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Chapter 10

Advancements in the Fractionation of Milk Biopeptides by Means of Membrane Processes

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1. Introduction

Nowadays the most common way to obtain bioactive peptides is by enzymatic hydrolysis of protein solutions. The most studied substrates used to produce bioactive peptides are milk proteins in the form of co-products from dairy industries: caseins, cheese whey, buttermilk, whey protein concentrates and isolates or even pure single proteins that can be obtained at a reasonable price on an industrial scale (e.g. β-lactoglobulin, β-Lg).

Different specific and non-specific enzymes are used to obtain hydrolysates (trypsin, pepsin, pancreatin and alcalase). The catalytic activity of some of them is quite specific and the composition of the hydrolysate is predictable when substrates are quite pure [1]. In other cases, the activity of the enzyme is non-specific and produces a complex mixture of peptides and amino acids in which individual effect of each molecule in the subsequent fractionation process is difficult to demonstrate and quantify. The design of an efficient fractionation methodology is then of paramount importance for peptides separation and even more, when the process must applied on an industrial scale. Separation technologies, which discriminate small differences in charge, size and hydrophobicity, can be employed to fractionate protein hydrolysates and obtain peptide fractions with higher functionality or higher nutritional value in a more purified form. Membrane separation techniques seem to be well suited for this purpose. These processes are based upon selective permeability of one or more of the liquid constituents through the membrane according to the driving forces.

2. Overview of techniques used for peptide fractionation

Due to the demonstration of their impact on human health, the market for functional food and nutraceuticals containing bioactive peptides is increasing very rapidly and, consequently, the food and bio-pharmaceutic industries are looking for processes allowing

the production of this kind of products from natural sources. Considering that most functional peptides are present in complex mixtures containing a large number of hydrolysed protein fractions, their separation and purification are required.

The methodologies commonly used for peptide fractionation and enrichment include: selective precipitation, membrane filtration, ion exchange, gel filtration technologies and liquid chromatography [1]. However, significant differences concerning the number and type of extracted peptides occur among extraction procedures. Additionally, undesired peptides, such as allergenic or bitter-tasting peptides, could be enriched in the process when using some of those techniques [2].

Fractionation methods involving precipitation steps are carried out by means of the addition of organic solvents like ethanol, methanol or acetone; adding acids like trichloroacetic acid (TCA), sulphosalicylic acid or phosphotungstic acid (PPTA); by means of the addition of salts (ammonium sulphate) or just by adjusting the pH to the isoelectric point. Precipitation often results in a selective fractionation of peptides depending on their solubility in the precipitating agent [3]; however the addition of chemical compounds causes in some cases peptide degradation and changes in the biological and physical properties.

Chromatographic methods for peptide separation are currently used at lab-scale: high performance liquid chromatography (HPLC), fast protein liquid chromatography (FPLC), isoelectric focusing (IEF) and ion exchange chromatography (IEC) are some of them. In most cases, one or two cycles of successive HPLC separation had been adequate to isolate peptides one by one. In the same way, IEC has been used for the enrichment of casein phosphopeptides from casein hydrolysates or for the isolation of cationic antibacterial peptides from lactoferrin. However, although chromatographic processes can provide good separation selectivity, the low productivity and high production costs involved in these processes make impossible its use at industrial scale.

Size exclusion chromatography (SEC) and more frequently Ultrafiltration-Nanofiltration (UF-NF) are the main techniques used to isolate peptides according to their molecular size [4-10]. In addition it is possible to obtain more purified hydrolysate samples by removing salts and other interfering components by means of UF membranes [11]. In fact, investigations into these methodologies under optimized conditions to reduce time and cost are ongoing [12].

Pressure-driven membrane-based processes, such as UF and NF, are used to fractionate peptide mixtures and amino acids [13]. These types of membrane have been widely used to fractionate milk protein hydrolysates with the aim of enhancing their biological or functional properties [14-15]. It has been shown that variations in operating conditions may favor the permeation of bioactive peptides [16-17].

Membrane technology has become an important separation technology in recent decades probably because their main advantages (it works without the addition of chemicals, with a relatively low use of energy, it has low processing costs, the scale-up is an easy subject and the process lines are well arranged) make it the ideal technology for use on an industrial

scale. In addition, membrane processes are especially suitable for the food industry, because of the mild working conditions, relatively easy scale up and low processing costs in comparison to chromatographic techniques.

The separation of peptides by UF mainly depends on the molecular weight (MW) cut-off (MWCO) of the membrane. However, when the MW of the peptides involved in the process is quite similar, their isolation is a hard subject; in these cases, NF is the best membrane separation technique [18]. The fact that NF membranes are usually charged offers the possibility of separating solutes through a combination of size and charge mechanisms.

3. Membrane technology applied to peptide fractionation

Membrane processes are now viewed as efficient tools for the development of new value-added products by separating minor compounds such as bioactive peptides [19]. These separation processes are based upon selective permeability of one or more of the liquid constituents through the membrane according to the pressure difference. Amongst the pressure-driven membrane techniques, which main features are summarized in Figure 1, UF and NF have been tested for the fractionation of protein hydrolysates due to the fact that the molecular weight of most bioactive peptides is within the normal pore size range of these membranes.

