USE OF OSMOTIC MEMBRANES FOR CONCENTRATION OF PROTEINS IN A SUSTAINABLE BIOREFINERY

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ABSTRACT

The increasing world population is estimated to reach a total of 10 billion people in the next decades, implying a raising demand of animal products and therefore animal feed. To reduce the current European dependency on imported soybean meal for livestock feed, several alternative sources of proteins such as grain legumes or leaves have been proposed, but economic feasibility needs to be achieved to become a viable alternative. In this context, the OrganoFinery project is a concept of green biorefinery aiming the production of organic protein for animal feed together with biogas and fertilizer.

In this study, a second separation step to increase the protein yield from a waste stream using osmotic membranes has been proposed to improve feasibility of the OrganoFinery. Firstly, two forward osmosis membranes, Porifera and Aquaporin, were tested in terms of water flux and reverse salt flux with deionized water as feed and 1 M NaCl as draw solution. Porifera achieved better results with higher water flux and lower reverse salt flux, 16 $l/m^2 \cdot h$ and 7,14 $g/m^2 \cdot h$ respectively.

Secondly, Porifera was used to concentrate BSA as standard protein using 1 M NaCl as osmotic agent and three different initial BSA concentrations. Similar values of water flux and enrichment factor were reported: circa 12 $l/m^2 \cdot h$ and 1,7 in 3 h, respectively. It was concluded that BSA concentration of the feed does not influence greatly the water flux, indicating no membrane fouling. Moreover, complementary experiments with extra addition of salt were performed showing no experimental change in water flux. Protein precipitation due to supersaturation was proposed, but concentration by forward osmosis did not lead to precipitation and hence ammonium sulfate was used.

Thirdly, Porifera was also used to concentrate brown juice, waste stream of the OrganoFinery, reporting low values of water flux; osmotic pressure of the feed was proposed as the main reason. Brown juice was then diafiltrated to reduce its sugar and organic acid content, achieving a 50% reduction and 63% rejection of proteins. To discard membrane fouling, new pieces of membrane were used to concentrate all fractions from diafiltration, but similar low water fluxes were obtained.

Comparing different feed solutions, it was shown that water flux is highly influenced by the feed composition. However, further experiments are needed to enhance feed dewatering and achieve an increase in protein recovery from the OrganoFinery.

AKNOWLEDGEMENTS

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ABBREVIATIONS

BJ: Brown juice

BSA: Bovine serum albumin

BSE: Bovine spongiform encephalopathy

CP: Concentration polarization

DS: Draw solution

DW: Deionized water

FO: Forward osmosis

FS: Feed solution

GBR: Green biorefinery

GJ: Green juice

MD: Membrane distillation

MF: Microfiltration

NF: Nanofiltration

PC: Press cake

RO: Reverse osmosis

RSF: Reverse salt flux

SMB: Soybean meal

SRSF: Specific reverse salt flux

TCA: Trichloroacetic acid

TKN: Total Kjeldahl Nitrogen

UF: Ultrafiltration

WF: Water flux

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STRUCTURE OF THE THESIS

This thesis is divided into 7 chapters which cover a literature review and different experimental work. Chapter 1 comprises an overview of the state of the art regarding protein production in Europe and membrane technology. Chapter 2 details the main objective of this thesis and the research questions proposed to perform the experimental work. Chapters 3 to 5 comprise the experimental work performed to answer the research questions, separated in two sections: materials and methods and results and discussion. They are divided by topics but following a line of research.

Evaluation of membrane performance is studied and compared in chapter 3 using two different osmotic membranes. Then, one membrane is employed to concentrate a standard protein in chapter 4; not only membrane performance but also protein enrichment is studied. Chapter 5 is dedicated to the study of brown juice, the stream of interest. First, a method for protein determination is presented and then protein concentration is studied, including pre-treatment of the sample before forward osmosis concentration.

Chapter 6 compares relevant results from these three different topics. Finally, chapter 7 contains the main conclusions and future perspectives of this project.

CHAPTER 1 – BACKGROUND

1.1. Protein production in Europe

1.1.1. Current state of protein production

The increasing world population and therefore the increase in energy and food demand, ligated to the depletion of fossil fuels and the climate change are some of the main concerns at a social, economic and political level. World population is estimated to reach a total of 10 billion people in the next decades, implying an increase in animal production: 1,5% per year for meat production and 1,3% for milk, according to the FAO agricultural outlook 2015-2030 (Bruinsma 2002).

In Europe, animal products such as meat, fish and milk are the most important source of protein for people together with plant products like wheat (Schreuder and De Visser 2014). This demand increase for animal products comes with an unavoidable raise in protein feed for livestock. Some years ago, the primary protein source in animal diets was meat and bone meal, but the FAO recommended a global ban on its use as an action plan to control the spread of bovine spongiform encephalopathy (BSE).

As a consequence, alternative protein sources such as soybean meal (SBM) were found to maintain quality protein levels and therefore livestock productivity (Hard 2002). Currently, the main source of protein in Europe is soybean meal, with a yearly import of around 20 Mtons of soybean meal and 12 Mtons of soybean (Visser 2013).

Soybean is a species of legume with several applications such as biodiesel production from the oil extraction process and soybean meal, the residual product. The initial processing of soybeans consists in cleaning, drying and cracking of the beans to remove the hulls and then flaking to extract the oil by solvent extraction (Pettersson and Pontoppidan 2013). From the resulting cake, the defatted flakes, protein concentrates can be prepared by removal of non-protein components such as soluble carbohydrates.

Removal can be achieved by water or aqueous alcohol washing. Soybean meal is produced from defatted flakes by protein extraction at alkaline pH; proteins are then spray dried. Membrane filtration can be used to increase protein yield by removing the non-protein solubles and recovering the soluble protein at the isoelectric pH (Krimpen *et al.*, 2013). **Figure 1** illustrates this process:

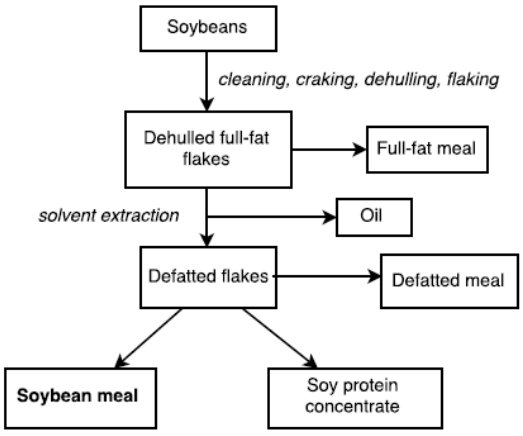


Figure 1. Overview of the process to obtain soybean meal and soybean protein concentrate. (Adapted from Krimpen et al., 2013; Pettersson & Pontoppidan, 2013).

Nevertheless, despite the high soybean meal consumption in Europe, around 30 Mtons, European production covers approximately 2,5% of the total demand. This is translated into a great dependency on soybean importation since protein crops in the EU are not competitive with the currently produced crops (Visser 2013). In addition to Europe's dependency, soybean meal price level has risen since 2009, with a cost of 361€/ton in 2014 (Schreuder and De Visser 2014).

For this reason, the European Parliament adopted in 2011 a motion to support research into breeding and supply of protein crop seeds, considering different alternatives to soybean meal proteins. However, the replacement of soybean meal by other alternative sources of proteins in Europe does not involve an increase in sustainability; the enlargement of European soybean for example may lead to a decrease in other cereal production (Visser 2013).

Along with the increase in animal production demand in Europe, the organic sector in the EU has been rapidly developing with a 13% yearly growth rate. However, the whole organic area represents only the 5,4% of total used agricultural area in Europe, being pasture (45%) and cereals (15%) the biggest share. Even though both animal and arable crop organic production follow an increasing trend, animal production may still be restricted due to challenges regarding the supply of organic feed (Rossi 2013).

Alternative sources of proteins

In order to be a feasible option to substitute soybean meal proteins, alternative sources of proteins have to meet some requirements such as a high protein content and quality. The current protein intake for livestock depends heavily on soybean meal (40% protein content), for this reason alternatives should have a high protein content; starch is also main compound of the feed, but it is largely available from other cereals.

Not only protein content but protein quality is also important to maintain livestock productivity, it is necessary the good digestibility of amino acids and the amino acid profile to match their dietary requirements. Alternative sources should in addition have a low price level or comparable to the soybean meal (Visser 2013). Some of these alternatives, presented in **Table 1**, were reviewed by Krimpen *et al*., (2013). However, FAO's Animal Feed Resources Information System, called Feedipedia, also provides information about the different animal feed resources.

Table 1. List of potential alternative sources of proteins (Adapted from Krimpen *et al.*, 2013).

Category	Protein source
Oil seeds	Rapeseed, sunflower seed
Grain legumes	Peas, beans, chickpeas
Aquatic proteins	Micro-, macroalgae, duckweed
Insects	Mealworm, housefly
Leaf proteins	Grass, alfalfa, sugar beet leaves

The first alternative to reduce Europe's dependency on imported soybean proteins is its replacement by European soybeans, but it is hardly cultivated in the EU. Its competitiveness to wheat and corn is not very good and yields need to improve, although its quality can match that of the imported soy (Visser 2013).

Within oil seeds, rapeseed and sunflower are also considered good alternatives. They are the two major oil producing crops in Europe, with a protein content around 23%. Rapeseed press cake, however, can contain up to 30-40% protein, and is used for animal feed. Similar processes to the used for soybean meal production can be applied to protein production from oil seeds, but the removal or inactivation of anti-nutritional factors (ANFs), linked to indigestibility of proteins, is important for its optimization (Krimpen *et al.*, 2013).

Grain legumes such as peas, beans and chickpeas are interesting alternatives to soybean meal; they have a high protein content (around 20%), but after protein preparation by wet fractionation, similar to the soybean meal process, it can increase up to 80-90%. Dry fractionation, on the other hand, reaches levels of about 60-70% protein besides two fractions enriched in fibres and starch. Nevertheless, these legumes crops are sensitive to pathogens and pests and can only be cultivated in crop rotation, reducing their competitiveness (Krimpen *et al.*, 2013).

Another alternative source of proteins comes with aquatic proteins from both micro- or macroalgae and duckweed. Micro- and macroalgae contain great amounts of proteins in the dry matter, 50-70% and 10-30%, respectively. Cultivation at high industrial scale of microalgae is not applied in Europe, although in the Netherlands there are pilot projects for macroalgae cultivation to obtain not only proteins but also colorants and polysaccharides (Krimpen *et al.*, 2013). Duckweed can be considered an alternative due to its high protein content in dry matter (25-35%), but the total dry matter content is around 6-8%, implying huge amounts to compete with soybean meal (Visser 2013). A drying step would then be required for aquatic protein production.

Insects have been studied as a source of proteins, with a crude protein level in the dry matter higher than 50% (Visser 2013). Different insects such as grasshoppers, crickets or cockroaches have been used as complementary food sources for poultry to supply the necessary amino acids (van Huis et al. 2013). So far they have been grown on waste material, but to be studied as a replacement for soya meal high production levels would need to be achieved (Visser 2013).

Lastly, plant leaves as a source of proteins have been studied (alfalfa, grass, sugar beet…). Protein levels are low, but protein extracts from alfalfa for example, with >50% protein content, are commercially available (Visser 2013). The basic steps are grinding and squeezing the leaves to obtain a protein rich juice followed by protein precipitation. However, the protein content depends on the plant species and conditions; freshly harvested leaves are not available all year. In addition, refinery of left-over plant materials from different crops could be a sustainable option to valorise all biomass from a crop. To overcome feasibility and availability, dried or ensiled material may also be used to operate all year round in the so-called integrated biorefinery, enhancing feasibility of the feedstock and sustainability of the process (Krimpen *et al.*, 2013).

1.1.2. Biorefineries

Sustainability can be seen as the destination of a process or journey called sustainable development (Delgado 2007), defined as “development that meets the needs of the present without compromising the ability of future generations to meet their own needs” (Brundtland GH & World Commission on Environment and Development, 1987).

According to Dr. Karl Henrik-Robert, founder of The Natural Step, “A transition to sustainability involves moving from linear to cyclical processes and technologies. The only processes we can rely on indefinitely are cyclical”. To enable this circularity, sustainable

processes should use renewable resources and produce biodegradable wastes, giving way to the called biorefineries.

The term biorefinery can be defined in several ways, but the definition by the International Energy Agency (IEA) Bioenergy Task 42 (IEA, 2008) is widely used: "Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy". This concept is analogous to a petroleum refinery, in which several products are produced from petroleum. A sustainable society should rely on bioenergy, biofuels and bio-based products as main pillars and biorefineries as the basis (Cherubini 2010).

Biorefineries may use different types of biomass as raw material, also called feedstock. Depending on these feedstocks, biorefineries are classified as Whole Crop Biorefinery, using raw materials such as cereals or maize; Green Biorefinery, using nature-wet biomasses such as green grass or clover; and the Lignocellulose Feedstock Biorefinery, using nature-dry raw materials such as cellulose-containing biomass and wastes (Kamm and Kamm 2004).

Green biorefineries

A Green Biorefinery (GBR) is an integrated concept to use green biomass as raw material for the manufacture of industrial products; a concept currently in advanced stage of development in some European countries such as Germany, Denmark and Austria. The main idea is the utilisation of whole green biomass such as grass or alfalfa to produce bio-based materials and even energy, possibly via biogas generation (Kromus et al. 2004).

Typical feedstock options investigated are different kinds of grass, clover and alfalfa; grass biomass has become a surplus raw material in many regions in Europe due to a reconstruction of the dairy farming and meat production. The valuable components of interest in grass are proteins, soluble sugars and the fibre fraction containing cellulose, hemicellulose and lignin; but the chemical composition may depend on the season and the mixture of grass species (Mandl 2012).

The first step in a GBR is a wet fractionation of the sample obtaining two different streams: a fibre-rich press cake (PC) and a nutrient-rich green juice (GJ). The objective of this step is the isolation of the content-substances in their natural form. PC contains cellulose and starch, but it may also contain valuable dyes and pigments or other organics. On the other hand, GJ contains proteins, free amino acids, organic acids, enzymes, dyes, etc.; the main objective is then the use of GJ to obtain different products such as proteins, lactic acid, amino acids or ethanol. PC can be used as raw material for production of different chemicals or conversion to syngas and hydrocarbons; the residues are suitable for the production of biogas and generation of heat and electricity (Kamm and Kamm 2004). A schema of GBR can be seen in **Figure 2.**

There exist different green biorefinery activities in Europe; for example, in 2000, Andersen and Kiel developed a concept to utilise plant juices coming from different feedstocks (Italian rye-grass, clover grass, alfalfa…) for production of fodder pellets as raw material in a fermentation process by lactic acid bacteria to make it storable. It was showed that juices from different feedstocks could be converted to a stable universal fermentation useful for lactic acid fermentation for production of other organic acids or amino acids in a second stage fermentation. The Dutch Grassa! Project is an example of leaf processing on pilot scale, aiming the production of both protein for feed and fibres for paper (Krimpen *et al.*, 2013).

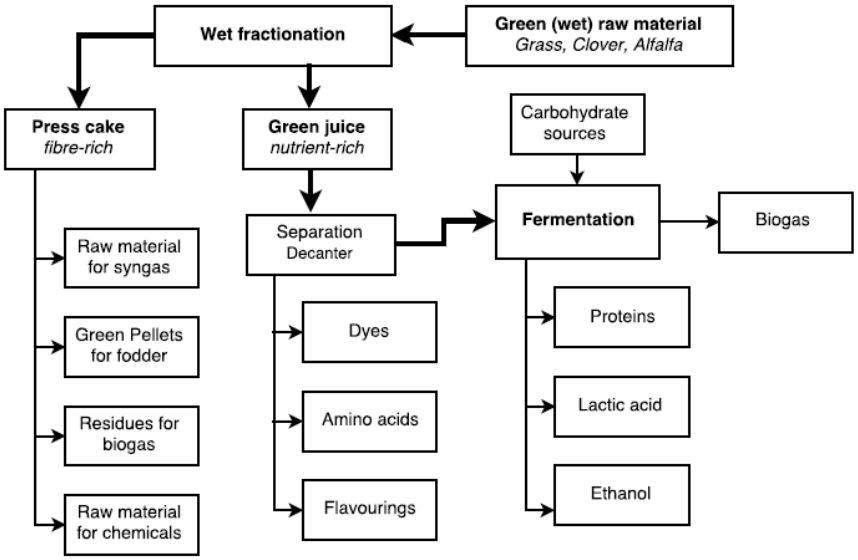


Figure 2. Overview of a green biorefinery (Adapted from Kamm & Kamm, 2004).

A pilot plant was established in 2006 in Germany to ferment agricultural residues in combination with hydrolysed cereals to lactic acid, which resulted to be a success producing 10t lactic acid in 200 days per year (Venus and Richter 2007). Another pilot plant was established in 2008 in Austria aiming the extraction of lactic acid and amino acids from grass silage. The concept of this plant was the use of fermented grass instead of fresh biomass to ensure year round availability. Silage juice was ultrafiltrated to remove impurities and softened to reduce the amount of cations, and then processed through a two-stage nanofiltration to separate amino acids and lactic acid (Ecker *et al*., 2012).

OrganoFinery project

The OrganoFinery is a project that aims the production of protein concentrate for animal feed, biogas and fertilizer as an example of green biorefinery, using organic feedstock such as clover or clover grass. Several biomasses have been tested on a laboratory scale, but red clover was chosen as model species since it is a monocrop easy to harvest and it can fix nitrogen avoiding the need of a fertilizer (De Vega et al. 2015).

The first process in this GBR is the fractionation of the biomass in a liquid fraction, the green juice, and a solid fraction, the press cake, using a screw press. The green juice is then inoculated with lactic acid bacteria (*Lactobacillus salivarius*) with the aim of decreasing its pH and precipitating proteins; they are then recovered through decantation. These proteins can provide a supply of organic protein feed for monogastric animals such as poultry or pigs, offering a solution to the current need of organically produced proteins (Santamaria 2015).

To increase feasibility of the project, different green biomasses have been studied to improve protein yield as well as different techniques to isolate proteins. However, only part of the proteins is collected by decantation; the remaining stream afterwards, the brown juice, contains the proteins that have not precipitated or been separated in addition to organic acids fermented by lactic acid bacteria and remaining sugars.

Brown juice is used as feedstock for anaerobic digestion together with the press cake to produce biogas and fertilizer. These fractions contain the rest of the lignocellulosic materials,

proteins and ash, resulting a proper input for biogas production. Nevertheless, current feasibility of the OrganoFinery is more dependent on biogas and fertilizer obtained after anaerobic digestion instead of protein concentrate, its main product (del Río, Fernando, and Grassino 2016). A scheme of the OrganoFinery project is presented in **Figure 3.**

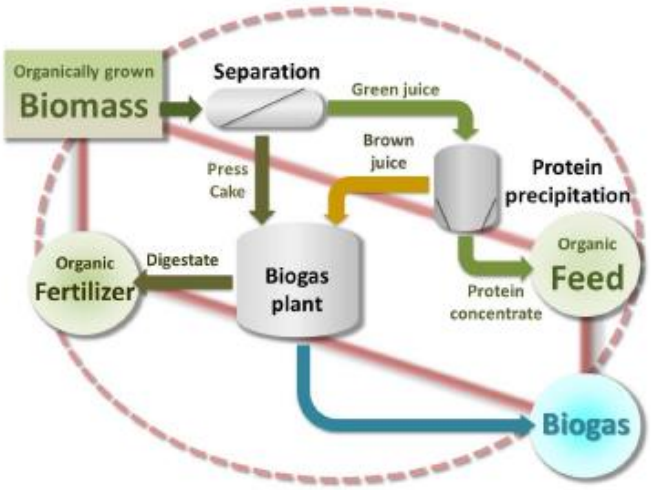


Figure 3. Scheme of the OrganoFinery project (Santamaría, 2015).

1.1.3. Extraction of proteins

To isolate proteins from a sample several different methods can be used, but the physical and/or chemical properties must be used. It is also important to consider the goal of the protein when choosing the method, since the purity depends on the final purpose; it is for example not the same to use an enzyme in a washing powder than for therapeutic use (Hedhammar, Karlström, and Hober 2006).

On the first place, proteins are brought into solution by sample homogenization. Secondly, proteins can be fractionated or isolated by precipitation and then polished by chromatographic methods depending on size or shape, isoelectric point or affinity.

Centrifugation can be used to separate different cell structures with different proteins of interest or to fractionate a protein mixture into different fractions, depending on the Svedberg units (S) (Martínez-Maqueda *et al.*, 2013). Another technique to fractionate proteins or peptides is the use of membranes for ultrafiltration or nanofiltration, which can remove compounds based on their molecular size.

A practical way to separate different types of proteins is their relative solubility; precipitation can be achieved by addition of salts or different solvents, variation of the pH, temperature… even fractional precipitation can be used to separate gross impurities or membrane proteins (Hedhammar, Karlström, and Hober 2006). Solubility of proteins can be described as the amount of protein that dissolves in a given amount of solution, and it is determined by different extrinsic factors including ionic strength, pH, temperature… and protein-protein or protein-water interactions (Trevino, Scholtz, and Pace 2008).

At laboratory scale, solubilisation of proteins has been achieved using organic solvents such as phenol or trichloroacetic acid (TCA)/acetone to extract proteins from different sources

(Natarajan *et al.*, 2005; Song *et al.*, 2006) for proteomic analysis. But alternative greener solvents are gradually substituting organic solvents due to their environmental impact, health effect and hazardous wastes (Sheldon 2005).

Ionic liquids, composed of cations and anions, are an alternative because of their ability to extract hydrophilic from hydrophobic compounds (Vanthoor-Koopmans *et al.*, 2013). They have already been used for protein extraction (Pei *et al.*, 2009) and as running electrolytes in combination to capillary electrophoresis to separate proteins (Jiang *et al.*, 2003). Aqueous extraction is also gaining attention, acid and alkaline protein extraction methods have been evaluated (Nadal *et al.*, 2011).

