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Svetlana Butylina

EFFECT OF PHYSICO-CHEMICAL CONDITIONS AND OPERATING PARAMETERS ON FLUX AND RETENTION OF DIFFERENT COMPONENTS IN ULTRAFILTRATION AND NANOFILTRATION FRACTIONATION OF SWEET WHEY

Thesis for the degree of Doctor of Science (Technology) to be presented with due permission for public examination and criticism in Auditorium 1382 at Lappeenranta University of Technology, Lappeenranta, Finland, on the 18th of May, 2007, at noon.

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ABSTRACT

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Effect of physico-chemical conditions and operating parameters on flux and retention of different components in ultrafiltration and nanofiltration fractionation of sweet whey

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In this thesis the effect of physico-chemical conditions and operating parameters on the fractionation of sweet whey was studied. In the beginning of the thesis the environmental impact of whey and the routes of its utilisation including the treatment with membranes were reviewed. The experimental part is divided into two main sections considering ultrafiltration and nanofiltration steps of sweet whey fractionation.

The selection of ultrafiltration membrane was made using the molar mass cut-off value determined with single polyethylene glycol solutions under conditions providing low concentration polarisation. The critical flux concept was used for protein concentration by ultrafiltration of sweet whey because whey solution contains proteins, which are well-known as fouling agents. Sieving characteristics of different whey components have been studied. Peptide fractions of whey ultrafiltration permeates were characterised by using size exclusion chromatography and MALDI-TOF mass spectrometry.

The average pore sizes of nanofiltration membranes were characterised using solutions of neutral solutes. The zeta potentials of the studied nanofiltration membranes were evaluated from streaming potential measurements. Model solutions of amino acids were applied to study the role of pore size and charge of the membranes in separation. The retention of amino acids was affected by pH and by the ionic strength of the solution, as well as by intermolecular interactions.

Whey ultrafiltration permeates containing low molar mass peptides, lactose and salts were tested in nanofiltration at acidic and alkaline pH. The nanofiltration of whey ultrafiltration permeates was found to be more feasible at alkaline pH, where the irreversible fouling decreased and the permeate flux was higher. At this pH the selectivity of lactose separation from peptides was increased, compared to that at acidic pH.

Keywords: sweet whey, ultrafiltration, nanofiltration, peptides, RP-HPLC, MALDI-TOF mass spectrometry

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CONTRIBUTION OF THE AUTHOR

All experimental work presented in this thesis has been performed by the author, except for:

Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analyses of peptides, which was performed by Elena González and José Maria Cerveró from the University of Oviedo. Analyses of metal ions by means of atomic absorption spectrometry and anions by ion-chromatography were performed by technician Helvi Turkia.

Some of the experimental data in Chapter 3 have been presented earlier, in an article:

Platt, S., Mauramo, M., Butylina, S., Nyström, M., (2003), Retention of pegs in cross-flow ultrafiltration through membranes, *Desalination*, 149, pp. 417 - 422.

Data on the fractionation of whey-derived peptides using a combination of ultrafiltration and nanofiltration (Chapter 4 and 7) have been presented in an article written by the author:

Butylina, S., Luque, S., Nyström, M., (2006), Fractionation of whey-derived peptides using a combination of ultrafiltration and nanofiltration, *Journal of Membrane Science*, 280, pp. 418 - 426.

Another paper written by the author and mentioned in the literature review section is related to one of the possible ways of whey utilisation through its fermentation by yeast cells and isolation of bioactive compounds from them:

Butylina, S., Shataeva, L.K., Nyström, M., (2007), Separation of nucleoprotein complexes with antioxidant activity from yeast *Saccharomyces cerevisiae, Separation and Purification Technology*, 53, pp. 64 - 70.

Besides results published in the papers above, the thesis contains work that has not yet been published, but will be published later.

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Most of this work has been performed in the Laboratory of Membrane Technology and Technical Polymer Chemistry at Lappeenranta University of Technology. Some experiments were performed in the Department of Chemical and Environmental Engineering at the University of Oviedo (Spain).

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Lappeenranta, March 2007

Svetlana Butylina

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SYMBOLS

A_p	Peak area in the permeate sample	AU s
A_r	Peak area in the retentate sample	AU s
C	Concentration	kg m^{-3}; g g^{-1}; M; %
C_b	Concentration in bulk	kg m^{-3}
C_m	Concentration at the membrane surface	kg m^{-3}
C_p	Concentration in permeate	kg m^{-3}
C_r	Concentration in retentate	kg m^{-3}
D	Diffusion coefficient	m^2 s^{-1}
d_h	Module effective diameter	m
G	Hydrophobicity	J mol^{-1}
H_F	Wall correction parameter	-
J_v	Permeate flux	s m^{-1}; L m^{-2} h^{-1}
J_w	Pure water flux	s m^{-1}; L m^{-2} h^{-1}
K_1, K_2	Constants	-
k	Mass transfer coefficient	m s^{-1}
L_p	Hydraulic permeability of the membrane	m s^{-1} Pa^{-1}; L m^{-2} h^{-1} bar^{-1}
M	Molar mass	kg mol^{-1}
P	Pressure	Pa
R	Retention coefficient	-
R_{calc}	Calculated retention coefficient	-
Re	Reynolds number	-
R_{exp}	Experimental retention coefficient	-
R_c	Resistance of reversible part of fouling layer	m^{-1}
R_f	Fouling layer resistance	m^{-1}
R_i	Resistance of irreversible part of fouling layer	m^{-1}
R_m	Intrinsic resistance of membrane	m^{-1}
R_{obs}	Observed retention coefficient	-
R_t	Retention time	s; min
r_p	Radius of pore	m
r_s	Radius of solute	m
Sc	Schmidt number	-
Sh	Sherwood number	-

S_{obs}	Sieving coefficient	-
S_y	Standard deviation	-
T	Temperature	^oC
u_{solute}	Velocity of the solute molecule	m s^{-1}
u_{water}	Velocity of the water molecule	m s^{-1}
v	Volume	m^3; L
w	Weight	kg
Z	Charge of the ions produced in MS	-

GREEK LETTERS

α	Degree of deprotonation	-
β	Constant	-
ΔE	Streaming potential	V
ΔP	Transmembrane pressure	Pa
ε_o	Permittivity of vacuum	F m^{-1}
ε_r	Dielectric constant of the medium	-
ζ	Zeta potential	V
θ	Contact angle	^{o}
κ	Conductivity of the solution	S m^{-1}
μ	Viscosity of the solution	Pa s
ν	Kinematic viscosity	m^2 s^{-1}
π	Osmotic pressure	Pa
σ	Reflection coefficient	-
υ	Cross-flow velocity	m s^{-1}
ψ	Selectivity	-

ABBREVIATIONS

Amino Acids

Three-letter code	Single-letter code	
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic acid
Cys	C	Cystein
Gln	Q	Glutamine
Glu	E	Glutamic acid
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

AAN	Alfa-Amino Nitrogen
AAS	Atomic Absorption Spectroscopy
ACE	Angiotensin I-Converting Enzyme
BSA	Bovine Serum Albumin
BOD	Biological Oxygen Demand
CN	Casein
COD	Chemical Oxygen Demand
DF	Diafiltration

HPLC	High Performance Liquid Chromatography
IEP	Isoelectric Point
Igs	Immunoglobulins
IU	International Units
α-LA	α-Lactalbumin
β-LB	β-Lactoglobulin
LCR	Low Concentrated Retentate
LF	Lactoferrin
LP	Lactoperoxidase
LYS	Lysozyme
MALDI	Matrix-assisted Laser Desorption Ionisation
MCR	Medium Concentrated Retentate
MF	Microfiltration
$[M + H]^+$	Protonated Ion
MMV process	Maubois, Mocquot and Vassal process
MPP	Microparticulated Protein Product
MS	Mass Spectrometry
NF	Nanofiltration
NPC	Nucleoprotein Complexes
NPN	Non-protein Nitrogen
PEG	Polyethylene Glycol
PWF	Pure Water Flux
RO	Reverse Osmosis
RP-HPLC	Reversed Phase HPLC
SEC	Size Exclusion Chromatography
SHP	Steric Hindrance Pore
TFA	Trifluoroacetic Acid
TOC	Total Organic Carbon
TOF	Time-of-light
UF	Ultrafiltration
UV	Ultraviolet
VRF	Volume Reduction Factor
WPC	Whey Protein Concentrate

1 INTRODUCTION

Whey is the waste remaining from the cheese-making industry. Cheese whey causes an important environmental problem because of the high volumes produced (145 * 10^6 tons of liquid whey per year) and because of its high organic matter content. One of the promising routes of whey utilisation is the purification of highly valuable components such as peptides and amino acids.

Membrane technology is widely used in the dairy industry and particularly for whey treatment. Among these applications the concentration of whey proteins is one of the most developed processes. Whey protein concentrates with different protein contents of 35 to 75 % (w/w) (on a dry matter basis) are the commercialised products. Nanofiltration has found applications in the demineralisation of whey. Although, membranes have been used in whey processing since the early 80s the fractionation of whey proteins or peptides is still challenging.

This thesis is focused on the separation of whey components using a combination of ultrafiltration and nanofiltration. Effects of operating conditions and physico-chemical parameters on permeate flux and retention of different whey components are examined. Whey permeates after ultrafiltration, containing peptides with molar masses in the range 570 to 1360 g mol^{-1}, were used as model substances in nanofiltration to study the possibility to separate these peptides from other organic molecules, such as lactose.

In the literature review the composition of bovine milk and sweet and acid whey produced from it are considered. The role of whey as the polluting agent and the possible routes of its utilisation, with an emphasis on the separation of whey proteins as concentrates or as individual components, have been presented. Various applications of membrane filtration processes in the dairy industry are reviewed. The selectivity of protein fractionation using membranes and the factors affecting the filtration performance have been discussed.

It is worth to notice that the measurement units presented in the literature review and in the experimental sections (Chapter 3-7) are different. Most of the data taken from literature

sources are given in units reported by authors, while SI units have been used for the experimental data obtained in this thesis.

In Chapter 3, characterisation of the molar mass cut-off of ultrafiltration membranes under conditions providing low concentration polarisation was studied. Chapter 4 focuses on concentration of proteins from sweet whey using hydrophilic ultrafiltration membrane under subcritical conditions. Sieving characteristics of different whey components have been studied. Special attention has been given to the study of low molar mass peptides in whey permeates after ultrafiltration later used as the feed for a nanofiltration step. In Chapter 5 the steric and electric characteristics of two polymeric nanofiltration membranes have been investigated. These membranes have been tested with model solutions of single amino acids at different pH values and ionic strengths (Chapter 6). The effect of pH on permeate flux and retention of components of whey ultrafiltration permeates nanofiltered using polymeric membrane is studied in Chapter 7. Data on the fractionation of whey-derived peptides using a combination of ultrafiltration and nanofiltration have been presented in an article written by Butylina et al. [2006].

In Appendix I the reproducibility in different experiments is covered.

2 LITERATURE REVIEW

2.1 Importance of milk

According to the pioneer of Finnish research in biochemistry and green silage making, the Nobel prize scientist A.I. Virtanen “Milk has a special position among other food stuffs, since it has the most versatile composition. As abundant a use as possible is economically advantageous from the viewpoint of both an individual and of a whole nation” [Maijala 2000]. Milk, intended as the first food for the mammalian neonatal, contains various components with physiological functionality.

2.2 Composition of whole milk

Milk contains approximately 5 % lactose, 3.2 % protein, 4 % lipid and 0.7 % mineral salts. The nutritional value of milk and milk products is due to these constituents. The maintenance of different compounds in milk depends on many factors, such as: place, breed of cow, animal health, season, environment and feed quality and lactation stage. Rattray and Jelen [1996] have presented data on the variation of protein content in cow milk in different countries, i.e. 3.11- 3.40 g (protein)/100 g of raw milk (Finland), 3.21-3.46 (Austria), 2.75-4.09 (Canada), 3.16-4.22 (New Zealand). Kelly et al. [1998] have studied the effect of different dietary oils on fatty acid composition of milk in cows. They reported that the protein content was greater (3.44 g/100 g of raw milk) during sunflower oil feeding, while the fat content was higher (2.31 g/100 g of raw milk) during linseed oil feeding. The number of somatic cells in milk is an indicator of the health of the cow; a high cell count is associated with an increase in proteolytic cleavage of caseins. The lactation stage has a great impact on the amount of bioactive constituents of milk. The colostrum produced during 4 days after parturition has a much higher concentration of immunoglobulins, lactoferrin, growth hormone and growth factors (transforming growth factor β and insulin-like growth factors) than mature milk [Pakkanen and Aalto 1997, Lindmark-Månsson 2005].

2.2.1 Caseins

The major protein fraction of milk consists of caseins that constitute approximately 76-86 % of the total milk proteins. The main function of casein in the milk system is seen, as the source of amino acids needed for growth and it is also a source of calcium and phosphorus. Holt [1997] proposed that the main physiological importance of the casein micelle system appears to be the prevention of pathological calcification of the mammary glands. Four major components: α_{s1}-, α_{s2}-, β- and κ-caseins (CN) are present in milk, and some additional heterogeneity arises from post-translation modification and limited proteolysis. Table 2.1 shows the concentrations and physical properties of individual bovine caseins. Caseins are neither globular, nor fibrillar proteins, molten globules or random coils. The word rheomorphic has been used to describe them [Swaisgood 2003]. The inability of casein molecules to form ordered secondary structures owes mainly to their high content of proline residues (Table 2.1).

Table 2.1 Physical characteristics of bovine caseins [[1]Creamer and Mac Gibbon 1996, [2]Swaisgood 2003, [3]Tremblay et al. 2003].

Protein	Content, kg m^{-3}	Molar mass calculated[3], g mol^{-1}	Molar mass measured[3], g mol^{-1}	IEP[3]	Proline/ Total amino acid residues[2]
α_{S1}-CN	9[1]; 12-15[2]	23614.8	23618±2	4.23-4.47	17/199
β-CN	9[1]; 9-11[2]	24092.4	24093±3	4.68-4.96	35/209
κ-CN	4[1]; 3-4[2]	19005.5	19006±2	5.54-6.12	20/169
α_{S2}-CN	3[1]; 3-4[2]	25228.4	25230±2	7.83-5.13	10/207

[3]The presented molar masses have been calculated using average molar masses of amino acid residues. The molar masses of caseins were measured from reconstructed mass-spectra.

Caseins also lack a tertiary structure, thus there is a considerable exposure of hydrophobic residues on their surface and as a result strong association reactions between the caseins, and they are insoluble in water. In addition, the lack of tertiary structure means that caseins are extremely heat stable, sodium caseinate at pH 7.0 can withstand heating at 140 ^{o}C for several hours [Fox 2003].

Unlike whey proteins, which are present in milk as individual proteins, caseins form large protein complexes with calcium and phosphate molecules included in them. There are several models describing the structure of the micelles, the first and most acceptable model being proposed by Morr [Fox 2003, Creamer 1996]. According to this model the micelle is composed of sub-micelles with molar masses approximately equal to 10^6 g mol^{-1} and with a diameter of 10-15 nm (Figure 2.1).

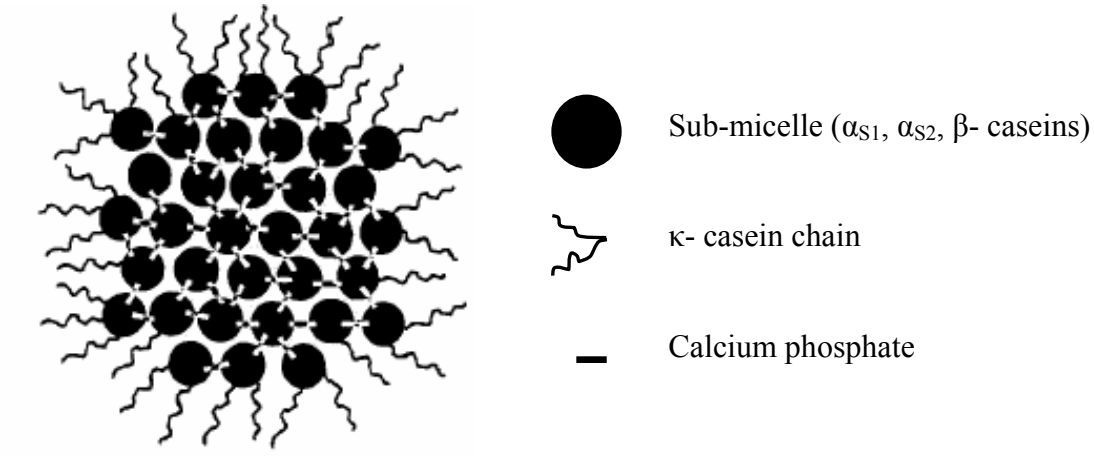


Figure 2.1 Sub-micelle model of the casein micelle [adapted from Creamer 1996].