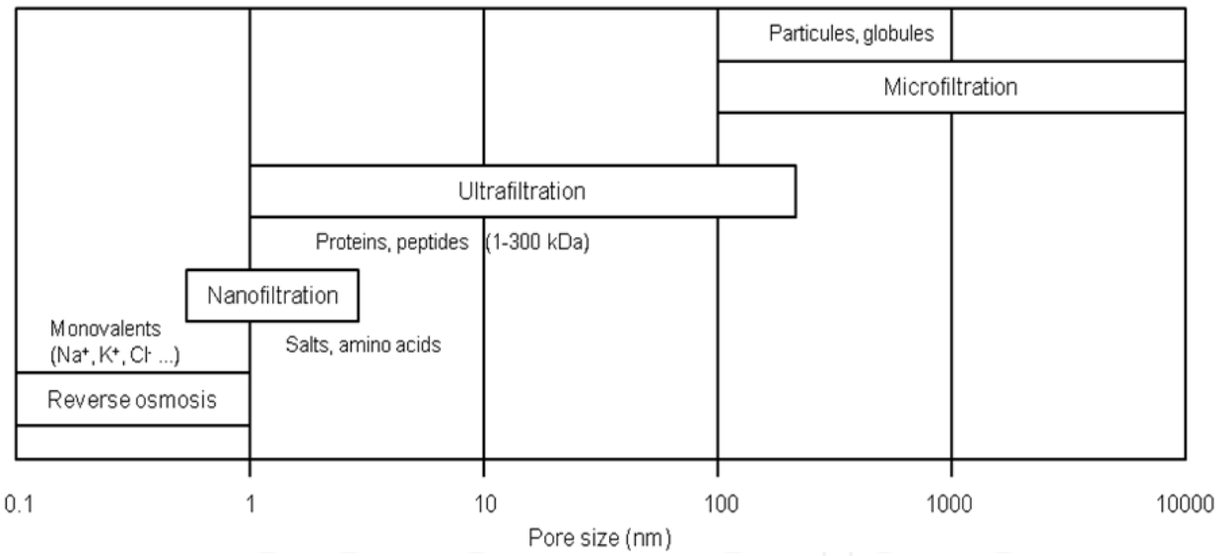

Figure 1. Pressure-driven membrane processes

UF is commonly applied to prepare enriched bioactive solutions from protein hydrolysates and improve the bioactivity of peptides. This process is also used to separate peptides with a size lower than 7 kDa [20]. The fractions are collected by subsequently filtration in two or three streams to obtain peptides with different size [21]. For example, amino acids and small peptides can be separated at pH 4.6 into four ranges of molecular mass (I<30 kDa, II>30 kDa (protein fraction), III>10 kDa (protein fraction), IV>0.3 kDa) [22]. Recent results on fractionation peptides by UF-membranes show that crude yoghurt fractions obtained after ion exchange can be separated into four fractions by successive UF using membranes with

molecular cut off sizes of 30, 10 and 3 kDa [23]; whereas UF membranes < 1 kDa are efficient for peptides fractionation from milk hydrolysates if the last permeate contains free amino acids [21].

The combination of membrane processes (UF and NF) is also often used to separation of peptides. The first step of these processes consists in the UF of the hydrolysate in order to obtain complete rejection of intact proteins and intermediate peptides. The resulting permeate fractions is then subjected to a fractionation by NF and a peptide fraction having a molar mass < 1 kDa is isolated of the mixture by means of these membranes.

In this case, permeates obtained after UF could be adjusted at two pH values (9.5 and 3.0) that corresponded to the different charged states of the membrane and of the peptides to improve of separation of polypeptides of molar mass < 1 kDa [23-24].

Recently a method that couple UF and HPLC has also been applied on milk hydrolysate samples for enhance the peptides separation. A current study showed that an UF-membrane was enough to concentrate peptides and subsequently, both permeate and retentate were fractioned by SE-HPLC to obtain small peptides with biological activity [25].

There are also other important UF-processes to separate specific compounds of whey as caseinomacropeptide (CMP). A first method was designed to obtain CMP fractions trough UF membranes with MWCO 20-50 kDa by two diafiltration steps [26]. The method is based on the ability of CMP to form non-covalent linked polymers with a molecular weight up to 50 kDa at neutral pH, which dissociate at acid conditions. The dissociated form of CMP permeates through the UF-membrane at pH 3.5, whereas the majority of whey proteins such as β-Lg, α-lactalbumin (α-La), immunoglobulins (IGs) and bovine serum albumin (BSA) are held back. At pH 7.0, permeate containing CMP can be concentrated by means of the same membrane; however a low permeation rate is obtained with this technique. A second method for separation of CMP can be seen in [27]. Thermal stability of CMP is used in comparison to that of the rest of whey proteins. Complete denaturation and aggregation of proteins is obtained by treating whey at 90°C for 1h; with this method, the denatured proteins can be removed by centrifugation at 5200 g and 4°C for 15 min and the supernatant containing CMP can be concentrated by UF with MWCO 10 kDa after pH adjustment to 7.0; however whey proteins lose part of their functionality due to the denaturation.

Another method for separation of CMP consists in the pretreatment of whey protein concentrate with the enzyme transglutaminase (Tgase) followed by microfiltration [28]. The amino acid sequence of CMP includes two glutamine and three lysine residues, whereby this peptide can be cross-linked by tranglutaminase. The covalent linked CMP aggregates can be removed be means of microfiltration or diafiltration to obtain CMP-free whey protein.

3.1. Enzymatic membrane reactor equipped with membranes: first step to peptide fractionation

Enzymatic membrane reactor (EMR) consists on a coupling of a membrane separation process with an enzymatic reaction. EMR allows the continuous production and separation

of specific peptide sequences by means a selective membrane, which is used to separate the biocatalyst from the reaction products and the peptides fractionation [29]. At present, EMR is used when working on an industrial scale. This technology for peptides separation is gaining interest, because it is a specific mode for running batch or continuous processes in which enzymes are separated from end products with the help of a selective membrane. By that way, it is possible to obtain complete retention of the enzyme without deactivation problems typical of enzyme immobilization. Furthermore, EMR have been shown to improve the efficiency of enzyme-catalyzed bioconversion and to increase product yields [13, 30-31].