To precipitate proteins the addition of salt may be used, ammonium sulfate is one of the most widely used salts because it is very stabilizing to protein structure, very soluble and relatively inexpensive (Burgess 2009). It increases the solubility of proteins, an effect called salting-in, and then at higher salt concentrations the solubility decreases leading to precipitation, this is called salting-out; process is showed in **Figure 4.**

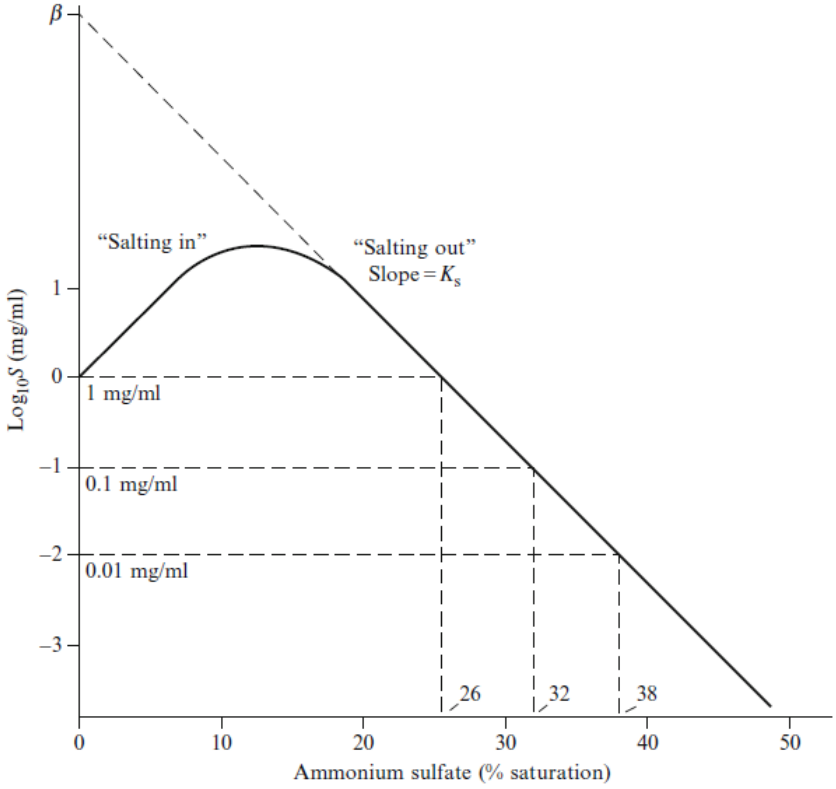


Figure 4. Ammonium sulfate solubility curve for a hypothetical protein (Burgess, 2009).

In **Figure 4**, log of the protein solubility is plotted as a function of ammonium sulfate concentration. In the region called salting out, log solubility decreases linearly with increasing salt concentration; this relationship is described in equation (1.1):

$$log_{10}S = \beta - K_s(\Gamma/2) \qquad (1.1)$$

Where S is the solubility of the protein in mg/ml, Γ/2 the ionic strength as ammonium sulfate percent saturation and β and K_S are constants characteristics of the protein. K_S measures the slope of the line and β is the log of the solubility if the salting-out curve is extrapolated to zero percent saturation (Burgess 2009).

In **Figure 4**, the upper horizontal dotted line relates the solubility line at logS=0 (in this hypothetical case, S = 1 mg/ml) and the percent saturation of ammonium sulfate, 26%. To decrease solubility to 0,1 mg/ml, ammonium sulfate saturation should increase to 32%, then 90% of the protein would precipitate (Burgess, 2009).

Nevertheless, supersaturation can be considered a driving force for protein aggregation and precipitation. A solution is considered supersaturated when it contains a higher amount of compound than what can be dissolved; the protein content of a solution can be concentrated so it reaches a state of supersaturation. This has already been studied using a dialysis membrane to concentrate the solution leading to protein crystallization (Thomas *et al.*, 1989).

1.2. Membranes for protein extraction

1.2.1. Introduction to membranes

According to Baker (2004), a membrane is nothing more than a discrete, thin interface that moderates the permeation of chemical species in contact with it. A physical barrier that completely rejects or reduces the flux of a given compound, so it can be separated from the rest of the influent stream (Tækker Madsen 2014). The volume flowing through the membrane per unit area and time is called the flux; the influent stream is called the feed and the filtrate permeate. Membranes can be classified in many ways, for example by nature (synthetic or biological) by morphology or structure (symmetric or asymmetric) or by driving force (pressure, concentration, etc.) (Mulder 1996).

Membranes can be molecularly homogeneous, uniform in composition and structure, or heterogeneous, containing pores for example. Two types of membranes can be distinguished based on morphology: symmetric and asymmetric. Symmetric membranes can be porous or nonporous, while asymmetric membranes are composed of a dense top-layer supported by a porous sublayer. Depending on the separation principle, membranes can be porous, separating by discriminating between particle size; nonporous, separating molecules of the same size by differences in solubility and/or diffusivity or carrier membranes, of which the transport is determined by a specific carrier-molecule (Mulder 1996).

The transport of molecules through the membrane can be caused by different driving forces: concentration difference, pressure difference or electrical potential difference between both sides (Jiang & Zhu, 2013). Two models are used to describe the mechanism of permeation through a membrane: the pore-flow model (A) and the solution-diffusion model (B), illustrated in **Figure 5**.

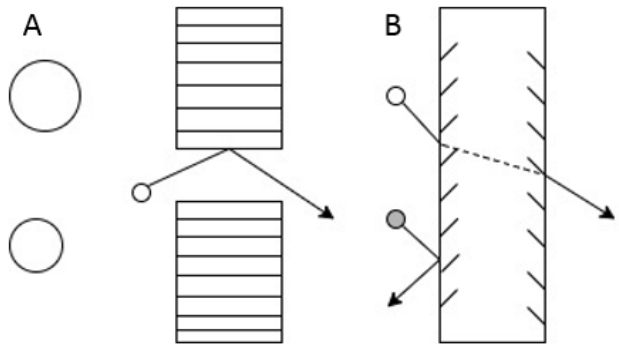


Figure 5. Models to describe the mechanism of permeation through a membrane (Adapted from Baker, 2004).

In the pore-flow model, permeants are transported by a pressure-driven flow through the pores; one of the permeants is excluded from some of the pores, and other permeants move. The basic equation for this type of transport is Darcy's law (eq. 1.2):

$$J_i = K' c_i \frac{\mathrm{d}p}{\mathrm{d}x} \tag{1.2}$$

Where Ji is the transfer rate of component i or flux (g/cm²·s), dp/dx is the pressure gradient existing in the porous medium, ci is the concentration of component i in the medium and K' a coefficient reflecting the nature of the medium (Baker 2004).

In the solution-diffusion model, permeants dissolve in the membrane material and diffuse through the membrane down on a concentration gradient; the permeants are separated because of differences in rates of diffusion and solubilities of the materials in the membrane. Fick's law of diffusion describes this type of transport (eq. 1.3):

$$J_i = -D_i \frac{\mathrm{d}c_i}{\mathrm{d}x} \tag{1.3}$$

Where Di is the diffusion coefficient (cm²/s), a measure of the mobility of the individual molecules, and dci/dx is the concentration gradient of component i (Baker 2004).

Membranes are divided in different groups depending on the size of the retained particles: Reverse Osmosis (RO), Nanofiltration (NF), Ultrafiltration (UF) and Microfiltration (MF) (Tækker Madsen 2014). The driving force in this case is the applied pressure, so the solvents and some solutes can permeate through the membrane and other particles are rejected, as explained in the pore-flow model. The smaller the pores, the higher the resistance of the membrane and hence the applied pressure should be increased to obtain the same flux. **Figure 6** shows the different membranes and particles that can be retained.

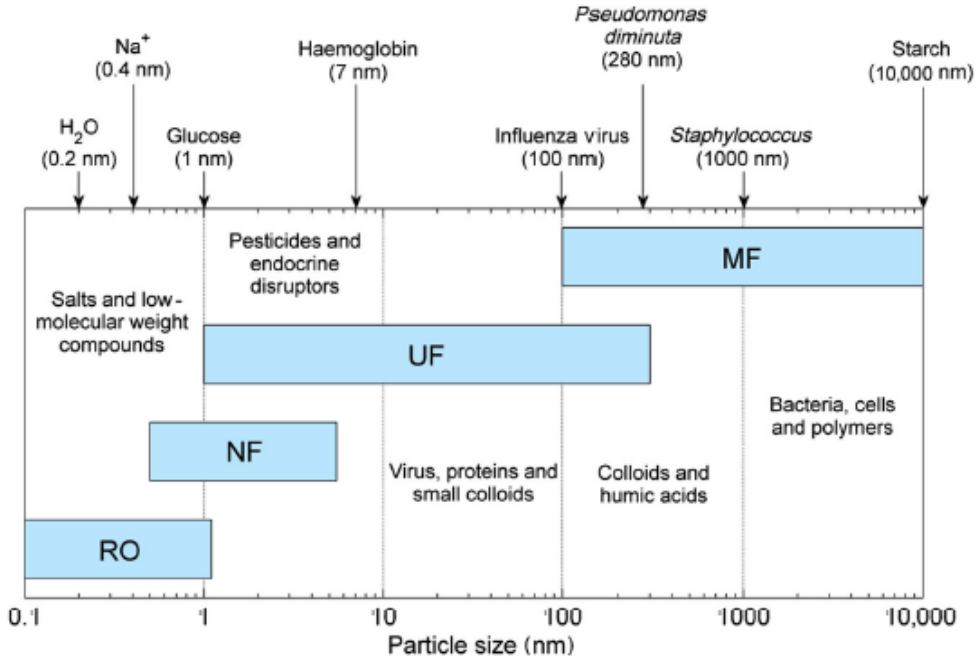


Figure 6. Particle size retention for different membranes (Tækker Madsen, 2014).

Membranes are differentiated depending on their molecular weight cut-off, term used to mean the molecular weight of the particle that is 90% rejected by the membrane (Baker, 2004). Rejection is used to describe the solute percentage retained by the membrane, calculated as (eq. 1.4):

$$Rejection = 1 - \frac{C_P}{C_F} \quad (1.4)$$

Where C_P is the concentration of the solute in the permeate and C_F the concentration in the feed (Tækker Madsen 2014). To calculate the percentage of the feed flow turned into permeate and recovered, the recovery is calculated (eq. 1.5):

$$Recovery = \frac{F_P}{F_F} \quad (1.5)$$

Where F_P is the permeate flow and F_F the feed flow. In a membrane filtration process, high recovery is a main objective, since it means a high amount of permeate relative to concentrate (Tækker Madsen, 2014).

Membrane processes which involve an electrical potential difference take place in processes such as electrodialysis, electro-osmosis or membrane electrolysis. In this processes, only charged molecules or ions are affected by the electrical field; uncharged molecules are not altered so electrically charged components are separated from their uncharged ones. There are two types of membranes: cation-exchange membranes, which allow positively charged cations to cross the membrane, and anion-exchange membranes, which allow negatively charged anions through them (Mulder 1996).

Several separation processes such as dialysis and Forward Osmosis (FO) are based on concentration differences as the driving force. In this processes, the difference in concentrations leads to an osmotic pressure that drives the process.

In a membrane processes, there is an influent stream called the feed and a filtrate called permeate. Two main types of filtration processes can be distinguished: dead-end filtration and cross-flow filtration, illustrated in **Figure 7**. Both processes contain a feed and a filtrate, but in dead-end filtration the feed crosses the membrane perpendicularly, while in cross-flow filtration it is parallel to the membrane. In this last process, the feed that does not cross the membrane is called retentate (Mulder 1996).

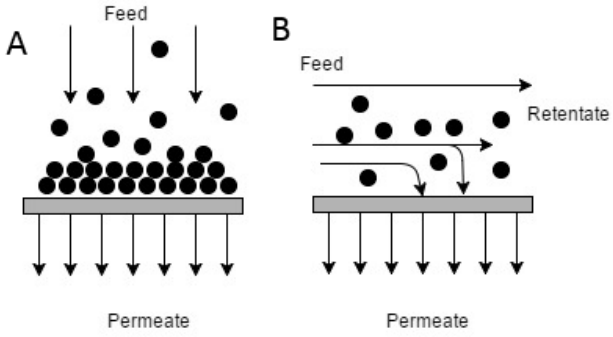


Figure 7. Comparison of filtration processes: dead-end (A) and cross-flow (B) filtration (Adapted from Tækker Madsen, 2014).

Working with dead-end filtration in a continuous process, the rejected solutes accumulate at the surface of the membrane leading to formation of a cake layer that reduces the flux through the membrane. On the other hand, in cross-flow filtration the feed flows tangential across the membrane surface allowing a theoretical continuous operation (Tækker Madsen 2014).

The deposition of particles on the membrane forming a cake layer is called fouling, a main challenge for operation of membrane filtration processes. When the deposited particles are

organic or microorganisms, it is called biofouling. When the membrane is fouled, the difference in pressure or concentration has to be increased to maintain a given flux since the fouling acts as resistance. Fouling may be removed by a backwash or a chemical cleaning (Tækker Madsen 2014).

Another main challenge is the formation of concentration gradients in the solutions on both sides of the membrane, since the feed contains a mixture of components that permeate at different rates; this is called concentration polarization (CP). The layer of feed solution adjacent to the membrane becomes depleted of the permeating solute, while it is enriched on the permeate side. The concentration difference across the membrane is then reduced and lowers the flux and membrane selectivity (Baker 2004).

Large membrane areas are usually required to apply membranes on a technical scale. The smallest unit into which the membrane area is packed is called a module, the central part of the process. But there exist two types of membrane configurations on which several module designs are based: flat sheet membranes and tubular membranes. Flat sheet membranes are stacked with spacer elements in a module called plate and frame, while they are wrapped around a central collection pipe in the module called spiral wound (Mulder, 1996).

On the other hand, a tubular membrane placed inside a porous tube, with several tubes put together is called a tubular module. The feed solution flows through the centre of the tubes and the permeate through the porous supporting tube. If the membranes, also called capillaries, are assembled together with their ends packed the module is called hollow fibre (Mulder, 1996).

Forward osmosis membranes

Osmosis is a physical phenomenon defined as the net movement of water across a selectively permeable membrane driven by a difference in osmotic pressure across the membrane (Cath, Childress, and Elimelech 2006). The concentration difference in both sides of the membrane leads to the difference in osmotic pressure, that acts as the driving force.

An osmotic pressure driven process operates based on the osmotic transport of water across the membrane from a diluted feed solution into a concentrated solution, illustrated in **Figure 8**. The membrane process that uses a semi-permeable membrane which separates two aqueous solutions of different osmotic pressures is called forward osmosis (Nayak and Rastogi 2010).

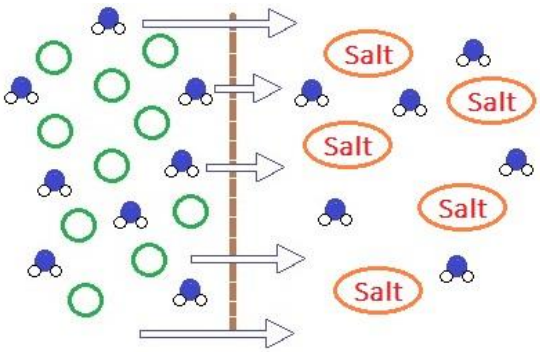


Figure 8. Representation of water transport through a membrane in an osmotic process. Green circles represent the solute of interest and orange ovals the osmotic agent.

FO processes concentrate a feed stream containing the solute of interest and dilute a highly concentrated stream called the draw solution (DS) or osmotic agent (**Figure 8**). The source of

the driving force in FO comes from the draw solution on the permeate side of the membrane. Different dissolved solutes can be used as DS, but the main criterion is that it has a higher osmotic pressure than the feed solution. A NaCl solution is a very frequent DS since it has high solubility and its relatively simple to re-concentrate with RO (Cath, Childress, and Elimelech 2006).

The osmotic pressure of both feed and draw solution can be estimated by the Morse equation (eq. 1.6):

$$\pi = iMRT \tag{1.6}$$

Where π is the osmotic pressure, i the Van't Hoff factor (it reflects the dissociation of the solute species), M the molarity of the feed or draw compound, R the ideal gas constant and T the temperature. Osmotic pressure is a colligative property, that means, it depends on the number ratio of solute particles to solvent molecules in a solution and not on the type of chemical present (ForwardOsmosisTech 2017).

In FO, solutes diffuse from the feed into the draw solution and from DS into the feed at the same time, called forward and reverse diffusion, respectively (Hancock and Cath 2009). Water flux through the membrane from FS to DS during osmosis can be estimated by eq. 1.7, assuming a well-stirred system:

$$J_w = A(\pi_D - \pi_F) \tag{1.7}$$

Where J_w is the water flux, A the water permeability coefficient, $\pi_D$the osmotic pressure in the draw solution and $\pi_F$the osmotic pressure of the feed solution (Cath et al. 2013). Similarly to water flux, reverse salt flux from draw to feed solution can be estimated based on eq. 1.3, Fick's Law:

$$J_s = B\Delta C \tag{1.8}$$

Where B is the solute permeability coefficient and ΔC the concentration gradient (Hancock and Cath 2009).

The employed membrane in FO is asymmetric and consists in two layers: a loosely bound support layer and a dense active layer. FO membranes should consist of a high density active layer with minimum porosity of the support layer and be hydrophilic. Water flux is influenced by the thickness of the membrane and the contact between the flowing solution and the membrane; the better the contact of the solutions, the faster the concentration (Rastogi and Nayak 2011).

In FO processes, there are two types of concentration polarization, internal and external; CP due to water permeation does not only take place in pressure-driven membrane processes. When the feed solution flows on the active layer of the membrane, solute concentration increases on that layer and at the same time DS is diluted at the permeate side of the membrane. These are respectively called concentrative external CP and dilutive external CP, and reduce the effective osmotic driving force.

On the other hand, there are two types of internal CP depending on the membrane orientation. When the feed solution faces the porous support layer, a polarized layer is established throughout the inside of the dense active layer as water and solute spread across the porous layer. This phenomenon is called concentrative internal CP. When the feed solution faces the active layer, water permeates the active layer and DS within the porous substructure becomes diluted; this is called dilutive internal CP. It is important to mention that the extent of external CP is much less than the internal CP (Cath, Childress, and Elimelech 2006).

As explained before, the membrane can be placed in two different orientations between the feed and DS. In the first one, called mode I, the feed solution is towards the support layer, while in mode II or reverse mode the feed is towards the active layer (Nayak and Rastogi 2010).

In mode I, the nature of the feed solution can lead to a significant internal or external CP. When the feed is water, it is diffused into the support layer and transferred to the DS on the other side through the active layer. In this case there is no significant CP, although there may be an insignificant external polarization on the DS side. When the feed solution contains low molecular weight compounds, it results in a significant concentrative internal CP and negligible external CP. But when the feed contains high molecular weight compounds, these are retained and accumulated on the support layer leading to a significant external CP on the feed side (Nayak and Rastogi 2010).

In mode II, when the feed solution faces the active layer and DS the support layer, water from the feed diffuses into the active layer and then the support layer. At the same time, since the solute used for DS is usually a low molecular weight compound, it can diffuse into the support layer to the interior surface of the active layer before flux can occur. Then, when water crosses the active layer into the support layer, the solute is diluted and concentration at the interior surface of the active layer is lower than in the bulk solution leading to an internal CP (Nayak and Rastogi 2010).

1.2.2. Use of membranes for protein extraction

Fractionation and isolation of proteins can be achieved by using different types of membranes; it is a biofriendly technique and the sample does not need to be diluted nor organic solvents are required, an energy efficient alternative. However, in some cases samples need pre-treatment procedures to improve the analyte concentration (Martínez-Maqueda et al. 2013).

Different types of pressure-driven membranes have been used for protein purification; Koschuh *et al*. (2005) used NF and UF membranes to filtrate silage juice from a green biorefinery as a first step to purify lactic acid and amino acids, showing NF satisfactory purification properties with relative low loss of amino acids.

Nanofiltration was then applied for the separation of lactic acid and amino acids in the same GBR. Different membrane materials and process parameters were tested, but it was not possible to produce two highly purified product streams. Quality of the separation, however, was enough for specific further product treatment (Ecker, Raab, and Harasek 2012).

Electric-driven membranes have also been used for fractionation and purification of proteins. Whey proteins were fractionated using a bipolar membrane with electroacidification, demonstrating feasibility of the technique to fractionate proteins. It was found that the initial protein concentration influences feasibility; at 10% whey protein isolate initial concentration, an enriched fraction containing 97,3% of β-lactoglobulin was obtained (Bazinet, Ippersiel, and Mahdavi 2004).

Isolation of amino acids, specifically separation of L-glutamic acid and L-aspartic acid from a mixture of amino acids by electrodialysis was also achieved with outstanding recoveries for Glu and Asp of 90% and 83%, respectively (Readi *et al.*, 2011). Electrophoresis, a technique that separates molecules by charge and polarity, was successfully used for the separation of a sample stream containing three proteins (BSA, myoglobin and cytochrome c) with high values of recovery and purity, around 85% and 95%, respectively (Melin and Poggel 2005).

Applications of FO membranes

Forward osmosis is one of the most promising membrane-based separation processes due to its advantages; it can be applied not only to food processing or protein concentration but also in wastewater treatment or water desalination. This process employs a semi-permeable membrane that separates two aqueous solutions, the feed and draw solution, with different osmotic pressures (Rastogi and Nayak 2011).

It is a process with a reduced energy consumption, since it can concentrate the feed solution at ambient pressure and temperature. In addition, it results in higher product quality in terms of less loss of fresh fruit flavours for example. However, the recovery of the draw solution can increase the energy and/or capital costs.

The applicability of forward osmosis has been demonstrated and promising results have been reported in different fields such as food processing (Petrotos and Lazarides 2001) and wastewater treatment (Holloway *et al.*, 2007). In 2001, Rodriguez-Saona *et al.* developed a process to concentrate red radish extract by forward osmosis and use it as food colorant. For that purpose, high fructose corn syrup was used as draw solution, reaching a 7,5-fold concentration in 11 hours. Other draw solutions such as NaCl have also been used for concentration of different juices, for example tomato juice (Petrotos, Quantick, and Petropakis 1998).

Forward osmosis membranes were first used for the concentration or dehydration of pharmaceuticals by Yang, Wang and Chung (2009). For the proof-of-concept demonstration, $MgCl_2$ was chosen for the draw solution and lysozyme for the feed solution as model of pharmaceutical product, achieving an enrichment factor of 1,85 in 3 h and 72 cm^2 of membrane area. Most pharmaceutical products are labile and heat sensitive, so athermal separation processes are preferred.

