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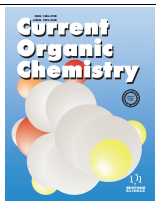
REVIEW ARTICLE



Membrane Processes for Whey Proteins Separation and Purification. A Review

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Abstract: ***Background*:** Whey is a valuable by-product obtained from cheese manufacture and employed as a source of proteins that present remarkable potential in food or pharmaceutical industries. Although chromatography is the common method to isolate whey proteins at preparative scale, it reports some disadvantages like its high operational cost.

***Objective*:** Membrane processes can play an important role in protein separation, as a consequence of the relatively lower energy consumption, good yields and easy scalability. Important effort has been done on the development of membrane chromatography as an innovative technique to obtain and isolate the different whey proteins. Pressure-driven membrane processes have become standard unit operations in different applications performed in the dairy industry. In this sense, membrane filtration can be applied for protein separation and isolation under adequate conditions.

***Conclusion*:** The application of an electrical field or the use of charged membranes combined with pressure-driven membrane processes, provide good results and decrease undesirable phenomena such as concentration polarization when compared to conventional membrane separations. This manuscript provides an overview of the main membrane methods employed in the literature to carry out the separation and isolation of whey proteins.

Keywords: Whey proteins, membrane chromatography, ultrafiltration, electrical field, charged membranes, membrane contactor.

1. INTRODUCTION

Whey is the most important by-product obtained in cheese manufacture [1]. Cheese whey contains about 93.0-94.0% of water, nutrients from the original milk such as lactose, soluble proteins, minerals, lactic acid and fats and other components, such as citric acid, non-protein nitrogen compounds (urea and uric acid) and vitamins (B group). Due to the nutritional and medical characteristics of the protein concentrates, the cheese whey valorization is receiving a growing interest. Additionally, the recovery of valuable compounds might contribute to reduce the environmental problem caused by high BOD and COD content of the dairy industry effluents [2, 3].

Whey proteins are largely used in food industry due to their high nutritional value, excellent functional properties availability and their low cost (in comparison with others sources of proteins) [4, 5]. Many reviews describe the properties and composition of whey [6-9] including the characteristics and applications of specific whey proteins of interest [10, 11]. Moreover, they are essential components for many products because of their ability to confer functional characteristics as foaming, emulsification, gelation and stabilization [1]. Whey protein composition, according to [12] is shown in Table **1**.

Many research groups are currently paying attention to whey protein separation, isolation and valorization [2, 13-17]. The separation of proteins from the original mixture is commonly carried out by processes that account more than 80.0% of the product costs [1]. Due to its efficiency, chromatography is the common method to obtain individual proteins at industrial scale, but it presents high operational costs. Therefore, the research and development of more efficient and scalable technologies for whey protein separation remains as a challenge [18]. In this framework, membrane technologies are promising tools for biotechnological downstream processing as they are simple, energy efficient and easy to scale-up [12]. Several works have been focused on membrane chromatography, especially on ion exchange and affinity chromatography [19-22]. Pressure-driven membrane processes, including ultrafiltration, are regularly used in dairy industries for whey treatment but their efficacy is still limited by either fouling phenomena or the difficulties to separate proteins with similar size [23]. Additionally, different authors [24-28] have studied the use of charged membranes to separate whey proteins achieving high purity and selectivity; however their industrial performance needs of the optimization of both the energy consumption and operational costs. Finally, the application of an electrical field during the filtration process facilitates the separation and reduces fouling.

Considering the interest and possibilities of membrane technologies in the field of protein separation, this paper is focused on the recent advances found in the literature dealing with the research and development of membrane technologies applied to whey proteins separation. In the following sections whey proteins characterization and properties are described. Then, an overview of the main membrane technologies of interest for whey protein separation namely membrane chromatography, pressure driven processes and electrically enhanced membrane separation are discussed.

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Table 1. Bovine whey protein composition [7, 12].

Protein	Content (% w/w)	Molecular Mass (kDa)	Isoelectric Point (pI)
β-lactoglobulin (β-lg)	35.0	18.4	5.2
α-lactalbumin (α-la)	12.0	14.2	4.5-4.8
Glycomacropeptide (GMP)	12.0	6.8	4.3-4.6
Proteose-peptone	12.0	4.0-22.0	-
Inmunoglobulins (Igs)	8.0	150.0-100.0	5.5-8.3
Bovine serum albumin (BSA)	5.0	66.5	4.7-5.0
Lactoferrin (Lf)	1.0	78.0	7.0-9.0
Lactoperoxidase (LP)	0.5	89.0	9.5

Table 2. Composition of whey from different sources [30].

Composition (%)	Sweet Whey	Acid Whey	Casein Whey
Total solids (%)	94.0-98.5	93.0-97.0	93.0-98.0
Lactose (%)	69.0-76.0	65.0-69.0	64.0-68.0
Proteins (%)	11.0-14.0	7.0-9.0	9.0-1.01
Fat (%)	0.5-2.0	-	-
Ash (minerals) (%)	6.0-9.0	9.0-12.0	10.0-13.0
pH	6.0-6.8	4.2-5.0	4.0-4.8

2. WHEY CHARACTERIZATION

Cheese whey is a green-yellowish liquid resulting from the precipitation and removal of milk casein during cheese-making that represents 85.0-95.0% of the milk volume [2]. The world whey production is estimated over 160 million of tones per year with an annual growth rate of 1.0-2.0% [13].

Whey is produced as consequence of the precipitation and removal of casein during cheese manufacture. This casein can be separated by several methods generating different types of whey. Sweet whey, which is the most common one, results from the casein precipitation by rennet at pH 6.5 [7, 13]. Acid whey is produced by the lactic acid precipitation during the manufacture fresh and curd cheese. Finally, casein whey is obtained in casein production when mineral acids are used for precipitation. Table **2** shows the general composition of the main types of whey [12, 29, 30].

As depicted in Table **2**, lactose is the main component of whey. Together with fat and proteins, it is responsible of the organic pollution generated by whey effluents [2, 15, 29]. Proteins represent between 7.0-11.0% of the total composition of whey. In spite of its low presence, proteins constitute and important by-product with a high added value [14].

Nowadays, about 50.0% of the total whey is treated and transformed into various food products: i) liquid product (45.0%), ii) dry whey powder (30.0%), iii) lactose and de-lactose by-products (15.0%) and iv) cheese whey protein concentrate (WPC) (10.0%) [7, 14, 15, 31]. Whey proteins are used by the pharmaceutical industry and in several applications in food industry to produce potential bioactive ingredients for dietary [14, 32]. Whey proteins are a mixture of natural components excluding Glycomacropeptide (GMP) that is obtained from casein during the first enzymatic step in cheese processing [7]. The major components are considered α-la, β-lg, GMP and the minor components are BSA, LF, LP and Igs (Table **1**) [4]. Because of theirs physicochemical and biological roles, the isolation of major whey proteins as β-lg and α-la has been satisfactorily achieved. However, minor proteins with similar size are difficult to isolate and their separation require more efforts to develop feasible separation techniques economically [14, 22, 33]. Some relevant characteristics and applications of whey proteins are included in Table **3** [5-8, 34-46].