EMR technology has been investigated for the production and separation of peptides since the 90´s. Antithrombotic peptides derived from hydrolysed CMP can be recovered by UF membranes [32-33] and Lactorphin have been successfully produced through continuous hydrolysis of whey in an UF-reactor [34-35]. Multicompartment EMR has also been designed for the continuous hydrolysis of milk proteins. Nowadays, this technique is operated under an electric field for continuous harvesting of some biologically active peptides, such as phosphopeptides and precursors of casomorphins from the tryptic digest of β-casein [36]. Special attention had also had the study of the hydrolysis of whey protein isolates (WPI) using a tangential flow filter membrane (TFF) of 10 kDa in EMR [37]. The factors influencing on the operation of the EMR (substrate concentration, ionic strength, and transmembrane pressure) have been studied and discussed in other research works [30, 38]. In recent years, the use of EMR has emerged as an exciting area of research due to their low production cost, product safety and easy scaled up [39]. Table 1 summarizes some examples of processes for the separation or concentration of bioactive peptides by means of UF membranes. UF offers possibilities for a large-scale production of bioactive peptides but seems limited because of fouling and poor selectivity. Another drawback of UF membranes is their pore size, because the large pores are not selective enough to fractionate small peptides MW of bioactive peptides is usually smaller than 1 kDa). To sum up, with the use of an EMR equipped with UF membranes, the first peptide fractionation is achieved but if a more purified permeate is required; NF membranes should be used as an additional step instead of UF membranes.

3.2. NF membranes and peptide fractionation

NF is a pressure-driven membrane technique in which the pore size of the membrane is in the nanometers range. As can be observed in Figure 1, this technique is an intermediate step between reverse osmosis (RO) and UF and it is useful to separate/fractionate solutes with MW lower than 5 kDa. Transmembrane pressure in NF is lower than in RO and the permeate flux is usually higher, which represents an important energetic advantage in industrial applications. NF membranes of cut-off < 1 kDa are particularly useful for the filtration of the smaller peptides from hydrolysates solutions.

The selectivity of NF membranes is based on both size and charge characteristics of the solutes and on the interaction between charged solutes and membrane surface. Hydrodynamic parameters (mainly transmembrane pressure and linear velocities) and

membrane material exert influence on membrane selectivity too. NF membranes have a slightly charged surface; because the dimensions of the pores are less than one order of magnitude larger than the size of ions [54].

Protein Hydrolysate Source	Biological Activity	References
Bovine caseinomacropeptide	Antithrombotic	[32-33]
	Calcium bioavailability improvement	[40]
	Bone and teeth mineralization	
Bovine whey β-lactoglobulin	ACE inhibitor	[41]
	Opioid	[42]
	Anti microbial	[43]
	Muscular contraction	[44]
Bovine whey α-lactalbumin	ACE inhibitor	[4]
Fish protein	ACE inhibitor	[45]
Alfalta white protein	ACE inhibitor	[46]
Alfalta leaf protein	Antioxidant	[47]
Wheat gluten	ACE inhibitor	[48]
Soybean protein	ACE inhibitor	[49]
Soybean β-conglycinin	ACE inhibitor	[50]
Sea cucumber gelatin	ACE inhibitor	[51]
Potato	Antimicrobial	[52]
Potato	Antimicrobial	[53]

Table 1. Bioactive peptides obtained by means of UF membranes

3.2.1. NF transport mechanism

The mechanism behind the selectivity of membrane processes is generally the size of the component. This mainly applies in the case of UF membranes and in the case of NF membranes with uncharged solutes. Charge effects are minimized in this case and the transmission of the solutes depends largely on the size exclusion effects of the membrane. This sieving effect is usually modeled and corrected [55] using continues hydrodynamic models such as originally proposed by Ferry. In this model, the membrane is assumed to be a network of perfectly cylindrical and parallel pores in which solvent velocity follows Poiseuille's law with a parabolic profile and solutes are assimilated to hard spheres. The transmission coefficient (*Tr*) of a given solute can be calculated according to equation (1) However, the selectivity of NF membranes is based on both size and charge characteristics of the solutes and on the interaction between charged solutes and membrane surface [56].

$$\mathrm{Tr} = (1-(\lambda(\lambda-2))^2 \exp(-0.7146\,\lambda^2) \quad (1)$$

Where λ is the relation between the radius of the solute and the radius of the pore.

The selectivity of the separation when using NF membranes is based on the following factors: a) Solute (peptide) size, shape and charge. b) Membrane pore size and surface charge (sign and surface charge density). c) Hydrodynamic conditions of the fractionation

process (transmembrane pressure, lineal velocities and solute concentration). d) Membrane characteristics (manufacture process, surface roughness, porosity, film layer material and hydrophilic/hydrophobic surface). All these aspects must be considered in order to estimate the viability of a peptide fractionation process.

Especially in NF membranes involving peptide fractionation from mixtures, charge exclusion mechanisms are predominant in the separation. The charge effects affect membrane-peptide and peptide-peptide interactions in the mixture or at the membrane surface. The transport mechanism through the pores is governed by convective and diffusive fluxes as well as by electromigrative flux. These phenomena make the prediction of the separation selectivity a difficult objective.

The current state of science the knowledge of the NF process is not sufficient to make a model fulfilling the requirements. The difficulties in modeling permeate flow rates and solute rejection come from the scale at which the different phenomena takes place at the membrane surface and through the membrane pores, where most of the hydrodynamic and macroscopic interactions begin to break down. However, simplified approaches could be used to explain qualitatively the experimental results obtained, as can be seen below.