Based on the same principle than pharmaceuticals concentration, bovine serum albumin (BSA) as standard protein has been concentrated using different systems. Wang *et al.* (2011) integrated a membrane distillation system to the forward osmosis process to re-concentrate the NaCl solution used as draw solution, with an enrichment factor around 2 in 3 h. The integrated system was found stable in continuous operation when the dehydration rate was the same as the water vapor rate. Ling and Chung (2011) used nanoparticles as intermediate draw solutes and reverse osmosis to re-concentrate the nanoparticle solution, achieving an enrichment factor of 1,1 in 3 h and 8 cm^2 of membrane area.

FO has several advantages as a process to concentrate beverages and liquid foods such as operation at low temperatures and low pressures, promoting high retention of sensory and nutritional values. In addition, it has high rejection of several contaminants and lower membrane fouling propensity than pressure-driven membrane processes, making it a very promising membrane separation process (Cath, Childress, and Elimelech 2006).

CHAPTER 2 – OBJECTIVES

The main objective of this project is to investigate the feasibility of using forward osmosis membranes for concentration of the protein content in brown juice, waste stream from the OrganoFinery project. The OrganoFinery is currently studying the production of organic protein concentrate for animal feed, biogas and fertilizer using organic clover as feedstock. However, it is economically feasible because of the production of biogas and fertilizer, which reports the major profits of this green biorefinery.

Brown juice, the remaining stream after decantation of the proteins in the green juice, is used for biogas production, but it still contains a high amount of proteins as well as lactic acid and water. To improve feasibility, a second separation step to increase the recovery of proteins is proposed using forward osmosis membranes. This recovery is based on protein concentration by brown juice dewatering and protein precipitation due to super saturation.

FO membranes have several advantages compared to other separation techniques, such as low energy consumption since they can work at ambient pressure and temperature. They are osmotic pressure-driven membranes, with less propensity to foul compared to pressure-driven membranes. In addition, the advantage of working at ambient conditions allows high retention of sensory and nutritional value in liquid food concentration and makes it an athermal dewatering solution for pharmaceutical products enrichment.

In this project, it will be investigated whether forward osmosis membranes can be applied and improve protein extraction from brown juice. For that purpose, the following research questions were formulated:

- How can the use of forward osmosis membranes increase the protein content of the brown juice and its value?
- Which are the optimal conditions to work with the FO system (osmotic agent concentration, flow rate)? Which is the best membrane in terms of water flux and reverse salt flux?
- How can a standard protein be concentrated by forward osmosis? Can it be precipitated due to super saturation? If not, can it precipitate by salt addition? Does protein initial concentration influence water flux? Which is the best technique to measure BSA concentration over time?
- How can brown juice protein content be concentrated by forward osmosis? Is water flux influenced by other components such as lactic acid? Can proteins precipitate due to super saturation? How can proteins of the brown juice be measured?

CHAPTER 3 – EVALUATION OF MEMBRANE PERFORMANCE

In this chapter, two different flat-sheet membranes, Aquaporin and Porifera, were evaluated in terms of water flux, reverse salt flux and specific reverse salt flux. In addition, water permeability coefficient was estimated with the difference in osmotic pressures and without applied hydraulic pressure, resulting in a lower value than in literature.

When evaluating performance of osmotic membranes, several parameters influence transmembrane flux such as the type of membrane, osmotic agent concentration, temperature or flow rate of feed and osmotic agent. This evaluation was performed based on two different parameters, concentration of osmotic agent and flow rate of draw and feed solution; temperature was not modified so room temperature was used. In both cases, Porifera reported higher values of water flux and lower of reverse salt flux, so it was chosen to perform further experiments involving protein concentration.

3.1. Materials and methods

3.1.1. Experimental set up

The experiments were carried out on a bench-scale laboratory system consisting in a membrane cell, a peristaltic pump and two beakers for feed and draw solutions. A sketch and a picture of the system are presented in **Figure 9**. Membranes were mounted in the cell with dimensions of the FS and DS channels being 85,65 mm long x 39,25 mm wide x 3,4 mm deep and an effective membrane area of 0,0034 m^2. A cell dead volume of 130 ml was estimated, so it was emptied before each experiment to avoid changes in the feed solution.

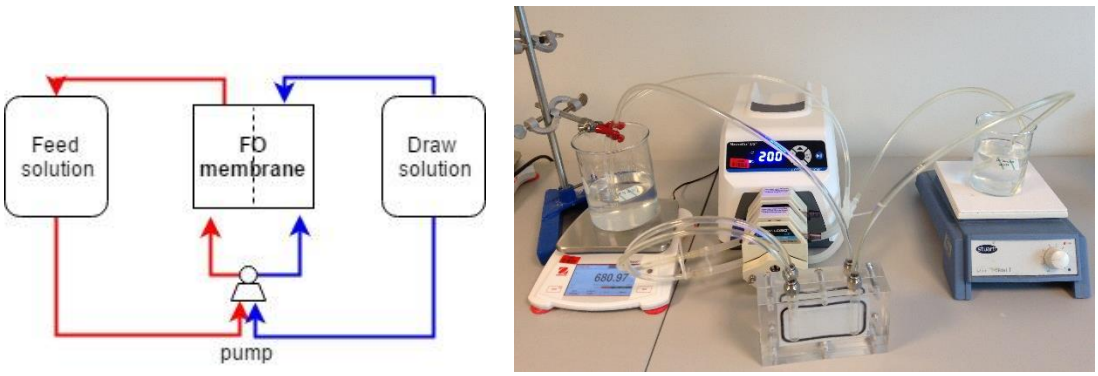


Figure 9. Sketch and picture of the experimental set up.

Deionized water was used as feed and different solutions of water and NaCl ranging from 0,5 M to 2 M were used as draw solution. 350 ml of FS and 500 ml of DS were run in a closed loop and pumped to and from a beaker using a Masterflex L/S variable speed peristaltic pump (Cole-Parmer Instrument Company, IL, USA) with two easy-load Masterflex L/S pump heads and tubing size 24 and 25. The beaker containing the DS was placed on a bench scale and the beaker with FS on a magnetic stirrer.

All experiments were performed at room temperature (23ºC) and the membrane was oriented in the FO mode, with the active layer facing the feed and the DS against the support layer. Experiments were conducted for 30 minutes and data were recorded every 5 minutes. There were two systems in total to perform experiments in duplicates; one contained tubing size 24 and the other one 25.

Reynolds number, flow velocity (m/s), volumetric flow rate (m^3/s) and flow rate (ml/min) were calculated for several speeds (rpm) of the pump and tubing size (**Appendix I**) and different flow rates varying from 240 to 650 ml/min were used. To calculate flow rate, water was pumped from one reservoir to another and the weight change was measured over time. Units were changed to calculate the volumetric flow rate, and then flow velocity and Reynolds number were estimated.

For these calculations, a cross sectional area of 0,00013 m^2, a hydraulic diameter of 0,00626 m, a density of 998,2 kg/m^3 and a dynamic viscosity of 0,001 uPa/s were utilized. To run the experiments with BSA and brown juice, a flow rate of approximately 504 ml/min and 508 ml/m were used for tubing size 24 and 25 respectively.

3.1.2. Forward osmosis membranes

Two commercially available flat-sheet membranes were used for this study: the Aquaporin Inside™ FO membrane with incorporated aquaporin proteins (Aquaporin A/S, Kongens Lyngby, DK) and the Porifera FO membrane with carbon nanotubes (Porifera Inc., Hayward, CA, USA). Prior to use, membranes were soaked in deionized water overnight and then run with fresh deionized water for at least 30 minutes to saturate with water the membrane's porous support layer, as specified by Aquaporin (AQUAPORIN 2017).

When necessary, membranes were cleaned using a 2wt% citric acid solution as recommended by HOLLOSEP engineering manual (Toyobo., n.d.). Citric acid was purchased from Sigma-Aldrich. Membranes were flushed with distilled water, then run with citric acid solution on both sides for 3 hours and left soaking overnight. The next morning, citric acid was removed, membranes were carefully flushed with abundant distilled water and run with fresh water for 30 minutes.

3.1.3. Water flux and reverse salt flux

Water flux (J_w) was calculated as the change in permeated water volume from the feed per unit membrane area per time ($l/m^2 \cdot h$). The mass of the draw solution, on a bench scale, increased with time because water permeated from the feed to the DS, then this mass increase was assumed to be the same as the feed reduction.

It was also assumed that 1 g of water equals 1 ml and no density difference was considered, then the flux was measured in litres instead of grams. The slope (g/min) resulted from plotting the feed mass reduction (g) vs. time (min) was used assuming a membrane area of 0,0034 m^2:

$$J_w \left(\frac{l}{m^2 \cdot h}\right) = \frac{slope\ (g/min)}{area\ (m^2) \cdot time\ (h)} \tag{3.1}$$

The corresponding units were changed from grams to litres and minutes to hours to have a final water flux in $l/m^2 \cdot h$.

Nevertheless, water flux was also calculated when a sample was taken every 5 minutes to have an overview of flux over time. In this case, the permeated water in 5 minutes was assumed to be the same as the weight increase in the DS and divided by membrane area and time.

To calculate the reverse salt flux, salt accumulation in the feed was plotted versus time. To calculate salt accumulation, salt concentration was multiplied by the feed volume at different times; salt concentration was estimated by correlation of conductivity and concentration. Different conductivity curves (**Appendix I**), depending on the concentration range, were created using the Seven2Go Conductivity Meter S3 (METTLER TOLEDO) to measure conductivity. Reverse salt flux (RSF, J_S) was calculated by dividing the slope (g/min) of salt accumulation vs. time by the membrane area and time:

$$J_s \left(\frac{g}{m^2 \cdot h}\right) = \frac{slope\ (g/min)}{area\ (m^2) \cdot time\ (h)} \tag{3.2}$$

As done for the water flux, the corresponding unit was changed from minutes to hours to have a final reverse salt flux in g/m²·h.

Specific reverse salt flux ($J_{specific}$) was defined as the ratio of salt flux (J_S) in the reverse direction of water flux (J_w); it is related to the water-salt selectivity of the membrane (Hancock and Cath 2009). It was calculated as the RSF divided by the water flux, with units of g/l. It is related to membrane efficiency and depends on active layer selectivity (Bell, Holloway, and Cath 2016).

$$J_{specific} \left(\frac{g}{l}\right) = \frac{J_S \left(\frac{g}{m^2 \cdot h}\right)}{J_w \left(\frac{l}{m^2 \cdot h}\right)} \tag{3.3}$$

The resulting units were g/l.

3.1.4. Determination of osmotic pressure and water permeability

The osmotic pressure (π) of feed and draw solution was calculated as eq. 1.6:

$$\pi = iMRT \tag{1.6}$$

Where i is the Van't Hoff factor, M the molarity of the feed or draw compound in mol/l, R the ideal gas constant in l·atm/K·mol and T the temperature in Kelvin, as explained in 1.2.1.

Osmotic pressure of the NaCl draw solution was calculated translating the conductivity in molarity, and then multiplying by room temperature (296 K), the gas constant (0,08206 l·atm/K·mol) and the Van't Hoff factor for NaCl, which is 2 due to ionization of NaCl into two ions in water. Osmotic pressure due to salt accumulation in the feed solution over time was also estimated measuring conductivity.

The water permeability coefficient, A, was calculated for both membranes. Membranes were not characterized using a reverse osmosis test apparatus, but parameters were estimated without applied hydraulic pressure and using the difference in osmotic pressure.

Water permeability was determined by dividing the water flux (J_w) by the difference in osmotic pressure (Δπ), calculated with molarity, temperature, R and the Van't Hoff factor (Cath et al. 2013).

$$A = \frac{J_w}{\Delta\pi} \tag{3.4}$$

Water fluxes used to estimate A were calculated every 5 minutes by dividing the weight increase by the membrane area and time A was calculated for four different NaCl molarities ranging from 0,5 to 2 M, and the average value was calculated. The corresponding unites were changed to obtain a final value in l/m²·h·atm.

3.2. Results and discussion

3.2.1. System set up

Since two different tubing sizes were used for the experiments, it was necessary to achieve the same flow rate to be able to compare experiments regarding membrane performance and protein concentration. To work under the same conditions, Reynolds number, flow velocity (m/s), volumetric flow rate (m³/s) and flow rate (ml/min) were calculated to characterize several pump speeds (rpm) and tubing sizes, as explained in 3.1.1. Detailed results are presented in **Appendix I**.

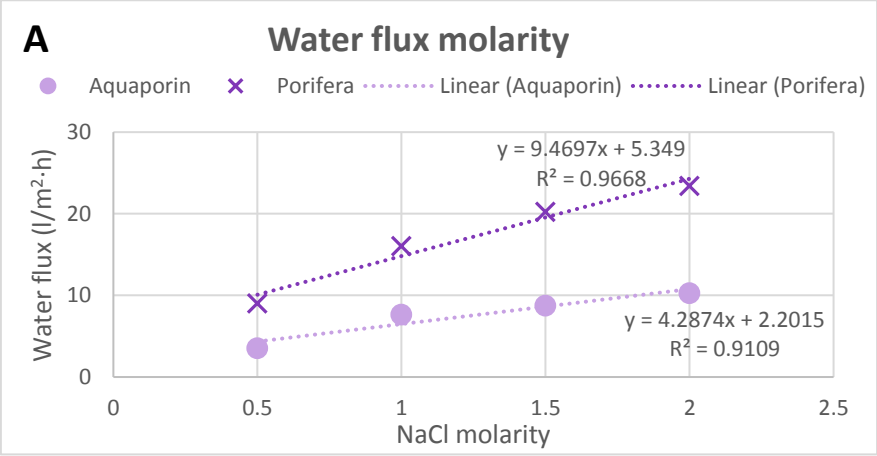
Table 2 shows the final speed chosen to work depending on the tubing size, although section 3.2.4. explains the final flow rate used to proceed with protein concentration experiments.

Table 2. Flow rate, flow velocity and Reynolds number for different speeds and tubing sizes.

Tubing size	*Speed (rpm)*	*Flow rate (ml/min)*	*Volumetric flow rate (m³/s)*	*Flow velocity (m/s)*	*Reynolds number*
24	200	504	8,40E-06	0.063	393,19
25	259	508	8,47E-06	0.063	396,32

3.2.2. Osmotic agent concentration

Calculated water fluxes and reverse salt fluxes for Aquaporin and Porifera depending on NaCl molarity are shown in **Figure 10**. Water flux, RSF and SRSF were calculated as described in section 3.1.3. Detailed values of water flux, reverse salt flux and specific reverse salt flux are presented in **Appendix I**. Experiments were conducted at 504 ml/min, and no duplicates were performed.



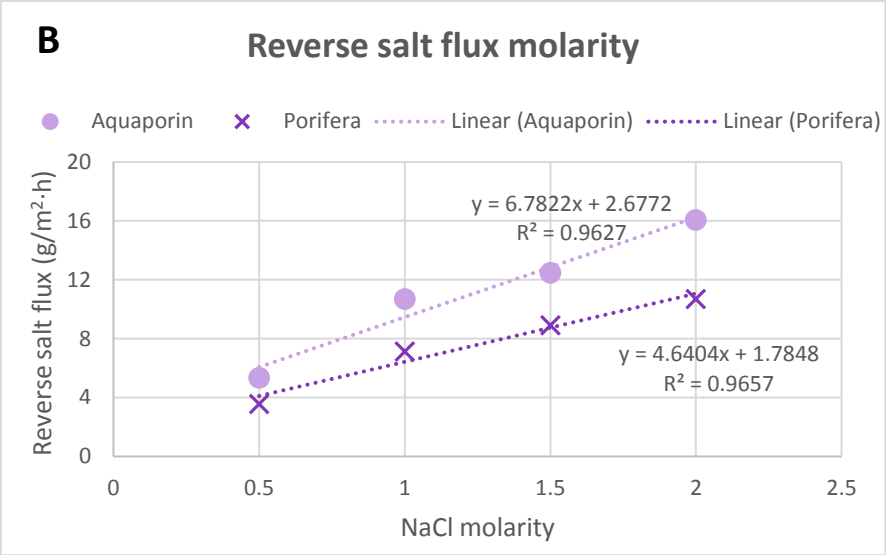


Figure 10. Water flux (A) and reverse salt flux (B) depending on NaCl molarity.

NaCl molarity is directly ligated to osmotic pressure, hence higher solute concentration increases osmotic pressure and therefore water flux. Porifera shows higher water flux and lower reverse salt flux than Aquaporin in all NaCl molarities, but the increasing ratio is quite similar. The NaCl leakage, RSF, increases with an increase in NaCl concentration in the draw solution.

When NaCl molarity raises up to 4x, from 0,5 M to 2 M, water flux increases almost 3x in both Porifera and Aquaporin membranes; they fit quite well a linear trendline with R^2 value of 0,9668 for Porifera and 0,9109 for Aquaporin. The same can be applied to reverse salt flux, which increases with higher solute molarity. Both membranes follow a linear trendline again, with R^2 value of 0,96.

Porifera water flux is almost double to Aquaporin while reverse salt flux is around 60%, but in both cases this proportion is maintained as NaCl molarity increases. Dova, Petrotos and Lazarides (2007) already indicated that an increase in draw solute concentration (NaCl) lead to an increase in transmembrane flux from the feed, in that case with a sugar solution as feed. It has also been reported that NaCl generates a higher flux than dextrose or sucrose when used as osmotic agent and deionized water as feed and FO mode (Gray, McCutcheon, and Elimelech 2006), but only sodium chloride was studied in this experiment.

Since water flux and RSF increase at a similar ratio with NaCl molarity, the specific reverse salt flux remains quite constant with an average value of 0,42 g/l for Porifera and 1,48 g/l for Aquaporin (see **Appendix I**).

3.2.3. Feed and osmotic agent flow rate

Another parameter that can affect the transmembrane flux is feed and osmotic agent flow rate. In this study, feed and draw solutions were pumped with the same pump, so the flow rate of both solutions was increased or decreased at the same time. Detailed values of water flux, reverse salt flux and specific reverse salt flux are presented in **Appendix I**.

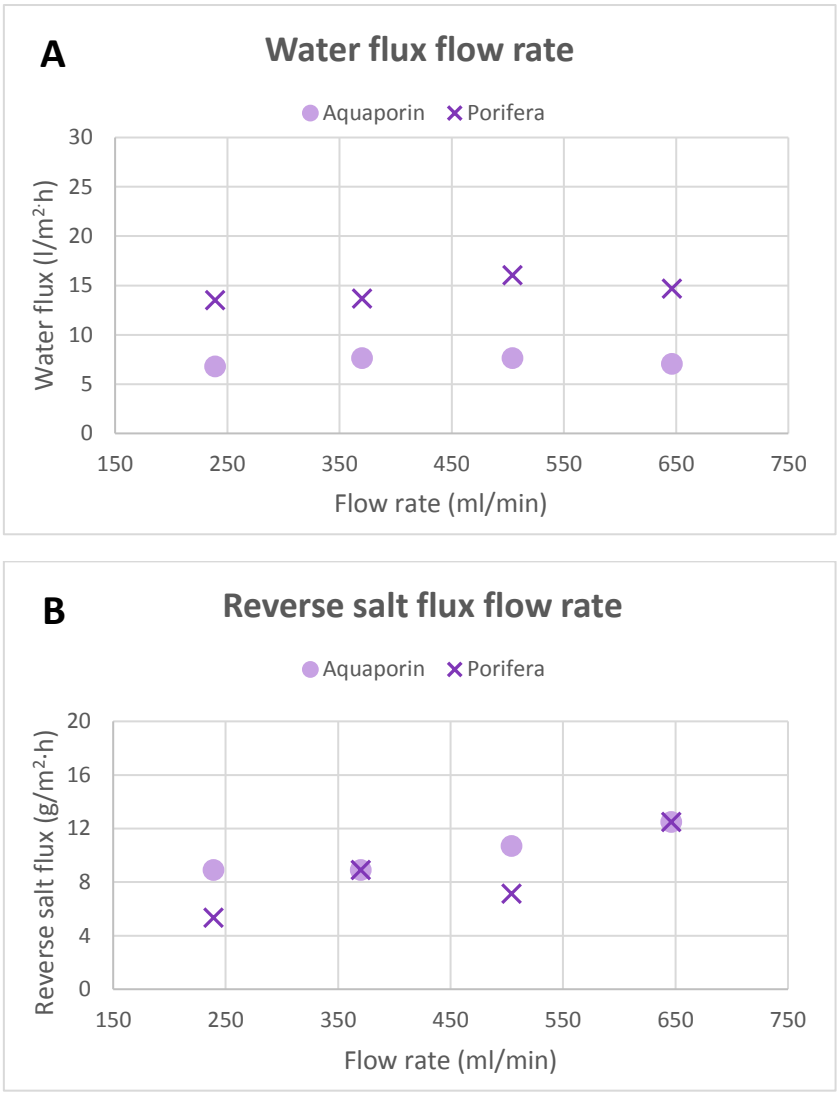


Figure 11. Water flux (A) and reverse salt flux (B) depending on flow rate.

Water flux and reverse salt flux for both membranes depending on flow rate are shown in **Figure 11**. All experiments with varying flow rate were performed using 1 M NaCl as osmotic agent. As demonstrated with varying NaCl molarity, Porifera has a higher water flux (almost two times) than Aquaporin as flow rate increases. However, reverse salt fluxes are more similar, being Porifera RSF around 60% of Aquaporin RSF (as found with varying NaCl molarity) at flow rates 240 and 504 ml/min but the same RSF at 370 and 645 ml/min.

These results reaffirm that Porifera has a higher transmembrane flux and lower reverse salt flux than Aquaporin, but these values do not increase greatly as flow rate becomes higher. In fact, with a 2,5x increase in flow rate water flux is almost the same for both Porifera and Aquaporin, with an increase of 1,09x and 1,04x respectively. Nayak and Rastogi (2010) already demonstrated that an increase in flow rate cannot be translated into a great water flux increase; with a 2,5x increase in feed flow rate, the transmembrane flux was found to increase from 4,2 to 4,7 $l/m^2 \cdot h$, around 1,12x increase. Nevertheless, this trend is not followed by the reverse salt flux; Porifera RSF is almost double and Aquaporin 1,5 times higher when flow rate is increased 2,5 times.

In this case, as water flux remains almost constant and RSF increases slightly when flow rate is higher, the specific reverse salt flux is not so stable. SRSF varies from 0,44 to 0,85 g/l with an average of 0,62 g/l for Porifera and from 1,17 to 1,77 g/l with an average of 1,41 for Aquaporin (see **Appendix I**).