3. MEMBRANE PROCESSES FOR WHEY PROTEIN SEPARATION

Membranes are defined as barriers that separate two fluid phases and selectively restrict the transport of several components. Transport can take place by convection or by diffusion of individual molecules, or it can be induced by an electric field or concentration, pressure or temperature gradient. A membrane separation system divides an influent stream into two effluent streams known as permeate and retentate. The permeate is defined as the fraction of the fluid that passes through the semi-permeable membrane, whereas the concentrate stream contains the constituents rejected by the membrane. These processes have numerous industrial applications and provide the following advantages: they offer energy savings, are environmental benign, replace technologies such as conventional filtration, distillation or ion-exchange, produce high-quality products, offer greater flexibility in system design and are considered as clean technologies and easy to operate [47].

Table 3. Main characteristics and applications of whey proteins.

Protein	Characteristics	Applications	Ref.
Inmunoglobulins (Igs)	Mixture of antibodies IgA, IgG, IgM, IgG1 and IgG2.	• Bacterial opsonization and agglutination. • Neutralization of toxins. • Viruses inactivation. • Reduction of the cholesterol level. • Manufacture of infant formulae or food products to reduce the viral and microbial infections.	[6, 34]
Bovine Serum Albumin (BSA)	BSA is synthesizedin mammary gland. It appears in milk by passive leakage from the blood stream.	• Bind reversibly with several fatty acids to participate in synthesis of lipids. • Act as emulsifying, gelling and foaming properties.	[34, 35]
Lactoferrin (Lf)	Found in cow or human milk, mucus, tears or saliva.	• Antimicrobial, anti-virus, anti-parasitic and anti-inflammatory activity. • Resistant to heat denaturation. • Pharma, cosmetics and hygiene products manufacture. • Supplement in food industry (infant formula).	[8, 36-38]
α-lactalbumin (α-la)	Protein synthesized in the mammary gland.	• Participation in the lactose synthesis. • Emulsifying, gelling and foam properties. • Infant formulas manufacture.	[6, 39]
Glycomacropeptide (GMP)	Protein realized during the enzymatic cleavage of k-casein in cheese manufacture.	• Gelling and foaming properties. • Benefit cardiovascular, digestive, immune or nervous system. • Tooth pastes manufacture (anticarciogenic properties). • Only protein that has no phenylalanine. Used in foods for phenylketoneuria (PKU).	[40-44]
Proteose-peptone	Composed by minor proteins and glycoproteins designed as component 3, 5, 8-slow and 8- fast.	• Employed in food products and as the prevention against dental caries.	[7, 45, 46]
Lactoperoxidase (Lp)	Found in animal secretions such as tears, saliva or milk.	• Protection against infectious microbes. • Prevention of several illnesses such as pneumonia. • Preservation of food, oral care or cosmetics manufacture.	[5, 6, 9]

The efficacy of a membrane system depends on both the required productivity and selectivity of the separation. Several drawback such as the high initial capital costs, fouling phenomena, achievable product purity, and limited long-term performance often limit its industrial development. However, numerous significant processes in the food processing and biotechnology industries employ membrane processes. Some examples include water desalination and purification, cold sterilization of beverages, recovery and fractionation of proteins from cheese whey, clarification of fruit juice, beer and wine and formulation of bio-products among many others [48].

Nowadays conventional chromatography remains as the most efficient technique to isolate or purify whey proteins at industrial scale [26]. Different chromatographic methods have been employed to carry out the protein separation such as affinity, ion exchange or hydrophobic iterations, but they present disadvantages such as fouling, long cycle times, and complicated process control systems [32, 36]. To overcome these disadvantages, more recently, macroporous membrane monolith columns have been employed in order to increase the separation rate and to reduce the backpressure thus avoiding the unspecific binding and product degradation. The advantages of these monoliths are similar to those derived from the use of conventional chromatography [17, 36].

Membrane pressure-driven processes are of great interest for the removal of mineral salts from whey. Whey is concentrated by evaporation or reverse osmosis (RO) and demineralized by electrodialysis or ion-exchange resins nowadays [49, 50]. Nanofiltration membranes (NF) can be used to carry out the demineralization because of their permeability to monovalent salts and organic compounds allowing at the same time, a reduction of the energy costs and wastewater disposal [51, 52]; RO leads to the retention of approximately 89.0% of lactose contain, thus facilitating the water evaporation or crystallization [53]; Microfiltration (MF) is mainly employed to remove microorganisms and residual lipids minimizing fouling and its effects on the subsequent processes [54]. Fig. (**1**) shows the main applications of membrane pressure-driven processes in the dairy industry.

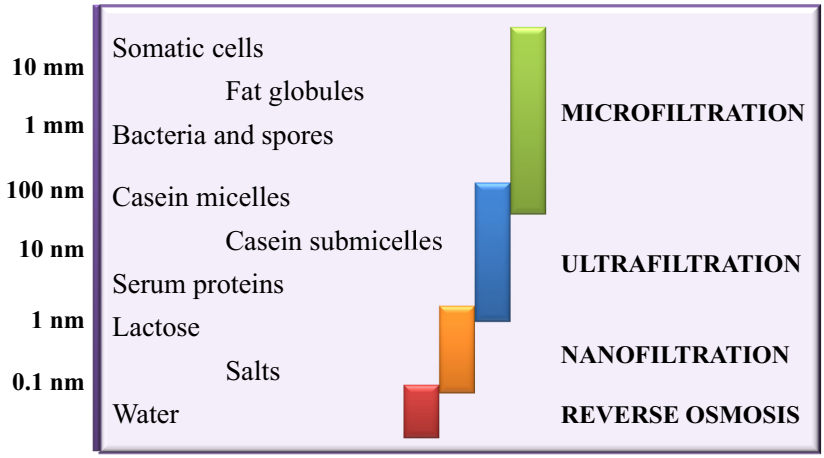

Fig. (1). Size indication and membrane processes of milk components separation [51].