The solute transfer through the membrane follow two main steps: distribution of ionic species at the selective interface according to their charge (both solutes and membrane) and transfer by a complex combination among diffusion, convection and electrophoretic mobility through the membrane, at least at low feed concentrations [13]. According to Donnan theory, the passage of charged solutes through a charged NF membrane is likely to be different whether they are considered to be co-ions, i.e. with the same charge of the membrane, or counter-ions, i.e. with a charge of opposite sign. In fact, due to electrostatic repulsive/attractive forces between the membrane and the solutes the concentration of co-ions will be lower in the membrane than in the solutions. On the contrary, the counter-ions have a higher concentration in the membrane than in the solution. This concentration difference of the ions generates a potential difference at the interface between the membrane and the solution, which is called Donnan potential. Under equilibrium conditions, electro-neutrality and equality of electrochemical potentials are maintained through the system. The Donnan equilibrium depends on the ion concentration, the fixed charge concentration in the membrane and the valences of the co-ions and counter-ions. Figure 2 shows an adapted schematic representation [57] of the influence of the electrostatic interactions in the transmission of charged peptides through a charged NF membrane.

Because of the electro-neutrality principle, and on the assumption that the charge density of the membrane is quite higher than the net charge of the co-ions, is possible to calculate the distribution of the co-ion resulting from a binary electrolyte $AB \rightarrow A^{z_A} + B^{z_B}$ between the membrane surface and the solution as a function of the charge density of the membrane.

$$K = \frac{C_B^m}{C_B} = \frac{(z_B \, . C_B)^{z_B/Z_A}}{(Z_B . C_B^m + Z_x \, . C_x^m)} \quad (2)$$

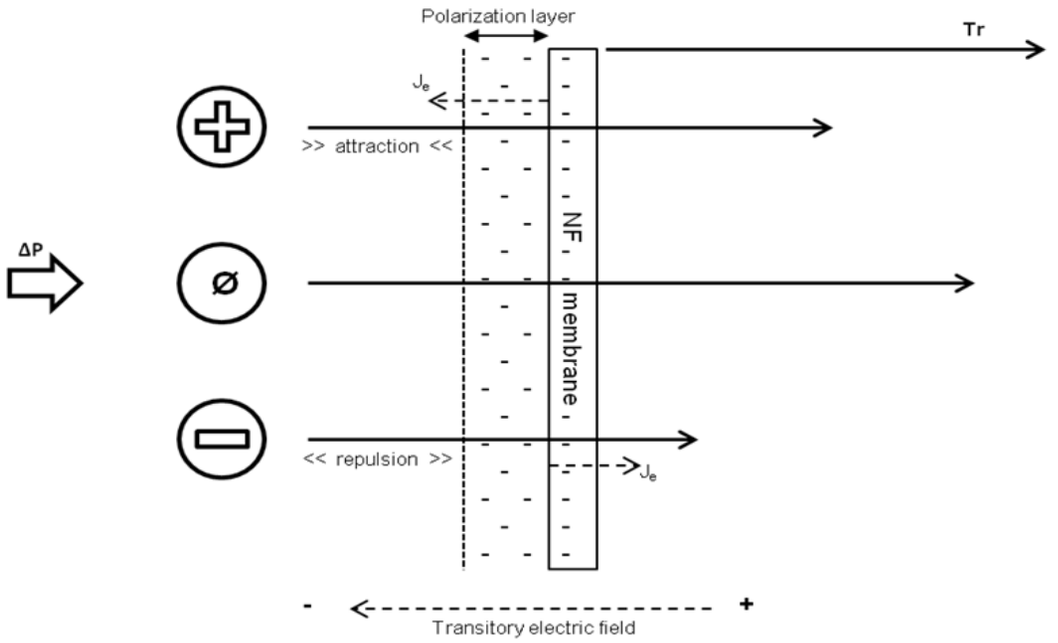

Figure 2. Schematic representation of solute flows across a negatively charged NF membranes. J_e: electromigrative flow as a consequence of the transitory electric field. Tr: transmission of the solute. Attractive (>> <<) and repulsive (<< >>) electrostatic interactions between charged solutes and the membrane are also represented.

C_B^m and C_B represent the concentration of co-ions B in the membrane and in the solution respectively. The coefficient of distribution, K, can be used to predict the rejection value of a binary electrolyte if the ionic transport is mainly due to convection and size exclusion effects are negligible. Under these conditions, K will mainly depend on: the co-ion valence (z_B), the counter-ion valence (z_A), the membrane charge (C_x^m), its valence (z_x) and the concentration of the co-ion in the solution (C_B).

According to equation 2, Donnan equilibrium predicts that an increase in the concentration of co-ions in the global solution and/or a decrease in the membrane charge density lead to a decrease in the exclusion of co-ions from the membrane surface (K is increased) and to a decrease in the retention of the binary salt (co-ion and cointer-ion) in order to maintain electroneutrality in both sides of the membrane [58-59]. The concentration of co-ions in the membrane will change according to the valence of the co-ion and counter-ions present in the solution. Thus, if the valence of the co-ion (z_B) has a lower value and the valence of the counter-ion (z_A) is increased, the concentration of co-ions in the membrane will be favored. For example, the retention of some common salts by descending order (Na_2SO_4 > NaCl > $CaCl_2$) through a negatively charged NF membrane can be predicted according to these principles [60-63].