3.2.4. Porifera vs. Aquaporin

Standard methodology to evaluate membrane performance was already proposed by Cath *et al*. (2006); it was suggested to work at 20ºC, with 1 M NaCl as draw solution, deionized water as feed and a flow velocity of 0,25 m/s. In this study, room temperature (23ºC), 1 M NaCl as DS, deionized water as FS and a flow velocity of 0,063 m/s were used to compare performance of Porifera and Aquaporin. In both cases, the active layer of the membrane was facing the feed stream. **Table 3** shows data calculated to compare the membranes: water flux, reverse salt flux and specific reverse salt flux.

Table 3. Water flux, reverse salt flux and specific reverse salt flux of Porifera and Aquaporin.

	M NaCl	*Flow rate (ml/min)*	*Water flux (l/m²·h)*	*Reverse salt flux (g/m²·h)*	*Specific reverse salt flux (g/l)*
Porifera	1	504	16,6	7,14	0,44
Aquaporin			7,64	10,71	1,40

As explained above, Porifera has a higher transmembrane flux and lower reverse salt flux than Aquaporin; a water flux of 16,06 l/m²·h was obtained using Porifera, while Aquaporin reached 7,64 l/m²·h. Higher water flux of Porifera compared to Aquaporin was described by Blandin *et al.*, (2016) but with higher values: 33 l/m²·h for Porifera and 9,5 l/m²·h for Aquaporin. Nevertheless, a water flux of >7 l/m²·h for Aquaporin together with a reverse salt flux of <2 g/m²·h was reported by Perry *et al.* (2015) as supplied by Aquaporin A/S. A water flux of 7,64 l/m²·h from this study is consistent with the provided data, but RSF is up to 5x higher. This may be due to an incorrect calibration of the conductivity meter used in this study.

Data from the Singapore International Water Week 2014 used for a Porifera technology overview available in Porifera homepage (Porifera 2017) describes water flux for Porifera as 33 l/m²·h and SRSF 0,4 g/l. Aquaporin water flux is described as 7 l/m²·h and SRSF 0,29 g/l. Even though water flux calculated for Porifera in this study is almost half, the specific reverse salt flux is quite similar, 0,44 g/l. Otherwise, water flux for Aquaporin in this study is very close to the provided data, but SRSF almost 5x, as RSF is quite higher.

Such great differences in water flux and reverse salt flux between membranes may be due to their different composition. Aquaporin membrane is a biomimetic membrane which integrates the aquaporin protein pores into the rejection or active layer (Zhao et al. 2012), described as Aquaporin Inside™ forward osmosis membranes in Aquaporin homepage. On the other hand, Porifera membranes are thin-film composite membranes which contain carbon nanotubes in the support layer to enhance membrane performance (Revanur *et al.*, 2012).

In addition to evaluate membrane performance, these experiments were conducted to select Aquaporin or Porifera for further experiments for protein concentration. Due to higher water flux and lower reverse salt flux, it was chosen to work with Porifera for BSA and brown juice

concentration. The same conditions used in these experiments, 1 M NaCl and 504 ml/min, were used for BSA concentration.

3.2.5. Determination of membrane characteristic parameters

Water permeability coefficient A, specific for every membrane, was estimated for Porifera and Aquaporin using water flux and the difference in osmotic pressure, as explained in 3.1.4. All NaCl molarities were used and an average value of A was estimated, presented in **Table 4**.

Table 4. Experimental water permeability coefficient of Porifera and Aquaporin.

	Water permeability (l/m²·h·atm)
Porifera experimental	0,305
Porifera literature	4,27
Aquaporin experimental	0,132

As shown in **Table 4**, experimental A calculated for Porifera differs greatly from the value obtained in the literature (Maisonneuve, Laflamme, and Pillay 2016), but it is important to take into account that no hydraulic pressure was applied to the membrane to estimate the coefficient. The difference in osmotic pressure was the only force considered, so any mistake regarding feed or draw solution can affect the final value. As expected, the water permeability coefficient of Aquaporin is quite lower than the coefficient of Porifera due to the differences in permeated water with varying NaCl molarities.

Water permeability coefficients from other commercially available membranes can be found in the literature (Maisonneuve, Laflamme, and Pillay 2016) with A values are quite close to Porifera, Nevertheless, commercial asymmetric CTA membrane from Hydration Technology Innovations showed an A value of approximately 0,7, closer to the calculated experimentally for Porifera.

CHAPTER 4 – ENRICHMENT OF A STANDARD PROTEIN

In this chapter, bovine serum albumin (BSA) was used as a standard protein to evaluate Porifera membrane performance in protein concentration with prospects of protein precipitation due to super saturation. Different BSA initial concentrations from 0,1 g/l to 20 g/l were used as feed and 1 M and 2 M NaCl as draw solution. Water flux, protein concentration and salt accumulation over time were measured and compared for the different concentrations to study protein enrichment. In addition, BSA concentration and water flux were estimated using a BSA mass balance in the feed tank and a model prediction for water flux based on osmotic pressure.

In all three initial BSA concentrations, reported water flux and reverse salt flux remained rather similar with varying values from 11 to 13,4 l/m²·h and from 5,4 to 8,9 g/m²·h, respectively. Compared to water and reverse salt flux values using deionized water as feed solution, they were quite close indicating no membrane fouling due to BSA. The enrichment factor of BSA experiments was also calculated over a period of 3 hours, resulting in similar values as well from 1,46 to 2 for all three different initial concentrations.

Complementary experiments with an addition of salt were performed to study water flux dependency on draw dilution over time, resulting in almost no experimental but theoretical dependency. Since no precipitation due to super saturation was found, after concentration by forward osmosis BSA was precipitated using ammonium sulfate at a saturation percentage around 60-70%.

4.1. Materials and methods

4.1.1 Experimental set up

Experiments were performed using a Porifera FO membrane. BSA, obtained from Sigma-Aldrich, was chosen as standard since it is a readily available and stable protein, in addition to having a similar size to proteins in the brown juice. Solutions of different initial BSA concentrations, ranging from 0,1 to 20 g/l, were used as FS while a solution of 1 M NaCl was used as DS in most of the experiments; 2 M NaCl was used for the extra addition of salt. Initial volumes of 500 ml and 350 ml were used for DS and FS respectively. As said in 3.1.1., a flow rate between approximately 504 and 508 ml/min was used to perform the experiments.

To prepare solutions, the BSA powder was left dissolving in deionized water overnight in the fridge to ease dissolution. Experiments were performed at room temperature during 3 hours, and DS weight, conductivity and a BSA sample were taken every 20 minutes. pH value was measured at the beginning and the end of each experiment.

BSA as standard protein

Serum albumin is a principal component from blood and the most multifunctional transport protein, besides having a great influence on blood pH maintenance. It is present in the body fluids and tissues of mammals, and has low molecular weights, is soluble in water and easily crystallized (Sigma-Aldrich, n.d.).

BSA is a serum albumin protein derived from cows, commonly used for quantitative measurement of other proteins by comparing an unknow quantity of the protein of interest with a known quantity of BSA. Albumins serve as transporter for fatty acids and binds water, Ca^{2+}, Na^{+} and K^{+}. Its main biological function is to regulate the colloidal osmotic pressure of blood (UniProtKB/Swiss-Prot).

Extraction of albumin from serum plasma is based on the differential solubility of albumin and other plasma proteins depending on factors such as pH, ethanol concentration or protein concentration. Albumin has the highest solubility and is the last precipitated product the so-called Fraction V, following the Cohn process for albumin precipitation.

4.1.2. Protein quantification

To estimate protein concentration, absorbance at 280nm was chosen as an easy and fast technique, since interference from other compounds for aqueous protein solutions is minimized at this wavelength (ThermoScientific 2002). Absorption of radiation at 280nm, near the UV by proteins depends on the tryptophan (Trp) and tyrosine (Tyr) content, and cysteine (Cys) to a lesser extent. BSA is frequently used as a protein standard and 1 mg/ml has a A_{280} of 0,66 (Walker 2002).

A calibration curve was created to correlate A_{280} and protein concentration (**Appendix II**). Protein concentration was estimated experimentally and theoretically, using absorbance values for the experimental concentration. Samples taken during the experiments were diluted with deionized water to be in the range of the calibration curve. To calculate the protein concentration theoretically, the volume of permeated water through the membrane over time was used to estimate the protein concentration assuming all the protein content stayed in the feed solution.

A_{280} was useful for BSA concentrations of 1 g/l and 10 g/l diluted to 1 g/l. When measuring absorbance of a 0,1 g/l solution, results were not very stable. For that reason, the Pierce BCA Protein Assay Kit was used to measure protein concentration when it was lower than 1 g/l.

The first experiments were performed measuring absorbance at 290 nm due to lack of material, but there were some problems so several hypotheses were analysed as detailed in 4.1.3. After these hypothesis, absorbance was measured at 280 nm with the proper cuvettes.

To compare membrane performance using different initial BSA concentrations, the enrichment factor (EF) was calculated based on Yang *et al.* (2009). It was calculated for both experimental and theoretical BSA concentration, prediction explained in 4.1.6. It is defined as the protein concentration (C_t) at a time interval over the initial feed protein concentration (C_o):

$$EF = \frac{C_t}{C_o} \tag{4.1}$$

4.1.3. Background absorbance

The first experiments to measure BSA absorbance with 0,1 g/l BSA in the feed were performed at 290 nm as explained before, but estimation of protein concentration by absorbance was not successful due to a background absorbance, so several hypotheses and experiments were proposed to find out the source of this absorbance, presented in **Table 5**.

Table 5. Hypotheses and experiments proposed for background absorbance.

	Hypothesis	Experiment
1	Error in the calibration curve	A new calibration curve was prepared to ensure the correlation between absorbance and BSA concentration. In addition, a scan was made to check that 280 nm was the best wavelength to measure BSA absorbance
2	Background from the salt accumulation	A series of NaCl solutions at different concentrations were prepared, and their A_{290} values were measured.
3	Background from the interaction between BSA and salt	A series of solutions of 0,1 g/l BSA were prepared and different amounts of NaCl were added to reach different salt concentrations. A_{290} was measured for each sample.
4	Background from the forward osmosis system	A blank experiment was performed using NaCl as draw solution and water as feed; A_{290} was measured during time.
5	Background from the membrane cell	A solution of BSA was pumped through the cell but without the membrane and A_{290} was measured over time
6	Background from the membrane	A piece of membrane was placed in a beaker with water and it was continuously stirring. At the same time, there was another beaker with water stirring and its A_{290} was measured as control.
7	Background from mechanical fractionation of BSA	A solution of 0,1 g/l BSA was pumped through the system but without the cell and the membrane, just recirculated to the beaker. A_{290} was measured during time.
8	Instability at 290 nm.	

4.1.4. BSA precipitation

Different methods were tried to induce protein precipitation besides super saturation. Firstly, the concentrated solution of BSA (>40 g/l) through forward osmosis was separated in two different beakers: the first one was left overnight in the fridge and the pH of the second one was adjusted to 4 with HCl and left in the fridge overnight. Secondly, a solution of 20 g/l BSA was prepared and BSA was added until concentration reached 100 g/l, but there was no precipitation.

Thirdly, ammonium sulfate, purchased from Sigma-Aldrich, was added since at high salt concentrations protein solubility decreases leading to precipitation, an effect called salting-out (Wingfield 2016). The addition of ammonium sulfate to a BSA solution to find the salt

saturation necessary for protein precipitation was based on the protocol from Duong-Ly and Gabelli (2014) and guidelines from Wingfield (2016).

To determine the amount of solid ammonium sulfate required to reach a specific saturation, the on-line calculator from EnCor Biotechnology Inc. was used. Calculation of the salt saturation percentage necessary for precipitation was performed preparing two BSA solutions of different initial concentration, 10 and 40 g/l. Then an amount of ammonium sulfate was added, solutions were stirred for 20 minutes and centrifuged for 20 minutes (10 000 x g, 4ºC). This procedure was repeated increasing the salt concentration in several steps until proteins precipitated.

4.1.5. Water flux and reverse salt flux

An overall water flux was calculated plotting the feed mass reduction versus time as explained in 3.1.3. But water flux (l/m²·h) was also calculated every 20 minutes, when a sample was taken. The permeated water in 20 minutes, assumed to be the same as the weight increase in the DS, was divided by the membrane area and time, changing the corresponding units when necessary.

Reverse salt flux was also calculated as explained in 3.1.3., plotting the salt accumulation versus time, but conductivity due to BSA concentration was not considered. The conductivity value measured at the beginning of each experiment was subtracted from the following measurements every 20 min.

4.1.6. Prediction of protein concentration and water flux

BSA concentration over time was estimated to be compared with the real protein concentration. This estimation was calculated using the total volume of the feed solution at a given time as proposed by Wang *et al.* (2011):

$$C_{BSA,t} = \frac{C_{BSA,0} V_{f,0}}{V_{ft}} \tag{4.2}$$

Where $C_{BSA,0}$ and $C_{BSA,t}$ are the initial BSA concentration and BSA concentration in feed tank at a given time, and $V_{f,0}$ and V_{ft} are the initial feed solution volume and feed solution volume at time t.

V_{ft} was calculated as the initial feed volume ($V_{f,0}$) subtracting the accumulated permeated water (ΔV):

$$V_{ft} = V_{f,0} - \Delta V \tag{4.3}$$

To estimate water flux over time, the general equation 1.7 described in 1.2.1. was used:

$$J_w = A(\pi_D - \pi_F) \tag{1.7}$$

Where A is the water permeability coefficient, $\pi_D$the osmotic pressure in the draw solution and $\pi_F$the osmotic pressure of the feed solution. Osmotic pressure of feed and draw solution were also estimated every 20 minutes using the molarity of NaCl in FS and DS, also explained in 3.1.4.

However, osmotic pressure of the feed was calculated as the addition of osmotic pressure coming from salt accumulation and from BSA concentration. The same water permeability

coefficient estimated for Porifera was employed. As done with experimental results, an overall water flux was calculated for BSA concentration, in this case as the average of all the predicted values.

4.2. Results and discussion

4.2.1. Background absorbance

During the first experiments with 0,1 g/l BSA as feed solution, A_{290} instead of A_{280} was measured due to lack of material. Nevertheless, protein estimation was not successful due to a background absorbance, so several hypotheses were proposed as detailed in 4.1.3. **Table 6** summarizes all the hypotheses, experiments performed and results obtained. Detailed results of the experiments can be consulted in the **Appendix II**.

Table 6. Summary of proposed hypotheses, experiments and results for background absorbance.

	Hypothesis	Experiment	Result
1	Calibration curve	New calibration curve. Scan of wavelength.	Calibration curve very similar to the first one. 280 is the best wavelength.
2	Salt accumulation	A_{290} of salt solutions.	A_{290} close to zero or lower.
3	BSA + salt accumulation	A_{290} of salt and BSA solutions.	A_{290} very stable independently of salt concentration.
4	FO system	NaCl as DS and DW as FS; A_{290} of FS.	A_{290} varies slightly over time.
5	Membrane cell	BSA pumped through the cell; A_{290} of BSA.	Something desorbing from the cell. Wash cell properly with water.
6	Membrane	Membrane stirred in water; A_{290}.	Something desorbing from the membrane. Flush membrane carefully with water.
7	Mechanical fractionation of BSA	BSA recirculated to the beaker without cell and membrane, A_{290}	A_{290} constant over time.
8	Instability at 290 nm		New cuvettes for A_{280}.

Several experiments were performed regarding FO system, membrane or cell desorption as well as BSA and salt interaction or BSA fractionation. No interaction was found between BSA or NaCl that could increase A_{290} and affect BSA experiments, since it remained very stable over time.

However, A_{290} was found to vary over time when a solution was pumped through the FO system; experiments with both the membrane cell and the membrane reported an increase in A_{290}, something seemed to be desorbing from them. Regarding the membrane, a possible reason for desorption is that membranes are usually covered in a protective layer of glycerol. It was then established that the membrane cell would be washed with water and the membrane would be carefully flushed with water when a new experiment was performed. In addition, when a new piece of membrane was used, it was washed and soaked in water as explained in 3.1.2.

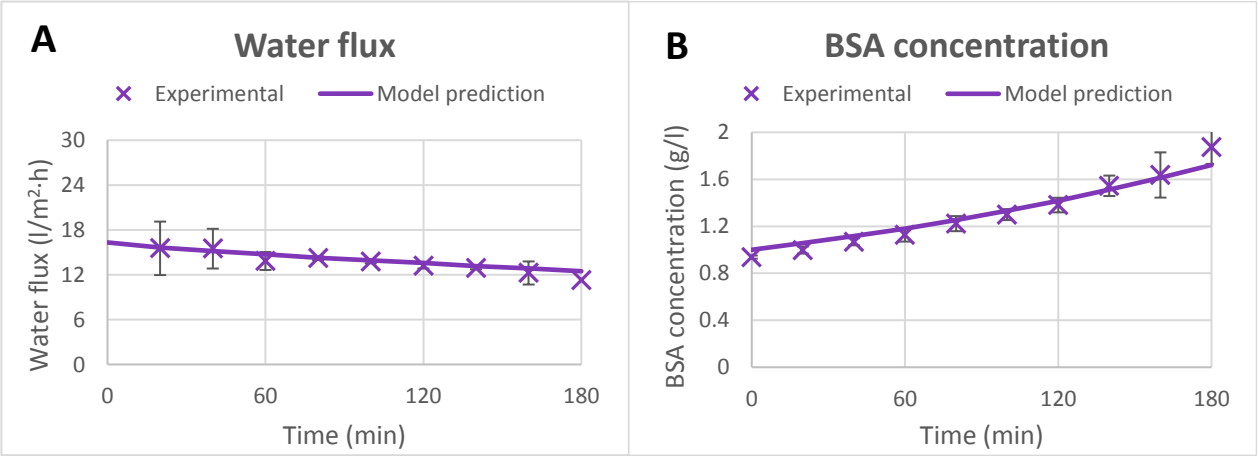
4.2.2. BSA concentration

As a representation, **Figure 12** shows the evolution of water flux, BSA concentration and salt accumulation over time in the feed tank with an initial BSA concentration of 1 g/l. Experiments were performed in duplicates so error bars with standard deviation is included. Graphs showing results of 0,1 g/l and 10 g/l BSA are included in **Appendix II**.

Firstly, the water flux decreases during time because the draw solution is diluted as water permeates the membrane from the feed solution, as expected. The first two samples of the experiments have a high standard deviation due to differences in duplicates, but after the third sample standard deviation decreases considerably.

Experimental water flux was calculated as the change in permeated water per area per time, described in 4.1.5. In addition, water flux was estimated by a model prediction using the calculated water permeability coefficient and the different osmotic pressures of draw and feed solutions, as detailed in 4.1.6. **Figure 12A** shows that the experimental and predicted water flux are very similar, which indicates the absence of BSA fouling and external concentration polarization, which could reduce the experimental water flux.

Secondly, BSA is concentrated by a factor of 2 after 3 h, from 1 to almost 2 g/l, as shown in **Figure 12B**. Both the experimental and model predicted concentration follow the same increasing trend and results are very similar, which indicates that the estimation of BSA concentration is quite accurate. BSA quantification using A_{280} was successful for both 1 and 10 g/l BSA, while it did not work so well for 0,1 g/l. In that case, protein concentration was measured using the Pierce BCA Protein Assay kit as explained in 4.1.2.



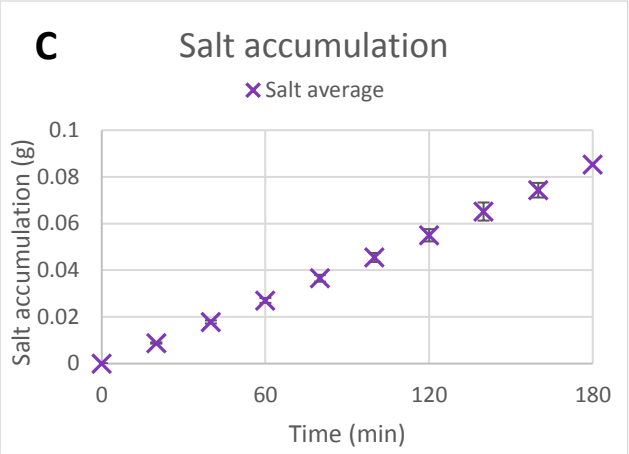


Figure 12. Water flux (A), BSA concentration in feed tank (B) and salt accumulation (C) as a function of time.

Thirdly, salt accumulation is shown in **Figure 12C**. These values were calculated measuring conductivity of the feed solution as a method to estimate salt leakage from the draw solution. In addition to salt accumulation, reverse salt flux from draw to feed solution was calculated as explained in 4.1.5. As done for water flux, reverse salt flux is calculated as a measure of salt permeability through the membrane, useful to characterize it and compare it with other membranes. In this study, the objective is the concentration of a standard protein to evaluate membrane performance, so reverse salt flux is not very harmful. However, if the aim was to purify proteins through FO, salt accumulation in the feed tank could be a disadvantage for the process depending on the protein final use.

The exact values of water flux, enrichment factor and reverse salt flux of the different BSA initial concentrations are presented in **Table 7**.

Table 7. Enrichment factor, water flux and reverse salt flux of BSA solutions. Standard deviation is shown in brackets. Exp. stands for experimental and The. for theoretical.

	Enrichment factor		**Water flux (l/m²·h)**		**Reverse salt flux (g/m²·h)**
BSA concentration (g/l)	*Exp.*	*The.*	*Exp.*	*The.*	*Exp.*
0,1	1,46 (0,04)	1,6 (0,02)	11,99 (0,29)	13,74 (0,01)	5,35 (0,00)
1	2 (0,12)	1,72 (0,12)	13,38 (0,74)	14,21 (0,20)	8,92 (0,00)
10	1,55(0,03)	1,52 (0,06)	11,07 (0,79)	14,42 (0,00)	7,14 (0,00)

Table 7 shows that there are no great differences in the enrichment factor, water flux or reverse salt flux between the different BSA concentrations. The enrichment factor was calculated as the initial protein concentration divided by the final concentration, as described in 4.1.2.

In 3 h, the feed solution has an EF varying from approximately 1,5 to 2, with an effective membrane area of 34 cm². In addition, the theoretical enrichment factor, calculated using the predicted BSA concentration, has a value very similar to the experimental one, which reaffirms

the accurate protein prediction. BSA concentration using NaCl as draw solution was already researched by Wang *et al.* (2011), but with a hydrophilic polybenziamidazole NF hollow fiber membrane. Two different initial BSA solutions were studied and concentrated from 0,1 to 0,19 g/l and from 1 to 2,1 g/l in 3 h, i.e. an enrichment factor of 1,9 and 2,1, respectively.