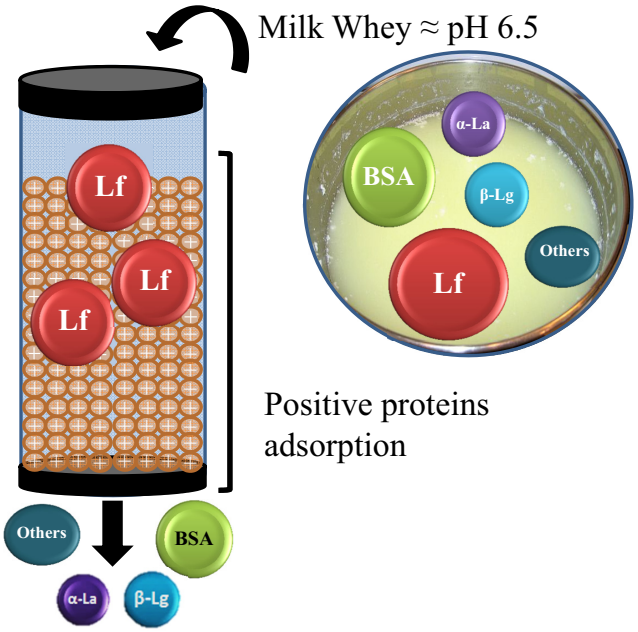

Fig. (2). Application of membrane chromatography to the separation of Lf from whey [16].

In the following sections a critical review including i) membrane chromatography, ii) pressure-driven membrane processes and iii) electrically-enhanced membrane separation processes, is reported and discussed.

3.1. Protein Separation by Membrane Chromatography

Chromatography is currently used for protein recovery and purification. However, the implementation of membrane adsorption technology provides the possibility to recover high value minor proteins from whey by optimizing the operational costs [32]. Therefore, membrane chromatography has been studied as a possible innovative technique to separate proteins based on the integration of filtration and liquid chromatography in a single step [22]. In addition, the use of adsorptive membranes provides high rates, short residence times at low trans-membrane pressure (TMP) and high binding capacities. In membrane chromatography separation processes, the ligand is immobilized on the membrane surface. When the solution with the compound of interest is filtered, the ligand binds the desired molecule selectively and reversibly, allowing the separation of the target molecules [21, 55]. Less strongly retained molecules are carried away by liquid phase [21]. A general scheme of those processes is depicted in Fig. (**2**). The predominant transport of solutes takes place by convection that improves the adsorption, washing, elution and regeneration steps, avoids the inactivation of proteins and reduces the mass transfer resistance [22]. Consequently, low residence time is required to show its binding capacity [19].

Membranes can be turned into adsorption materials by grafting functional exchange groups on the inner surface of the micropores, which determine the kind of system obtained such as ion-exchange, affinity, hydrophobic or metal systems [56]. Particularly, ion-exchange and affinity chromatography are the most suitable methods to separate or purify proteins [17].

3.2. Ion-Exchange Membrane Chromatography

Ion-exchangers consist of an insoluble matrix covalently bounded to charged groups. These functional groups are associated with mobile counter-ions that are reversibly exchanged with other ions with the same charge than proteins [57]. The principle of protein separation by ion-exchange membranes is based on the electrostatic interaction between the charged proteins and the adsorption surface [55]. Ion-exchange membranes are widely employed to separate amino acids or proteins. The target biomolecule is able to displace the counter-ion associated to the active surface group being further stripped with the help of complementary buffer salt. In order to achieve high yields of separation it is necessary to choose an appropriate buffer to shield the native protein [21, 57]. The Dynamic Binding Capacity (DBC) of exchangers depends on both the molecular size of the target protein and adsorption conditions such as pH, ionic strength or protein concentration [55].

Several authors employed ion-exchange membrane chromatography to separate target proteins such as Lactoferrin (Lf) and Lactoperoxidase (Lp). Plate *et al*., used cationic exchange membranes and 1.0 M NaCl as elution buffer, obtaining 84.0% of Lf (with a purity of 97.0%) and 10.0% of Lp. On the other hand, when the salt concentration is reduced to 0.1 M, 80.0% of Lp and 1% of Lf were eluted thus confirming the importance of salt concentration on the protein elution [32]. Similar results were obtained by Chiu *et al*., working with similar salt concentrations and low volumes (200 ml); In this work they obtained an elution of Lf about 80.0%, while the recovery decreased when the initial volume was increased. They

also studied the influence of the number of cycles in the separation process concluding that the membrane module was able to operate for 12 cycles without a decrease in the production due to the removal of the bounded proteins from the membrane surface during elution [58]. Fractionation of whey proteins using ionic exchange membranes was also studied by Voswinkel and kulozik [59]. Whey was treated in a first step using cationic exchange membranes at pH 7.0 able to uptake negatively charged proteins, except α-la. During the first stage, β-lg and BSA were eluted at 1.0 M and 0.1 M of NaCl, respectively. The effluent was treated in a second step of anionic exchange membranes in order to recover Lp, Lf and IgG at 0.35 M, 0.1 M and 0.025 M of NaCl with a purity of 100.0 % approximately. However, only about 30.0 % of the total Lf was recovered with this process and about 88.0 % of α-La was obtained in the effluent stream.

Avramescu [55] and Saufi and Fee [60] reported the synthesis method of ion-exchange mixed matrix membranes (MMM) with a particular material entrapped in a porous matrix for the separation of different proteins. This method avoids the need to modify chemical conditions to carry out the separation process [60]. The main ligands employed to synthesize the membranes are alkyl (*e.g.* butyl and octyl) and aryl (*e.g.* phenyl) groups, in order to obtain cationic or anionic membranes for protein separation [61]. On the other hand, due to the low pressure drop and suitable operation conditions, biomolecules can be obtained in native and biological form during the purification or isolation processes [56]. Phenyl sepharose mixed matrix membranes were used by Saufi *et al.* [61] to isolate β-lg, α-La, Lf and BSA using 2.0 M $(NH_4)_2SO_4$. Results demonstrated a lower binding percentage of β-lg in comparison with anionic exchange media, achieving a 76.0% of elution recovery. In addition, this method can be also used to isolate α-La from whey protein with a high purity [61]. Avramescu *et al*., [62] studied the separation of BSA/ hemoglobin (Hb) and lysozyme from chicken egg white with ion-exchange mixed matrix membranes. In the case of cation exchange membrane, BSA/Hb is recovered with a separation factor (which is defined by the target solute concentration divided by the concentration of other solute and the undesired) of 40.0 at pH 7.0. Differents anion exchange membranes were also studied and reported the total adsorption of BSA. The separation factor achieved with these membranes is around 50.0 at pH 5.5 [62]. On the other hand, hollow fiber membranes were prepared to separate lysozyme from chicken egg white. The ion exchange resins were incorporated into a PES polymeric matrix achieving recoveries about 95.0% of lysozyme with a concentration of 0.5 M NaCl [63].