The internal structure of the casein sub-micelles is composed of the calcium-sensitive caseins (β-casein, α_{s1}- and α_{s2}-caseins). The sub-micelles are aggregated through calcium and calcium phosphate cross-links with phosphoserine residues on the outer surface of the sub-micelles. Walstra proposed that the hydrophilic C-terminal of κ-casein is forming a 10-15 nm thick hairy surface layer [Fox 2003]. This hairy layer is responsible for micelle stabilisation due to strong electrostatic repulsion (zeta potential –20 mV) and steric stabilisation.

Milk contains 10^{14}-10^{16} micelles mL^{-1}, which are generally spherical particles with diameters ranging from 50-500 nm and the molar mass of the hydrated micelles are ranging from 10^6 to 3×10^9 g mol^{-1} [Fox 2003]. Casein micelles are very stable at high temperatures. There are several factors, which affect the micelle stability, such as: the salt content, pH, low temperatures (cryodestabilisation), dehydration and the presence of chymosin-rennet or other proteolytic enzymes (cheese manufacturing).

2.2.2 Whey proteins

The remaining portion of milk proteins (14-24 %) consists of whey proteins (Table 2.2), which besides the nutritional value also provide immunological protection and biological activity. The nutritional value of whey proteins is higher than that of the caseins. For example, the essential amino acid cystein in milk is derived almost exclusively from whey proteins, β-lactoglobulin (β-LG) and α-lactalbumin (α-LA). The cysteine is particularly important in the baby formula because infants lack the enzymatic capacity to convert methionine to cysteine [Hambræus 2003]. β-lactoglobulin is the dominant whey protein in bovine milk. No definite biological function has been identified in it, although several proposals have been suggested. The amino acid sequence of β-lactoglobulin has been reported to be homologous to serum retinol-binding proteins, thus it might play a role in retinol transport [Kontopidis et al. 2002]. This protein is often responsible for infant milk allergy [Exl and Fritsche 2001, Sharma et al. 2001]. Bovine β-lactoglobulin at neutral pH exists as a dimer (M = 36.4 kg mol^{-1}) of two identical or near identical units, but between pH 2 and 3 it tends to dissociate into monomers [Sawyer and Kontopidis 2000]. α-Lactalbumin plays a regulatory role in the lactating mammary gland via enhancing the binding of glucose to galactosyltransferase [Brew 2003]. It is a strong binder of calcium and the calcium ion affects the folding and structure of α-lactalbumin [Creamer and Mac Gibbon 1996, Brew 2003]. Milk antibodies in the form of immunoglobulins (IgG_1, IgG_2, IgA and IgM) protect the mammary glands and newborn babies. Immunoglobulin G is the predominant immunoglobulin in milk. The concentration of immunoglobulins is highest in the colostrums, but decreases with time (i.e. colostrums contains 32-200 mg of IgGs mL^{-1}, milk 0.72 mg mL^{-1}) [Hurley 2003, Pakkanen and Aalto 1997, Korhonen et al. 1998]. Lactoferrin (LF) was identified as the iron-binding protein that possesses antibacterial effect and immunomodulatory activity, and it also enhances iron absorption and acts as a growth factor [Korhonen et al.1998, Lönnerdal 2003]. The other two milk proteins having antimicrobial functions are lysozyme (LYS) and lactoperoxidase (LP) [Farkye 2003, Pakkanen and Aalto 1997].

Table 2.2 Physical characteristics of whey proteins in bovine milk [[1]Creamer and Mac Gibbon 2001, [2]Swaisgood 2003, [3]Konrad et al. 2000, [4]Bhattacharjee et al. 2006, [5]Brans et al. 2004, [6]Korhonen et al. 1998, [7]Zydney 1998, [8]Pakkanen and Aalto 1997 [9]Sawyer and Kontopidis 2000, [10]Farkye 2003, [11]Lindmark-Månsson et al. 2005].

Protein	Concentration, kg m^{-3}	Isoelectric point	Molar mass, kg mol^{-1}
β-lactoglobulin	3[1]; 2-4[2]; 3.2-3.4[3]; 3-4[4]; 3.2[5]; 3.3[6]; 2.7[7]	5.2-5.4[4]; 5.2[7]; 5.4[9]	18.3[4, 5, 7, 9]
α-lactalbumin	0.7[1]; 1-1.5[2]; 1.2-1.5[4]; 1.2[6]	4.2[4]; 4.5-4.8[7]	14.2[4, 5, 7]
Immunoglobulins	1[1]; 0.6-1[2]; 0.6-0.9[4]; 0.8[5]; 0.7[6]; 0.65[7]	5.8-7.3[4]; 5.5-8.3[7]	150-900[4, 5]
Serum albumin	0.3[1]; 0.1-0.4[2]; 0.3-0.6[4]; 0.4[5, 7]	4.9-5.1[4]; 4.7-4.9[7,11]	66.0[4, 5]; 69.0[7]
Lactoferrin	0.05[4]; 0.1[5, 6, 7]	8.0[4]; 9.0[7]; 8.0-9.0[11]	78[4, 7]; 86[5]; 78-86[11]
Lactoperoxidase	0.06[4]; 0.03[6]; 0.02[7]; 0.01-0.03[8]	9.6[4, 7]; 9.2-9.9[11]	78[4]; 89[7]; 76-82[11]
Lysozyme	0.0004[6]; 0.00007-0.0006[8]	9.5[11]	18[10, 11]
Glycomacropeptide	1.2[6]		7.0[7]
Proteose-peptone	0.6-1.8[2]; 0.8[5]		4-40[5]

2.2.3 Bioactive peptides derived during milk processing

In the late 1960s, Lahov and Regelson observed that hydrolysis of bovine milk casein by heating and chymosin treatment at neutral pH leads to the formation of polycationic low molar mass peptides with antimicrobial properties, the so-called casecidines [Liepke 1996]. Casecidin 1 (from the α_{s2}-casein fragment (165-203)) exhibits activity in vitro against *Staphylococcus, Sarcina, Bacillus subtilis, Diplococcus pheumoniae*, and *Streptococcus pyogenes*. Partially or extensively hydrolysed milk proteins have found increasing use in hypoallergenic infant formulas and dietary products [Korhonen et al. 1998]. The extensively hydrolysed formula contains more oligopeptides having molar masses of less than 2000 to 3000 g mol^{-1} and it is recommended for therapeutic indication, while the partially hydrolysed

formula contains more high molar mass fractions and is recommended mainly for allergy prevention [Exl and Fritsche 2001].

Peptides may be released from milk proteins as a result of proteolytic activity attributed to the presence of indigenous enzymes (plasmin, bacterial enzymes and somatic cell enzymes) and of enzymes used in milk processing (rennet, enzymes of starter and non-starter microflora). Table 2.3 shows the examples of active peptides derived from milk proteins. Among milk peptides caseins are more susceptible to proteolysis because they have more flexible chains than typical globular proteins [Swaisgood 2003]. The hydrolysis of α_{S2}- and β-caseins by residual chymosin and native plasmin plays a role in the ripening and flavour development of cheeses. The most commonly used enzyme in the production of whey hydrolysates is pepsin. Pepsin digestion of bovine lactoferrin releases the peptide fragment (17-41) with 100 to 1000 times the bactericidal activity of intact lactoferrin [Schanbacher et al. 1998].

Table 2.3 Bioactive peptides derived from milk proteins [[1]Pihlanto-Leppälä 2001, [2]Meisel 1997 and 1998, [3]Clare and Swaisgood 2000, [4]Liepke et al. 2001, [5]Schanbacher et al. 1998, [6]SWISS-PROT].

Peptide	Sequence	Molar mass[6], g mol^{-1}	Mode of action	Protein	Enzyme
[1,2]α-lactorphin	Y-G-L-F	498.3	ACE inhibitor; opioid	α-LA	Pepsin
[1,2]β-lactorphin	Y-L-L-F	555.3	ACE inhibitor; opioid	β-LG	Pepsin, trypsin
[2]β-casokinin-10	Y-Q-Q-P-V-L-G-P-V-R	1157.6	ACE inhibitor; immunomodulatory	β-CN	Pepsin, chymosin
[3,4]Isracidin	K-N-T-M-E-H-V-S-S-S-E-E-S-I-I-S-Q-E-T-Y-K-Q-E	2684.9	Antimicrobial	α_{s1}-CN	Chymosin, chymotrypsin
[2,3,5]Lactoferricin B	F-K-C-R-R-W-Q-W-R-M-K-K-L-G-A-P-S-I-T-C-V-R-R-A-F	3125.8	Immunomodulatory; antimicrobial	LF	Pepsin
[2]Casoplatelin	M-A-I-P-P-K-K-N-Q-D-K	1269.5	Antithrombotic	κ-CN	Chymosin (rennin)

2.2.4 Lactose and oligosaccharides

Lactose (4-(β-D-galactopyranosyl-D-glucopyranose) is the major carbohydrate in milk and it comprises 4.8 to 5.2 % (w/v) of it. Pharmaceutical and food industries utilise lactose to large proportions. Some people suffer from lactose intolerance owing to the absence of the lactase enzyme, which splits lactose to monosaccharides (galactose and glucose). Bovine milk contains trace amounts of ten acidic oligosaccharides and eight neutral oligosaccharides (Table 2.4).

Table 2.4 Oligosaccharides of bovine milk [Gopal and Gill 2000].

Neutral oligosaccharides	Acidic oligosaccharides
3'-Galactosyl-lactose	3-Sialyl-lactose
6'-Galactosyl-lactose	6-Sialyl-lactose
3-Fucosyl N-acetyl-lactosamine	6-Glucolylneuraminyl-lactose
N-Acetyl-lactosamine	6-Sialyl-lactosamine
N-Acetylgalactosaminyl-glucose	6-Glucolylneuraminyl-lactosamine
N-Acetylgalactosyl-lactose	3-Sialyl galactosyl-lactose
Lacto-N-novopentaose	Disialyl lactose
α-3'-Galactosyl lactose	Sialyl-lactosamine-1-phosphate
	Sialyl-lactosamine-6-phosphate
	3-Glucolylneuraminyl-lactose

Neutral oligosaccharides do not contain any charged carbohydrate residues, while acidic oligosaccharides contain one or more residues of N-acetylneuraminic acid (sialic acid). Oligosaccharides are important bioactive constituents, however, they do not play a role as nutrients for energy but they provide protection from pathogenic bacteria. Neutral oligosaccharides act as growth enhancers for probiotic micro-organisms (*Bifidubacterium)* in infants [Gopal and Gill 2000]. Sialyted oligosaccharides have been reported to inhibit adhesion of the ulcer-causing human pathogen *Helicobacter pylori* to epithelial cells [Simon et al. 1997].

2.2.5 Lipids

The lipid fraction of milk is composed mainly of triacylglycerols (95.80 %) with minor contributions of diacylglycerols (2.25 %), monoacylglycerols (0.08 %), sterols (mostly cholesterol, 0.46 % or 100-200 mg L^{-1}), phospholipids (1.11 %), fat-soluble vitamins and β-carotene [Jensen 1991]. The triacylglycerols of milk fat include a variety of fatty acids, which vary in concentration depending on a range of factors such as the stage of lactation and the diet of the cow. Almost 400 different fatty acids have been identified in milk fat, but only 10 of them (Table 2.5) are present at concentrations higher than 1 % [Creamer 1996]. The *cis*-9, *trans*-11 octadecadienoic acid (or conjugated linoleic acid) has been reported to possess anticarcinogenic, anti-atherogenic, antidiabetic and antiobesity activity [Jensen 1991, Henning et al. 2006]. As shown in Table 2.5 the concentration of *cis*-9, *trans*-11 octadecadienoic acid was highest in milk of cows consuming sunflower oil.

Two major groups of phospholipids, which form the membrane around fat globules, have been reported to be present in milk: phosphoglycerides and sphingolipids. Phosphatidyl ethanolamine (34 % of the membrane lipids) and phosphatidyl choline (25 %) are the major phosphoglycerides, while sphingomyelin is the major sphingolipid in milk fat (24 % of membrane lipids) [Sprong 2002]. The gangliosides are glycosylated sphingolipids comprising 0.5 % of the membrane lipids and they inhibit enterotoxins from *Vibrio cholerae* and *Escherichia coli* at concentrations of 1 μg L^{-1} of milk (i.e. monosialo-ganglioside, G_{M1}) [Jensen 1991].

Table 2.5 Fatty acid composition of milk from cows with different dietary treatment [Kelly et al. 1998].

Fatty acid		Concentration of fatty acids, g/100 g fat		
C	Systematic name	Peanut oil	Sunflower oil	Linseed oil
6:0	Hexanoic	1.37	1.09	1.32
8:0	Octanoic	0.65	0.54	0.66
10:0	Decanoic	1.33	1.20	1.38
12:0	Dodecanoic	1.77	1.71	1.83
14:0	Tetradecanoic	7.80	7.59	7.87
14:1	Tetradecenoic	1.23	1.22	1.27
15:0	Pentadecanoic	0.71	0.68	0.74
16:0	Hexadecanoic	23.01	20.84	19.35
16:1	Hexadecenoic	2.30	2.74	2.19
18:0	Octadecanoic	12.97	12.39	11.44
18:1	Octadecenoic	38.70	40.62	36.99
18:2	Octadecadienoic	2.36	2.78	3.27
18:2*c*	*Cis*-9, *trans*-11-octadecadienoic	1.33	2.44	1.67
18:3	Octadecatrienoic	0.18	0.19	0.44

2.2.6 Vitamins and minerals

Milk is also an important source of dietary fat-soluble vitamins (A, D, E, and K) and water-soluble vitamins (thiamin, riboflavin, pyridoxine, cyanocobalamin, niacin, pantothenic acid). All 22 minerals considered to be essential to the human diet are present in milk. Table 2.6 shows the average content of vitamins and minerals of raw milk and sweet whey remaining after cheese making from this milk. According to Table 2.6 most of the vitamins and minerals are remaining in sweet whey. The high amount of panthotenic acid in sweet whey compared to raw milk could be explained by the presence of yeasts. Yeasts are applied with lactic acid bacteria in cheese-making processes. They have been reported to support the growth of lactic acid bacteria through the synthesis of panthotenic acid and other vitamins [Viljoen 2001].

Table 2.6 Average content of vitamins and minerals in milk and sweet whey obtained from it [Sienkiewicz 1986, Zall 1992].

Component	Milk	Sweet whey
Water-soluble vitamins, μg/ 100 g		
Thiamin (B_1)	43.3	38.3
Riboflavin (B_2)	227	160
Pyridoxine (B_6)	46.0	42.3
Cyanocobalamin (B_{12})	0.34	0.28
Pantothenic acid	308	426
Biotin (H)	1.40	1.70
Ascorbic acid (C)	466	231
Fat-soluble vitamins		
(vitamin A), IU/ 100 g	115	84.3
Minerals, mg/ 100g		
Calcium	116	38.1
Magnesium	15.3	9.67
Sodium	41.7	45.0
Potassium	141	147
Phosphorus	95.3	45.7
Chloride	99.3	106

1 International unit (IU) Vitamin A is the biological equivalent of 0.3 μg retinol, or of 0.6 μg of beta- carotene.

2.3 Whey as a by-product of cheese production

Whey is the liquid remaining following precipitation and removal of milk casein during cheese-making. The two main whey varieties produced are acid (pH <5) and sweet (pH 6-7) whey, according to the procedure used for casein precipitation.