Donnan theory is generally used to describe the permeability and selectivity of NF membranes using solutions containing only one amino acid. For example, for an amino acid co-ion and its associated counter-ion, in accordance with the Donnan equilibrium, the amino

acid is electrically rejected by the charged active layer of the membrane. Simultaneously, the counter-ion is retained to ensure the balance of charges as the consequence of the electromigrative flow that opposes the convective one. However unfortunately, the extrapolation of Donnan theory to predict the behavior of individual solutes in mixed solutions containing several negative, neutral and positive solutes is very limited, mainly because of coupling and competitive effects. For this reason, NF process of complex mixtures of amino acids and peptides is a difficult object for mathematical modeling [64].

3.2.2. NF Applied to amino acid and peptide fractionation: Review

To clarify the mechanisms involved in the separation of biomolecules by NF membranes several fundamental researches have been published. Table 2 shows relevant NF studies involving amino acids and peptides. The data obtained are relative at different factors affecting the separation of single amino acid (AA) solutions, peptides mixtures and protein hydrolysates. For example, the influence of pH in the retention of amino acids through NF membranes was studied to analyse the separation of small peptides (only two amino acids). In this case, different isoelectric points (pI) by adjusting the pH of the mixture were considered in peptides rejection [65]. Another report showed the separation of a mixture of nine amino acids on the basis of electrostatic interactions of solutes-membrane [66]. According to results, pH has the greater influence on membrane selectivity. In addition the content of inorganic ions compared to the content of ionized amino acids affects also the separation. Therefore these variables are crucial for optimization of membrane selectivity.

Reference	**Solution**	**Experiments**	**Membrane**
[65]	Single AA solutions Mixtures of dipeptides	pH variation experiments Separation experiments of mixed dipeptides	Flat-sheet membranes **Materials:** Phosphatidic Acid (PA), Thin Film Composite (TFC), Sulfonated Polyethersulfone SPES) and Sulfonated Polystyrene (SPE) **MWCO**: 0.2-3 kDa **Charge at pH 7**: negative (SPES, SPE and TFC) or amphoteric (PA)
[66]	Mixtures of AA	Separation of a mixture of 9 AAs on the basis of differential electrostatic interactions with the membrane Membrane selectivity as a function of pH, AA concentration and Ionic Strength	**Material:** Inorganic membrane, chemical modification of the ZrO_2 layer of a UF membrane with cross linked Polyetherimide (PEI) **Charge:** positive

[13]	Single AA solutions AA mixtures Peptides (from protein hydrolysate)	NF of charged AA (single solutions and mixtures) and peptides(similar MW but different pI)	**Material:** ZrO2 filtering layer on a mineral support **Charge:** weakly negative charge at pH 8.0
[67]	Single AA solutions AA mixtures	Influence of concentration and ionic composition (salt concentration and kind of salt added) on single AA retention. Separation of AA mixtures	**Material:** cellulose acetate, SPES, SPS and Polysulfunate (PS) **MWCO:** 35-45% (NaCl retention), 1 kDa, 3, 6 kDa respectively
[68]	Protein hydrolysate	Separation of a mixture of 10 small peptides Influence of physicochemical conditions (ionic strength and pH) on the fractionation (permeate flux and *Tr*)	**M5+PEI:** ZrO_2 modified with PEI **Kerasep Solgel:** microporous active layer of ZrO_2
[16]	Protein hydrolysate	Effect of adjusting pH and ionic strength in the fractionation of the hydrolysate.	Flat sheet TFC membranes. **Material and MWCO:** PA (2.5 kDa), cellulose acetate (0.5, 0.8, 1-5 and 8-10 kDa). **Charge:** anionic characteristics
[69]	Single AA solutions AA mixtures	Influence of experimental conditions on the steady-state regime pH effect on retention coefficients of single AA solutions and AA mixtures Influence of ionic strength and transmembrane pressure on retention coefficients of an AA mixture	Cross-flow NF membrane **Material:** ceramic alumina γ with an average pore radius of 2.5 nm. **Charge:** zero point charge in the range of pH 8-9. Positively charged in the pH range tested.
[70]	AA mixtures	Separation performance of two different NF membranes. Influence of pH and operation pressure on the selectivity of the separation. Simulation NF process system for separation and concentration of L-Phe and	CTF membranes with asymmetric structure **Material:** aromatic PA and SPS

		L-Asp	
[71]	Protein hydrolysate	Concentration polarization phenomena: effect of hydrodynamic conditions on the *Tr* of selected peptides from the hydrolysate	Flat sheet membrane **Material:** cellulose acetate **MWCO:** 2.500 kDa **Charge:** anionic charge characteristics at basic pH
[72]	Single AA solutions Fermentation broth	Effect of pH, concentration and physicoquemical environment (ionic strength and kind of salt added) on single AA rejection Effect of operating pressure and concentration of fermentation broth on NF (selectivity and AA rejection)	**Material:** SPES **Charge:** high negative charge at neutral pH
[14]	Protein hydrolysate	Effect of feed concentration, pH, transmembrane pressure and feed velocity in the ability of a "loose" composite NF membrane to fractionate acid, neutral and basic peptides. Evaluation of the effect of peptides fouling on sieving and electrostatic characteristics of the membrane: PEG and Effect of aggregating peptides on the fractionation of a protein hydrolysate	Flat sheet membrane **Material:** PA (proprietary) **MWCO:** 2.5 kDa **Charge:** negatively charged at alkaline pH
[57]	Protein hydrolysate	NaCl retention measurements.	Flat sheet membrane **Material:** PA (proprietary) **MWCO:** 2.5 kDa **Charge:** negatively charged at alkaline pH
[23]	Peptide mixture	Selectivity estimation in the separation peptides from lactose and effect of pH in fouling	**Material:** SPES **MWCO:** 1 kDa **Charge:** negatively charged at neutral pH
[73]	AA mixtures	Separation of neutral AA using multilayer polyelectrolyte NF	**Material:** Bilayers of Phosphatidylserine synthase (PSS) on porous