3.3. Membrane Affinity Chromatography

Affinity chromatography has been widely used for protein purification. This technique takes advantage of the strong interaction between ligands and target proteins based on biological functions. The global process includes three steps: loading, washing and elution [64]. The main advantages of membrane chromatography in comparison with traditional chromatography are the low diffusion time (due to the interactions between molecules occur inside the pores), low-pressure drop, high flow-rate and high productivity [20, 36]. This technique is more suitable for larger proteins because they rarely enter into the pores as they are bounded on the external surface area. Several studies also suggest that membrane chromatography is appropriate for processing large volumes of liquid with low concentration of the target protein [17].

Chromatographic membranes have been developed by supporting a matrix on the surface of a polymeric scaffold with the desired physical or chemical properties [19]. Hydrophilic materials are suitable for affinity membrane chromatography due to their low non-specific adsorption, their chemical stability, good mechanical properties and easiness to form microporous or macroporous structure. Therefore, materials like chitosan and chitin are mainly employed in membrane affinity manufacture. Macroporous chitin membranes prepared with silica as porogen were employed by Ruckenstein and Zengto [65] to separate lyzosyme from ovalbumin and lysozyme from egg white. A high purity of lysozyme (>99.0%) and yields around 61.5% were achieved due to the hydrogen bonding and the van der Waals interactions between lysozyme and chitin oligosaccharide. Large volumes of lysozyme can be treated achieving satisfactory selective separations in both mixtures [66]. A broad description of commercial chromatographic membranes is included in Table **4**.

Despite the good results obtained to isolate or separate proteins using membrane chromatography, this technology is not implemented at large-scale since adsorption with membranes presents lower binding capacity per volume protein than resin columns. Therefore, development of membranes with higher binding capacity without modification of mechanical strength, hydraulic permeability or pore size distribution, is still a challenge for further research [17, 67].

3.4. Protein Separation by Pressure-driven Membrane Processes

Pressure-driven membrane processes employ the difference of pressure between the feed and the permeate side to force the transport of the solvent through the membrane and include, Microfiltration (MF), Ultrafiltration (UF), Nanofiltration (NF) and Reverse Osmosis (RO) [68]. Fig. (**3**) shows the classification of pressure-driven membrane processes according to the type and size of the solute or particle [69].

Pressure-driven membrane processes are the major membrane processes that have become standard unit operations in the dairy industry. MF is mainly employed for clarification and defatting of cheese whey. The use of ceramic and polymeric membranes for skim milk microflitration has been deeply studied by Adams and Barbano [70], Hurt and Barbano [71] and Adams *et al*., [72]; RO is used in the partial demineralization or pre-concentration of whey and NF is able to satisfactorily perform the simultaneous demineralization and concentration of whey [73].

3.4.1. Ultrafiltration

UF is used for the concentration of species with a molecular weight between 10.0 and 1000.0 kDa [74] which involves the protein separation. It is widely used in dairy industry because it avoids the need of a phase change (*i.e.* evaporation) thus leading to more economical processes [75]. One of the most important applications of UF is the production of whey protein concentrates due to its ability to retain proteins and permeate minerals, lactose, water and other compounds of low molecular weight. A general description of this process is depicted in Fig. (**4**) [76].

The main drawbacks associated with protein separation with UF are the reduction of selectivity and filtration efficiency during the process due to membrane fouling [16, 23]. The membrane material and the molecular conformation of whey proteins exert a considerable influence on the flux. Opposite charges between proteins and membrane induce protein-membrane electrostatic attractions that promote protein adsorption on the membrane surface. This may also produce an undesirable denaturation and aggregation of the

Table 4. Characteristics of commercial chromatographic membranes [19] (adapted).

Product Name	Functionality	Pore Size (μm)	Membrane Material	Capacity (10.0%)
QyuSpeed D[a]	Anion-exchange	n/a	GMA grafted polyethylene	>40.0 mg mL^{-1} BSA
QyuSpeed A[a]	Anion-exchange	n/a	Ultra-high molecular weight polypropylene	≥50.0 mg mL^{-1} BSA
Natrix Q[b]	Anion-exchange	0.45	Hydrogel	>300.0 mg mL^{-1} BSA
Natrix S[b]	Cation-exchange	0.45	Hydrogel	>250.0 mg mL^{-1} Lysozyme
Natrix C[b]	Cation-exchange	0.45	Hydrogel	>80.0 mg mL^{-1} IGg
Natrix IMAC-Ni^{2+}[,b]	Immobilized metal affinity Cromatography (IMAC)	0.45	Hydrogel	>70.0 mg mL^{-1} GFP
Natrix Protein A[b]	Affinity	n/a	Hydrogel	n/a
Natrix Aldehyde[b]	Affinity coupling	n/a	Hydrogel	n/a
Mustang Q[c]	Anion-exchange	0.8	Modified PES	56.0 mg mL^{-1} BSA
Mustang S[c]	Cation-exchange	0.8	Modified PES	47.0 mg mL^{-1} Lysozyme
Mustang E[c]	Anion-exchange	0.2	Modified PES	Endotoxin
Sartobind Q[d]	Anion-exchange	>3.0	RC	29.0 mg mL^{-1} BSA
Sartobind D[d]	Anion-exchange[(e)]	>3.0	RC	21.0 mg mL^{-1} BSA
Sartobind STIC[d]	Anion-exchange	>3.0	RC	50.0 mg mL^{-1} BSA
Sartobind S[d]	Cation-exchange	>3.0	RC	25.0 mg mL^{-1} Lysozyme
Sartobind C[d]	Cation-exchange	>3.0	RC	21.0 mg mL^{-1} Lysozyme
Sartobind Phenyl[d]	Hydrophobic interaction	>3.0	RC	15.0 mg mL^{-1} IgG
Sartobind IDA[d]	Immobilized metal affinity	>3.0	RC	3.6 mg mL^{-1} His-tagged protein
Sartobind Protein A[d]	Affinity	0.45	RC	5.0-7.5 mg mL^{-1} IgG
Sartobind epoxy[(5)]	Affinity coupling	0.45	RC	n/a
Sartobind aldehyde	Affinity coupling	0.45	SRC	n/a

GMA—glycidyl methacrylate. PES—Polysulfone. RC—*Regenerated cellulose*. STIC—salt tolerant interaction chromatography. SCR—solvent-resistant regenerated cellulose. a: manufacturer: Asahi Kasei Bioprocess. b: manufacturer: NATRIX Separations, Inc. c: manufacturer: Pall Corporation. d: manufacturer: Sartorius AG. e: Need a separate column from manufacturer Pallmakes Mustang® Sartorius. n/a not available.