Ling and Chung (2011) also studied BSA enrichment by forward osmosis reporting an enrichment factor of 1,1 in 3 h but using a substantially smaller membrane effective area, 8 cm^2, and highly hydrophilic nanoparticles as draw solution instead of NaCl. However, Yang, Wang and Chung (2009), the pioneers concentrating protein through forward osmosis membranes, achieved an enrichment factor of 1,85 in 3 h with 72 cm^2 of membrane and an average water flux of 12.7 $l/m^2 \cdot h$.

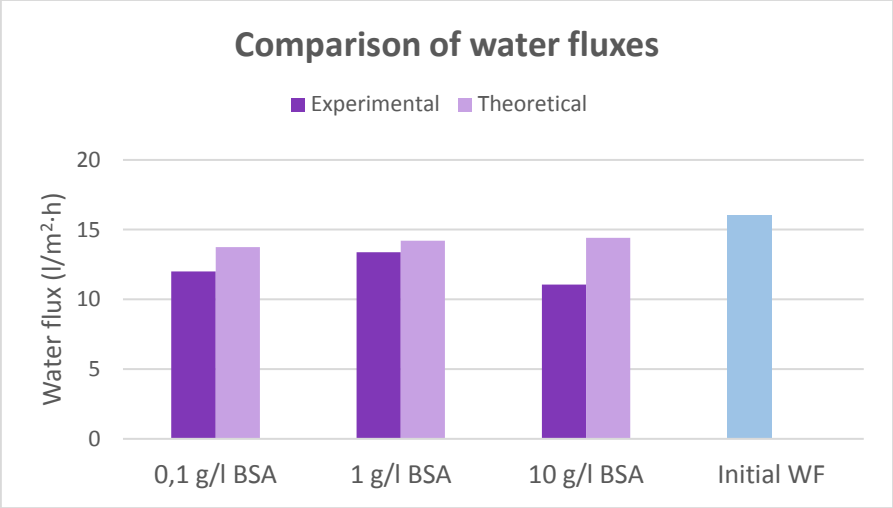


Figure 13. Comparison of experimental and predicted water flux depending on BSA concentration.

In this study, an experimental water flux ranging from 11 to 13,4 $l/m^2 \cdot h$ was calculated, while the predicted water flux is a little higher, from 14 to 14,7 $l/m^2 \cdot h$ (**Figure 13**). Predicted water fluxes are very similar because the same draw solution was used for all the experiments, 1 M NaCl, but the differences in conductivity and thus osmotic pressure affect the final value. That means, the preparation of the experiments was the same, but conductivity values vary from 1 to 1,1 M NaCl; detailed conductivity measurements can be seen in **Appendix II**.

In addition to different conductivity of the draw solutions, differences in BSA concentration entail different osmotic pressures of the feed solutions. But although 10 times increase in BSA concentration implies 10 times increase in osmotic pressure, its value is negligible compared to the osmotic pressure of the draw solution and thus predicted water flux does not vary greatly.

Comparing the experimental water fluxes, it can be seen that the water permeated through the membrane is very similar regardless of BSA concentration, which reaffirms the low impact of BSA on osmotic pressure. Besides osmotic pressure, similar water fluxes may be due to the low fouling tendency of BSA, as proposed by Wang *et al.* (2011).

Along with the similar water fluxes, reverse salt fluxes of the different BSA concentrations are close, but RSF at 1 g/l is higher than at 0,1 or 10 g/l. RSF calculation is based on the salt accumulation in the feed tank, coming from the salt leakage from the draw solution. In this

sense, any setback during conductivity measurement may lead to a higher or lower reverse salt flux.

4.2.3. Extra addition of salt

In addition to the previous BSA enrichment, two more experiments with initial concentrations 10 and 20 g/l were performed including an extra addition of salt. **Figure 14** shows protein concentration, water flux and salt accumulation over time for both experiments. The extra addition of salt is indicated with a red arrow. The experiment with 10 g/l was performed with 1 M NaCl as draw solution, and 20 g/l with 2 M NaCl. **Table 8** contains the calculated value.

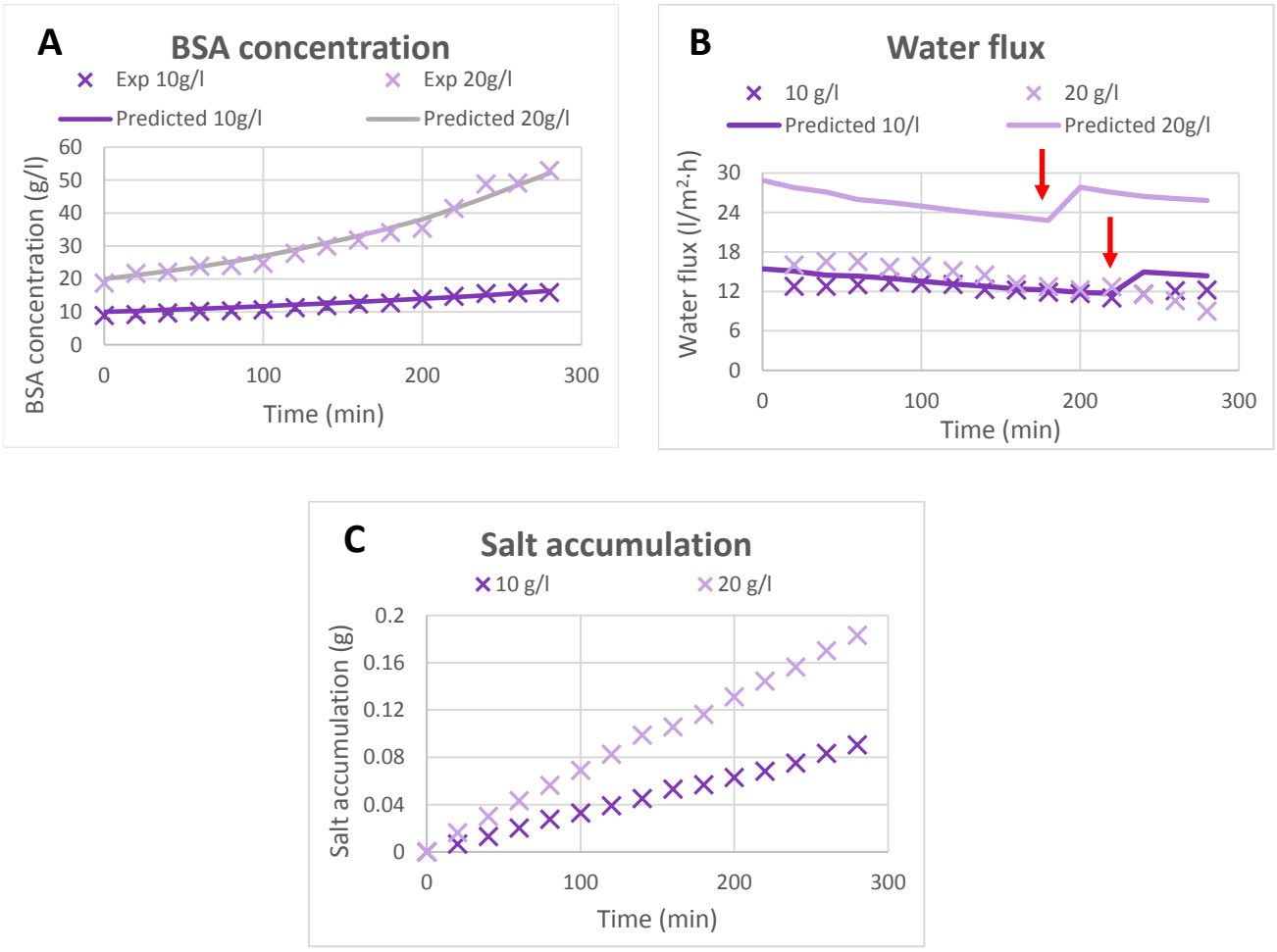


Figure 14. BSA concentration in feed tank (A), water flux (B) and salt accumulation (C) as a function of time with extra addition of salt (red arrow).

Table 8. Enrichment factor, water flux and reverse salt flux of 10 g/l and 20 g/l BSA solutions. Exp. stands for experimental and The. for theoretical.

	Enrichment factor		**Water flux (l/m²·h)**		**Reverse salt flux (g/m²·h)**
BSA concentration (g/l)	*Exp.*	*The.*	*Exp.*	*The.*	*Exp.*
10	1,78	1,64	12,31	13,67	3,57
20	2,83	2,61	13,65	25,86	10,71

To avoid a great decrease in water flux as seen before, extra NaCl was added while the experiments were running. As shown in **Figure 14B**, this addition did not imply a remarkable increase of water flux, but it remained almost the same even though osmotic pressure of the draw solution increased and therefore the predicted water flux. Water flux of the experiment with 20 g/l seems to increase slightly at minute 220, but afterwards it keeps decreasing as before salt addition. Water flux of 10 g/l however, has a light increase at minute 240 and remains quite stable the next measurements.

To maintain constant NaCl concentration in the draw solution, water should be removed at the same rate as it permeates from the feed solution, allowing then a constant water flux only dependant on protein fouling, concentration polarization or osmotic pressure of the feed solution. An integration of a FO system together with membrane distillation was studied by Wang *et al.* (2011) reporting promising results. When working with 0,5 M NaCl as osmotic agent at 57ºC for the distillation, it was successfully coupled reporting similar and constant water fluxes for both FO and MD processes after 50 minutes of operation. It is then a technique with great potential that can increase feasibility of protein concentration.

When comparing experimental water flux of 10 and 20 g/l, both are very similar remaining between 12-17 $l/m^2 \cdot h$; however, predicted water fluxes are quite different due to the different concentration of draw solution. The experiment with 20 g/l has a predicted water flux around 25 $l/m^2 \cdot h$, while the one for 10 g/l is around 15 $l/m^2 \cdot h$. The differences in osmotic pressure, estimated with the molar concentration calculated by conductivity, imply a higher water flux for a DS of 2 M NaCl, as expected.

Despite the high concentration of BSA, 20 g/l, it does not have a great impact on the osmotic pressure, but the water flux is higher due to NaCl molarity. Nevertheless, when calculated experimentally, water flux for 20 g/l BSA results quite lower than predicted.

The experiment with 10 g/l BSA has a close water flux to 0,1 or 1 g/l BSA, as explained before, but 20 g/l BSA does also have a similar water flux even though molarity of the draw solution is double. Assuming the BSA osmotic pressure is discarded as an influence on the water flux, concentration polarization or protein fouling can contribute to the low water flux of 20 g/l. Wang *et al.* (2011) already proposed that BSA has a low fouling tendency resulting in similar water fluxes for 0,1 and 1 g/l BSA, but 20 g/l may have a greater impact.

Figure 14A shows protein concentration over time, both experimental and predicted for 10 and 20 g/l BSA. In both cases, predicted and experimental concentration are almost the same, reaffirming that the A_{280} estimation is quite accurate. The experiment with 10 g/l reaches an experimental and predicted enrichment factor of 1,78 and 1,64, respectively, while the experiment with 20 g/l reaches 2,83 experimentally and predicts 2,61. Once again, the higher enrichment factor of the second experiment, 20 g/l, is due to a higher osmotic pressure of the draw solution, but also because of the higher BSA concentration.

Nevertheless, when protein enrichment of the experiment with 10 g/l BSA is compared to the first experiment of 10 g/l BSA without salt addition, the different predicted and calculated EF are very similar with values around 1,5 – 1,6. This similarity can be expected due to the close values of water flux, not quite affected by salt addition.

Regarding salt leakage, the slope of salt accumulation over time is higher when the draw solution is 2 M NaCl implying a higher reverse salt flux, as expected. It is two times higher, as NaCl concentration, so it can be concluded that reverse salt flux is directly ligated to NaCl molarity. Experiments of 10 g/l BSA with and without salt addition do have a similar salt accumulation as well, but reverse salt flux of the second one is a bit higher.

4.2.4. Precipitation with ammonium sulfate

At the beginning of the study, the main objective was the precipitation of BSA due to super saturation after dewatering with forward osmosis membranes. Nevertheless, protein precipitation was not successful by BSA concentration with this FO system, reaching concentrations of 50 g/l when starting with 20 g/l. No previous data regarding BSA solubility in water were found, but experiments of BSA concentration were not very long since the feed became very foamy and it was hard to work with it.

Different methods were tried then to precipitate BSA, as explained in 4.1.4. The beaker with BSA concentrated left overnight in the fridge did not precipitate, and neither did BSA with pH 4. Since these methods did not work, a solution of 20 g/l BSA with continuously added BSA up to 100 g/l was prepared, but no precipitation was found.

Then, BSA precipitation was achieved by salting-out with ammonium sulfate, gradually added to increase the percentage of saturation from 10 to 10%. In two different experiments with initial BSA concentrations of 10 and 40 g/l, a final percentage saturation of 60% and 70% was needed to obtain a white precipitate. Odunuga and Shazhko (2013) already precipitated BSA using ammonium sulfate, but they found a peak in BSA precipitation when percentage saturation ranged between 50% and 60%.

Nevertheless, in both cases BSA precipitation is dependent on percentage saturation of ammonium sulfate instead of BSA concentration. In the experiments with 10 and 40 g/l, the same salt saturation was needed to precipitate the proteins even though protein concentration was 4 times higher in one of the experiments.

As explained by Odunuga & Shazhko (2013) and Svensson (1941), BSA purification by salt precipitation is not common event though it is not expensive and easy. The use of ammonium sulfate to precipitate proteins is common due to its high solubility, low price and availability.

CHAPTER 5 – BROWN JUICE CONCENTRATION

In this chapter, the Porifera membrane was used to perform experiments regarding brown juice concentration. Different batches of brown juice were used with initial protein concentrations ranging from 4 to 9 g/l and draw solutions varying from 1 to 5 M NaCl. As done with BSA, water flux, enrichment factor and salt accumulation over time were calculated to study membrane performance and protein enrichment. Moreover, protein concentration and water flux were estimated using a mass balance of brown juice in the feed tank and a model prediction for water flux based on osmotic pressure.

Together with nitrogen measurement by Total Kjeldahl Nitrogen, a method to determine protein concentration by absorbance was developed. Low values of water flux, around 4.5 l/m²·h with 5 M NaCl as draw solution, were reported due to osmotic pressure of the feed solution, so brown juice was pre-treated by diafiltration to reduce the sugar and organic acids content. A mass balance of diafiltration was performed showing great reduction of sugar and organic acids content but also proving the presence of small peptides in the feed. However, pre-treatment did not lead to any significative increase in water flux giving room to future improvements.

5.1. Materials and methods

5.1.1. Experimental set up

Experiments were performed using a Porifera membrane, and several 500 ml NaCl solutions with different molarities, from 1 M to 5 M, were used as draw solution. Frozen brown juice was provided by the OrganoFinery Project, so it was unfrozen in the fridge for 2 days to maintain the cold chain. Brown Juice does not only contain proteins but also sugars, lactic acid, etc., so it was centrifugated (3 600 rpm, 10 min., 4ºC) to avoid blockage of the membrane cell.

After centrifugation, 350 ml of brown juice were used as feed solution for experiments regarding water flux, reverse salt flux and enrichment factor calculation. To compare water flux of the different brown juice fractions after the pre-treatment (see 5.1.3), an initial volume of 150 ml was used as feed. Experiments were performed at room temperature during 3 hours, and DS weight, conductivity and a brown juice sample were taken every 30 minutes. As said in 3.1.1., a flow rate between approximately 504 and 508 ml/min was used for tubing size 24 and 25 respectively to perform the experiments

5.1.2. Chemical characterization

In every experiment, the initial and final feed solution was analysed in terms of pH, conductivity and Total Kjeldahl Nitrogen (TKN). pH and conductivity were measured immersing the glass electrode from the pH-meter and the electrode from the conductivity meter, respectively, in the beaker containing the liquid. Protein concentration was estimated using TKN results as well as A_{280}-A_{310}, as explained in 5.1.4.

After the pre-treatment, the three different fractions were analysed in terms of pH, conductivity and TKN. In addition, free sugars, ethanol, lactic, citric, acetic and succinic acids were determined by High Performance Liquid Chromatography (HPLC). Samples for HPLC

were prepared without dilution and centrifugated at 10 000 rpm for 10 min. The supernatant was then filtered using a 45 μm filter and transferred into a HPLC vial. The samples were analysed on a Dionex Ultimate 3000-LC system with an Aminex HPX-87H column coupled to a refractive index detector. H_2SO_4 (4 mM) was used as mobile phase with an elution rate of 0,6 ml/min, kept at 60ºC. HPLC results are listed in **Appendix III**.

5.1.3. Brown juice pre-treatment

The first experiments with brown juice showed a very low water flux compared to BSA or water, so it was decided to pre-treat it after centrifugation (3 600 rpm, 10 min., 4ºC) to reduce the salt and sugar content. A measured volume of brown juice was diafiltrated using a flat-sheet ultrafiltration membrane made of polyethersulphone with a molecular weight cut-off 5 kDa (Alfa Laval Ultrafiltration Membranes – PP series). Two membranes were placed in the support disk in the Alfa Laval LabStack M20 mounted in the Alfa Laval TestUnit M20 (Alfa Laval, Glostrup), with an effective membrane area of 0,036 m^2.

A beaker was placed on a scale to weight the permeate over time, and the retentate was recirculated to the feed. The experiment was conducted until half of the total volume, the measured brown juice plus the retained water inside the unit, permeated through the membrane to the permeate beaker. Three brown juice fractions were differentiated after the diafiltration: initial feed, permeate and retentate.

These fractions were chemically analysed as detailed in 5.1.2. and used to measure water flux as explained in 5.1.5. In addition, separation percentages of the different compounds were calculated.

5.1.4. Protein quantification.

Protein concentration in Brown Juice was estimated using two different methods, UV absorbance and Total Kjeldahl Nitrogen. UV absorbance was measured at two different wavelengths (280 nm and 310 nm); A_{310} was subtracted from A_{280} as an approximate correction for turbid solutions (Walker 2002). Even though there are not only proteins in the samples of brown juice, it was assumed that only proteins absorb light at 280 nm. Protein concentration was then calculated following Beer's Law, assuming that the absorbance is proportional to concentration:

$$A_\lambda = \varepsilon \cdot c \cdot L \tag{5.1}$$

where A_λ is the absorbance at a given wavelength (without units), ε is the molar extinction coefficient (units of $L \cdot mol^{-1} \cdot cm^{-1}$), c is the concentration of the compound (expressed in $mol \cdot L^{-1}$) and L the path length of the cuvette, in cm.

However, the extinction coefficient used as reference was the percent solution extinction coefficient ($\varepsilon_{percent}$) of Rubisco, with an estimated value of 17 (Hudson 1994). $\varepsilon_{percent}$ has units of $(g/100mL)^{-1} \cdot cm^{-1}$ instead of $L \cdot mol^{-1} \cdot cm^{-1}$, and is used when the absorbance (A_{280nm}) values are for 1% (=1g/100mL) solution measured in a 1cm cuvette. Consequently, the units for concentration in the general formula are percent solution (1g/100mL).

$$A/\varepsilon_{percent} = percent\ concentration \tag{5.2}$$

To report concentration in mg/ml or g/l, and adjustment factor of 10 has to be made (ThermoScientific 2002):

$$(A/\varepsilon_{percent}) \cdot 10 = concentration\ (\frac{mg}{ml}) \tag{5.3}$$

Different dilutions of brown juice and deionized water were prepared from two brown juice samples, one directly unfrozen and the other one unfrozen and centrifuged. Absorbance values of these dilutions were measured to find the optimal dilution factor for the experiments.

Total Kjeldahl Nitrogen (TKN) was measured following the procedure from APHA Standard Methods (2005). Samples were diluted before TKN determination and an appropriate series of alanine standards were used. Nitrogen concentration was then calculated taking the dilution factor into account, and crude protein content was estimated using a conversion factor of 6,25, assuming that the nitrogen content of proteins is 16% (Mariotti, Tomé, and Mirand 2008).

As done in experiments for BSA concentration, the enrichment factor was calculated to evaluate membrane performance in protein concentration. For brown juice, three different EF were calculated: theoretical based on prediction of protein concentration, explained in 5.1.6, experimental based on absorbance concentration and experimental based on TKN results. It was calculated dividing the protein concentration at the end of the experiment by the initial feed protein concentration, as detailed in 4.1.2.

5.1.5. Water flux and reverse salt flux calculation

As done for the BSA, an overall water flux was calculated plotting the feed mass reduction versus time as explained in 3.1.3. But in the experiments with brown juice water flux (l/m^2·h) was also calculated when a sample was taken every 30 minutes. For this calculation, the permeated water in 30 minutes was assumed to be the same as the weight increase in the DS and divided by the membrane area and time, changing the corresponding units when necessary.

Reverse salt flux was also calculated as detailed in 3.1.3, but conductivity due to the brown juice was not considered and the initial value of every experiment was subtracted from the rest of measurements every 30 minutes.

5.1.6. Prediction of protein concentration and water flux

For brown juice experiments, protein concentration over time was estimated using the initial protein concentration calculated with TKN:

$$C_{BJ,t} = \frac{C_{BJ,0}V_{f,0}}{V_{ft}} \tag{5.4}$$

Where $C_{BJ,0}$ and $C_{BJ,t}$ are the initial brown juice concentration and concentration in feed tank at a given time, and $V_{f,0}$ and V_{ft} are the initial feed solution volume and feed solution volume at time t. V_{ft} was calculated as the initial feed volume ($V_{f,0}$) subtracting the accumulated permeated water (ΔV) in equation 4.3.

Water flux over time was estimated as described in 4.1.6 for BSA experiments and osmotic pressure in 3.1.4. Osmotic pressure of the feed solution was calculated as the sum of osmotic pressures from the different compounds: salt accumulation, protein content, organic acids and sugars. A molecular weight of 70 kDa was estimated for the proteins, and a Van't Hoff factor

of 1 for all components except for NaCl, with i = 2. As explained for BSA concentration in 4.1.6, an average of predicted water fluxes was calculated as overall.

5.2. Results and discussion

5.2.1. Protein quantification

Before the study of brown juice concentration using forward osmosis membranes, a simple method to follow protein enrichment over time was developed, as explained in 5.1.4. Protein absorbance was first measured at 280nm and then concentration was estimated. Then, protein concentration was calculated with the total crude protein content and applying the dilution factors used to measure the absorbance.