		membranes	alumina support
[74]	Protein hydrolysate	Fractionation of small peptides using a 1 kDa NF membrane. Influence of pH and ionic strength on *Tr*	Cross-flow filtration **Material:** cellulose acetate **MWCO:** 1 kDa
[75]	Single AA solutions	Permeation of single AA solutions in the whole range of their solubility with a stepwise pH scan ranging from 0 to -1 total net charge	Membrane discs **MWCO and material:** 0.15-0.30 kDa (proprietary), 1 kDa (proprietary), 2.5 kDa (proprietary), 0.15-0.30 kDa (permanently hydrophilic PES) and 1kDa (permanently hydrophilic PES)
[76]	Single AA solutions	Study solute rejection versus concentration of 5 different AA. Comparison of experimental data against a combined steric and charge rejection model.	**Material:** SPES **MWCO:** 1kDa

Table 2. NF studies involving amino acids and peptides

Influence of concentration and ionic composition (salt concentration and type of salt added) on single amino acid retention and on the separation of amino acid mixtures was also studied to explain peptides rejection [67]. The different results show that both parameters have a negative impact on the selectivity of the membrane when size effects are not dominant. Under these conditions, the membrane seems to be more permeable to charged components due to saturation of its charged sites which makes that repulsive/attractive force between the membrane and the charged peptides become weaker.

Other studies have showed that the mixture of amino acids and their concentration affect also the behavior of NF membranes. However very few works have focused on concentrated amino acid or peptide mixtures. The most NF studies involve highly diluted amino acid solutions, which are the most likely to be found in industrial processes, and the results obtained to date are not completely understood due to at the difference in the data. For example, the results of the separation of l-glutamine (l-Gln) from Gln fermentation broth by NF, showed the effects of various experimental parameters such as transmembrane pressure, pH and concentration of broth on the rejection of l-Gln and l-glutamate (l-Glu). However, the rejection of fermentation broth from a single l-Gln or l-Glu solution was mainly caused by the complex ionic composition of the real fermentation broth [72]. Increase of I-GIn rejection was

reported as a function of concentration in a concentration range from 0.3 to 3% (w/v) while the rejection of I-Glu decreased in the range from 0.1 to 0.85%.

Permeation experiments of aqueous solutions of diprotic amino acids (L-glutamine and glycine) showed different data [75]. Amino acid rejection became more concentration dependant at higher pH values due to the increased net charge of the solutes. In this high concentration regime (up to 2 M of glycine) and under alkaline conditions, an important decrease in amino acid rejection was observed in all tests.

Recent results were also found in the experiments of rejection of five amino acids by NF membranes, where experimental data were compared against a combined steric and charge rejection model [76]. Only positive charged amino acids showed good agreement with the model in all the concentration range studied while the behavior of negatively charged peptides only agree with the model at the highest concentration values and rejection of neutral amino acids was decreased due to its smaller net charge. Despite these data, the separation of bioactive peptides from natural sources and the prediction of their individual behavior require previous NF studies of complex mixed solutions.

At the other hand, the study of separation of tryptic β-casein peptides trough UF membranes showed that the separation of peptides is also affected by ionic strength by means a controlled dual mechanism: size exclusion and electrostatic repulsion [77]. Electrostatic interactions affect the peptides transport, especially if the ionic strength of the solution is low.

Another subsequent work reported the interesting potential of NF membranes for separating peptides in the range of 0.3-1 kDa [68]. Specific conditions of ionic strength and especially pH promoted the separation of peptides because the membrane and peptides showed amphoteric properties. Three categories of peptides (acid, basic, neutral) were separated according to their pI. At optimum pH 8 this led to high transmissions of basic peptides (even over 100%), intermediate transmissions for neutral peptides, and low transmissions for acid peptides. The addition of multicharged cationic and anionic species in the hydrolysate induced a markedly enhanced selectivity when the polyelectrolyte was a membrane co-ion and a complete reversion of selectivity when it was a membrane.

An additional research was later performed in order to understand the separation of peptide mixtures through NF membranes [13]. In this case, the solution tested was a mixture of 4 small peptides (4-7 residues) obtained by trypsin hydrolysis of caseinomacropeptide. From above results, it was proposed the first comprehensive approach concerning at filtration of mixtures of peptides, under two principles: (i) electro-neutrality of the solutions is always recovered, which means that all charged solute transmission are interdependent, and (ii) the number of charges along the peptide sequence, rather than the global net charge, has to be considered in order to explain the transmission of a given peptide.

Afterwards, it was investigated the potential of organic NF membranes with a MWCO between 1 and 5 kDa for the fractionation of whey protein hydrolysates. The effect of adjusting pH and ionic strength on the separation properties of the membranes was also characterized in these tests [16]. Highest selectivity between basic and acidic peptides was

found at alkaline conditions without the addition of NaCl. In addition the authors demonstrated that two peptides differing by only one amino acid are transmitted differently. Consequently a single change in the amino acid sequence can affect peptides transmission.

Influence of peptide interactions on peptide separation was also established in some studies of NF membranes. The data show that the same peptide could be transmitted differently when issued from different hydrolysates, reflecting the importance of surrounding peptides, and, hence, the possible occurrence of peptide-peptide interactions [78]. Therefore hydrophobic interactions between peptides when the pH of the solution is close to their pI can lead to their aggregation and subsequent fouling of the NF membrane.