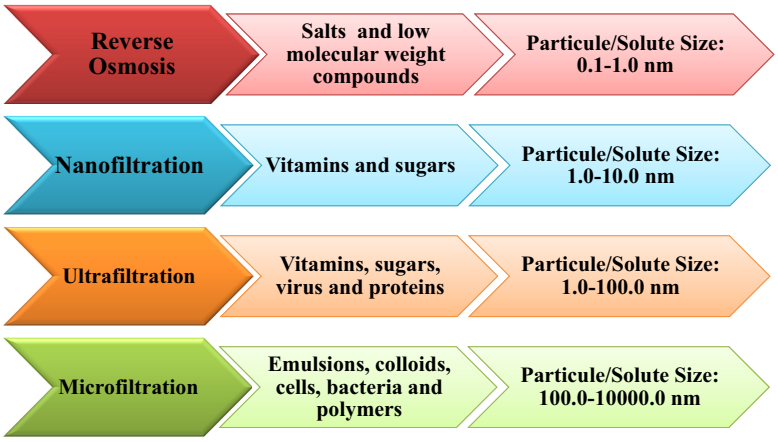

Fig. (3). Classification of pressure-driven membrane processes [71].

adsorbed proteins [70]. UF membranes, that can be manufactured in different geometries, such as hollow-fibers, flat-sheet cassettes, spiral-wound cartridges or tubular modules, are generally synthetized using hydrophobic polymers such as polyacrilonitrile, regenerated cellulose membranes or cellulose acetate. These materials are prone to increase the fouling due to the deposition of proteins on

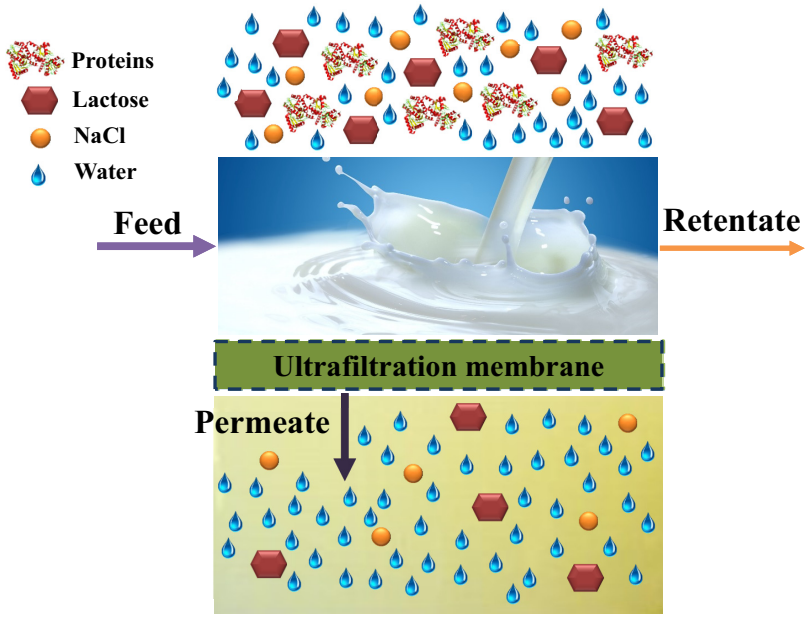

Fig. (4). Production of whey protein concentrate by UF [76].

the surface or the adsorption into membrane pores producing a flux decrease [52]. Ceramic membranes can be also employed as an alternative because of their high chemical resistance [51]. Doyen *et al*., [77] studied the ultrafiltration of whey for protein recovery with ceramic (ZrO_2) and organo-mineral (ZrO_2/PSf) membranes and compared their performance with a conventional polymer membrane (PSF/PVP) with molecular cut-off values between 25.0-50.0 kDa. All of them showed similar flux and concentration factors, permeability values were higher for organic-mineral membranes due to their much higher surface porosity [42]. Almécija *et al*., [78] used tubular ceramic cross-flow ultrafiltration membranes of 300.0 kDa to clarify whey proteins. α-La and β-Lg were eluted with low permeate yields at pH 4 and 5 while BSA, IgG and Lf were retained at pH 9.0 reporting improvements on the selectivity up to 60.0% as compared with original whey.

However, polymeric polysulfone membranes remain the most widely used membranes in whey UF, to avoid the protein denaturation, due to their low cost, good thermal stability and mechanical properties. These membranes are commonly employed in the spiral bound or hollow-fiber configurations. The availability of industrial hollow-fiber modules is limited by their low transmembrane pressure (TMP) ratings. Spiral bound modules, which can withstand higher pressures and are able to minimize the occurrence of concentration polarization phenomena, are the configurations with the lowest capital and operating costs compared with other configurations [70].

In order to improve the production and to reduce the concentration polarization, UF is sometimes operated in the so called diafiltration (DF) mode where deionized water is continuously added to the retentate to wash out the impurities thus increasing its purity [23, 75]. Energy consumption can be also reduced by selecting the optimal operation conditions [79].

Several limitations of UF for whey treatment such as low selectivity, difficulty to separate proteins with similar size and concentration polarization, reduce the useful life of membranes and the permeate flux, and contribute to increase the cost of the process [80].

3.4.2. High-Performance Tangential Flow Filtration

Conventional flow filtration is limited to the separation of solutes that differ ten-fold in size. However, an emerging technology known as High-Performance Tangential Flow Filtration, HPTFF, has been developed to enhance the separation and prevent the formation of fouling [28]. HPTFF is a two-dimensional purification method that is based on the size and charge differences of biomolecules. Separation of BSA and antigen binding fragment (Fab) derived from a recombinant DNA antibody was studied achieving a high rejection of Fab using a positively charged membrane. Protein sieving decreased when the protein is highly charged at pH near to isoelectric point of BSA [81, 82]. A similar method was employed by Lucas *et al*. [83] to separate α-La from acid casein whey protein (WPC) with polyethyleneimine membranes. The selectivity was three times higher than the obtained with unmodified membranes. HPFTT was also used to separate monomers from oligomers based on their size differences and to remove impurities in purification processes such as DNA, proteins or endotoxins [16]. Selectivity has been demonstrated to be dependent on both the transmembrane pressure and the local filtrate flux. The electrostatic interactions between charged proteins and charged membranes control the HPFTT separations. Further, the process efficacy can be improved by optimizing the feed flow rate, ionic strength, bulk protein concentration and membrane pore size [28, 84]. Cheang and Zydney [85] also studied the separation of α-La and β-Lg with a diafiltration process using a tangencial flow filtration system. Selectivity was about 55.0 as a result of the strong retention of β-Lg molecules and the high transmission of α-La. In comparison with previous studies, the application of this technology provides better results and lower the operational costs. Several methods have been employed to prevent membrane fouling and to enhance the protein separation such as boundary layer controls, membranes with new materials or modifications and the use of an electric field [86]. In this last case, protein degradation can take place when an electrical current in applied. In order to prevent this situation, electrodes can be shielded by ion exchange membranes or dialysis membranes, allowing the development of new separation techniques [87]. Frac-