Acid whey is formed during the manufacture of cottage cheese or industrial casein, when lactic acid or mineral acid is used for casein precipitation. The acids coagulate the casein micelles by decreasing their charge to the isoelectric point. In addition, the acids increase the solubility of organic calcium and phosphorus that results in disintegration of the micelles and in casein precipitation. The acid type of whey contains a higher amount of ash and a lower amount of protein than sweet whey (Table 2.7). The non-protein nitrogen (NPN) fraction contains most, if not all, of the low molar mass nitrogen-containing molecules present in whey: amino acids, small peptides, nucleotides, vitamins, urea, creatinine, ammonia, etc.

Table 2.7 Composition of sweet-type (Cheddar cheese) and acid-type whey [Sienkiewicz 1986].

Composition	Sweet whey	Acid whey
Water, %	93.3	95.6
Dry matter, %	6.70	6.42
Total protein, mg g^{-1}	0.60	0.53
Non-protein nitrogen, mg g^{-1}	0.34	0.34
Lactose, %	5.00	4.40
Ash, %	0.52	0.60
pH	6.10	4.70

For the principal family of cheeses (approx. 75 % of the total cheese, [Hyslop 2003]) coagulation is achieved by adding a small amount of rennet or some other milk clotting enzyme or a combination of enzymes to the milk. The coagulation reaction includes two stages:

1st stage: κ-casein + rennet → para-κ-casein + glycomacropeptide

The cleavage of the Phe_{105} – Met_{106} peptide bond in the κ-casein molecule is the first step in the coagulation of milk [Creamer 1996]. It results in the loss of the hydrophilic, negatively charged glycomacropeptide domain from the micelle surface.

2nd stage: para-κ-casein + Ca^{++} → casein curd

The second stage does not require enzymes. It results from the reaction of the exposed chemical groups in para-κ-casein with calcium for polymerisation to a gel.

2.3.1 Utilisation of whey

To make 1 kg of cheese, 9 kg of whey is generated [Jelen 1979]. Cheese whey causes an important environmental problem because of the high volumes produced (145 * 10^9 kg of liquid whey per year) and because of its high organic matter content (BOD_5 = 30-50 kg m^{-3} and COD = 60-80 kg m^{-3}) [Gonzales Siso 1996]. The whey itself is not a balanced source of nutrients because of its very high concentration of water (93.5 %) and lactose (4.5-5.0 % (w/v)). Liquid whey without any treatment can be used for feeding of farm animals. Whey is a nutritious protein source, but its application in food products without demineralisation is limited.

Possible waste minimisation routes of whey have been described by e.g., Zall [1992]:

1. Application of techniques minimising whey production during cheese-making
2. Usage of whey powder as a valuable by-product in the food industry
3. Production of petrochemicals such as methane and ethanol
4. Utilisation of lactose
5. Considering whey as a valuable by-product seems to be a very promising future route development for membrane technology

2.3.1.1 Application of techniques minimising whey production during cheese-making

Different types of milk ultrafiltration retentate, which have found application in cheese-making, are presented in Table 2.8. The low concentrated retentates (LCR) result in a slight increase in yield, it makes easy implementation of continuous cheese-making and increases plant capacity. Most types of hard cheeses have been made by the LCR process. Medium concentrated retentates have been reported to yield high-and consistent-quality cheese and result in a 6-8 % increase in cheese yield. However, it requires special cheese-making equipment in opposite to the LCR process, which allows the use of conventional equipment. Ultrafiltration can be directly included in the cheese-making process, the so-called MMV (Maubois, Mocquot and Vassal) process [Zydney 1996].

Table 2.8 Applications of ultrafiltration milk retentates in the cheese industry [Rattray and Jelen 1996].

Type of ultrafiltration milk retentate used in cheese-making	Concentration factor	Cheese varieties
Low concentrated retentate (LCR)	1.2-2	Cheddar, cottage cheese, mozzarella, quarg, edam
Medium concentrated retentate (MCR)	2-5	Cheddar, feta, gouda, blue cheese
Liquid pre-cheese (MMV process)	5-7	Camembert, quarg, ricotta, feta, mozzarella, cream cheese.

Currently more than 95 % of the Danish Feta cheese is produced using the MMV process (UF-FETA-2.5 and UF-FETA-5). The cheese yield in the UF-FETA-5 process is approximately 32 % greater than in the traditional process due to the complete retention of the whey proteins in the final cheese. The usage of ultrafiltered milk retentate has been shown to have an undesirable effect on texture and flavour of cheese, such as Cheddar [St-Gelais et al. 1997, Mistry and Pulgar 1996]. Textural and sensory characteristics of cheese varieties are affected among other factors by the extent of proteolysis. The slower rate of proteolysis in cheeses made from ultrafiltered milks has been explained by the lower rennet-to-casein ratios,

inhibition of plasmin by the retained β-lactoglobulin retained, the increased levels of proteinase/peptidase inhibitors and/or the resistance of undenaturated whey proteins to degradation in cheese [Guinee et al. 1995].

2.3.1.2 Usage of whey powder as a valuable by-product in the food industry

Different kinds of condensed or powdered whey are prepared and they have found applications on a commercial scale, including condensed whey, acid or sweet-whey powders, demineralised whey powder, and delactosed whey powder. Whey-based microparticulated protein products (MPP), such as Simplesse® and Dairy-Lo®, are fat replacers [McMahon 1996]. Fat replacers reduce the calorie levels while maintaining some of the desirable qualities that fat brings to food, such as "mouth feel", texture and flavour. These whey-based MPP can be used in reduced-fat versions of butter, sour cream, cheese, yoghurt, salad dressing, margarine, baked goods, coffee creamer, soups and sauces [Kurtzweil 1996].

2.3.1.3 Production of petrochemicals such as methane and ethanol

Anaerobic digestion producing methane has been employed in industrial waste treatment. Production of ethanol from non-concentrated cheese whey is not economically feasible because the level of ethanol obtained reaches only about 2 % making the distillation too expensive [Gonzalez Siso 1996].

2.3.1.4 Utilisation of lactose

The milk sugar, lactose, can be purified from cheese whey or ultrafiltrate formed after whey protein concentrate (WPC) separation by crystallisation. It is used as a supplement in baby foods and as an excipient for pharmaceutical products. One of the most important functions of lactose is its possible utilisation as a fermentation substrate (Table 2.9).

Lactic acid bacteria produce lactic acid from lactose, which is the beginning of many fermented dairy products. The historical development of the Valio Hydrolysis Process for

hydrolysing lactose in whey using enzymes from *Aspergillus niger* has been initiated by Heikonen [Markula 2001]. The enzymatic lactose hydrolysis by the β-galactosidase enzymes is an example of a modern industrial process aimed at producing a more physiologically acceptable milk for consumers experiencing the well-known problem termed "lactose intolerance". The main barrier to a more widespread use of the lactose hydrolysis process is its high cost. However, from the standpoint of consumer needs, the production of lactose-free or lactose modified dairy foods should be considered as one of the most important aims of modern nutraceutically oriented dairy processing as the potential market worldwide is very significant [Jelen and Lutz 1998].

Table 2.9 Micro-organisms used for whey fermentation and their products [Sienkiewicz 1986].

Micro-organism	**Product**
Yeasts	
Candida curvata	Fats, butter
Kluyveromyces fragilis	Single cell protein, ethanol, β-galactosidase
Kluyveromyces lactis, Saccharomyces cerevisiae, Candida	Single cell protein, ethanol
Moulds	
Penicillium cyclopium, Penicillium javanicum, Morchella	Microbial protein
Geotrichum candidum, Geotrichum lactis	Fats
Aspergillus niger	Citric acid, fats, β-galactosidase
Bacteria	
Propionibacterium	Vitamins B_6 and B_{12}
Clostridium acetobutylicum	n-butanol
Xanthomonas campestris	xanthan

Yeast biomass has been produced commercially from whey since 1940 [Gonzalez Siso 1996]. Whole cheese whey is not used for this purpose because yeast cells cannot metabolise its

proteins. Thus, processes producing WPC, lactose and yeast cells can be combined (Figure 2.2). Following yeast-biomass separation, the BOD (35-45 kg m^{-3}) of the effluent obtained is reduced to a similar value as that following anaerobic treatment of whey (9-11 kg m^{-3}) [Mawson 1994].

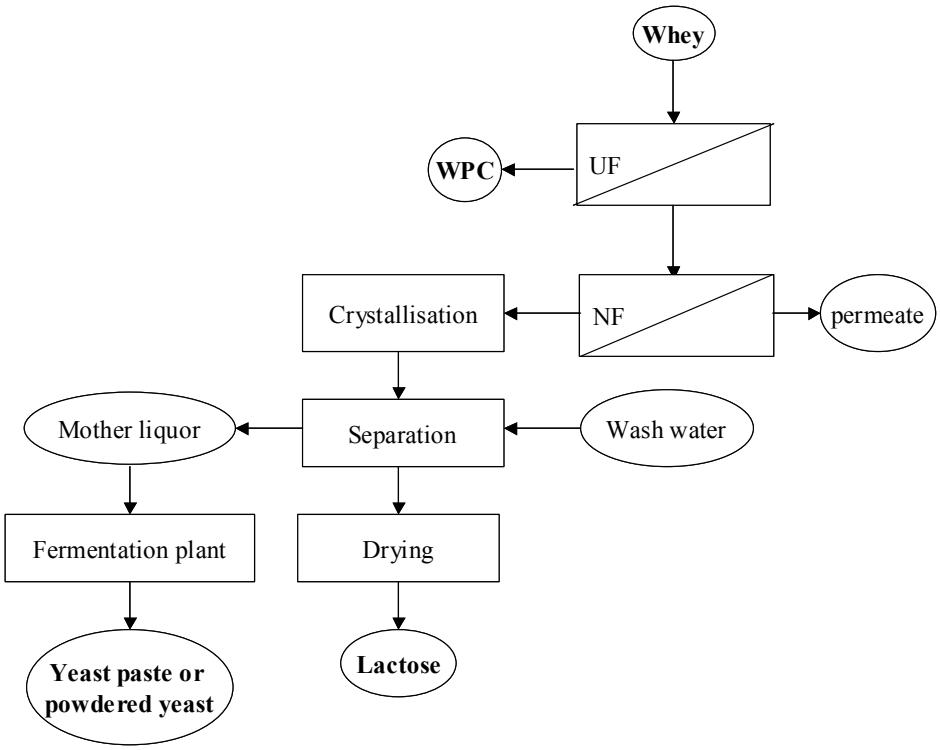


Figure 2.2 Scheme of a whey purification process integrated with other manufacturing processes [Di Giacomo et al. 1996, Helakorpi et al. 2001].

The dried yeast biomass (e.g. "Protibel") is a commercial product, which is used, in human dietetic nutrition. However, not only yeast cells by themselves are interesting as dietary supplements but also the bioactive constituents isolated from the cells e.g., nucleoprotein complexes (NPC). NPC were isolated from yeast cells by mild alkaline extraction followed by precipitation with acetic acid, and the high molar mass fractions were separated using cross-flow microfiltration. The high molar mass nucleoprotein complexes have been shown to affect the survival of oxidatively stressed yeast cells and they also possess the immune stimulating effect on human T-lymphocytes [Butylina et al. 2005, 2007].

2.3.1.5 Considering whey as a valuable by-product seems to be a very promising future route development for membrane technology

Since whey is a by-product from the cheese industry and may create an environmental waste problem, it is of fundamental importance to find new applications of these highly valuable nutritional proteins and peptides. The biological activities of whey proteins have been reviewed in section 2.2.2. Purified lactoferrin and lactoperoxidase are well-known commercial realities. As has been shown in Table 2.3 peptides released from milk proteins by enzymatic digestion can modulate various physiological functions. These peptides may be used not only in the food industry as dietary supplements, but also in the pharmaceutical industry, for example in the treatment of hypertension (casokinins), thrombosis (casoplatelins), dental and bone diseases (casein phosphopeptides) [Meisel 1997]. Achievements in separation techniques, particularly membrane filtration, in the dairy industry offer the opportunities to isolate, to concentrate or to modify these compounds, so that their application in the food industry has become possible.

2.4 Application of membrane filtration in the dairy industry

Membrane based separation processes are generally classified on the basis of the membrane pore size or on the type of material being filtered. There are four categories of membrane processes mainly used in the dairy industry: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). The main features of these pressure-driven processes are presented in Table 2.10.

Table 2.10 Characteristics of pressure driven membrane processes.

Process	Pore size	Type of transport
MF	0.05-10 μm	Convective
UF	2-100 nm	Convective
NF	0.5-3 nm	Convective/diffusive
RO	Not relevant	Diffusive

2.4.1 Traditional application of membranes in the dairy industry

Since the early 80s membrane filtration started to be applied in the dairy industry. Cross-flow microfiltration has become an industrial separation technology for the removal of milk bacteria, the reduction of the fat content of whey and the enrichment of milk with micellar casein in cheese making. The characteristics of these milk microparticles are presented in Table 2.11. A microfiltration system capable of removing 99.7 % of the bacterial load from skim milk containing 30 000 colony-forming units (CFU) per mL has been introduced by Alfa-Laval, Lund, Sweden [Rosenberg 1995].

Table 2.11 Milk particles [Jensen 1991, Saboya 2000, Fox 2003].

Component	Major constituents	Content per mL	Diameter, μm
Casein micelles	α_{s1}-, α_{s2}-, β- and κ-caseins, Ca^{2+}, PO_4^{3-}	10^{14}-10^{16}	0.03-0.50
Bacteria			0.20-15.0
Fat globules	Triacylglycerols, fat-soluble vitamins, cholesteryl esters	1.1×10^{8}	0.20-6.00
Somatic cells	Macrophages, neutrophils, lymphocytes, epithelial cells, leukocytes	10^{2}-10^{3}	6.00-15.0

Ultrafiltration is a well-established process for the preparation of whey protein concentrates with moderate (35 % (w/w)) to high (75 % (w/w)) protein concentration (on a dry matter basis) and with a significantly reduced lactose and mineral content. Ultrafiltration offers the possibility of adjusting the mass ratios of different milk constituents without adversely affecting their physicochemical characteristics and it can be used in milk protein standardisation [Rosenberg 1995, Rattray and Jelen 1996]. The applications of ultrafiltration can be used to standardise the nutritional value of consumer's milk or to prepare standardised milk powders, thus overcoming natural variations in milk composition. Ultrafiltration is commonly applied in the manufacturing of fresh cheese varieties like quarg, ricotta and Camembert, or brine cheeses (such as feta).

An ion-exchange membrane technique has been employed successfully for the separation of lactoferrin and lactoperoxidase from cheese whey [Chiu 1997]. Both lactoferrin and

lactoperoxidase are recovered on an industrial scale and have found applications in food, veterinary and pharmaceutical production.

Nanofiltration has been developed for the removal (84 %) of salt from salty whey (Cheddar cheese production), the partial removal (42 %) of acid from acid whey, and the partial demineralisation of sweet whey in the manufacture of lactose or lactose-fermented products.

The advantages of membrane techniques compared to other types of separation techniques (chromatography and crystallisation) are:

- Inexpensive and relatively easily scalable processes
- High throughput of products
- Ease of equipment cleaning and sanitation

Although chromatographic systems can provide effective protein purification, they are typically unacceptable at large scale because of the high processing costs and the difficulties associated with the disposal of large quantities of undesirable effluents [Zydney 1998]. Given the attractiveness of membrane systems for large-scale dairy processing, it is not surprising that there has been a number of attempts to use membrane systems for actual whey protein fractionation.

2.4.2 Fractionation of whey proteins and peptides

The current trend in the food industry is to produce special products with a high added value, especially nutraceuticals. Consequently, there is an increasing need for isolation and purification of high-value components (e.g., bioactive proteins, peptides and amino acids) from complex solutions such as whey. Zydney [1998] has reviewed a number of complete separations of whey proteins in model solutions (BSA from lysozyme – selectivity 170; BSA from haemoglobin – 140; IgG from BSA - 50). A principle scheme of whey protein fractionation is presented in Figure 2.3.