These calculations were performed in duplicates with two different samples, brown juice and centrifuged brown juice. Protein value with TKN was assumed to be the real one, while protein concentration calculated through absorbance was considered a rough or estimated concentration. **Figure 15** shows both protein estimations.

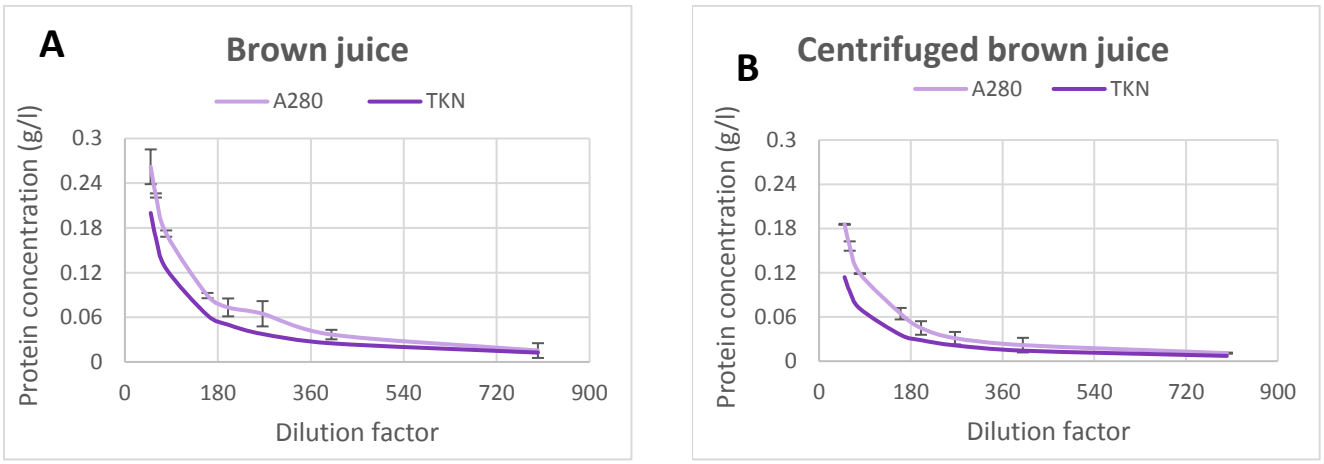


Figure 15. Protein estimation using A_{280} and TKN for brown juice (A) and centrifuged brown juice (B).

As seen in graphs A and B, protein estimation using A_{280} and TKN are not the same but follow the same trend. When using A_{280}, protein concentration is always higher than TKN when the dilution factor is lower than 400, but as the dilution factor increases, this difference is reduced. However, the higher the dilution factor, the higher the error.

This experiment was used to prove a reliable and easy method to follow protein concentration in brown juice during membrane experiments. Although absorbances do not provide an accurate value of protein concentration, it is enough to estimate its increase.

5.2.2. Concentration with different NaCl molarities

Three different NaCl molarities ranging from 1 to 5 M were used to concentrate brown juice and calculate water flux, protein concentration and reverse salt flux. Water flux depending on NaCl concentration can be seen in **Figure 16**. Model predicted water flux could not be estimated accurately because of all the different components in brown juice, thus only experimental results are presented.

As shown in **Figure 16B**, using 1 M NaCl as draw solution a water flux around 1,5 l/m²·h is achieved, a low value taking approximately 12 l/m²·h obtained with BSA as reference. To study the increase of water flux depending on NaCl molarity, 2 M and 5 M NaCl draw solutions were also employed. It was found that water flux increases as NaCl molarity is higher, as expected. Nevertheless, this increase is not very remarkable.

A 1 M NaCl DS leads to a water flux of around 1,5 l/m²·h, while 2 M NaCl reaches around 3 l/m²·h and 5 M NaCl close to 4,5 l/m²·h, which means water flux increase is not proportional to NaCl molarity. A NaCl molarity increase of 2 does mean a double of water flux, but when DS molarity is 5 times higher, water flux increases three times. In all cases, however, water flux decreases slightly over time remaining quite stable (**Figure 16A**).

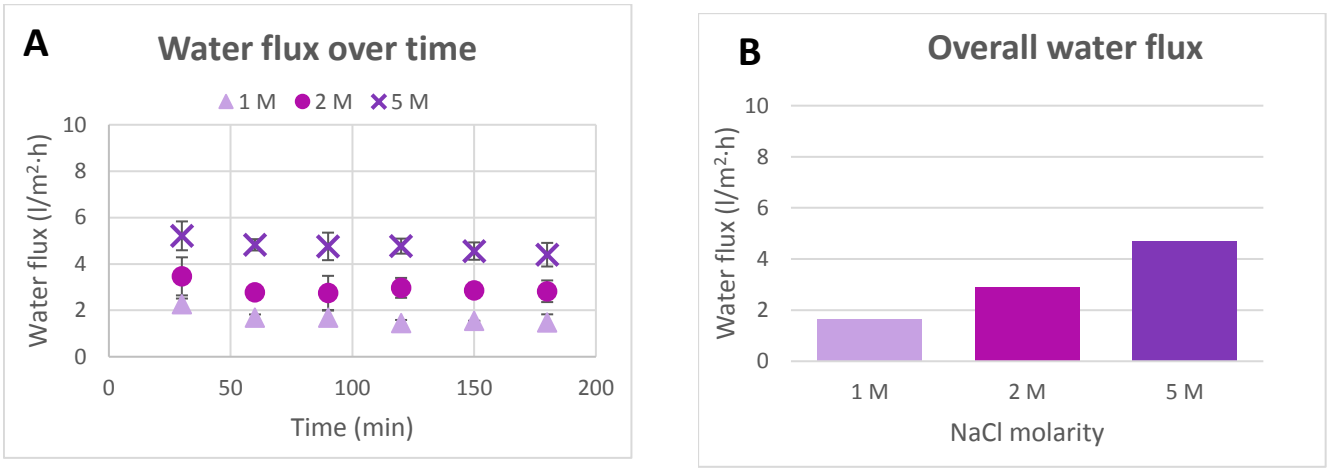


Figure 16. Water flux over time (A) and general (B) of brown juice depending on NaCl molarity.

Two different reasons were proposed to the low water flux, membrane fouling and osmotic pressure. Fouling of the membrane could be possibly caused by lactic acid bacteria present in the feed solution, but this idea was discarded because all the experiments were performed during 3 hours and this amount of time was not considered enough for a biofilm creation. Osmotic pressure should then be triggering a low water flux.

As already found when working with 1 and 2 M NaCl for BSA concentration, even though a higher NaCl molarity increases water flux, this increase is not as significative as expected. Water flux is based in two different parameters: A, the water permeability coefficient intrinsic to the membrane, and the difference in osmotic pressure of the feed and draw solution. Water permeability was not considered because the same membrane, Porifera, was used for BSA and brown juice experiments.

In the same way, in both BSA and brown juice concentration a draw solution consisting in deionized water and NaCl was used, but water flux resulted quite higher when working with BSA. It can then be inferred from these data that brown juice is causing a low water flux.

As already said in 5.1.2, brown juice does not only contain proteins but also free sugars, lactic acid, etc. These components influence the osmotic pressure of the feed solution, therefore the difference in osmotic pressure of feed and draw solutions is lower and this may lead to a reduction in water flux.

The increase in osmotic pressure of the feed may not be just caused by the sugar content, but also by lactic acid and small peptides. The purpose of this diafiltration was to reduce the sugar

and organic acid content to test if they were causing a decrease in water flux, using a membrane with a molecular weight cut-off of 5 kDa, as detailed in 5.1.3.

Protein concentration and salt accumulation over time was also studied in brown juice using different NaCl molarities, shown in **Figure 17**. In addition, protein concentration was predicted using water permeated through the membrane over time, as explained in 5.1.6. For these experiments, the same batch of brown juice was used.

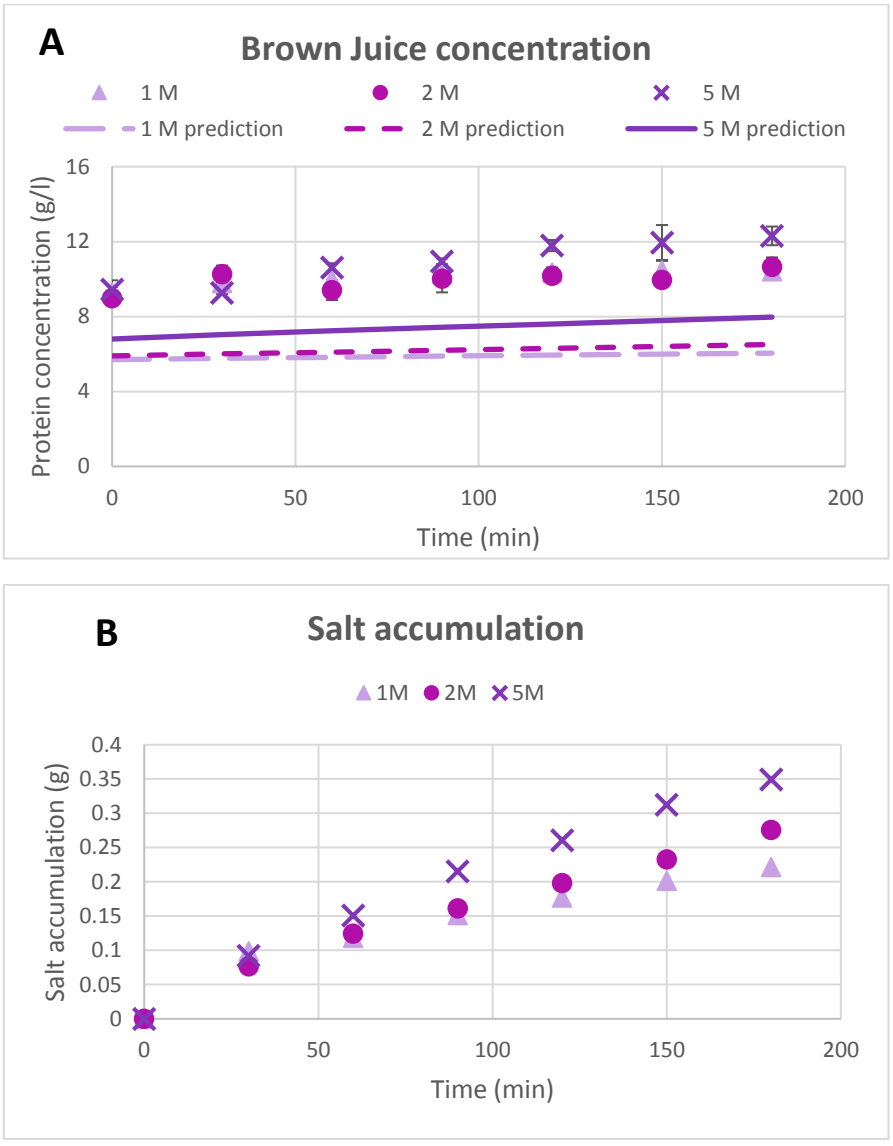


Figure 17. Protein concentration (A) and salt accumulation (B) of brown juice depending on NaCl molarity.

Regardless of NaCl molarity, protein concentration calculated experimentally is always higher than the predicted. This was expected because protein concentration calculated using absorbance was already shown to be higher than concentration calculated using TKN, and predicted protein concentration was estimated with the initial TKN results. However, both protein concentrations, experimental and predicted, remain quite stable over time increasing

slightly. More detailed data of protein concentration can be seen in **Table 9**, where enrichment factor is presented.

Table 9. Enrichment factor, water flux and reverse salt flux of brown juice concentration using different NaCl molarities. Standard deviation is shown in brackets.

	Enrichment factor			Water flux (l/m²·h)	Reverse salt flux (g/m²·h)
M NaCl	*Exp. Abs*	*Predicted*	*The.*	*Exp.*	*Exp.*
1	1,12 (0,00)	1,06 (0,01)	1,14 (0,02)	1,66 (0,18)	19,53 (2,52)
2	1,19 (0,04)	1,1 (0,02)	1,12 (0,00)	2,91 (0,48)	25,88 (1,26)
5	1,30 (0,02)	1,17 (0,01)	1,21 (0,04)	4,71 (0,07)	33,91 (0,00)

Enrichment factor of proteins in brown juice was calculated using three different methods, as explained in 5.1.4. When compared, however, the three EF are quite similar. The enrichment factor calculated using TKN results is taken as the real one, while the other two (Abs. and predicted) are considered as rough estimations. Except for the real EF, in all cases it increases simultaneously with water flux as NaCl molarity is higher.

As the predicted EF was calculated using water permeated through the membrane, its increase is ligated to the raise of water flux. The real EF used TKN results, so any mistake conducting the experiments may have caused a lower EF for 2 M NaCl, although experiments were performed in duplicates.

Salt accumulation over time can be seen in **Figure 17B**; these data were used to calculate reverse salt flux, presented in **Table 9**. Nevertheless, these values may be considered too high taking into account Porifera results in experiments for membrane performance, with a RSF of 7,14 g/m²·h when working with 1 M NaCl, and BSA concentration, with varying RSF from 5,35 to 8,92 g/m²·h.

RSF in these experiments was calculated not considering conductivity of the brown juice as explained in 5.1.5, but subtracting the initial conductivity of the feed to the following measurements. In this way, a possible increase in conductivity as brown juice is concentrated over time is discarded and calculated RSF can result higher than it actually is.

5.2.3. Performance recovery after cleaning of the membrane

After the experiments of brown juice concentration were performed, the membranes turned brown and had a stale appearance. To evaluate how brown juice concentration had influenced membrane performance, water flux was measured after BJ (before cleaning) and after cleaning with citric acid. Results are presented in **Figure 18**.

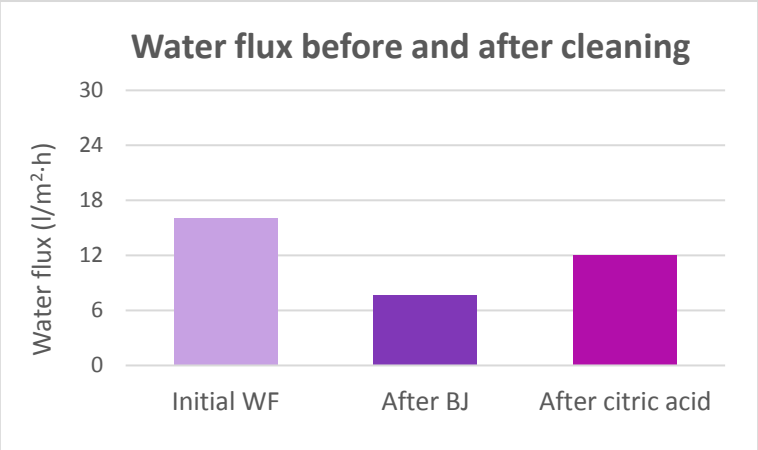


Figure 18. Initial water flux, after BJ concentration and membrane cleaning.

The maximum water flux achieved using Porifera and a draw solution of 1 M NaCl was 16,06 l/m²·h, as explained in 3.2.3. This value was used as the initial reference to compare water flux after brown juice concentration and membrane cleaning.

As seen in **Figure 18**, water flux after brown juice experiments is almost half of the reference value, meaning a decrease in membrane performance. Membrane fouling due to bacteria accumulation was already discarded because as the experiments did not last more than 3 hours and this amount of time was not considered enough. However, fouling due to small peptides or organic acids was possible.

2wt% citric acid was chosen as cleaning agent due to its easy preparation as recommended by HOLLOSEP engineering manual (Toyobo., n.d.), although it may not be the best option to remove organic fouling. Nevertheless, water flux after membrane cleaning was found to have increased from approximately 8 to 12 l/m²·h, around 50 %. After cleaning, membranes had then around 75 % of the initial performance, based on 16,06 l/m2/h. These membranes were used for the first concentration of the different fractions after the diafiltration.

5.2.4. Pre-treatment: mass balance

Low water fluxes in experiments of brown juice concentration were attributed to the different components present in the feed such as free sugars and lactic acid. To overcome this, brown juice was pre-treated by diafiltration using an ultrafiltration membrane with molecular weight cut-off 5 kDa. As detailed in 5.1.3, the experiment was conducted until half of the feed volume permeated through the membrane, during almost 3 h.

After diafiltration, samples were analysed by HPLC to measure several components such as sugars, lactic and acetic acid, etc. Total Kjeldahl Nitrogen was also measured to estimate the crude protein content. **Table 10** presents results regarding proteins, sugars, lactic and acetic acid in the three fractions together with their separation percentage. Free sugars are presented as the sum of the different sugars measured by HPLC, and proteins were calculated using TKN and a conversion factor of 6,25, explained in 5.1.4. Results are shown in total grams of each fraction. Detailed data can be consulted in the **Appendix III**.

Table 10. Chemical composition of feed, retentate and permeate together with separation percentage.

Volume (l)	2,8	4,61	2,305	% separation	
	Feed	*Retentate*	*Permeate*	*Retentate*	*Permeate*
Proteins	24,64	18,44	10,95	62,75	37,25
Free sugars	21,11	13,46	11,06	54,89	45,11
Lactic acid	55,78	32,82	30,66	51,71	48,29
Acetic acid	10,42	5,90	5,81	50,39	49,61

As said before, the increase in osmotic pressure may be due to sugars, organic acids and small peptides, which can pass through the pores of the ultrafiltration membrane. Proteins and polysaccharides, on the contrary, do not cross the membrane. The separation percentage of these main fractions are presented in **Table 10**.

In the case of sugars, lactic and acetic acid, almost half of the initial content permeated through the membrane, resulting in a similar final amount of the component in each fraction. The protein content, however, is higher in the retentate than in the permeate. Diafiltration started with an initial feed volume of 2,8 l, but 1,81 l of water were inside the equipment so the feed was diluted. In total, 2,305 l of water were added, the same volume as the permeate. Since the permeate contained half of the total initial volume, it was expected to contain half of the components that could pass the membrane as well. Sugar, lactic and acetic acid content prove this assumption: there was almost a 55%, 52% and 50% rejection, respectively.

The nitrogen content of brown juice calculated by TKN was considered to be contained completely in proteins instead of small peptides. Proteins in the brown juice have an estimated size of around 70 kDa, but after centrifugation some may have degraded to small peptides. If the TKN value of the permeate is used to calculate the amount of proteins in the fraction, it means that around 37% of the initial content of the feed has permeated the membrane, with 67% rejection. However, the molecular weight cut-off was 5 kDa to avoid protein permeation; this separation percentage confirms the presence of small peptides in the feed.

5.2.5. Water flux of the different DF fractions

After pre-treatment of the brown juice, the three different fractions, feed, retentate and permeate, were used to measure water flux using two sets of membranes: feed and retentate were measured with the already used for the first experiments of brown juice, and a new piece of membrane for each fraction to exclude membrane fouling as a reason for low water flux.

- **Concentration with the same membranes**

The first experiments for water flux determination of feed and retentate from the diafiltration were performed using the same membranes employed for brown juice concentration with previous citric acid cleaning. In order to measure if the membrane was fouled after each experiment, water flux using deionized water as feed and 1 M NaCl as draw solution was measured. Water flux for the different fractions and water flux as fouling control can be seen in **Figure 19** A and B, respectively. Experiments of brown juice concentration were performed in duplicates with 5 M NaCl as draw solution.

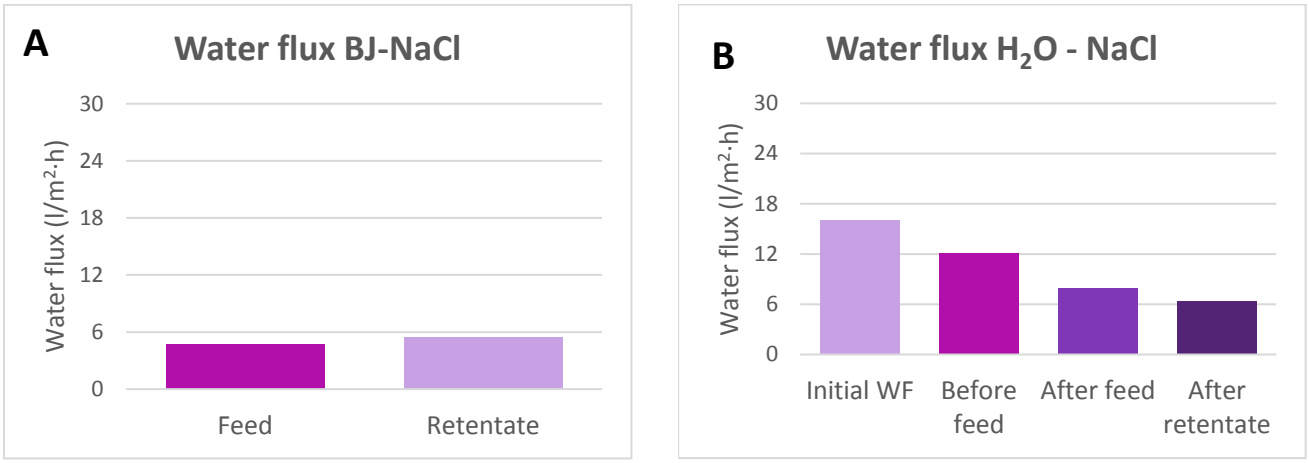


Figure 19. Water flux of feed and retentate (A) and as a control of membrane fouling (B).

Brown juice used for these experiments was obtained from a second batch; protein concentration was a bit higher than the first batch, but composition was estimated to be similar.

The feed of diafiltration was the first fraction to be concentrated; initial membrane performance with deionized water and 1 M NaCl is shown in **Figure 19B**, calculated after cleaning with citric acid, and water flux from feed concentration in **Figure 19A**. The membrane performance before feed concentration was estimated around 65% of the initial water flux (16 l/m²·h), while for retentate it was around 50%. However, calculated water flux for both fractions was very similar, around 5 l/m²·h as shown in **Figure 19A.**

Water flux obtained with retentate as feed solution is slightly higher than the feed of diafiltration used as feed solution, but this difference is not very significative. Similarity of both feed solutions can be due to membrane fouling or stale, so experiments were repeated using a new piece of membrane for each fraction.

- **Concentration with new membranes**

As said above, a new piece of membrane was used for each fraction in order to discard membrane fouling as the cause for low water flux. In these experiments feed, retentate and permeate of the diafiltration were used as feed solutions. Experiments were performed in the same conditions as the previous experiments with the first membranes, with 5 M NaCl as draw solution. Calculated water fluxes for each fraction are presented in **Figure 20** together with water fluxes from the previous experiments with the first membrane, while **Figure 21** compares them with predicted water fluxes.