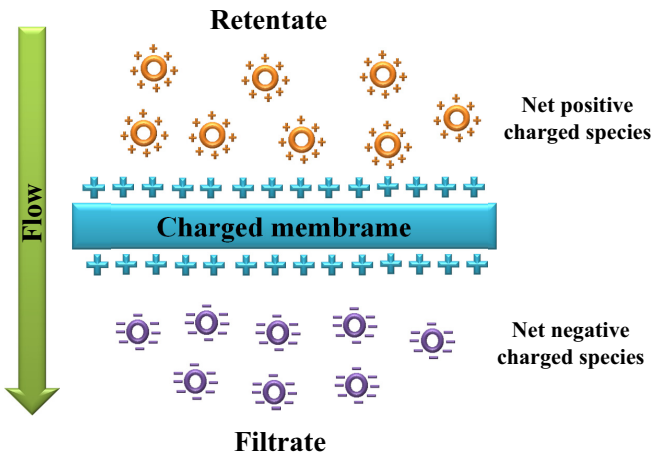

Fig. (5). Scheme of the separation process with UF charged membranes [98].

tionation of bovine α-La from β-Lg in milk serum permeate using tangential flow ultrafiltration has been studied by Arunkumar and Etzel [88]. By incorporating positively charged functional groups on the surface of a 300.0 kDa regenerated cellulose ultrafiltration membrane, it was possible to increase the selectivity for fractionating α-La and β-Lg by 180.0% compared to an uncharged membrane [88]. Thus, like-sized proteins that differed only on the isoelectric point, and were about 15-20 times smaller than the membrane molecular weight cutoff, were fractionated using charged ultrafiltration membranes. Electrostatic repulsion was solely responsible for the improved selectivity. Protein sieving coefficients were explained by the net charge on the protein and the stagnant film model. Arunkumar and Etzel [89] also examined the use of wide-pore negatively charged ultrafiltration membranes for whey protein concentration. It was concluded, that the incorporation of negative charges on the surface of an ultrafiltration membrane, improved the rejection of negatively charged proteins due to the combination of sized based sieving and electrostatic repulsion. Therefore, the surface functionalization allows the use of wide-pore membranes that provide higher fluxes without affecting the protein recovery. It was found that negatively charged 100.0 kDa ultrafiltration membranes had the same protein recovery than 10.0 kDa unmodified membranes used in the dairy industry, but offered a flux that was at least two-fold higher. The new membranes were used for a 40-fold concentration of whey with subsequent diafiltration to mimic the industrial process for making whey protein concentrate.

3.5. Electrically Enhanced Membrane Separation

Although pressure-driven membrane processes are widely used in dairy industry to concentrate or isolate proteins, they are not effective enough when the proteins present in the mixture have a similar size. Taking advance of the electrostatic charge of protein molecules, the use of charged membranes can increase the selectivity and efficiency of the separation processes [1, 25].

The use of pressure-driven membrane processes combined with the application of an electrical current has received the interest of the scientific community due to their ability to separate proteins based on its size or charge with high purity and throughput [16, 36, 90]. Electrically-enhanced membrane filtration (EMF) takes advantage of the superposition of an electrical field and conventional filtration techniques [91, 92]. Three different effects can be considered when an electric field is applied: electrophoresis, electroosmosis and electrolysis. Electrophoresis is defined as the migration of particles/ions driven by the charge differences when an electric field is applied (electrophoretic mobility) [93]. On the other hand, electroosmosis is the motion of solvent induced by an applied potential across a porous material. Finally, electrolysis is carried out using electrodes to promote chemical reactions leading to the formation of new substances [94]. Protein separation by means of EMF can be performed by two different techniques: electro-ultrafiltration (EUF) and electrodialysis combined with ultrafiltration (EDUF).

3.5.1. Charged UF Membranes

UF membranes can be modified to increase their superficial charge. The process selectivity can be improved by the proper selection of pH and ionic strength conditions since they have an effect on the interactions between charged protein and functional groups on the membrane surface [95-97]. Charged ultrafiltration membranes with a defined pore structure have been employed in the selective separation of proteins [18]. Fouling is also prevented as a consequence of the electrostatic repulsion between membrane surface and foulants [16]. The scheme of the separation process with UF charged membranes is shown in Fig. (**5**) [98].

Several authors have studied the separation of different proteins employing charged membranes. Nakao *et al*. [99] separated cytochrome-c and myoglobin (MYO) at the isoelectric point of one of the proteins using surface-modified polysulfone membranes. The proteins were separated using negatively charged membranes produces by sulfonation and positively charged membranes functionalized by cholormethylation, followed by quaternization of the amino group. At the isoelectric point of the cytochrome-c, the protein permeates completely and 80.0% of myoglobin is rejected with negatively charged membranes. In the opposite case, 20.0% of cytochrome-c is rejected and all myoglobin permeate through the membrane [99]. Other applications of charged membranes were developed by different research groups [100, 101]. In example, Saksena and Zydney [84] improved the separation between BSA and IgG more than 20-field by adjusting the pH using charged membranes. Valiño *et al*. [1] studied the separation of BSA and Lf using an initial concentration ratio of 4.0/1.0 and positively charged membrane. BSA recovery was 70.0% and a permeate flux of 30.31 g m^{-2} h^{-1} at pH value of 5.0. These results show that it is possible to obtain optimal separation of the proteins with the appropriate process conditions.