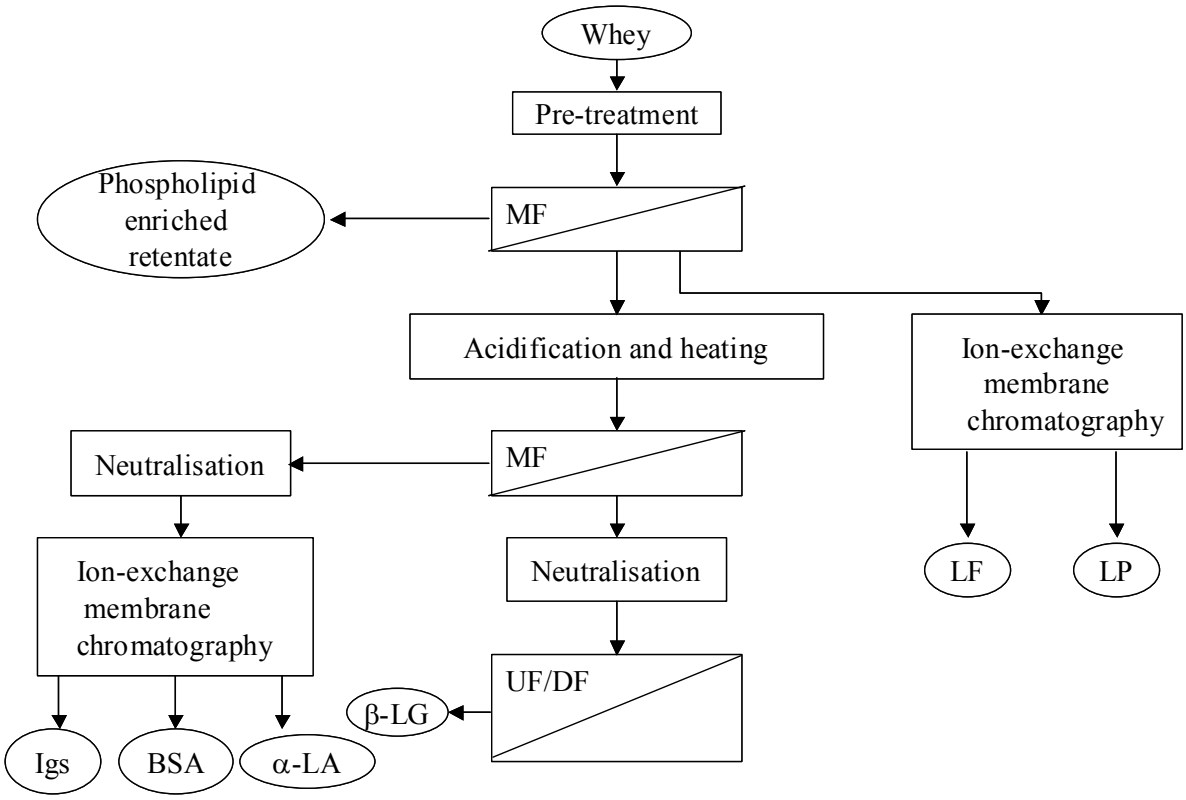


Figure 2.3 Fractionation of whey proteins: immunoglobulins (Igs), bovine serum albumin (BSA), α-lactalbumin (α-LA), β-lactoglobulin (β-LG), lactoferrin (LF), and lactoperoxidase (LP) [Rosenberg 1995, Chiu and Etzel 1997].

Experiments with complex multicomponent mixtures are more limited, e.g., Muller et al. [1999] purified α-lactalbumin from acid casein whey only by 50 %; Bottomley et al. [1991] purified α-lactalbumin from cheddar whey, with a final product having nearly 25 % β-lactoglobulin. Metsämuuronen et al. [2006] reported a result of α-lactalbumin fractionation from 2.65 % (w/v) whey to have a selectivity of 45.

One of the most important recent advances in the separation of small charged biomolecules, like amino acids and peptides, was the development of nanofiltration membranes because of their high selectivity in the separation of components in the molar mass range 300-1000 g mol^{-1} [Martin-Orue 1998]. Nanofiltration fractionation of milk protein enzymatic hydrolysates can lead to peptide mixtures having improved functionality, lower salt content, or simply a modified peptide content [Pouliot et al. 2000]. Bargeman studied the selective separation of α_{s2}-casein fragments (183-207) with proven antimicrobial activity against Gram-

positive and Gram-negative micro-organisms from hydrolysate of α_{s2}-casein using batch-wise membrane filtration [Bargeman 2002]. The positively charged α_{s2}-casein fragment (183-207) was accumulated in the permeate, and a maximum concentration, 25 % in the permeate, was achieved compared to 7.5 % in the initial feed.

2.5 Factors affecting the selectivity of a separation process

Fouling is the major limiting factor at all stages of milk and whey fractionation. Membrane fouling is described as causing an irreversible flux decline and it is different from the concentration polarisation effect, which is reversible [Cheryan 1986].

In the pressure controlled region permeate flux (J_v) can be described using Equation (2.1):

$$J_v = \frac{\Delta P}{\mu R_m} \tag{2.1}$$

where ΔP is the transmembrane pressure, μ is the viscosity of permeate and R_m is the intrinsic membrane resistance determined using pure water as the feed. According to Equation (2.1), flux is directly proportional to the applied pressure and inversely proportional to viscosity. For Newtonian liquids, such as whey (at protein concentrations less than 12 %, w/w) [Carr 2003], viscosity is affected by two factors: the solids concentration and temperature.

If fouling occurs Equation (2.1) can be rewritten to take into account additional resistances associated with fouling:

$$J_v = \frac{\Delta P}{\mu (R_m + R_f)} \tag{2.2}$$

where R_f is the sum of the hydraulic resistances of the irreversible (R_i) and the reversible (R_c) part of the fouling layer. Irreversible fouling can include adsorption/deposition on the membrane surface, internal pore blocking or pore constriction due to adsorption of solute on

the pore wall. Concentration polarisation, gel and cake formation are considered as reversible flux decline.

Fouling is a complex phenomenon affected by three significant groups of factors: feed material properties, membrane material properties and chemical engineering aspects.

2.5.1 Feed material

Almost every component in the feed stream can foul a membrane to some extent. Proteins, lipids, salts, and micro-organisms have been frequently mentioned in the literature. The composition of typical sweet and acid whey streams was presented in Table 2.7. Proteins are known to be the major foulants in ultrafiltration of food and biological streams. Their content in sweet and acid whey (Table 2.7) is 8-9 % (w/w) of dry matter.

Fouling by proteins has been described as a two-step process, including a rapid protein monolayer adsorption and a slower deposition on top of the first deposited layer. Fouling is a result of specific interactions between membrane and protein. Electrostatic interactions, charge transfer and hydrophobic interactions, which are resultants of long range Lifshitz – van der Waals and short range interactions caused by hydrogen bonding, are included.

Protein adsorption is affected by pH and ionic strength. In general, flux is lowest and adsorption is highest at the IEP of protein. The high selectivities seen in the studies reported above were consistently obtained by operating the membrane device near the isoelectric pH of the lower molar mass protein and far from the IEP of the larger molar mass component to maximise the difference in effective hydrodynamic volume. In addition, relatively low salt concentrations (1-20 mM) were used to enhance the magnitude of the electrostatic interactions [Zydney 1998].

Salts and small organic molecules such as sugars, small peptides, amino acids, urea, etc. are characterised by very high osmotic pressure. For sucrose ($M = 342$ g mol^{-1}) at a concentration of 50 kg m^{-3} and 20 ^{o}C the osmotic pressure has been reported to be 0.356 MPa [Belfort et al. 1994]. Mineral salts also have a significant effect on fouling. They can interact with the membrane directly or precipitate on the membrane, which causes a flux decline. For the

cheese whey system in particular, it appears that both the calcium-caseinate complex and the calcium phosphate complex play major roles in membrane fouling [Cheryan 1986]. Some evidence seems to indicate that the removal of lipids by whey centrifugation has a beneficial effect on flux. Also, low molar mass sugars have been reported to cause “crowding” of the membrane pores [Cheryan 1986].

2.5.2 Membrane properties: molar mass cut-off, charge and hydrophobicity

The selectivity of a membrane can be described by a sieving curve. The curve is usually presenting the retention coefficients as a function of molar mass of the solutes. A widely used membrane characteristic is the molar mass cut-off defined as the molar mass of a solute molecule that would be retained at 90 %. However, in order to achieve good selectivity in protein fractionation using only the steric exclusion mechanism very big differences in the sizes of the proteins to be separated are needed. A broad pore size distribution of the asymmetric ultrafiltration membranes negatively affects the selectivity of the fractionation process.

Membranes having fixed charges were shown to be more useful than uncharged membranes because they can repel molecules with the same sign of charge even if the sizes of these molecules are much smaller than the pore size of the membrane. The highest permeate fluxes were obtained in the presence of repulsive electrostatic forces between molecules and the membrane surface [Metsämuuronen 2002]. The main advantage of charged membranes compared to uncharged membranes is that the former may separate charged from uncharged solutes by an electric effect even though they have the same molecular size. The charged nanofiltration membranes were successfully applied for separation of amino acids [Garem et al. 1997, Grib et al. 2000, Gotoh et al. 2004]. Lapointe et al. [2005] have reported a separation of positively charged peptides from β-lactoglobulin hydrolysates using negatively charged G-10 nanofiltration membrane.

Many researchers have followed the idea of increasing the hydrophilicity of a membrane material with a goal of reducing fouling [Brink and Romijn 1990, Nyström 1992, Möckel et al. 1999]. The principle behind this is that hydrophilic surfaces preferentially adsorb water rather than solutes, leaving the membrane surface unchanged. On hydrophobic surfaces

protein molecules tend to unfold and expose their hydrophobic interior sites, which then lead to adsorption due to hydrophobic interactions. Sheldon et al. [1991] studied the adsorption of BSA onto 10 kg mol^{-1} cut-off polysulphone and regenerated cellulose membrane. They observed that the amount of adsorbed protein was three times higher on the polysulphone membrane, than on the regenerated cellulose membrane. The resistance of the polysulphone membrane increased by over 5 times as much as that of the regenerated cellulose membrane. One factor influencing adsorption was the hydrophobic nature of polysulphone. The interaction between BSA and the hydrophobic surface of polysulphone membranes causes the molecule to unfold and expose its hydrophobic sites. Filamentous molecules were not seen in the fouling layer of the regenerated cellulose [Sheldon et al. 1991]. Metsämuuronen et al. [2002] found that regenerated cellulose membrane was less prone to fouling and it had higher critical flux values for both yeast and myoglobin.

2.5.3 Chemical engineering aspects: transmembrane pressure, hydrodynamics of systems

Fouling can be controlled by using the critical flux concept proposed by Field et al. [1995]. According to this concept a flux exists below which a decline of flux with time does not occur. The strong form of critical flux is the flux below which the same transmembrane pressure (TMP) is required to maintain a similar flux as for pure water with the “working” solution (Figure 2.4). Sometimes even though the flux of the “working solution” is lower than the pure water flux it increases linearly with pressure up to a critical value. In that case the point of deviation is called the weak form of critical flux. For optimal selectivity the isolation of whey proteins is restricted to the subcritical regime [Brans et al. 2004].

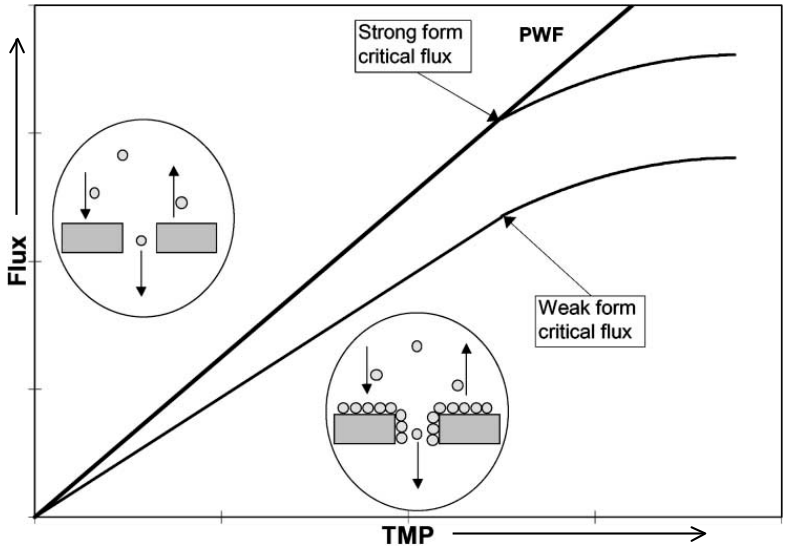


Figure 2.4 Schematic representation of a weak form and a strong form critical flux [adapted from Metsämuuronen et al. 2002].

An increase in pressure above the critical value generates a growth of irreversible deposit until a decrease in flux reaches its limiting value. It is the so-called mass transfer controlled region. In this regime flux can be independent of the transmembrane pressure and the pore size of the membrane, and in the absence of any physicochemical interaction, only the polarisation layer controls the permeate flux. Osmotic pressure and film theory models are used to predict the flux.

Another parameter that is important is the cross-flow velocity. By promoting turbulence (high cross-flow velocities) concentration polarisation can be reduced due to the increase in shear rate. This hinders the proteins from adsorbing onto the membrane and helps in transporting them back to the bulk solution [Muller et al. 2003]. The critical flux was observed to increase with increasing cross-flow velocity and decreasing concentration of solute [Metsämuuronen et al. 2002]. Ghosh [2003] has defined that the selectivity in protein fractionation in ultrafiltration was independent of the cross-flow velocity at very low and very high permeate fluxes. In the intermediate permeate flux range (1 μm s^{-1} to 10 μm s^{-1}), the selectivity increases with an increase in cross-flow velocity.

3 CHARACTERISATION OF ULTRAFILTRATION MEMBRANES

The performance of ultrafiltration membranes is dependent on their molar mass cut-offs. The molar mass cut-off value of a membrane may be defined as the molar mass of the solute, which is to 90 %, retained by the membrane. Manufacturers of membranes usually specify a nominal cut-off for their products. Their cut-off values are often defined in different ways and the results are also dependent on their test conditions such as pressure, circulation flow, apparatus etc [Trägårdh 1985]. However, detailed information regarding the test substance and the test conditions are often not found in the manufacturer specification sheets [Schock et al. 1989].

The molar mass cut-off can be determined by filtration of reference polymer solutes (e.g., proteins, polyethylene glycols or dextrans) of known molar mass at standard conditions. An ideal test substance applied for characterisation should have a narrow molar mass distribution and be a rigid spherical molecule which is unaffected by pH and shear. Proteins, being globular molecules, are generally considered to be the best model substances. However, their conformation is strongly affected by pH, ionic strength and shear. In addition, membrane fouling caused by their adsorption on the membrane is a well-recognised phenomenon in protein filtration. Proteins cannot always be obtained at all types of molar masses to a reasonable price. Dextrans and polyethylene glycols are widely used as test solutes, because they are water-soluble and can be obtained with narrow molar mass distributions.

Some authors recommend the use of a feed solution containing a polymer mixture with a wide molar mass distribution [Norbega et al. 1989]. This procedure allows the determination of the retention coefficients and the molar mass cut-off value of a given membrane by performing only one ultrafiltration experiment. However, this method has a significant disadvantage: when a mixture of polymer molecules of different sizes is filtered, the large molecules may form a dynamic membrane thus apparently increasing the retention of the smaller molecules [Tam and Tremblay 1991, Alargova et al. 1998]. Thus, characterisation of membranes using single polymer solutions of different molar masses is preferred. Automation of test techniques provides comparable testing times to mixed solute characterisation methods.

The objective of this chapter is to check if the cut-off reported by the manufacturers is a realistic value and to verify the use of PEGs for membrane characterisation. Single PEG solution experiments were performed under conditions which minimise concentration polarisation in order to determine the correct value for the molar mass cut-off for the studied membranes (C 5F, C 10F, PES 4H, P 5F, PES 30H and C 30F). That is, the experiments were performed at close to subcritical flux.