By means of NF experiments on fractionation of β-Lg tryptic hydrolysate, it was shown that peptide-peptide interactions are mainly driven by hydrophobic interactions and that some peptides are aggregated at acidic pH [14]. The morphology of these aggregates avoids the neutralization of the negative charge of the membrane surface with the alkaline peptides in the bulk. Therefore, higher permeability and higher transmission of small positive peptides is obtained under these conditions.

Furthermore peptide aggregates contribute at the polarization concentration on the membrane surface. In this case, the peptides can interact in the polarized layer during the filtration process and their transmission decreases with the time under specific conditions [79].

Other successive tests demonstrated that although physico-chemical parameters such as pH and ionic strength are the dominant ones in the case of NF membranes, operational parameters which determine permeate flux through the membrane, and in particular transmembrane pressure, have also an important influence on the retention of peptides and therefore on the selectivity of the membrane [71]. Furthermore, it should be noted that the resulting sieving properties of some NF membranes could depend on the fouled peptide layer and the composition of this layer interacting with the membrane is pH dependant [28].

The combination of membrane processes (UF and NF) was also recently used in the fractionation of whey hydrolysates to study peptides transmission [29]. The first step of this process consisted in the UF of the hydrolysate in order to obtain complete rejection of intact proteins and intermediate peptides. The resulting permeate fractions were then subjected to a fractionation by NF and a peptide fraction having a molar mass range of 5-2 kDa was isolated in this step. Transmission of peptides, amino acids and lactose were found to be mainly affected by the permeability of the fouling layer showing the effect of peptide aggregates.

Comparison of results of NF peptides using a single amino acid solutions, amino acid mixtures and peptide mixtures, had enabled to conclude that whatever the complexity of the solution: the charge is the most important criterion for the separation of peptides having similar molecular weight. The pH value of the solution is the parameter, which has the greatest effect on the separation. Addition of salts (increase of ionic strength) could decrease the intensity of charge effects. The determination of both the membrane and the mixture

characteristics are of paramount importance in order to predict and optimize the performance of NF membranes for the fractionation of complex peptide mixtures.

3.2.3. Main parameters influencing peptide fractionation using NF membranes

The interactions of peptide-peptide and peptide-membrane affect the separation process performance and thus it is difficult to predict the selectivity of the membranes when the objective is the fractionation of complex peptide mixtures. According the literature, the most important parameters that cause effect on membrane selectivity are pH, ionic strength, polarization layer and fouling.

1. The pH of solution is an important control variable in NF processes for the fractionation of complex peptide mixtures, because peptides are molecules that have at least one carboxylic group ($R-COOH \leftrightarrow R-COO^-$ and one amine group ($R-NH_3^+ \leftrightarrow R-NH_2$). The total number of acid and basic groups depends on its primary structure (amino acid sequence) and it determines the pH value at which the peptides have the same number of negative than positive charges, i.e., its pI. Peptides can be classified in three different groups according to their pI: acidic peptides (pI ≤ 5), neutral peptides (5 < pI ≤ 7) and basic peptides (pI > 7). Their net charge depends on the pH of the solution, as well as the charge density of the NF membrane. This last value will vary because of the ionization of its functional groups (acidic and basic).

In NF, the transmission of amino acids and peptides reaches its maximum value when the pH is equal to the pI. Under these conditions, repulsive electrostatic interactions are minimized. That way, the modification of NF membranes transmission is possible by changing the pH of the mixture.

In the case of protein hydrolysates, which composition is more complex, there will be a pH value at which the fractionation of acid, neutral and basic peptides is maximized. For example, it has been shown that the separation factor between basic and acid peptides reaches its maximum value when the pH of the mixture is alkaline [16]. However, literature published on this topic only describes the behavior of "tracer" peptides in the hydrolysate and this limits the scope of the separation factor calculated.

2. The ionic strength of peptides solution affects the selectivity of NF membranes. In an aqueous medium the increase of the ionic strength, for example by the addition of NaCl, results in a decrease of zeta potential of the NF membrane [80-83] as well as a decrease in the electrophoretic mobility of proteins and peptides [84]. According to these observations, electrostatic interactions between the membrane and the peptides become less intense, which usually leads to better transmission values of the peptides.

Several authors have demonstrated the preponderance of a selectivity based on electrostatic interactions at low ionic strength values [16, 68, 85]. The fact that electrostatic interactions membrane-peptide lose significance at high ionic strength values results in a decrease of the double selectivity size/charge in processes involving NF membranes. In addition to the effect over the charge density of the membrane, ionic strength also influences the effective

hydrodynamic volume of charged proteins and peptides [86]. A charged protein is surrounded by a diffuse ion cloud, typically called the electrical double layer, and the thickness of this layer is characterized by the Debye length (L_D):

$$L_D = 0.304\, I^{-1/2} \tag{3}$$

Where I is the ionic strength (mol/L) and L_D is in nm. According to equation 3 the higher the ionic strength the narrower the Debye length.

In addition, the effect of the electrical double layer could be described in terms of an increase in the effective protein radius R_{eff}:

$$R_{eff} = r_s + 0.045\, Z^2\, \frac{L_D}{r_s} \tag{4}$$

Where r_s is the hard-sphere radius of the uncharged protein or peptide (in nm) and z is the surface charge of the protein (in electronic charge units).

Equations (3) and (4) indicate that relatively low salt concentration is needed in order to enhance the magnitude of the electrostatic interactions. However, the increase in the ionic strength leads to an increase in the transmission of charged peptides though the membrane.

This last observation, which is well known and it has been applied to explain the selectivity of several protein separation processes using UF membranes, is not usually mentioned in works involving the separation of peptides by NF membranes. The effects of the ionic strength over the charge density of the membrane and over the effective hydrodynamic volume of charged peptides are complementary and both of them contribute in the explanation of experimental results.