When comparing the water flux obtained using the feed of the diafiltration, it can be seen that both experiments, first and new membrane, resulted in similar values (water flux close to 5 l/m²·h), although the new membrane reported a slightly higher water flux.

A similar situation applies to water flux using the retentate as feed solution, although in this case water flux with the first membrane is a little higher than the calculated with a new membrane. For both fractions, however, the use of a new piece of membrane does not impact significantly the water flux. Taking this into consideration, membrane fouling could not be a major cause to low water flux, since the new pieces of membranes where just hydrated with deionized water.

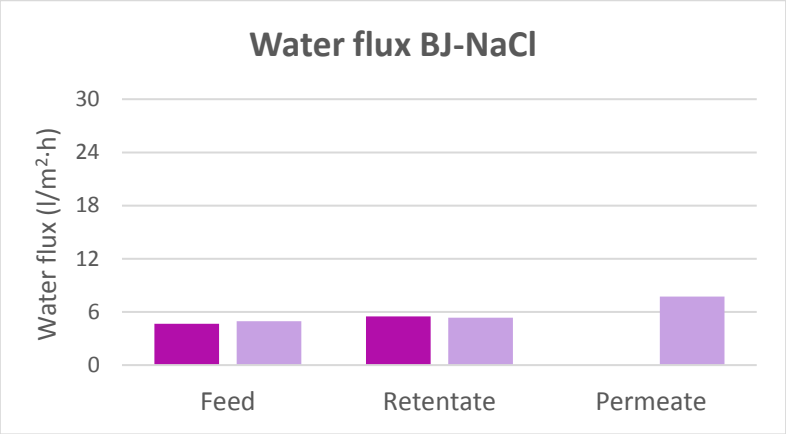


Figure 20. Water flux of feed, retentate and permeate using new pieces of membrane compared to previous experiments.

Figure 20 also shows water flux obtained using the permeate as feed solution; in this case, water flux was only measured with a new piece of membrane. Once membrane fouling has been discarded, it is necessary to focus on brown juice composition, which affects the osmotic pressure.

As said before, brown juice contains not only proteins but also sugars and organic acids, measured by HPLC. BJ was diafiltrated with the aim to reducing sugar content, since sugars were considered to increase the osmotic pressure due to their high concentration. Nevertheless, HPLC results reported values not so remarkable, around 3,6 g/l of glucose and 2,1 g/l of xylose in the feed; detailed data can be consulted in **Appendix III**.

Even though sugar content was reduced around 45% in the diafiltration, having the retentate as the product of interest, water flux using 5 M NaCl as draw solution was still low. However, small peptides and organic acids can also contribute greatly to an increase in osmotic pressure.

Different organic acids are present in the brown juice such as lactic, acetate or citric acid, although lactic and acetic acid are the major components. pH of the samples was around 3.6-3.7, so some proportion of the organic acids was dissociated into the ions. The presence of ions such as lactate or acetate has an impact in the osmotic pressure, since it increases as a compound is dissociated into ions.

In addition to organic acids, small peptides might have an impact in osmotic pressure as well, although results of estimated osmotic pressure (**Appendix III**) do not concur. Analysis of the different amino acids of the brown juice were not performed, but the presence of ionisable amino acids such as aspartate or glutamate may lead to an increase in osmotic pressure.

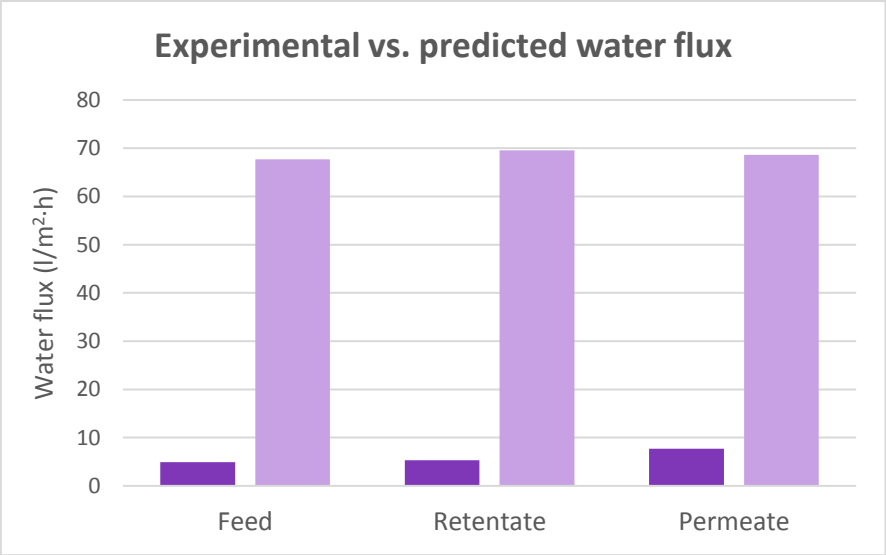


Figure 21. Water flux of feed, retentate and permeate using new pieces of membrane compared to predicted water fluxes.

A rough estimation of water flux for each fraction is presented in **Figure 21** being very similar for all fractions. This estimation was calculated taking into account the osmotic pressure of the different components in the feed solution, as discussed before, but results do not indicate a great impact of those components though.

All experiments were performed with 5 M NaCl as DS having therefore a very close osmotic pressure and predicted water flux. Differences in these predictions are due to the concentration of the different components in the fractions. But even though brown juice pre-treatment reduced the content of both sugars and organic acids and osmotic pressure reduced compared to the feed, it is not translated into a higher water flux.

CHAPTER 6 – Influence of feed on water flux

In this chapter, water flux obtained with 1 M NaCl as draw and the three different feed solutions from chapters 3 to 5 are compared: deionized water, BSA and brown juice. In addition, brown juice was used as draw solution to study whether its osmotic pressure can establish a comparable water flux.

When compared, it was found that water flux is highly dependent on the components of the feed, which influence the osmotic pressure and therefore water permeated through the membrane.

A summary of the experimental water fluxes obtained using DW, 10 g/l BSA and BJ as feed solutions and 1 M NaCl as DS is presented in **Figure 22**. In addition, water flux using brown juice as draw solution and deionized water as feed is also showed. Water flux was calculated as explained in 3.1.3 for DW, 4.1.5 for BSA and 5.1.5 for brown juice; feed mass reduction was also plotted against time when BJ was used as draw solution. Both experiments with 10 g/l BSA and BJ as feed solution were conducted during 3 h and experiments with DW as FS and BJ as DS during 30 minutes.

Figure 22 shows water flux is highly influenced by the composition of feed solution, reaching its maximum using deionized water, 16 l/m²·h. When a solution of 10 g/l BSA initial concentration is used as feed, water flux is still relatively high with an approximate value of 11 l/m²·h, using DW as the reference. On the contrary, BJ used as feed solution does not achieve even 2 l/m²·h, but when it performs as DS water flux is approximately 3.5 l/m²·h, almost double.

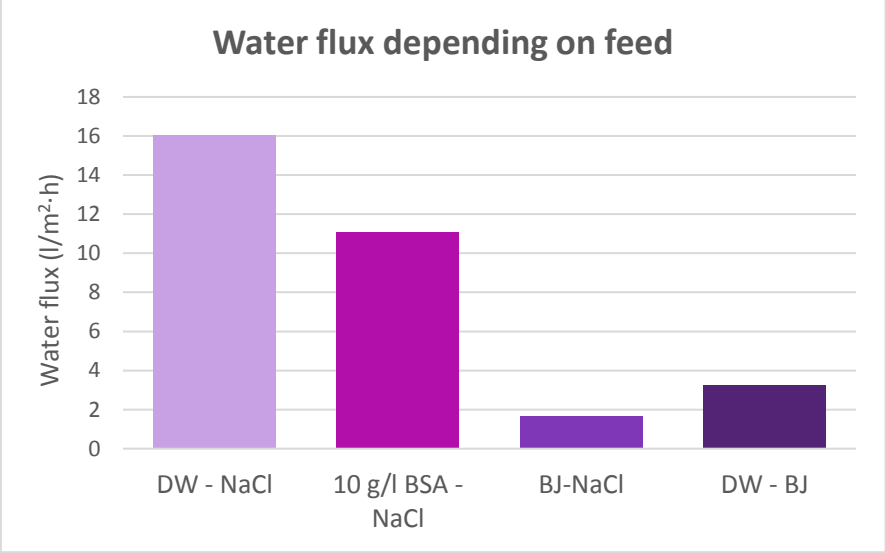


Figure 22. Comparison of water fluxes using DW, BSA and BJ as FS with 1 M NaCl and BJ as DS with DW.

As said before, in all cases 1 M NaCl was employed as draw solution except when BJ was used as such, so differences in water flux are likely caused by differences in osmotic pressures of the feed solutions. Fouling was discarded when working with BSA, as close water fluxes were obtained with different BSA initial concentrations in 4.2.2, from 0,1 to 10 g/l. In 5.2.5, experiments with brown juice using a new piece of membrane were compared with an old

membrane, showing water fluxes very similar and discarding as well fouling as the main cause for low water flux.

A rough estimation of osmotic pressure was calculated as explained in 3.1.4. In the first case, DW as feed, osmotic pressure is influenced by salt accumulation in the feed over time, but it is a negligible amount. In the second case, BSA as feed, osmotic pressure of the feed is composed by both salt accumulation over time and dissolved BSA. Salt accumulation is again very low compared to salinity in the draw solution, and BSA does not influence highly the osmotic pressure either.

In the third case, brown juice was used as feed solution. Osmotic pressure in this case may be highly influenced by the different components such as sugars and organic acids, although it was not calculated due to lack of data. However, chemical analysis to measure sugars and organic acids were performed when brown juice was pre-treated (5.2.4), so similar results could be used to discuss about osmotic pressure even though there were different batches of BJ.

Brown juice was found to contain different organic acids such as lactic or acetic acid, besides free sugars and small peptides that permeated the membrane during diafiltration. When using BSA, on the contrary, the feed solution can be estimated to contain just BSA protein in its natural conformation, not degraded. As osmotic pressure is dependent on dissociation of solute particles, a higher dissociation of components in brown juice than in BSA is estimated, reducing the difference in osmotic pressure and thus water flux.

Similarly, when brown juice is used as draw solution, its different components create a difference in osmotic pressure between feed and draw solution resulting in a relatively low water flux compared to DW or BSA, but higher than when using BJ as feed.

CHAPTER 7 – CONCLUSIONS AND FUTURE PERSPECTIVES

In this study, a second separation step to increase the protein recovery of the OrganoFinery project using osmotic membranes was evaluated. Brown juice is a highly hydrated waste stream from this green biorefinery used for anaerobic digestion, but it still contains a high amount of proteins as well as free sugars and organic acids. The proposed strategy was protein concentration through forward osmosis and precipitation due to supersaturation.

In the first place, two forward osmosis membranes, namely Porifera and Aquaporin, were evaluated in terms of water flux and reverse salt flux using different osmotic agent concentration and flow rate of feed and draw solution. This evaluation showed higher performance of Porifera, with a maximum water flux of 16 $l/m^2 \cdot h$ and reverse salt flux of 7,14 $g/m^2 \cdot h$ when working with deionized water as feed and 1 M NaCl as osmotic agent. The Porifera membrane was then chosen to perform further experiments involving protein concentration.

Before proceeding to brown juice concentration, bovine serum albumin was used as standard protein in the feed solution to study membrane performance. Three different initial BSA concentrations were employed reporting similar water fluxes and enrichment factors in 3 h, with average values circa 12 $l/m^2 \cdot h$ and 1.7, respectively. Complementary experiments with salt addition were performed to study water flux over time as draw solution dilutes, but no relevant experimental results were obtained. Water flux and enrichment factor were estimated in both cases using a prediction model, showing results quite close to the experimental ones except for water flux in experiments with salt addition.

BSA was concentrated up to 50 g/l through forward osmosis, but no precipitation was found due to supersaturation. As alternative, protein precipitation by salt addition was studied using ammonium sulfate, and a percentage saturation between 60 and 70% was needed to obtain a precipitate after centrifugation. However, further studies are needed to study the feasibility of BSA concentration through forward osmosis coupled to its precipitation by supersaturation or salt precipitation.

Once membrane performance was studied using a standard protein, brown juice was used as feed and its concentration evaluated. 1 M NaCl reported a very low water flux compared to BSA and deionized water, so NaCl molarities up to 5 M were used as draw solution. However, water flux was still low, then osmotic pressure of the feed solution was proposed as the main reason. To study that hypothesis, brown juice was pre-treated by diafiltration and its sugar and organic acid content was reduced almost 50%. In addition, the protein content was not completely retained and circa 37% permeated the membrane, meaning 63% rejection.

New pieces of membranes were used to concentrate each fraction and discard membrane fouling, but water fluxes remained quite low between 5-6 $l/m^2 \cdot h$ except for the permeate, with almost 8 $l/m^2 \cdot h$. Since both the retentate and permeate had a similar organic acids and sugar content, differences in water flux were mainly attributed to the protein content, assumed to consist in both proteins and small peptides. Water flux was also estimated using the chemical composition of brown juice, but values were quite higher than the experimental ones.

Moreover, water flux of the different feed solutions was compared when using 1 M NaCl as draw solution, showing high influence of the feed composition. Nevertheless, no protein precipitation was obtained using either BSA or brown juice. To achieve this, water flux should be improved in the first place to enhance feed dewatering. In addition, recovery of additional by-products such as lactic acid should be studied to increase feasibility of the OrganoFinery.

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APPENDIX

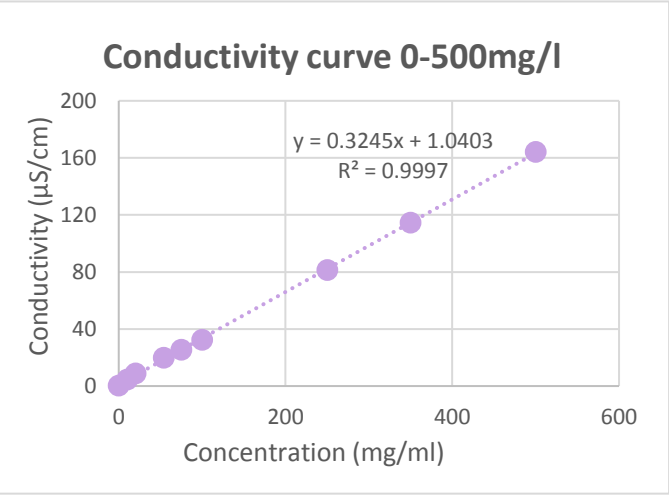
I – EVALUATION OF MEMBRANE PERFORMANCE

- **Reynolds number, flow velocity, flux and flow rate**

Tubing size	*Speed (rpm)*	*Flow rate (ml/min)*	*Volumetric flow rate (m^3/s)*	*Flow velocity (m/s)*	*Reynolds number*
24	50	118,85	1,98E-06	0,015	92,72
	75	178,13	2,97E-06	0,022	138,97
	100	239	3,99E-06	0,030	186,77
	150	370	6,16E-06	0,046	288,26
	200	504	8,40E-06	0.063	393,19
	250	646	1,08E-05	0,081	503,98
	300	790	1,32E-05	0,099	616,32
	350	963	1,61E-05	0,120	751,28
	400	1128	1,88E-05	0,141	880,01
25	200	376,7	6,28E-06	0,047	293,86
	250	496	8,27E-06	0,062	386,95
	259	508	8,47E-06	0.063	396,32
	300	592	9,87E-06	0,074	461,85

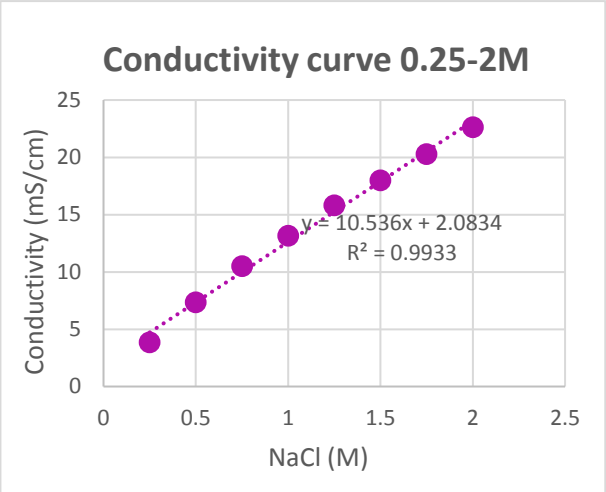
- **Conductivity curves**

Curve 1: 0 – 500 mg/l NaCl



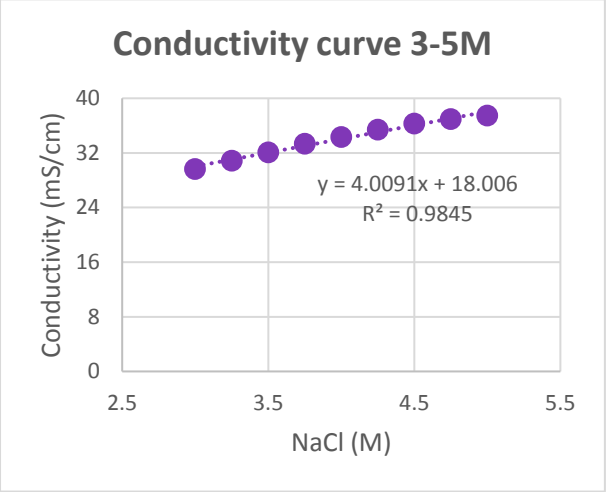
mg/l	*Conductivity (µS/cm)*			*Average*
0	0,17	0,17	0,18	0,17
10	4,37	4,36	4,35	4,36
20	8,78	8,77	8,77	8,77
54	19,82	19,80	19,79	19,80
75	25,37	25,32	25,31	25,33
100	32,38	32,34	32,34	32,35
250	81,24	81,20	81,20	81,21
350	115,10	114,20	113,80	114,37
500	164,40	163,90	163,70	164

Curve 2: 0.25 – 2 M NaCl



NaCl (M)	*g/l*	*Conductivity (mS/cm)*			*Average*
0,25	14,60	3,84	3,84	3,85	3,84
0,5	29,29	7,34	7,34	7,34	7,34
0,75	43,85	10,49	10,48	10,49	10,49
1	58,43	13,16	13,14	13,14	13,15
1,25	73,04	15,80	15,81	15,81	15,81
1,5	87,64	17,95	17,97	18	17,97
1,75	102,25	20,27	20,28	20,29	20,28
2	116,85	22,61	22,62	22,62	22,62

Curve 3: 3 – 5 M NaCl



NaCl (M)	*g/l*	*Conductivity (mS/cm)*			*Average*
3	175,32	29,64	29,6	29,63	29,62
3,25	189,93	30,85	30,86	30,86	30,86
3,5	204,54	32,08	32,08	32,07	32,08
3,75	219,15	33,37	33,35	33,34	33,35
4	233,76	34,35	34,34	34,33	34,34
4,25	248,37	35,45	35,43	35,41	35,43
4,5	262,98	36,3	36,28	36,28	36,29
4,75	277,59	36,96	36,97	36,92	36,95
5	292,2	37,46	37,47	34,46	37,46

- **Osmotic agent concentration**

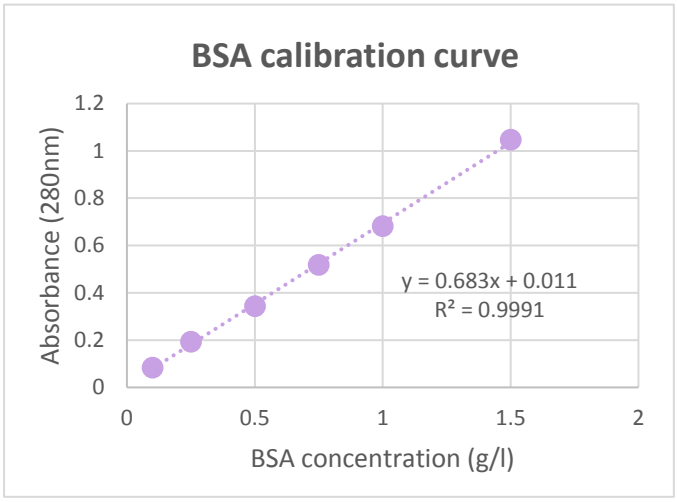
	M NaCl	*Water flux (l/m²·h)*	*Reverse salt flux (g/m²·h)*	*Specific reverse salt flux (g/l)*
Porifera	0,5	9,03	3,57	0,40
	1	16,06	7,14	0,44
	1,5	20,23	8,92	0,44
	2	23,42	8,92	0,38
Aquaporin	0,5	3,53	5,35	1,52
	1	7,64	10,71	1,40
	1,5	8,78	12,49	1,42
	2	10,30	16,06	1,56

- **Feed and osmotic agent flow rate**

	ml/min	*Water flux (l/m²·h)*	*Reverse salt flux (g/m²·h)*	*Specific reverse salt flux (g/l)*
Porifera	239	13,52	5,35	0,40
	370	13,68	8,92	0,65
	504	16,06	7,14	0,44
	646	14,70	12,49	0,85
Aquaporin	239	6,80	8,92	1,31
	370	7,66	8,92	1,17
	504	7,64	10,71	1,40
	646	7,01	12,49	1,77