3.1 Materials and experimental set-up

In the experimental work the six types of membranes presented in Table 3.1 were investigated.

Table 3.1 Commercial characteristics of the membranes as reported by the manufacturer Microdyn (former NADIR) [Väisänen 2004].

Membrane	Material	Cut-off, kg mol^{-1}	Permeability, L m^{-2} h^{-1} bar^{-1}	pH 20 °C	T_{max}, °C
C 5F	Regenerated cellulose	5	8.3 - 20	1 - 11	70
C 10F	Regenerated cellulose	10	13 - 22	1 - 11	70
C 30F	Regenerated cellulose	30	100 - 200	1 - 11	70
P 5F	Polyethersulphone	5	14.4	0 - 14	95
PES 4H	Polyethersulphone	4	7-17	0 - 14	95
PES 30H	Polyethersulphone	30	35 - 85	0 - 14	95

The measurements were carried out in a cross-flow flat-sheet laboratory-scale ultrafiltration cell made of polycarbonate with a filtration area of 21.6 cm^2 (Figure 3.1). The channel height of the module was 0.001 m, the width 0.02 m, and the effective diameter of the module (d_h) 0.002 m. The two pressure gauges (WIKA) were placed before and after the membrane cell in the retentate loop (range 0-0.5 MPa). The retentate was circulated with a magnetic pump (IsmaTec MV-Pump system, Type MV-Z, Switzerland). In order to measure the retentate flow a flow-meter (Bailey-Fischer-Porter, Germany) was used. The permeate flow was measured by timing and weighting with a high precision electronic balance (Mettler PM 600, GMB).

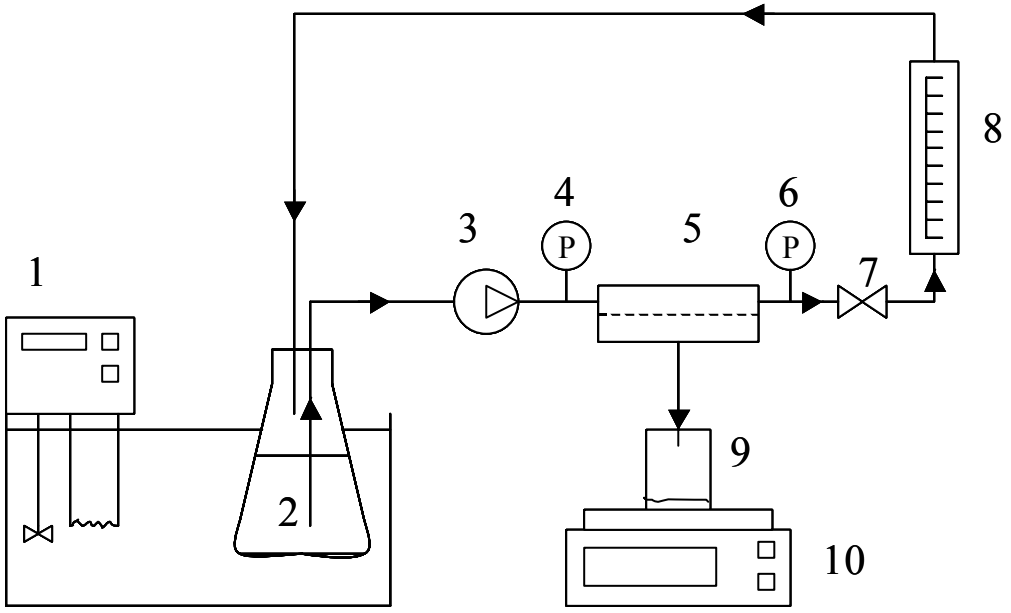


Figure 3.1 Experimental set-up with: 1 thermostat, 2 feed reservoir, 3 magnetic pump, 4 and 6 pressure gauges, 5 membrane cell, 7 needle valve, 8 flow-meter, 9 permeate reservoir, and 10 balance.

All experiments were made at a constant temperatures of 20, 25 or 50 °C, which was kept using thermostat (LAUDA Dr. R. Wobser GmbH&Co. KG). In order to avoid any change during operation the membranes were pressurised at 0.3 MPa for 1 hour at the working temperature (20, 25 or 50 °C, respectively) before being used. This time was enough to obtain a stable pure water flux (PWF). After stabilisation the PWF was measured as a function of transmembrane pressure. In all cases linear dependences of PWF on pressure were observed. The operating conditions used were a cross-flow velocity of 2 m s^{-1}, which corresponds to the turbulent regime (*Re* = 4480). Flux was adjusted to 11.1 μm s^{-1} (40 L h^{-1} m^{-2}), except when the most permeable C 30F membrane was used, then the filtration was carried out at the lowest flux, which was possible to get without applying pressure on the permeate side of the membrane cell.

Aqueous solutions of several polyethylene glycols (PEGs) with molar masses of 0.6, 1.5, 2, 3, 4, 6, 8, 10, 12, 17.5, 20 and 35 kg mol^{-1} at a concentration of 0.2 kg m^{-3} were used. These relatively small concentrations assure minimal solute-solute interactions [Tam and Tremblay 1991]. The PEGs were purchased from Fluka. The PEGs were filtered one at a time and each filtration run was repeated three times, according to the procedure described by Platt et al. [2003]. The solute concentrations were determined by total organic carbon analysis (TOC)

with an automated carbon analyser, Shimadzu. In the permeation experiment, the observed retention coefficient of solute by membrane was calculated using Equation (3.1),

$$R_{obs} = 1 - C_p/C_r \tag{3.1}$$

where C_p and C_r are the solute concentrations of permeate and retentate, respectively.

3.2 Theory

Most ultrafiltration data are so highly influenced by concentration polarisation that a determination of the membrane transport properties is very uncertain [Jonsson 1985]. Concentration polarisation is increasing the solute concentration at the membrane surface. It causes a decrease in permeate flux by a reduction of the transmembrane pressure because of the increase of a counteracting osmotic pressure. The concentration polarisation model used is illustrated in Figure 3.2.

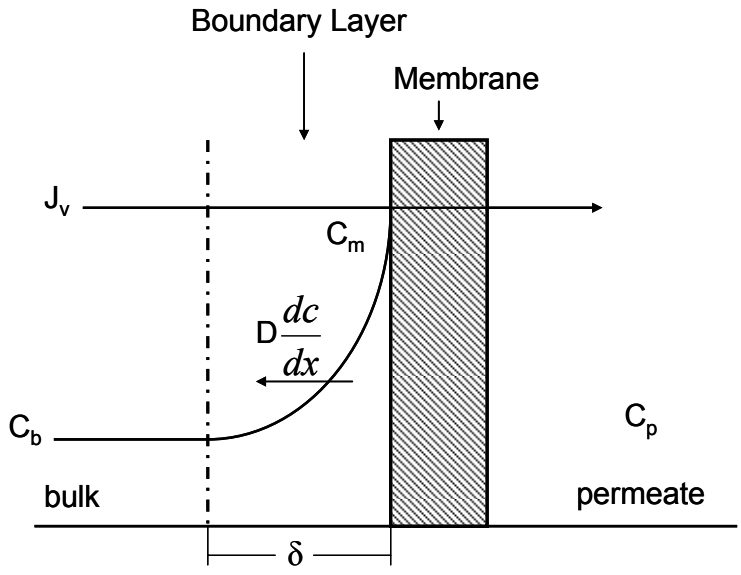


Figure 3.2 Schematic representation of concentration polarisation [Nakao 1994].

Gekas et al. [1993] provide the following differentiation between three types of retention coefficients:

1. The observed retention, $R_{obs} = 1 - C_p/C_r$, where C_p is the measurable permeate concentration and C_r is the measurable retentate concentration. This retention coefficient is dependent on concentration polarisation (external diffusion) and internal diffusion.

2. The actual retention coefficient, $R_m = 1 - C_p/C_m$, where C_m is the solute concentration at the membrane wall ($C_m > C_r$). This retention coefficient is free of concentration polarisation effects but may include contribution of diffusive solute transport across the membrane.

3. The intrinsic or true retention coefficient, R_∞, is the limit of the actual retention coefficient when the internal Peclet number approaches infinity that is in the purely convective case.

The concentration polarisation modulus C_m/C_r can be calculated from the well-known film theory equation:

$$(C_m - C_p)/(C_r - C_p) = \exp(J_v/k) \tag{3.2}$$

where, J_v is the volumetric permeate flux and k is the mass transfer coefficient in the polarised layer. When the solute is completely retained by the membrane ($C_p = 0$) the concentration polarisation Equation (3.2) reduces to $C_m/C_r = \exp(J_v/k)$. Jonsson [2001] reported that the value J_v/k should be in the range of 0.405-0.693 (J_v close to subcritical flux) in order to avoid the formation of a concentration polarisation layer when doing membrane characterisation experiments. This range was also verified to be good in the work reported by Platt et al. [2003].

The mass transfer coefficient can be estimated from correlations for the Sherwood number (*Sh*) in terms of the Reynolds number (*Re*) and the Schmidt number (*Sc*).

In the case of turbulent flow (*Re* > 2000), the Dittus-Boelter correlation can be used [Ghosh 2003]:

$$Sh = 0.023\ Re^{0.8}\ Sc^{0.33} \tag{3.3}$$

$$Sh = k\ d_h/D \tag{3.4}$$

$$Sc = \nu/D \tag{3.5}$$

$$Re = d_h\ \upsilon/\nu \tag{3.6}$$

where, ν is the kinematic viscosity ($m^2\ s^{-1}$), d_h is the module effective diameter (m), D is the solute diffusion coefficient ($m^2\ s^{-1}$) and υ is the cross-flow velocity ($m\ s^{-1}$).

3.3 Results and Discussion

3.3.1 Characterisation at standard conditions

The diffusion coefficients of the PEGs used were calculated for the same temperature using the relation between diffusion coefficient (D) and molar mass (M) given by Pradanos et al. [1992]:

$$D = 9.82*10^{-9}\ *M^{-0.52} \tag{3.7}$$

According to the CHARMME project [2001] the optimal operating regime, which allows for minimising fouling was found to apply at the constant flux 11.1 $\mu m\ s^{-1}$ (40 $L\ h^{-1}\ m^{-2}$). This value was chosen for the calculation of J_v/k for all PEGs. Table 1 shows the calculated D, *k* and J_v/k values. The results show that for most of the PEGs this flux value will give a J_v/k within the range suggested by Jonsson [2001].

Table 3.2 The diffusion coefficients, mass transfer coefficients and J_v/k values for the PEGs used in the experiment. The effective diameter of the module was 0.002 m, the cross-flow velocity 2 m s^{-1}, and the temperature 20 ^{o}C.

Molar mass, kg mol^{-1}	D, 10^{-11} m^2 s^{-1}	k, 10^{-5} m s^{-1}	J_v, 10^{-6} m s^{-1}	J_v/k
0.6	35.3	4.49	11.1	0.25
1.5	21.9	3.26	11.1	0.34
2	18.9	2.95	11.1	0.38
3	15.3	2.56	11.1	0.43
4	13.2	2.32	11.1	0.48
6	10.7	2.01	11.1	0.55
8	9.2	1.82	11.1	0.61
10	8.2	1.68	11.1	0.66
12	7.4	1.58	11.1	0.70
17.5	6.1	1.39	11.1	0.80
20	5.7	1.32	11.1	0.84
35	4.3	1.09	11.1	1.02

The sieving curves generated by the six membranes used are shown in Figure 3.3. The transmembrane pressures applied for the studied membranes in constant flux experiments and determined versus nominal molar mass cut-off values of these membranes are presented in Table 3.3.

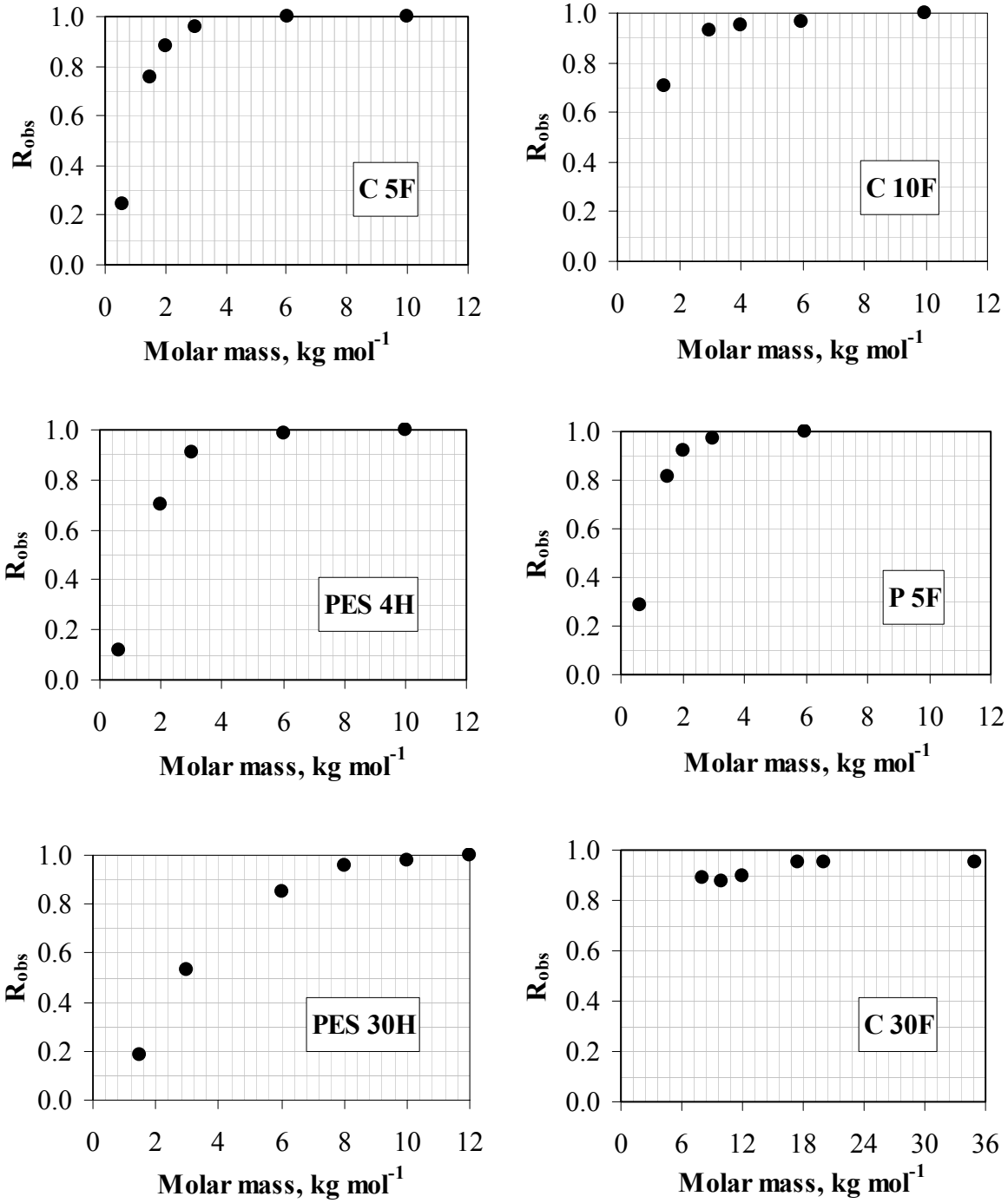


Figure 3.3 Sieving curves for the studied membranes: C 5F, C 10F, PES 4H, P 5F, PES 30H and C 30F. J_v = 11.1 μm s^{-1}, T = 20 ^{o}C, and υ = 2 m s^{-1}.

Table 3.3 Characteristic performance of ultrafiltration membranes used in this study during constant flux experiments (T = 20 ^{o}C, υ = 2 m s^{-1}).