3.5.2. Electro-ultrafiltration

Electro-ultrafiltration (EUF) which is based on the application of an electric force field across the membrane, is a promising method to reduce fouling and concentration polarization [100]. The electric field exerts an electrophoretic effect on the charged molecules dragging them away from the membrane surface. The concentration polarization layer is thereby reduced and the flux increases. However, in order to achieve a good separation, the isoelectric point of proteins to be separated must be different [102]. It is also important to take into account that solutions with high salt have a detrimental effect on the effectiveness of EUF since the electric field is shared between electrolyte and proteins mobility [87]. Song *et al*. [86] studied the application of EUF to separate BSA using membranes with different molecular weight cut-off, 50.0 and 100.0 kDa. Results showed that membrane fouling can effectively diminished and permeate flux increased when the membrane of 100.0 kDa was used. Sarkar *et al*. [103] showed the effects of varying electric field during ultrafiltration of BSA and lysozyme in aqueous

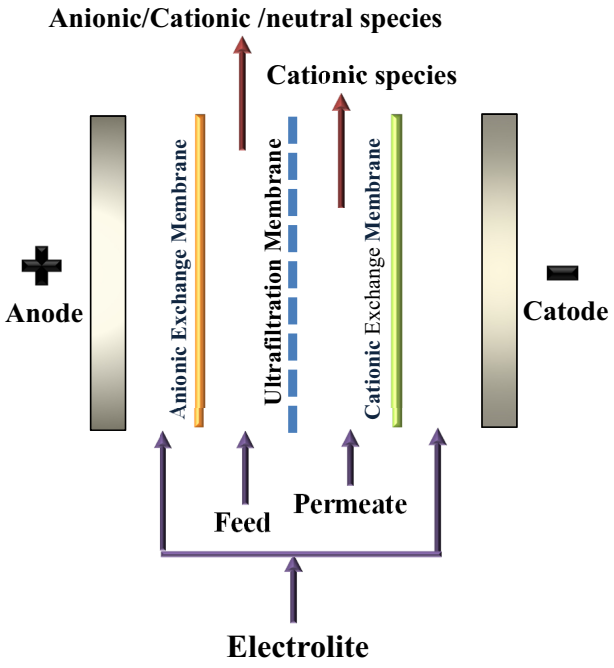

Fig. (6). ED cell with ultrafiltration membrances: EDUF technology [12].

solution. Higher permeate flux (36.7 L $m^{-2} \cdot h^{-1}$) and low retention of lysozyme (20.0%) were achieved working at its isoelectric point (pH=7.4) when an electric field were applied [103].

EUF is commonly applied under a constant electric field, but recent studies have demonstrated that the use of a pulsed electric field (PEF) can reduce the energy consumption and improve the permeate flux [87]. Zumbusch *et al*., [93] concluded that permeate flux of BSA increases when an alternating current is applied. They also observed that the effect of the electric field is more pronounced at low frequencies. The effect of pulsed electric field in an ultrafiltration module with a BSA solution was studied by Oussedik *et al*. [104] They reported that permeate flux increased about 300.0% when a pulsed electric field was applied and membrane damage by abrasion was avoided. PEF has been employed in numerous applications in food industry such as pre-treatment of carrot purees to improve the sugar and polyacetylene content [105], pretreatment and microbial growth controller in wine, beer and rice wine productions [106] or treatment of food by-products or wastes to recover valuable compounds [107].

3.5.3. Electrodialysis Combined with Ultrafiltration (EDUF)

Electrodialysis (ED) is an electro-membrane process based on the selective transport through ion exchange membranes by means of an electric potential gradient. When an electric field is combined with the use of ion exchange and ultrafiltration membranes, the separation of charged species takes place by mechanisms that depend on their molecular size and electrical charge. This alternative is commonly known as electrodialysis combined with ultrafiltration (EDUF). The typical EDUF cell is constituted by parallel ion-exchange and UF membranes placed between two electrodes known as cathode and anode [108, 109], (Fig. **6**). This method uses an external field as driving force providing the removal of salts and proteins at the same time [92, 102].

Several authors have studied the application of EDUF to separate whey proteins. Chen and *et al*. [102] studied the separation of BSA and lysozyme. Concentration polarization decreased as the current intensity increases; higher recoveries of lysozyme (72.9%) were achieved when the pH was not buffered. The permeate flux decreased as consequence of the increase on the protein concentration during EDUF being the best value 17.0×10^{-8} m s^{-1} with concentrations of 1.0 g l^{-1} of BSA and 0.01 g/l of lysozyme. EDUF can be also employed to separate bioactive peptides and other charged molecules with industrial interest as demonstrated by Poulin *et al*. [110]. They studied the separation of peptides from β-Lg hydrolysate achieving the migration of 40.0 of these peptides from the raw material Some relevant studies reported in the literature related to the use of EDUF for whey protein separation are included in Table **5**.

EDUF is usually performed by means of Electrophoretic membrane contactors (EMC). The driving force in EMC is provided by two electrodes located in the compartments that are separated by ion-exchange membranes or porous membranes with low molecular weight cut-off [113]. The pH of the different compartments is kept at the desired value by continuous addition of adequate buffer solutions [114]. The selectivity of the separation can be optimized by changing the characteristics of the background electrolyte or the membrane pore size and charge [115]. UF membranes should also take an average pore size larger than the target protein size, to retain other components present in the mixture, or smaller than the protein size to permit the pass of smaller species [116]. The process can be performed in two operating modes: separation mode and elution mode. The separation mode is showed in Fig. (**7a**) where two compartments are fed with the same solution containing two solutes, A and B. In the elution mode, depicted on Fig. (**7b**), the initial solution containing A and B is fed in only one compartment and in the other is fed with a buffer solution. The use of 'separation mode' or 'elution mode' depends on the specific separation objective. In terms of production, 'separation mode' would be preferable and for achieving higher purification, the 'elution mode' [117-119].

Both operation modes were studied by Galier and Roux-de Balmann [119] to isolate α-La and bovine hemoglobin. The best results were achieved working in elution mode at an initial concentration of 0.1 g/l, obtaining a concentrate enriched in α-La with a purity of 99.0%. However, the yield was higher in separation mode than in elution mode with values of 86.0% and 66.0%, respectively. Galier and Roux-de Balmann [120] proposed a methodology to select the best operating conditions to separate biomolecules in an EMC. Single component solution experiments and theoretical analysis were performed to predict the influence of the process parameters (pH, membrane MWCO) on the separation factor. They studied the separation of α-La and β-lg using an electrophoretic membrane contactor with cellulose acetate membranes of 30.0 kDa and 100.0 kDa. These proteins were only separated by membranes with a pore size of 100.0 kDa and pH=4.8 reaching a separation factor (SF) of 1.2. A value higher than unity means that separation can be achieved whereas a value equal to unity reveals no selectivity [108].

Horvath and *et al*. [121] designed a new device based on EMC, known as "*Gradiflow*". This design combines the hydraulic flow of the protein mixture through the separation channels with orthogonal electrophoretic transport of proteins across de single separation membrane of polyacrylamide which divides both compartments. The use of this technology avoids the protein denaturation and enables the process to be scaled-up [122]. Membranes are placed in a cartridge, parallel to the flow and electrodes. Separated reservoirs of sample solution and buffer are used which were circulated using

Table 5. Relevant applications of EDUF for whey protein separations compiled in the literature [12].