Variation of these parameters has been applied by some authors [86-88] to obtain good selectivity values in the fractionation of different proteins with similar sizes. The wise combination between membranes, pH and ionic strength is called HPTFF (High-performance-tangential flow filtration) and it is effective when proteins or peptides to be fractionated show different pI and when low or medium protein concentration is processed.

3. Concentration polarization and fouling is also a condition affecting the peptides separation. Physico-chemical parameters such as pH and ionic strength are of paramount importance in NF processes because they modulate the electrostatic interactions on which the selectivity of these membranes is supported. In addition electrostatic interactions may partly explain the distribution of a peptide between the whole solution and the membrane interface [89-90]. However, when using porous membranes, peptides are involved in a convective transport flux and its rejection is therefore the result of (i) electrostatic interactions between the membrane and the peptides plus (ii) a steric mechanism through the porous. In this sense, hydrodynamic parameters have influence in peptide rejection [91]. Thus, for example, when the MWCO of the membrane and the molecular weight of the peptide have similar values or in the presence of electrostatic interactions, an increase in transmembrane pressure will result in an increase of amino acid retention.

Concentration polarization is one of the consequences of selective solute transport through membranes. The constituents of the solution that are retained by the membrane tend to accumulate over its surface and this creates a concentration gradient in the area called polarization boundary layer. This phenomenon is quickly established at the beginning of the process and leads to a modification in the efficiency of membrane processes as well as a change in the composition of the permeate stream. The management of hydrodynamic conditions could minimize its effects. In addition size exclusion properties related to the pore size of the membrane could be completely modified due to pore blocking by the peptides. Fouling is a general term for any accumulation of deposits and materials over the membrane surface or within the pores. Two kinds of fouling can be defined: reversible fouling, the one which can be reduced by adjusting hydrodynamic conditions (velocity or transmembrane pressure), and irreversible fouling, which effect can´t be avoid by cleaning procedures.

In practice, the series resistance model is widely used for fouling quantification in membrane processes. This approach derives from Darcy's phenomenological equation. The clean water flux rate (J_W) through a membrane is defined by equations (5).

$$J_w = \frac{P_T}{\mu_w R_M} \tag{5}$$

Where P_T is the transmembrane pressure, μ_W the water viscosity and R_M the intrinsic resistance of the membrane.

The measurement of water flux rate through the same membrane after being used (J_W') can be expressed as:

$$J_W' = \frac{P_T}{\mu_w (R_M + R_f)} \tag{6}$$

Equation 6 allows the calculation of the resistance associated to fouling (R_f).

Studies involving peptides transmission or retention don´t usually take into account the polarization and fouling phenomena but it has been demonstrated that these phenomena are crucial in the case of protein hydrolysates, especially at acid pH values [16, 68]. Complex peptide mixtures contain peptides, which with different physicochemical characteristics (pI, hydrophobicity, charge) promote the creation of strong interactions with filtration membranes [92-93].

4. Future potential of peptides fractionation by means of membrane techniques

Currently, conventional membrane separation techniques can be employed to obtain peptide fractions in purified form with higher functionality and higher nutritional value. Special properties of the NF membranes make possible novel peptide separations. However, the specific separation of one or more peptides from a raw hydrolysate is a difficult subject because ionic interactions between peptides and membranes can markedly influence on peptides fractionation. In addition these pressure-driven processes involve the accumulation of particles on membrane leading formation of a fouling and to the modification of the

membrane transport selectivity. Therefore, it is clear that NF still has to grow more in terms of understanding, materials, and process control. In addition modeling studies are necessary to predict of the process performance in all circumstances.

Alternatively the application of an external electrical field, which acts as an additional driving force to the pressure gradient, can be seen as a technique that could improve the efficiency of the conventional membrane processes for the separation of charged bioactive molecules. In this sense, two different configurations can be distinguished: electrically-enhanced filtration, which can be used with conventional pressure driven membrane filtration, and forced-flow membrane electrophoresis, which is conducted in an electrophoretic cell. Intensive researches on these membrane processes have been carried out including electromembrane filtration (EMF) [94-95], electrodialysis with UF membranes (EDUF) [96-99] and forced-flow electrophoresis (FFE) [100] for the separation of charged bioactive molecules.

EDUF couples size exclusion capabilities of UF membranes with the charge selectivity of electrodyalysis (ED) allowing separation of molecules according to their electric charges and to their molecular mass (membrane filtration cut-off). The feasibility of peptide fractionation by EDUF was demonstrated notably with β-Lg tryptic hydrolysate solutions and was suggested to improve the separation between basic and neutral peptides [97]. Actually, EDUF process also allowed a selective and a simultaneous separation of anionic and cationic peptides presents in an uncharacterized concentrated polypeptide mixture of snow crab by-products hydrolysate [101].

Recently a comparative study on NF and EDUF was performed in terms of flux and mass balance [102]. The results showed that NF provides a greater mass flux while when using EDUF a wider range of peptides and more polar amino acids are recovered. EDUF can be seen to be a promising separation technology, but further scale-up developments will be necessary to confirm its feasibility at large scale.

EMF combines the separation mechanisms of membrane filtration and electrophoresis. Ion exchange membranes are replaced by UF in a conventional electrodialysis cell. In electrophoretic separators, a porous membrane is used to put into contact two flowing liquids between which an electrically driven mass transfer takes place. During this process the mass transport is affected by electrostatic interactions taking place at the membrane solution interface. The perspectives in the field of peptide fractionation will be the complete understanding of the interactions of peptides and membrane as well as the development of new membrane materials of gels limiting or increasing these interactions to improve the selectivity and the yield of production of specific peptides [100].

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