Membrane	Pressure (MPa), corresponding to the permeate flux 11.1 μm s^{-1}	Measured molar mass cut-off, kg mol^{-1}	Molar mass cut-off reported by manufacturer, kg mol^{-1}
C 5F	0.191	2	5
C 10F	0.185	2.5	10
PES 4H	0.235	3	4
P 5F	0.163	2	5
PES 30H	0.05	7	30
C 30F	0.025	12	30

The comparison of measured molar mass cut-offs and nominal cut-offs given in Table 3.3 shows that the experimental values were lower. The cut-off for the C 30F membrane was determined as 12 kg mol^{-1}, because then retention is about 90 %. The sieving curve for the C 30F membrane deviates from the others. This could be explained by the wide pore size distribution typical for this membrane. About 5 % of the pores have a size, which is large enough to pass the polyethylene glycol molecules with a molar mass of about 35 kg mol^{-1} through it. Metsämuuronen et al. [2005] reported that the C 30F membrane did not show any dependency between the critical flux and the solute flux. Thus, they made an assumption that the broad pore size distribution of this membrane was responsible for it.

In all cases the cut-offs were lower (retention was higher) than those quoted by the manufacturer. The high measured retention could have been the result of either pore blocking/fouling or concentration polarisation. If so, pore blocking reduces the number of pores having high permeability leaving only smaller pores open for transport. In the reported experiments, though, the pure water flux was measured before each PEG filtration (Figure 3.4 and 3.5), and as seen from these figures the pure water flux was more or less constant and no flux decline was observed. Therefore, the assumption of pore blocking seems not to be valid in this case.

Fouling of membranes by PEG solutions might also have been the reason for the higher observed retention coefficients. A membrane surface and pores altered by fouling result in permeate flux decline. However, polyethylene glycols are known to exhibit low membrane-

solute interactions at neutral pH [Tam and Tremlay 1991]. As was also shown before (Figure 3.4 and 3.5) the clean water flux did not show a decline, and thus irreversible fouling did not occur during the PEG experiments.

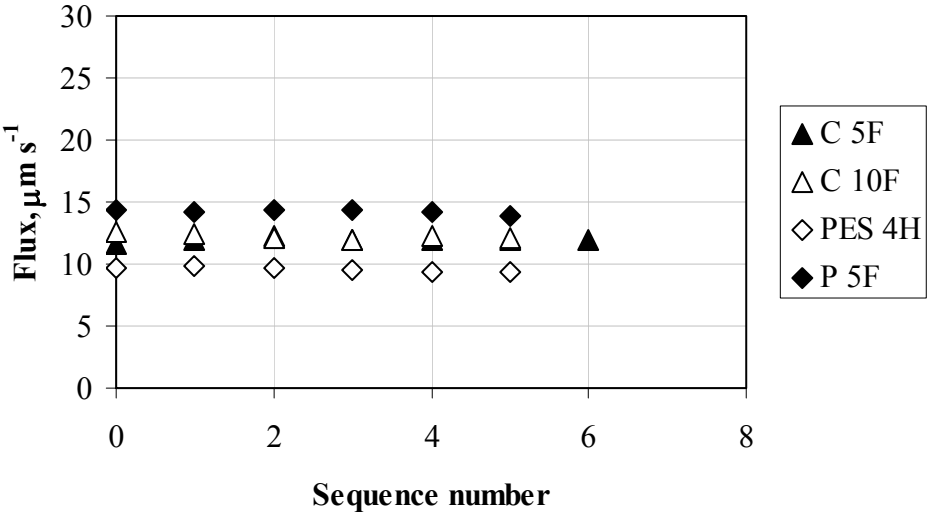


Figure 3.4 Pure water flux before each PEG stage for four different membranes measured at a pressure of 0.2 MPa, T = 20 °C, and υ = 2 m s^{-1}.

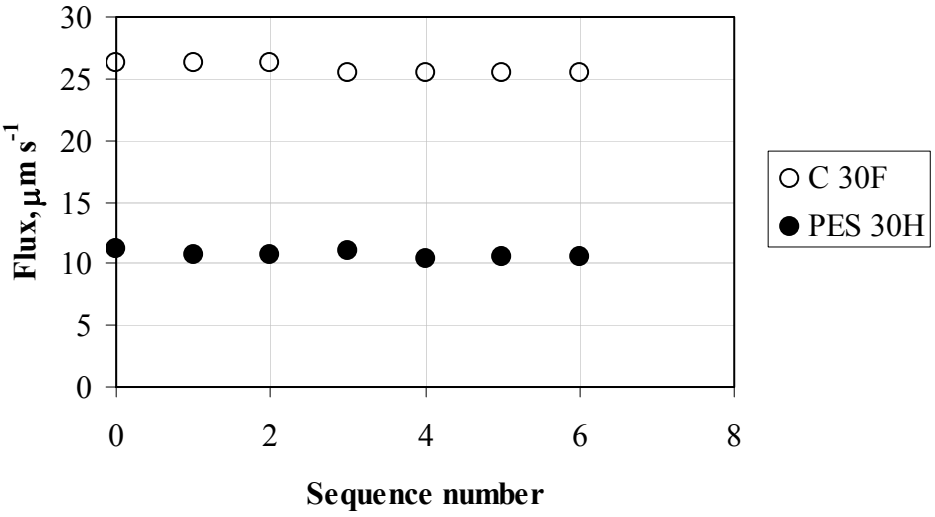


Figure 3.5 Pure water flux before each PEG stage for C 30F and PES 30H membranes measured at pressures of 0.025 MPa and 0.05 MPa, respectively. T = 20 °C, and υ = 2 m s^{-1}.

Concentration polarisation is the third factor, which could explain the higher retention. However, concentration polarisation, as was mentioned above, should result in a permeate

flux decline. Flux reduction was not observed during the PEG experiments though the filtration was carried out for one hour. This proves that the selection of the operating parameters, such as flow velocity (2 m s^{-1}) and flux (11.1 μm s^{-1}) at the given module configuration (d_h = 0.002 m) is very important in the minimisation of concentration polarisation (C_m/C_b).

It was, therefore, considered that the cut-off values measured were the true membrane cut-offs. Earlier, discrepancies between the measured membrane cut-off and the value reported by the manufacturer were also observed by Schock et al. [1989]. These authors have been performing the molar mass cut-off analyses for the CA 10, G 20, G 50, CA 30 and CA 100 membranes (from Kalle and DSI) by using 1 % (w/v) dextran solutions containing 0.30 % (w/v) sodium chloride under the following conditions: pH 7.1, temperature of 25 ^oC, pressure of 0.05 MPa and stirrer speed 600 min^{-1}. The conditions applied by Schock et al. [1989] were enabling them to minimise concentration polarisation, and the molar mass cut-off values determined were 2 kg mol^{-1} (CA 10), 3 kg mol^{-1} (G 20), 5 kg mol^{-1} (G 50), 9 kg mol^{-1} (CA 30), and 45 kg mol^{-1} (CA 100), respectively.

Pore size distribution data were collected for four pieces of C 10F membrane (Figure 3.6). It can be seen that for two membrane pieces the measured cut-offs were 3 kg mol^{-1} and for two other pieces the cut-offs were close to 2.5 kg mol^{-1}. All PEG filtrations were repeated three times at the same conditions (flux = 11.1 μm s^{-1}, T = 20 ^oC, cross-flow = 2 m s^{-1}) for each membrane piece. The standard deviations among experiments with the same membrane piece were small (< 0.9 % for PEGs 4, 6 and 10 kg mol^{-1}, and < 2 % for PEGs 1.5 and 3 kg mol^{-1}).

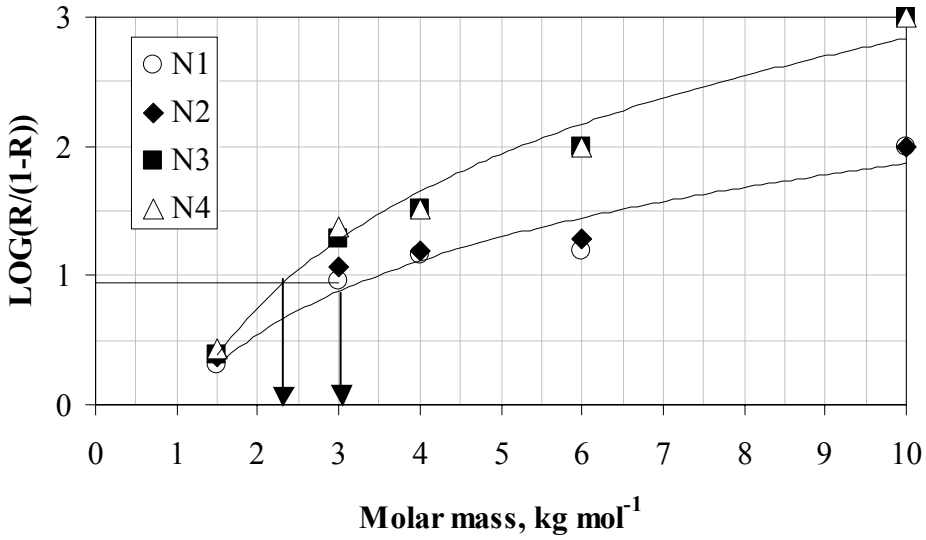


Figure 3.6 Average cut-off curves for 4 pieces of C 10F membrane (N1, N2, N3 and N4) using PEG as indicator of retention. Operating conditions: J_v = 11.1 μm s^{-1}, T = 20 °C, and υ = 2 m s^{-1}.

Concentration polarisation could explain the higher retention observed for two of the membrane pieces. However, concentration polarisation results in flux reduction, which was not found in any PEG filtration run. Thus, it was concluded that the difference in the cut-offs between these pieces of C 10F membrane was the result from the membrane not being very homogeneous.

3.3.2 Effect of temperature on retention characteristics of C 10F membrane

The regenerated cellulose C 10F membrane was chosen from the set of characterised membranes for further work. The selection of this membrane is based on several factors. First, a membrane with a molar mass cut-off of 10 kg mol^{-1} is traditionally used in the dairy industry for the production of whey protein concentrates (WPC). The second reason is its hydrophilicity, which prevents protein adsorption and fouling. Protein adsorption is a well-recognised fouling factor in filtration of various streams used in the dairy industry. Thus, both the cut-off and the non-fouling characteristics of the C 10F membrane should be ideal.

A temperature of 50 ^{o}C is commonly used in filtration of streams produced in the dairy industry. This temperature prevents bacterial growth and also enhances the flux because of the reduction of viscosity. Therefore, the study of the impact of high temperature on retention characteristics of the C 10F membrane is important. Two different temperatures were used, 25 ^{o}C and 50 ^{o}C. The characterisation method described in section 3.1 was used which enables working at constant mass transfer conditions. The operating constant flux conditions used were: cross-flow velocity = 2 m s^{-1} and flux = 11.1 μm s^{-1} (40 L m^{-2} h^{-1}). The results are presented in Figure 3.7

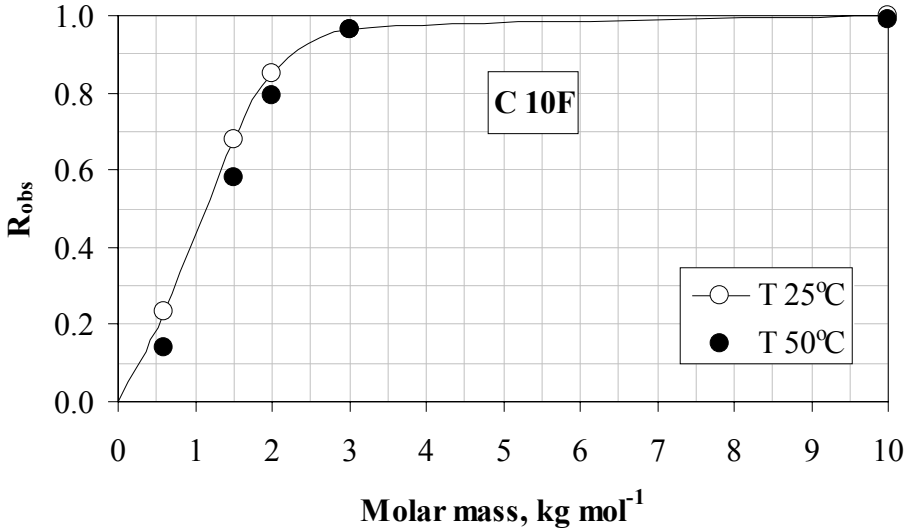


Figure 3.7 Determination of molar mass cut-off of the C 10F membrane at two temperatures using PEG solutions. Operating conditions: J_v = 11.1 μm s^{-1}, and υ = 2 m s^{-1}.

Figure 3.7 shows that a molar mass of 2.5 kg mol^{-1} corresponded to a 90 % retention of the PEG solution and was thus considered as the cut-off of the membrane at both temperatures. The observed retention coefficients for polyethylene glycols with molar masses up to 2 kg mol^{-1} at 50 ^{o}C were lower than at 25 ^{o}C. According to the data provided by the manufacturer (Table 3.1) the C 10F membrane is resistant to high temperature up to 70 ^{o}C. Therefore, the difference in retention of polyethylene glycols at the two temperatures studied was thought to depend on the intrinsic properties of the solute. An increase in diffusion coefficients of these PEGs with temperature could explain the decrease in their retention. According to the data published by Chin et al. [1991] the diffusion coefficients of

polyethylene glycols were found to increase linearly with temperature but decrease exponentially with molar mass.

3.4 Conclusion

For all membranes investigated the measured cut-offs were smaller (2 kg mol^{-1} (C 5F), 2.5 kg mol^{-1} (C 10F), 3 kg mol^{-1} (PES 4H), 2 kg mol^{-1} (P 5F) and 7 kg mol^{-1} (PES 30H)) than the values reported by the manufacturers. The values calculated to be used for flux (11.1 μm s^{-1}) and cross-flow velocity (2 m s^{-1}) so that concentration polarisation was at a minimum proved to be correct since no concentration polarisation was observed during any of the PEG filtrations.

The molar mass cut-off value for the C 10F membrane, selected for further research work, was found not to change when increasing the temperature from 25 ^{o}C to 50 ^{o}C. However, the retention coefficients of polyethylene glycols with molar masses up to 2 kg mol^{-1} were found to decrease at enhanced temperature. This behaviour was explained by increased diffusivity of low molar mass polyethylene glycols with temperature, and it will affect the membrane molar mass cut-off value if it falls in this range.

4 SEPARATION OF WHEY PROTEIN CONCENTRATES BY UF UNDER SUBCRITICAL CONDITIONS

Ultrafiltration is the method widely used for protein diafiltration, clarification, and concentration. The main problem in concentration of proteins is to get a satisfactory permeate flux because it tends to decrease with an increase in protein concentration. At high concentrations proteins tend to form gels. According to the results presented by Jonsson [1984] a gel formation during whey filtration occurs when the wall protein concentration reaches 37 % ("gelation concentration"). Taking into account the high fouling potential of proteins the examination of subcritical conditions for whey filtration is very important.

An ideal membrane for protein concentration should totally retain the proteins while allowing a high water permeability through it. According to Table 2.2 (Chapter 2) the molar masses of whey proteins are in the range 900 to 14.2 kg mol^{-1}. The selected C 10F membrane has a molar mass cut-off value of 2.5 kg mol^{-1}, which is lower than the molar mass of the smallest whey protein (α-lactalbumin). However, a particular membrane can show quite different retention characteristics for two solutes having the same molar mass. As has been mentioned earlier, the proteins are quite sensitive to the environmental conditions (e.g., pH value, salt concentration, and presence of other proteins in solution). The retention of proteins may also be altered by adsorption and fouling [Ghosh 2003].

The objective of this chapter was to study the relevance of the critical flux concept in ultrafiltration of the sweet whey. The hydrophilic regenerated cellulose C 10F membrane with a molar mass cut-off of 2.5 kg mol^{-1} was used to concentrate proteins in sweet whey. The sieving properties of this membrane to the different whey components were studied. In addition, the molar mass distribution of peptides in the permeates obtained after ultrafiltration of the sweet whey was determined using the matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS).

4.1 Experimental

4.1.1 Whey preparation

Whole milk pasteurised and enriched in calcium produced by Valio (Finland) was used for the preparation of whey samples according to the procedure described below. First, the milk was coagulated using a rennet/chymosin mixture (Sigma Chemical Co.) at +35 ºC for 12 hours. Then the whey solution was separated from the coagulated casein micelles by filtration through a 60-100 mesh (mesh openings 275 – 160 μm) cotton material. The results on the average physico-chemical analyses of the two separately prepared whey samples are presented in Table 4.1.