Ref	Compound ($g \cdot L^{-1}$)	Contactor	Membrane	Conditions	Flux ($L \cdot m^{-2} \cdot h^{-1}$)	Selectivity
[110]	β-lg=0.2 α-la=0.1	EMC 4.4-17.6 V	CMX-SB cationic AMX-SB anionic Cellulose acetate 30.0 and 100.0 kDa	Tris-Mes pH 8.0 Meshistidine pH 6.0 L-alanine-acetic acid pH 4.8 140.0-220.0 μS cm^{-1}	-7.1 β-Lg -28.6 (total)	1.25
[111]	Hb= 1.0 BSA= 1.0	*IEM-FFIEF 60.0-200.0 V	Cation exchange Anion exchange Homemade Polysulfone-based Cation-exchange	pH 4.8	-9.3 Hb -31.1 (total)	Total BSA
[112]	BSA= 5.0 MYO_{blood}= 5.0	*IEM-FFIEF 200.0 V	Cation exchange Anion exchange Homemade Polysulfone-based cation-exchange	0.02 M acetic acid-Tris Buffer pH 4.8 No conductivity data	-2.1 BSA -20.0 (total)	Total BSA
[92]	Synthetic and real whey + commercial bovine lactoferrin (Lf)	EDUF 20.0 V	CMX-SB cationic AMX-SB anionic Polyethersulfone 500.0 kDa	2.0 $g \cdot L^{-1}$ KCl pH 3.0 3.3 mS cm^{-1}	-8.9 Lf -8.0 α-la -41.0 β-lg -2.0 BSA	Lf/α-la=1.1 Lf/BSA=4.45 Lf/β-lg=0.2
[89]	Mixture: 1.3 Lf + 1.3 whey Proteins isolated: β-lg+α-la	EMF 5.0-10.0 V	Polyvinylidene fluoride 0.5 μm	Deionized water pH 7.0 16.0 μS cm^{-1}	130.0	$*S_{Lf/\beta\text{-}lg}$=3.0-6.7 $S_{Lf/\alpha\text{-}la}$=9.0-62.2

*Ion Exchange Membrane Partitioned Free-Flow Isoelectric Focusing (IEM-FFIEF). Emerging separation process combining both membrane technologies and electrophoresis technologies in a series of separated chambers.
**$S_{x/y}=(C_{permeate}/C_{Feed})$

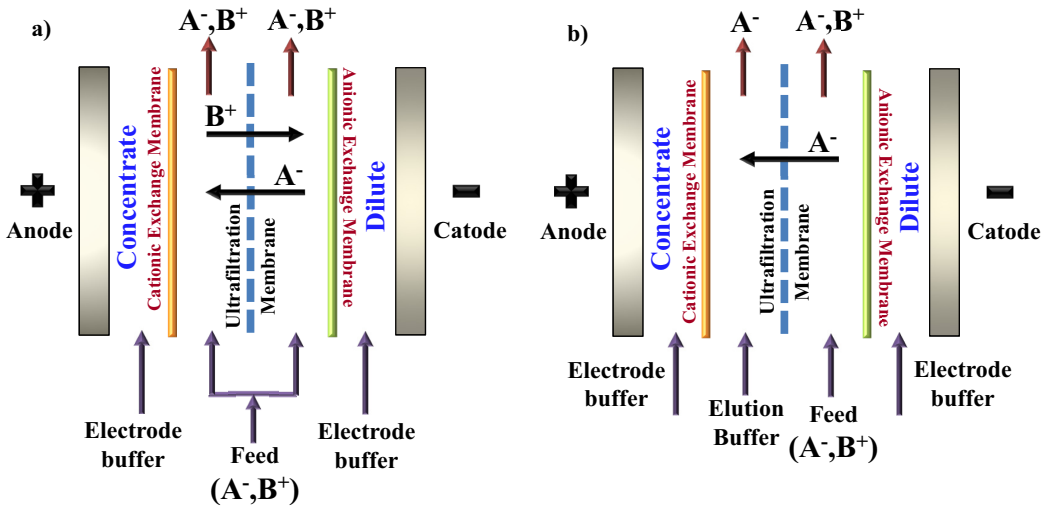

Fig. (7). Electrophoretic membrane contactor (EMC): a) separation mode and b) elution mode [117].

a two-channel peristaltic pump housed within the device [123]. This technology has been employed to separate proteins from complex biological solutions such as plasma, algal extracts, and milk or to separate monoclonal antibodies from ascetic fluid [111]. Cheung *et al*., [124] achieved the separation of Fab/F(ab')$_2$ antibodies applying a voltage of 250 V. Rothemund *et al*. [125] recovered the 76.0% and 65.0% of basic protein from mixtures with egg yolk and lysozyme, respectively, at pH=10.2 and 250 V. Catzel *et al*. [126] purified recombinant human growth hormone from CB515 at pH=7.5 and 8.5 achieving in both cases high yields and purity (90.0% and 98.0% respectively).

CONCLUSION

The high nutritional and functional properties of whey proteins have increased the interest in the use of these macromolecules,

mainly in isolate form, in food and pharmaceutical industry. This input has encouraged the development of more advanced and efficient separation and isolation techniques.

Chromatography has been the most widely used method to separate whey proteins at preparative scale but it presents several disadvantages which entail high operational costs and hinder the full scale implementation. Membrane processes represent an opportunity to overcome these drawbacks due to their easiness to scale up, low operational costs and relatively small energy consumption.

Membrane chromatography presents several advantages compared to traditional chromatography, among them, lower diffusion times, low pressure drop, high flow-rate and high productivity. Despite the good results obtained to isolate or separate proteins using membrane chromatography, this technology has not been implemented at large-scale because they reported lower binding capacity per volume protein than resin columns. Therefore, the development of membranes with higher binding capacity without modification of mechanical strength, hydraulic permeability or pore size distribution, is still a challenge for further research.

On the other hand, pressure-driven membrane processes are nowadays essential unit operations in the dairy industry. In particular, UF is used for the concentration of species with molecular weights between 1.0 and 10.0^3 kDa, including protein separation. This technology is one of the pioneers in the development of new and more economical processes since they do not involve change phases. Nevertheless the limitations of UF in whey processing, namely low selectivity, difficulty to separate proteins with similar size and concentration polarization, reduce the useful life of membranes and the permeate flux, thus claiming for new advances addressed to overcome these limitations.

Taking advantage of the electrostatic charge of protein molecules, the use of charged UF membranes is a promising alternative to increase the selectivity towards target proteins. Moving a step forward, protein separation by means of EMF, usually performed by two different techniques: electro-ultrafiltration (EUF) and electrodialysis combined with ultrafiltration (EDUF), combines the use of an electric field with ion exchange and ultrafiltration membranes allowing the separation of proteins according to their molecular size and electrical charge. Results reported in the literature present promising possibilities for these technologies applied to whey proteins purification although further research is needed to develop industrial applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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