II - ENRICHMENT OF A STANDARD PROTEIN

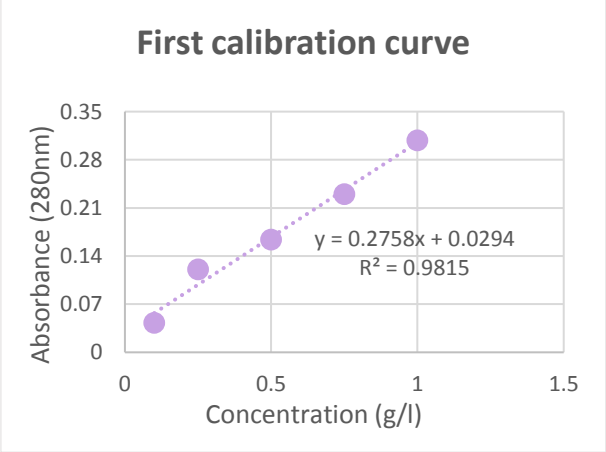
- **Calibration curve A_{280}**

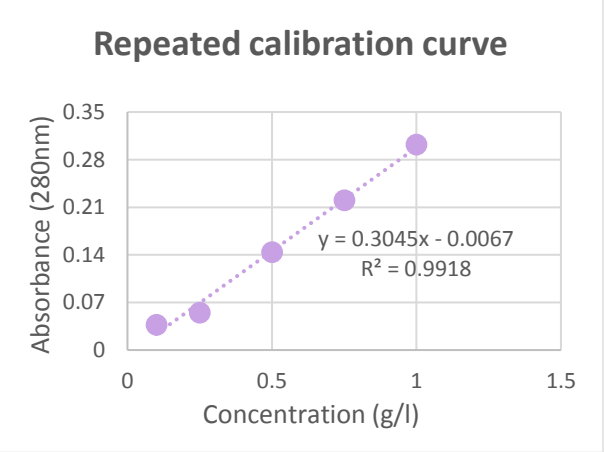


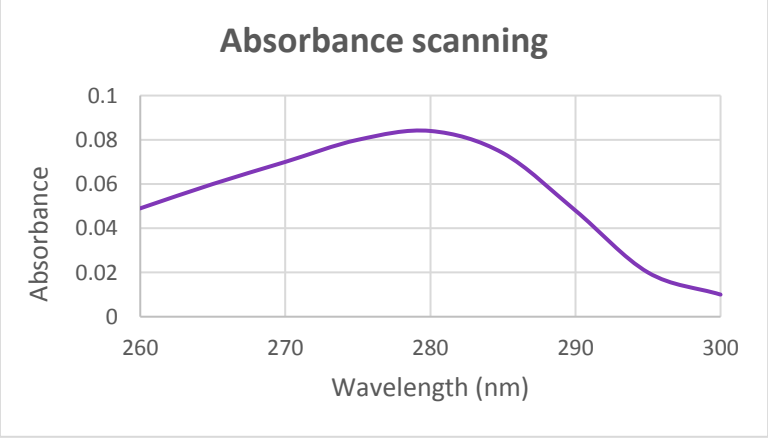
g/l	*A_{280}*			*Average*
0	0,001	0,001	0	0,001
0,1	0,084	0,083	0,082	0,083
0,25	0,194	0,194	0,192	0,193
0,5	0,344	0,344	0,342	0,343
0,75	0,518	0,517	0,516	0,517
1	0,683	0,682	0,681	0,682
1,5	1,048	1,048	1,046	1,047

- **Background absorbance**

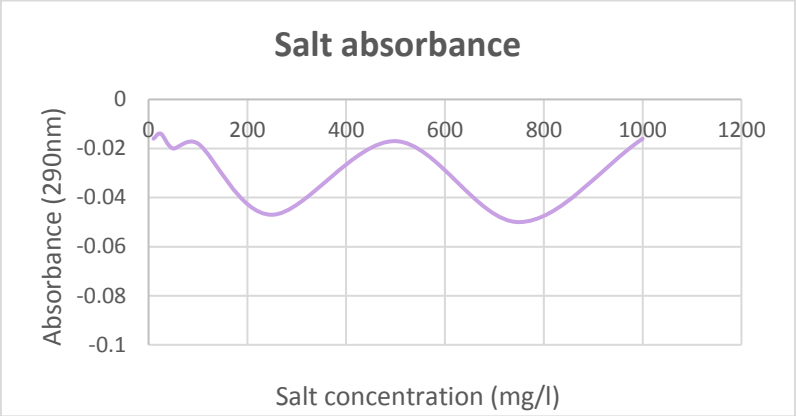
Hypothesis 1 –Calibration curve



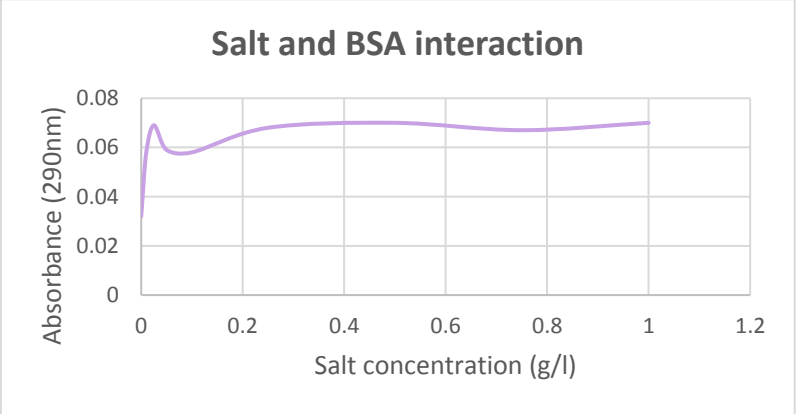




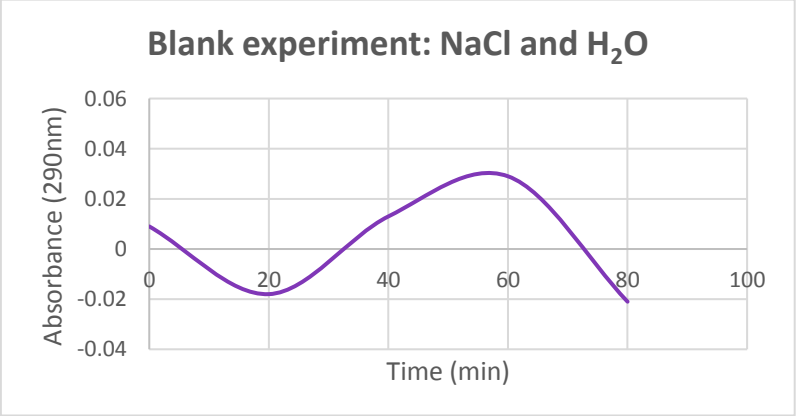
Hypothesis 2 – Salt accumulation



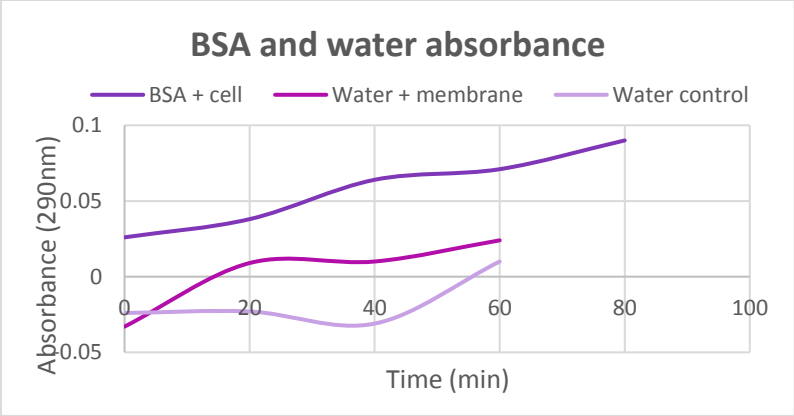
Hypothesis 3 – BSA + Salt accumulation



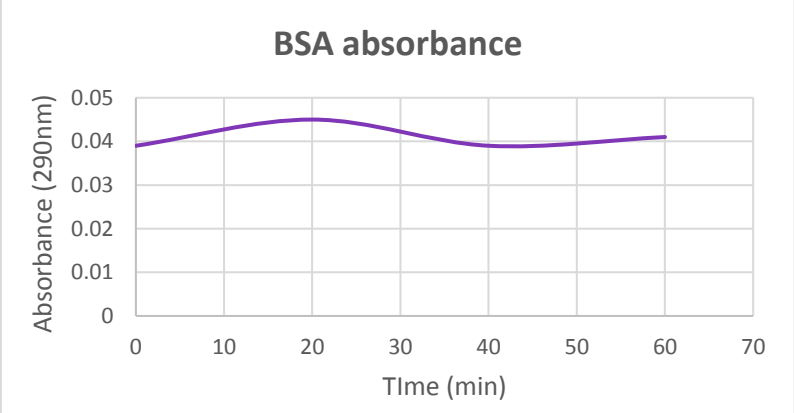
Hypothesis 4 – FO system



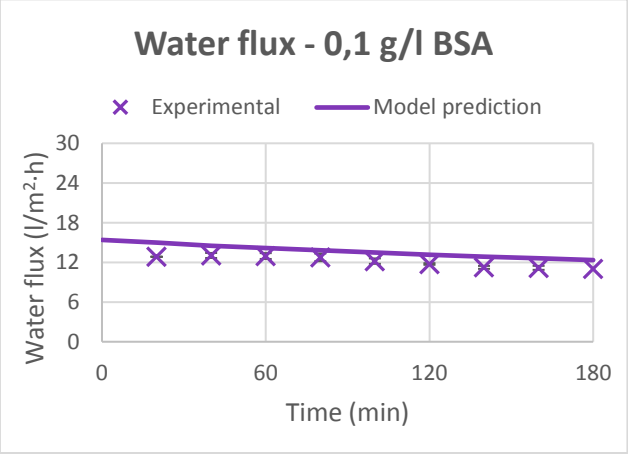
Hypothesis 5 & 6 – Membrane cell & membrane

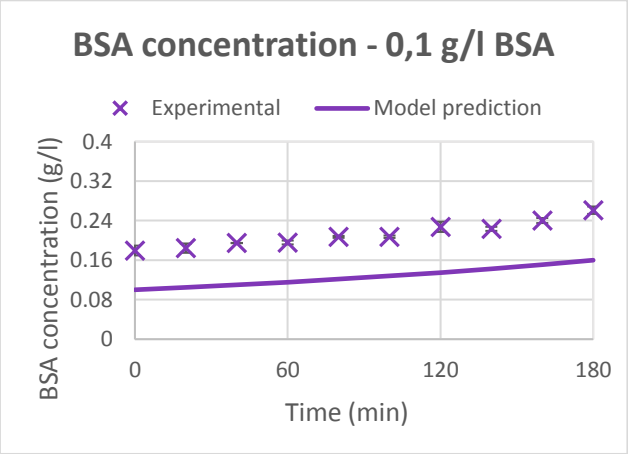


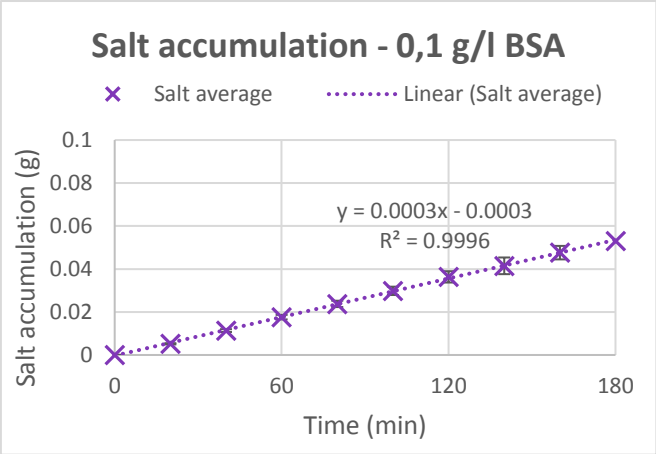
Hypothesis 7 – Mechanical fractionation of BSA

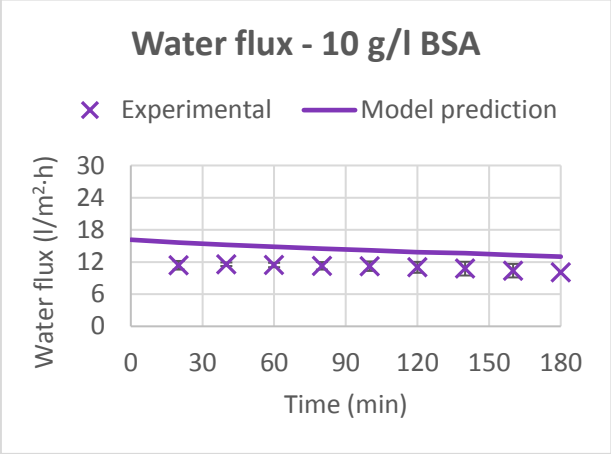


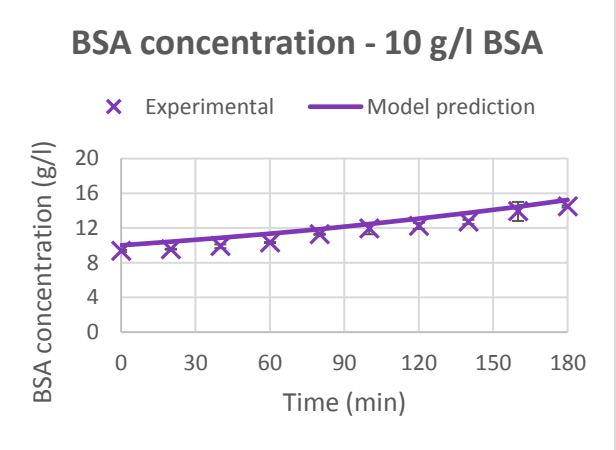
- **0.1 and 10 g/l BSA concentration**

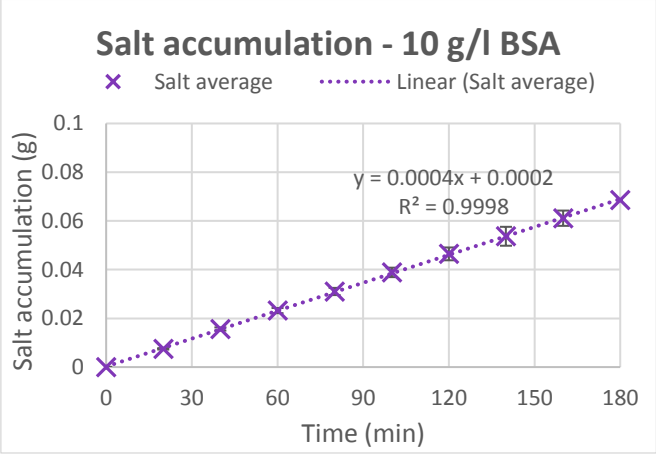





Salt accumulation - 0,1 g/l BSA
Salt average
Linear (Salt average)
y = 0.0003x - 0.0003
R² = 0.9996
Salt accumulation (g)
0.1
0.08
0.06
0.04
0.02
0
0
60
120
180
Time (min)


Water flux - 10 g/l BSA
Experimental
Model prediction
Water flux (l/m²·h)
30
24
18
12
6
0
0
30
60
90
120
150
180
Time (min)


BSA concentration - 10 g/l BSA
Experimental
Model prediction
BSA concentration (g/l)
20
16
12
8
4
0
0
30
60
90
120
150
180
Time (min)


Salt accumulation - 10 g/l BSA
Salt average
Linear (Salt average)
y = 0.0004x + 0.0002
R² = 0.9998
Salt accumulation (g)
0.1
0.08
0.06
0.04
0.02
0
0
30
60
90
120
150
180
Time (min)

- **Pierce BCA Protein Assay kit**

	Concentration (g/l)	
time (min)	A	B
0	0,187	0,172
20	0,191	0,178
40	0,195	0,194
60	0,199	0,193
80	0,206	0,208
100	0,209	0,206
120	0,220	0,235
140	0,220	0,226
160	0,237	0,244
180	0,266	0,256

- **Conductivity measurements**

0,1 g/l BSA	*Feed solution (µS/cm)*						*Draw solution (mS/cm)*					
Minute	A			B			A			B		
0	1,21	1,21	1,21	2,31	2,32	2,32	12,95	12,96	12,98	13,10	13,10	13,10
20	7,37	7,37	7,37	8,32	8,32	8,32	12,75	12,71	12,68	12,78	12,75	12,74
40	13,57	13,58	13,58	15,10	15,09	15,08	12,41	12,40	12,40	12,45	12,44	12,43
60	20,62	20,58	20,54	22,71	22,70	22,70	12,26	12,22	12,18	12,13	12,16	12,14
80	28,15	28,12	28,10	31,02	31,03	31,01	11,95	11,92	11,90	11,98	11,95	11,93
100	36,32	36,28	36,24	40,16	40,14	40,13	11,73	11,70	11,67	11,70	11,73	11,68
120	45,80	45,77	45,75	50,79	50,79	50,81	11,48	11,44	11,43	11,46	11,44	11,42
140	54,28	54,26	54,24	60,96	60,95	60,92	11,27	11,26	11,24	11,29	11,27	11,24
160	64,53	64,50	64,50	74,26	74,23	74,20	11,13	11,11	11,09	11,13	11,10	11,05
180	77,09	77,05	77,04	86,10	86,11	86,11	10,95	10,94	10,92	10,90	10,88	10,85

1 g/l BSA	*Feed solution (µS/cm)*						*Draw solution (mS/cm)*					
Minute	A			B			A			B		
0	3,99	3,99	3,99	2,90	2,90	2,90	13,68	13,67	13,66	13,70	13,72	13,69
20	15,64	15,66	15,67	10,65	10,68	10,69	13,24	13,21	13,17	13,24	13,24	13,23
40	26,54	26,57	26,58	19,49	19,55	19,58	12,86	12,83	12,79	12,93	12,91	12,90
60	38,66	38,64	38,65	29,83	29,81	29,79	12,51	12,49	12,46	12,66	12,64	12,63
80	52,88	52,87	52,91	41,32	41,31	41,31	12,17	12,16	12,13	12,38	12,37	12,35
100	67,43	67,30	67,35	54,21	54,20	54,20	11,93	11,93	11,88	12,12	12,10	12,08
120	85,18	85,17	85,19	68,59	68,62	68,61	11,73	11,69	11,65	11,87	11,85	11,83
140	107,30	107,30	107,30	84,93	84,84	84,88	11,37	11,32	11,29	11,62	11,65	11,63
160	127,50	127,40	127,20	104,70	104,50	104,20	11,12	11,10	11,07	11,49	11,47	11,44
180	152,10	152,40	151,80	129,80	129,80	129,40	10,82	10,82	10,81	11,26	11,24	11,22

10 g/l BSA	*Feed solution (µS/cm)*						*Draw solution (mS/cm)*					
Minute	A			B			A			B		
0	22,68	22,70	22,71	22,86	22,83	22,87	13,61	13,62	13,62	13,52	13,52	13,52
20	31,37	31,40	31,40	30,68	30,70	30,70	13,27	13,25	13,23	13,13	13,12	13,11
40	39,93	39,91	39,90	39,18	39,21	39,20	12,94	12,93	12,93	12,89	12,89	12,87
60	49,00	48,97	48,95	47,44	47,45	47,47	12,64	12,63	12,63	12,66	12,65	12,63
80	58,96	58,95	58,94	57,03	57,01	57,01	12,41	12,40	12,39	12,43	12,41	12,40
100	70,08	70,05	70,04	67,30	67,32	67,29	12,20	12,19	12,16	12,23	12,22	12,20
120	82,34	82,33	82,32	77,94	77,93	77,92	11,93	11,93	11,92	12,00	11,99	11,96
140	95,33	95,32	95,31	89,17	89,18	89,15	11,85	11,82	11,78	11,83	11,81	11,79
160	111,00	111,20	110,80	100,60	100,60	100,60	11,63	11,61	11,59	11,58	11,56	11,54
180	127,40	127,10	126,80	114,70	114,50	114,30	11,37	11,37	11,36	11,42	11,41	11,40

III - BROWN JUICE CONCENTRATION

- **HPLC results**

g/l	*Cellobiose*	*Glucose*	*Xylose*	*Arabinose*	*Lactate*	*Glycerol*	*Acetate*	*Ethanol*	*Citric acid*	*Succinic acid*
Feed	0,27	3,57	2,09	1,61	19,92	0,56	3,72	0,89	1,06	0,78
Retentate	0,10	1,35	0,81	0,66	7,12	0,21	1,28	0,21	0,38	0,39
Permeate	0,07	3,47	0,19	1,07	13,30	0,38	2,52	0,62	0,80	0,52

- **Mass balance diafiltration**

Component (g)	*Feed*	*Retentate*	*Permeate*
Proteins	24,640	18,440	10,949
Cellobiose	0,756	0,461	0,161
Glucose	9,996	6,224	7,998
Xylose	5,852	3,734	0,438
Arabinose	4,508	3,043	2,466
Free sugars	21,112	13,461	11,064
Lactate	55,776	32,823	30,657
Acetate	10,416	5,901	5,809
Ethanol	2,492	0,968	1,429
Citric acid	2,968	1,752	1,844
Succinic acid	2,184	1,798	1,199

- **Prediction of osmotic pressure (feed solution)**

Feed - Osmotic pressure (π) atm										
Time (min)	*NaCl*	*Peptides*	*Lactic acid*	*Citric acid*	*Succinic acid*	*Acetic acid*	*Cellobiose*	*Glucose*	*Xylose*	*Arabinose*
0		0,003	5,371	0,134	0,160	1,505	0,019	0,481	0,338	0,260
30	0,631	0,003	5,823	0,145	0,174	1,631	0,021	0,522	0,367	0,282
60	0,977	0,004	6,267	0,156	0,187	1,756	0,022	0,562	0,395	0,304
90	1,422	0,004	6,710	0,167	0,200	1,880	0,024	0,602	0,422	0,325
120	1,831	0,004	7,208	0,180	0,215	2,019	0,026	0,646	0,454	0,350

Retentate - Osmotic pressure (π) atm										
Time (min)	*NaCl*	*Peptides*	*Lactic acid*	*Citric acid*	*Succinic acid*	*Acetic acid*	*Cellobiose*	*Glucose*	*Xylose*	*Arabinose*
0		0,001	1,920	0,221	0,080	0,518	0,007	0,182	0,131	0,107
30	0,343	0,002	2,081	0,240	0,087	0,561	0,008	0,198	0,142	0,116
60	0,672	0,002	2,240	0,259	0,094	0,604	0,008	0,213	0,153	0,125
90	1,001	0,002	2,398	0,277	0,100	0,647	0,009	0,228	0,164	0,133
120	1,283	0,002	2,576	0,297	0,108	0,695	0,010	0,245	0,176	0,143

Permeate - Osmotic pressure (π) atm										
Time (min)	*NaCl*	*Peptides*	*Lactic acid*	*Citric acid*	*Succinic acid*	*Acetic acid*	*Cellobiose*	*Glucose*	*Xylose*	*Arabinose*
0		0,002	3,586	0,101	0,107	1,019	0,005	0,468	0,031	0,173
30	0,739	0,002	3,888	0,110	0,116	1,105	0,005	0,507	0,033	0,188
60	1,085	0,002	4,184	0,118	0,125	1,189	0,006	0,546	0,036	0,202
90	1,765	0,002	4,480	0,126	0,134	1,273	0,006	0,584	0,038	0,216
120	2,478	0,003	4,812	0,136	0,144	1,368	0,007	0,628	0,041	0,232