Table 4.1 Basic physico-chemical properties of whey samples prepared in this study.

Protein content, kg m^{-3}	8.50 ± 0.22
Lactose, kg m^{-3}	52.0 ± 0.50
Ash, kg m^{-3}	5.60 ± 0.16
Dry matter, kg m^{-3}	67.3 ± 0.07
Viscosity (μ), mPa s at 25 ^{o}C	1.10 ± 0.03
pH at 25 ^{o}C	6.6 ± 0.1

4.1.2 Ultrafiltration of whey processed from milk in this study

Before filtration the membranes were compressed under a pressure of 0.3 MPa for 1 hour at a temperature of 50 ^{o}C. A working temperature of 50 ^{o}C was chosen in order to prevent bacterial growth and to obtain conditions as close as possible to industrial operation. The flow rate was set to 2 m s^{-1} (Reynolds number = 4480), which means a slightly turbulent flow. The pure water fluxes (PWF) through the regenerated cellulose C 10F membrane were measured under the working conditions (T = 50 ^{o}C, υ = 2 m s^{-1}) in the pressure range 0.05-0.3 MPa both before and after filtration of whey solution and were used as indicators of membrane fouling.

The observed sieving coefficients (S_{obs}) for the whey constituents were calculated according to:

$$S_{obs} = C_p/C_{r,} \quad (4.1)$$

where C_p is the concentration in the permeate and C_r is the concentration on the feed/retentate side of the membrane.

4.1.3 Analyses of carbohydrates, ions and proteins/peptides

The carbohydrate content was determined using the Anthrone (9,10-dihydro-9-ketoanthracene) reaction, as described by Hodge et al. [1962]. Lactose was used as a reference substance due to the dominant role of this disaccharide in milk/whey solutions. Anions were analysed by ion chromatography and metal ions were analysed by atomic absorption chromatography (AAS). The protein content was determined by Lowry's method using bovine serum albumin (BSA) as the reference [Lowry et al. 1951]. The total content of peptides/amino acids was determined by the ninhydrin method as α-amino nitrogen (AAN) [Diamond and Denman 1973, Friedman 2004]. Dry matter was determined by drying in an oven at 105 ºC overnight. Ash was then determined in the samples by burning in a muffle furnace at 550 ºC for 5 hours. Viscosity and pH measurements were conducted at a temperature of 25 ºC. Viscosity was measured using a capillary Ostvald viscosimeter.

4.1.4 Reversed phase chromatography of whey concentrate and permeate fractions

Reversed phase chromatography analyses were performed on a Jupiter 5u C18 300 Å (150 x 4.6 mm) (Phenomenex) column using a Shimadzu HPLC system. The analyses were performed at 35 °C with a flow rate of 16.7 μL s^{-1}, using a linear acetonitrile gradient, starting with 75 % (v/v) of buffer A (Milli-Q water, 0.1 % (v/v) trifluoroacetic acid (TFA)) ending at 55 % (v/v) of buffer B (acetonitrile, 0.1 % (v/v) TFA) in 20 minutes. Then the 55 % (v/v) of buffer B was kept for 5 minutes. After analysis the column was equilibrated for 10 minutes with buffer A. The column was calibrated with the whey protein standards (20 to 400 g m^{-3}):

α-lactalbumin, β-lactoglobulin, lactoferrin, BSA, and lysozyme (all from Sigma). The signal was monitored at 280 nm.

4.1.5 Peptide characterisation by SEC and MALDI-TOF MS

For peptide purification two subsequent steps were carried out. In the first purification step by size exclusion chromatography on a Superose 12 (10 x 300 mm) column (GE Healthcare) whey permeate samples were separated to three fractions. The fractions were eluted with 10 mM ammonium acetate at a pH of 6.0 and a flow rate of 8.3 μL s^{-1}, because low molarity volatile buffers are good for desalting of the fractions collected from the column [Zhao et al. 1997, Hancock and Prestidge 1987], and they were needed for the following MS analysis. The elution was monitored at 220 and 280 nm. The molar mass markers were dextran blue (2000 kg mol^{-1}), lactalbumin (14 kg mol^{-1}), aprotinin (6.5 kg mol^{-1}) and L-pro-leu-gly (280 g mol^{-1}). The fractions were collected from at least ten chromatographic runs followed by lyophilisation.

The analysis of peptides by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has recently developed into a valuable tool in the biosciences for obtaining both accurate mass determinations and primary sequence information. For that purpose, the dried samples were solubilised in 1 mL of water containing 0.1 % (v/v) trifluoroacetic acid and diluted with the same solution. Millipore ZipTip pipette tips packed with reversed phase resin were used for purification and concentration of peptides prior to MALDI mass spectrometry. Aliquots of 5 μL were added to 5 μL of matrix. Alfa-cyano-4-hydroxy cinnamic acid resuspended in distilled water was used as the matrix. 1 μL of the resulting mixture was deposited on the MALDI-TOF sample holder. It is important that neither the matrix nor the analyte precipitates when the two solutions are mixed. Once the sample is applied to the sample support, the sample is allowed to evaporate. Non-volatile solvents are to be avoided because they can interfere with the crystal growth. MALDI mass determination was carried out using a Voyager-DE time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA) following procedures described by Fedele et al. [1999]. Once inserted in the mass spectrometer, a 337-nm nitrogen laser pulse is applied to each individual spot thus ionising molecules of the matrix, which in turn transfers a proton to the peptides.

The peptide ions are accelerated under 25 kV through the flight tube under vacuum and in most cases in a reflector mode, which basically makes the flight path longer than the actual tube, and the ions arrive at the detector based on their mass to charge ratio (M/Z). Using calibration peptides, the actual masses of the peptides are determined. Each charge-to-mass spectrum is the sum of 100 laser pulses. The collected data was analyzed using the ProTS Data™ software (Efeckta Technologies Corporation, Steamboat Springs, CO, USA).

4.2 Results and Discussion

4.2.1 Critical flux of sweet whey

Figure 4.1 presents the plot of flux as a function of transmembrane pressure for the prepared whey solution and for pure water. The fluxes of the whey solution at the same applied pressure were lower than the fluxes of pure water at all measured pressures. A linear dependence of flux on transmembrane pressure was found with the whey solution in the region under 0.2 MPa (Figure 4.1). The behaviour of flux would imply a critical flux at 0.2 MPa. This type of critical flux could be called the weak form according to the classification given by Field et al. [1995]. Possible explanations for this lower flux include [Metsämuuronen 2003] adsorption of proteins and viscosity effects. Irreversible adsorption could not have taken place in this case, because the pure water fluxes before and after the experiments were the same. Therefore, other reasons to the deviation from pure water flux in the pressure dependent regime have to be sought.

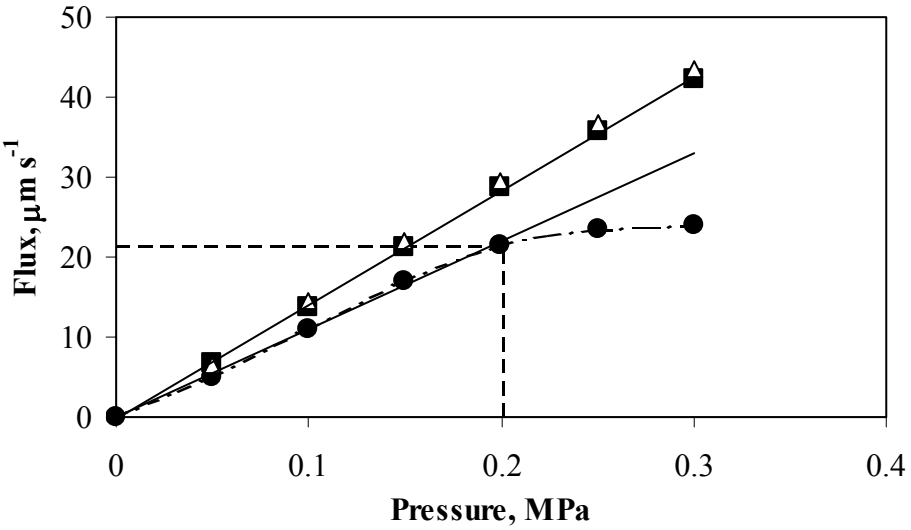


Figure 4.1 Critical flux behaviour of sweet whey. Flux is shown as a function of transmembrane pressure for: (–△–) the filtration of water at the beginning of the experiment, (--●--) the flux of prepared sweet whey, and (■) the flux of water at the end of the experiment. Cross-flow velocity of 2 m s^{-1}, T = 50 °C.

4.2.2 Viscosity correction of water flux to simulate the natural solvent

In the pressure controlled region viscosity could be one reason for the discrepancy between the pure water flux and the flux of the whey solution. As shown in Table 4.1 the whey contained 8.5 kg of proteins per 1 m^3 and the viscosity was 1.10 mPa s, while the viscosity of pure water measured at the same conditions is 0.89 mPa s. Figure 4.2 presents a model correction for viscosity of the pure water flux curve to simulate the background natural solvent for whey containing ions and lactose. When the correction for viscosity has been done the experimental points in the pressure controlled region fit to the line corresponding to pure water. The pressure, 0.2 MPa, corresponding to a flux of 22 µm s^{-1} (79.2 L m^{-2} h^{-1}) is, therefore, the critical point, and above this point an increase in pressure is observed to have less effect on flux. This region was identified as the mass-transfer controlled region of whey protein in natural solvent.

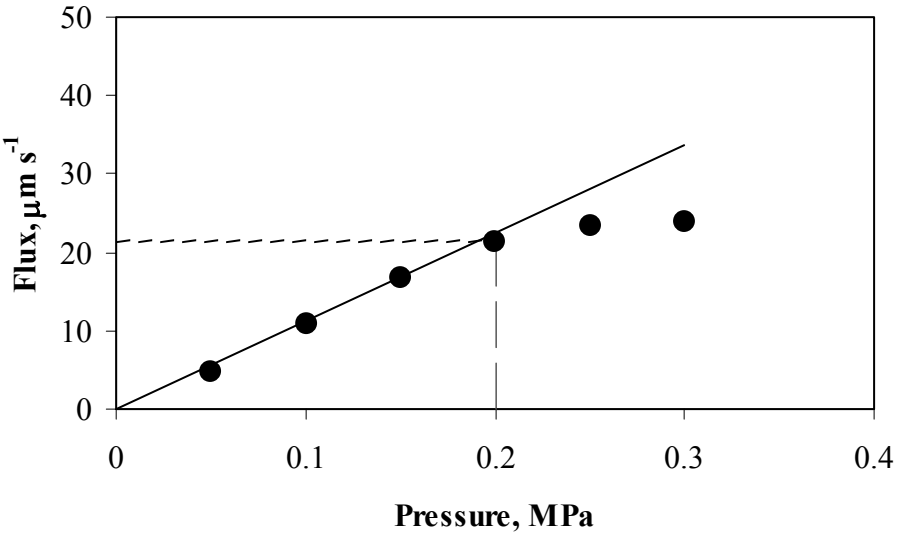


Figure 4.2 Critical flux behaviour of sweet whey after a viscosity correction made for the pure water flux. Flux as function of transmembrane pressure for (–) the flux of water after a viscosity correction, (•) the flux of sweet whey. Cross-flow velocity = 2 m s^{-1}, T = 50 °C.

4.2.3 Concentration of whey under subcritical conditions

For ultrafiltration (Figure 4.3) the pressure was adjusted to a value of 0.1 MPa that gives a flux for sweet whey of 11.1 µm s^{-1} (40 L m^{-2} h^{-1}). The pressure was kept constant at all times of the concentration experiment. Figure 4.3 shows that no significant changes occurred in the flux of the prepared sweet whey for more than 3 hours. The filtration was stopped when a volume reduction factor (VRF) of 2 had been reached.

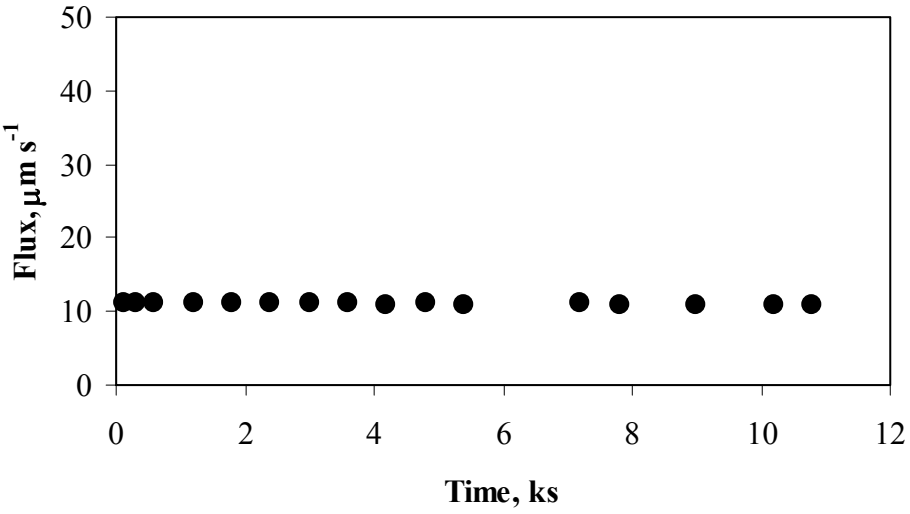


Figure 4.3 Flux of sweet whey solution versus time during a 10.8 ks (3 hours) concentration experiment up to a VRF = 2. Operating conditions: T = 50 °C, υ = 2 m s^{-1}, transmembrane pressure = 0.1 MPa.

4.2.4 Sieving coefficients of components in whey samples from ultrafiltration

The analysis of the fractionated whey is shown in Table 4.2. The concentration of whey proteins in the permeate was negligible compared to the protein concentration of the retentate. As expected, proteins could not penetrate the C 10F membrane, and therefore, they accumulated in the retentate. According to the protein concentrations measured by Lowry's method, the ratio of proteins in the feed solution before filtration (8.5 kg m^{-3}), to that after (17.3 kg m^{-3}), was estimated to be equal to 2. The values of viscosity for the permeate and the retentate were found to differ from each other by 19 %. The ninhydrin method gave more precise values of the peptide and amino acid contents of whey permeate samples than the Lowry method. Ninhydrin reacts with free amino groups, while in Lowry's method only peptide bonds containing an aromatic amino acid (particularly tyrosine or tryptophan) participate in the reaction [Diamond and Denman 1973].

Table 4.2 Characteristics of prepared sweet whey retentate and permeate samples collected after a two-fold ultrafiltration concentration experiment at subcritical flux, 11.1 μm s^{-1}, P = 0.1 MPa, using C 10F membranes and the observed sieving coefficients for the different whey constituents (mean of 2 experiments).

Component	Retentate (C_r)	Permeate (C_p)	$S_{obs} = C_p/C_r$
Proteins, kg m^{-3}	17.3 ± 1.3	0.40 ± 0.02	0.02
Peptides (AAN), kg m^{-3}	3.62 ± 0.31	1.00 ± 0.01	0.28
Carbohydrates (as lactose), kg m^{-3}	50.6 ± 1.2	51.0 ± 0.04	1.01
Citrate, kg m^{-3}	2.01 ± 0.09	1.62 ± 0.08	0.80
Mineral content, kg m^{-3}:			
Calcium	0.39 ± 0.03	0.22 ± 0.03	0.57
Chloride	1.03 ± 0.01	0.98 ± 0.07	0.94
Magnesium	0.11 ± 0.03	0.09 ± 0.00	0.80
Phosphate	0.95 ± 0.10	0.83 ± 0.01	0.88
Potassium	1.67 ± 0.08	1.68 ± 0.12	1.01
Sodium	0.45 ± 0.02	0.41 ± 0.01	0.90
Sulphate	0.28 ± 0.05	0.13 ± 0.00	0.47
Ash, kg m^{-3}	6.12 ± 0.11	6.10 ± 0.06	
Viscosity, mPa s at 25 ^{o}C	1.22 ± 0.02	0.99 ± 0.01	

Peptides, free amino acids and compounds that are consisting of non-protein nitrogen, e.g., nucleotides, creatin, creatinine, urea, etc., were able to penetrate the membrane because of their low molar masses. In opposite to the behaviour of large protein molecules, lactose was found to be similarly distributed between the retentate and permeate sides of the C 10F membrane, and consequently, the sieving coefficient for this carbohydrate was close to unity. Monovalent ions were shown to have sieving coefficients close to unity.

