adsorption of milk proteins on to hydroxyapatite particles. *Food Biophys.*, **2014**, *9*(4), 341-348.
116. Nagasaki, T.; Nagata, F.; Sakurai, M.; Kato, K. Effects of pore distribution of hydroxyapatite particles on their protein adsorption behavior. *J. Asian. Ceram. Soc.*, **2017**, *5*(2), 88-93.
117. Kawachi, G.; Watanabe, T.; Ogata, S.I.; Kamitakahara, M.; Ohtsuki, C. Protein adsorption on needle-shaped hydroxyapatite prepared by hydrothermal treatment of mixture composed of  $\text{CaPO}_4$  center dot  $2\text{H}_2\text{O}$  and  $\beta\text{-Ca}_3(\text{PO}_4)_2$ . *J. Ceram. Soc. Jpn.*, **2009**, *117*(1367), 847-850.
118. Cetinkaya, S.; Akkaya, B. Selective and single step adsorption of  $\alpha$ -lactalbumin from whole cow's milk on hydroxyapatite microbeads. *Mat. Sci. Eng. C-Mater.*, **2016**, *68*, 573-578.
119. Moreno-Castilla, C. Adsorption of organic molecules from aqueous solutions on carbon materials. *Carbon*, **2004**, *42*(1), 83-94.
120. Ribeiro Alves, M.R.; Zuñiga, A.D.G.; Sousa, R.d.C.S.; Zacchi Scolforo, C. The process of separating bovine serum albumin using hydroxyapatite and active babassu coal *orbignya martiana*. *Sci. World J.*, **2016**, *2016*, 2808241.
121. Gecgel, U.; Uner, O. Adsorption of bovine serum albumin onto activated carbon prepared from elaeagnus stone. *B. Chem. Soc. Ethiopia*, **2018**, *32*(1), 53-63.
122. Pereira, R.G.; Veloso, C.M.; da Silva, N.M.; de Sousa, L.F.; Bonomo, R.C.F.; de Souza, A.O.; Souza, M.O.D.; Fontan, R.D.I. Preparation of activated carbons from cocoa shells and siriguela seeds using  $\text{H}_3\text{PO}_4$  and  $\text{ZnCl}_2$  as activating agents for BSA and  $\alpha$ -lactalbumin adsorption. *Fuel Process. Technol.*, **2014**, *126*, 476-486.
123. Andrade, S.N.; Veloso, C.M.; Fontan, R.C.I.; Bonomo, R.C.F.; Santos, L.S.; Brito, M.J.P.; Diniz, G.A. Chemical-activated carbon from coconut (*cocos nucifera*) endocarp waste and its application in the adsorption of beta-lactoglobulin protein. *Rev. Mex. Ing. Quim.*, **2018**, *17*(2), 463-475.
124. Batista, G.A.; Silva, M.L.M.; Gomes, W.D.; Neves, I.C.O.; Mol, P.C.G.; de Resende, J.V.; Verissimo, L.A.A.; Soares, J.R. Preparation of mesoporous activated carbon from defective coffee beans for adsorption of fresh whey proteins. *Acta Sci.-Technol.*, **2020**, *42*.
125. Seredych, M.; Mikhalovska, L.; Mikhalovsky, S.; Gogotsi, Y. Adsorption of bovine serum albumin on carbon-based materials. *Journal of Carbon Research*, **2018**, *4*(1).
126. Guo, Z.Y.; Zhang, D.D.; Song, S.F.; Shu, Y.; Chen, X.W.; Wang, J.H. Complexes of magnetic nanospheres with amphiprotic polymer-zn systems for the selective isolation of lactoferrin. *J. Mater. Chem. B*, **2018**, *6*(35), 5596-5603.
127. Guo, P.F.; Wang, X.M.; Wang, M.M.; Yang, T.; Chen, M.L.; Wang, J.H. Boron-titanate monolayer nanosheets for highly selective adsorption of immunoglobulin G. *Nanoscale*, **2019**, *11*(19), 9362-9368.
128. Chen, Q.; Zhang, D.D.; Wang, M.M.; Chen, X.W.; Wang, J.H. A novel organic-inorganic hybrid polyoxometalate for the selective adsorption/isolation of beta-lactoglobulin. *J. Mater. Chem. B*, **2015**, *3*(34), 6964-6970.
129. Soleimani, M.; Ghaderi, S.; Afshar, M.G.; Soleimani, S. Synthesis of molecularly imprinted polymer as a sorbent for solid phase extraction of bovine albumin from whey, milk, urine and serum. *Microchem. J.*, **2012**, *100*, 1-7.
130. Jimenez-Guzman, J.; Mendez-Palacios, I.; Lopez-Luna, A.; Del Moral-Ramirez, E.; Barzana, E.; Garcia-Garibay, M. Development of a molecularly imprinted polymer for the recovery of lactoferrin. *Food Bioprod. Process.*, **2014**, *92*(C2), 226-232.
131. Wang, Y.F.; Zhou, J.J.; Wu, C.; Tian, L.; Zhang, B.L.; Zhang, Q.Y. Fabrication of micron-sized BSA-imprinted polymers with outstanding adsorption capacity based on poly(glycidyl methacrylate)/polystyrene (PGMA/PS) anisotropic microspheres. *J. Mater. Chem. B*, **2018**, *6*(37).
132. Wang, Y.F.; Zhou, J.J.; Gu, H.M.; Jia, X.K.; Su, K.H.; Zhang, B.L.; Zhang, Q.Y. Preparation of anti-nonspecific adsorption chitosan-based bovine serum albumin imprinted polymers with outstanding adsorption capacity and selective recognition ability based on magnetic microspheres. *Macromol. Mater. Eng.*, **2019**, *304*(4).