Among the mineral constituents calcium and sulphate ions showed the strongest tendency to accumulate on the retentate side of the membrane. A probable explanation of the higher content of these two types of ions compared to others in the retentate could be the formation of sparingly soluble calcium sulphate salts. Even though a flux decline due to calcium

deposition was not found in this study, calcium precipitation could be a serious problem in prolonged filtrations. In addition, whey proteins are reported to possess mineral binding activity, e.g. α-lactalbumin is reported to be a strong binder of calcium [Creamer and MacGibbon 1996]. As a conclusion, a further desalting of the feed/retentate solution as well as a reduction of its lactose content using C 10F membrane can be reached by conducting the filtrations in the diafiltration mode.

4.2.5 Characterisation of whey proteins and peptides in ultrafiltration retentate and permeate samples

Reversed phase chromatographic profiles of whey retentate and permeate samples after ultrafiltration are shown in Figures 4.4a and 4.4b. Single whey proteins were applied to evaluate the retention time of these proteins running a linear gradient from 25 to 55 % (v/v) of buffer B (acetonitrile, 0.1 % (v/v) TFA) for 1.2 ks (20 minutes). The retention times of the standards were used for further identification of the proteins in the whey samples (Table 4.3).

Table 4.3 Retention times of individual major whey proteins in standard solutions (mean of 5 measurements) and peak heights (A_{280}) corresponding to these retention times in whey ultrafiltration retentate (r) and permeate (p) samples eluted from a reversed phase Jupiter C_{18} column. Samples were collected after a two-fold concentration of sweet whey at 0.1 MPa and 11.1 μm s^{-1} (40 L m^{-2} h^{-1}) (subcritical flux) using the C 10F membrane. See also Figure 4.4a.

N	Standards	Retention time (R_t), ks	Peak height, mAU (at 280 nm)		Molar mass, kg mol^{-1}
			Retentate	Permeate	
1	Lysozyme	0.82 ± 0.01	5.77	–	14
2	BSA	0.94 ± 0.01	12.7	–	67
3	Lactoferrin	0.97 ± 0.01	105	–	77
4	α-lactalbumin	0.99 ± 0.01	659	–	14
5	β-lactoglobulin	1.10 ± 0.01 1.11 ± 0.01	385 320	– –	18 chain B 18 chain A

Analysis of the data presented in Table 4.3 and Figure 4.4b led to the conclusion that the major identified whey proteins were not present in the permeate samples obtained after ultrafiltration. Other large whey proteins such as immunoglobulins (M = 150 kg mol^{-1}) and lactoperoxidase (M = 86 kg mol^{-1}) which are present in minute concentrations were thought not to be able to penetrate the membrane due to their great size compared to the membrane pores and they were not analysed in this study. Caseinomacropeptide (M = 8 kg mol^{-1}) was not identified in this study either (it does not absorb UV light at 280 nm), but it was also suggested to be rejected by the applied ultrafiltration membrane. According to the data reported [Kawasaki et al. 1993] caseinomacropeptide at neutral pH exists in the form of oligomers (trimers to pentamers). In our experiment a neutral pH was used, and thus the hypothesis of oligomer formation seems to be valid. The distribution of standard molecules on the reversed phase column was found be affected by the hydrophobicity of the separated molecules rather than by their molar mass. Comparison of the amino acid composition of the identified proteins showed that lysozyme, which was eluted the first, had the lowest content of hydrophobic amino acids among the standard proteins [SWISS-PROT database]. Several small peaks were eluted at the initial parts of the chromatogram both in the retentate and permeate samples (Figure 4.4). These peaks were suggested to correspond to relatively hydrophilic protein related molecules.

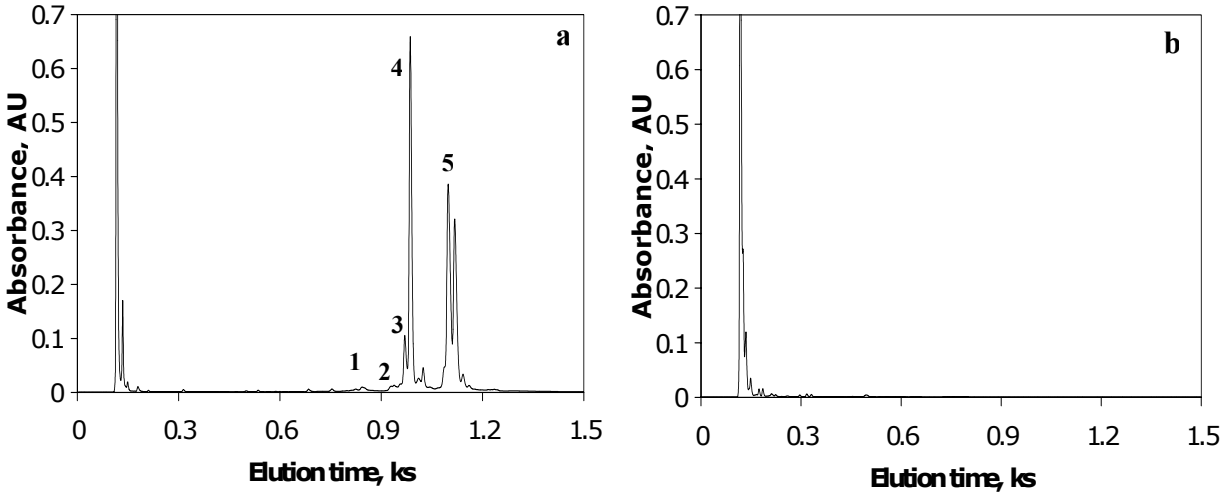


Figure 4.4 Chromatographic profiles of whey retentate (a) and permeate (b) samples eluted from a Jupiter C_{18} (4.6 x 150 mm) column using a linear acetonitrile gradient, starting with 75 % (v/v) of buffer A (Milli-Q water, 0.1 % (v/v) TFA) ending with 55 % (v/v) of buffer B (acetonitrile, 0.1 % (v/v) TFA) in 1.2 ks (20 minutes). Then the 55 % (v/v) of buffer B was kept for 0.3 ks (5 minutes). Analyses were performed at 35 °C with a flow rate of 16.7 μL s^{-1}. UV-detection was monitored at 280 nm. See Table 4.3 for peak identification.

The permeates obtained after ultrafiltration were further investigated by size exclusion chromatography (SEC) in order to identify the protein related molecules in Figure 4.4b. However, due to the low ionic strength of the eluent used, significant hydrophobic interactions take place between the column matrix and the peptides, resulting in longer elution times and lower (observed) molar masses. Even though, it is not possible to obtain an accurate molar mass from this SEC chromatogram. Figure 4.5a shows that the applied conditions give a possibility to resolve three amine-containing fractions from the permeate samples. These fractions were collected and analysed for their molar mass by MALDI-TOF MS. The MALDI-TOF spectrum for the second fraction collected from size exclusion chromatography is shown in Figure 4.5b. The mass values for various peaks in the MALDI-TOF spectra of peptide fractions collected from the size exclusion column are reported in Table 4.4.

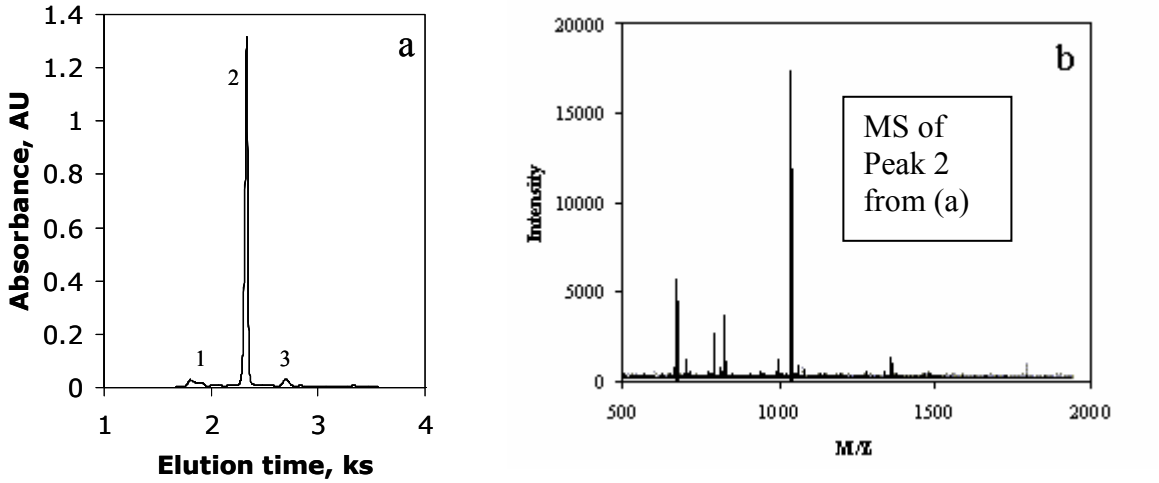


Figure 4.5 (a). Chromatographic profiles of permeate samples eluted from a Superose 12 (10 mm x 300 mm) column using 10 mM ammonium acetate with a pH of 6.0 at a flow rate of 8.3 μL s^{-1}. UV-detection was monitored at 280 nm. (b). MALDI time-of-flight mass spectrum of positive ions acquired at 25 kV from fraction 2 deposited on the sample holder using α-cyano-4-hydroxy cinnamic acid as the matrix.

Table 4.4 Molar masses of the most abundant $[M + H]^+$ species as determined by MALDI-TOF MS analyses performed on three peptide fractions obtained by SEC of the whey UF permeates.

Fraction	Mono-isotopic fragment ion masses, g mol^{-1}	Charge, Z
1	640, 662, 668, 679, 696, 704, 792, 902, 1180 and 1252	+1
2	672, 679, 701, 792, 826, 997, 1038 and 1362	+1
3	566, 662, 679, 701, 792, 830 and 851	+1

Matrix-assisted laser desorption ionisation time of light (MALDI-TOF) generally leads to the formation of singly charged $[M + H]^+$ ions from peptides. However, the mass of peptides alone does not provide sufficient information to identify them. The peptides (Table 4.4) present in the permeates obtained after ultrafiltration of sweet whey were suggested to be a product of the complex hydrolysis of milk proteins by endogenous proteases such as plasmin

(milk protease) and of the enzymatic activity of rennet (chymosin/pepsin mixture) that was applied for coagulation of casein micelles. Due to the similarity of the molar mass of the obtained peptides with the potentially bioactive peptides listed in Table 2.3 it seems to be interesting to study the behaviour of these peptides using nanofiltration. The selectivity of nanofiltration membranes is based on both solute charge and size. Thus, it is assumed to be a useful tool for the separation of whey solutions containing peptides and amino acids with molar masses in the range 100 to 2000 g mol^{-1}, which have a small number of ionogenic and hydrophilic groups.

4.3 Conclusion

In the work described in this chapter, the proteins of sweet whey (pH 6.6 ± 0.1) were concentrated by ultrafiltration using regenerated cellulose C 10F membrane. This membrane shows no flux alterations in subcritical conditions up to a VRF of 2. Protein identification from collected permeate and concentrate samples was performed using reversed phase chromatography. No major whey proteins were found in the permeate samples. Three peptide fractions were resolved from the whey permeates using size exclusion chromatography. Analysis of these fractions using MALDI-TOF mass spectrometry resulted in multiple signals corresponding to peptides with molar masses in the range 570 to 1360 g mol^{-1}.

5 CHARACTERISATION OF NANOFILTRATION MEMBRANES

Nanofiltration is a pressure driven membrane process, which lies between ultrafiltration and reverse osmosis. Nanofiltration membranes are generally accepted to have pores, and the sieving mechanism is very important as well as the charge effects [Nakao 1994]. The solute transfer includes the two following steps [Bowen and Mukhtar 1996]:

1. Distribution of ionic species at the selective interface, according to their charge
2. Transfer by a combination of diffusion, convection and electrophoretic migration

Useful models for process performance of membrane separation utilise available physical property data of a process stream and a membrane. For NF membranes, this would involve structural parameters such as the pore radius and electrical parameters such as surface charge density. Ferry's law [Ferry 1936] describes how the size effect relates to the transmission of molecules, that is, to their radius relative to the pore radius of the membrane. In the case of uncharged solute molecules, mainly size exclusion is thought to be responsible for the separation.

The surface charge can have a significant effect on the transport of charged molecules. Understanding the electrical interactions between the solute molecules and the membrane can significantly enhance the effectiveness of a membrane separation process.

The objective of this chapter was to estimate the average pore size for two polymeric nanofiltration membranes (NTR 7450 and NF 270) from the retention coefficients of neutral molecules with molar masses in the range 75 to 1355 g mol^{-1}. Different models for the retention of uncharged organic molecules were applied: the Ferry's model, the Zeman and Wales model, and the steric hindrance pore (SHP) model. The streaming potential measurements were carried out to determine the zeta potential and the IEP of the membranes.

5.1 Materials and Methods

5.1.1 Nanofiltration membranes

Two commercially available nanofiltration membranes (NF 270 and NTR 7450) were used in this investigation. The key properties of these membranes, which have been described by their manufactures and several researchers, are summarised in Table 5.1.

Table 5.1 Membrane characteristics [[1]Braeken et al. 2006, [2]Timmer et al. 1998, [3]Mänttäri and Nyström 2003, [4]Mänttäri et al. 1997, [5]Schaep and Vandecasteele 2001, [6]Nghiem et al. 2005 B, [7]Nyström et al. 1995, [8]Own measurements].

Property	NTR 7450	NF 270
Manufacturer	Nitto Denko	Dow
Membrane material	Sulphonated polyether sulphone	Polyamide
Molar mass cut-off, g mol^{-1}	600-800[1,5]; 1000[2]	180[1]
Operating pH	2-11[1,2]	3-10[1]
Max temperature, ^{o}C	40[2]	45[3]
Permeabilty, (10^{-11} m $s^{-1}Pa^{-1}$)	3.3[1]; 2.6[4]; 2.8[7]; 3.0[8]	3.1[1]; 5.2[8]
Contact angle (θ), ^{o}	70[1]	26[1]
Membrane surface charge density (10^{-3} C m^{-2}) at pH 7.0	-11.0[1]	-14.9[1]
IEP	3.5[4]	3.5[6]
Retention, %		
NaCl	50[5,7]	65.8[8]
Na_2SO_4	92[7]	98.5[8]
$MgCl_2$	13[7]	72.0[8]

5.1.2 Filtration procedure

A laboratory-scale, cross-flow membrane filtration unit was used in this study. The effective membrane surface area was 21.6 cm^2. Prior to each experiment, the membrane was stabilised at 0.4 MPa using water (Millipore grade) for at least 1 hour until the permeate flux attained a constant value at 37 ^{o}C. The feed reservoir temperature was kept at 37±1 ^{o}C throughout all

experiments. After stabilising the membrane, the water permeability was determined at a cross-flow velocity of 2 m s^{-1} under working conditions (T = 37 ^{o}C, υ = 2 m s^{-1}) in the pressure range 0.1-0.4 MPa. All filtration runs were conducted at an applied pressure of 0.35 MPa and a cross-flow velocity of 2 m s^{-1}.

5.1.3 Test substances

The molar mass cut-off values for the nanofiltration membranes were determined by using model solutions at a concentration of 0.2 kg m^{-3}. Glycine solution at neutral pH, carbohydrates (glucose, lactose and raffinose) and vitamin B_{12} solutions were used.

The concentration of a single amino acid was determined by the ninhydrine colorimetric method [Friedman 2004, Diamond 1973]. The concentrations of vitamin B_{12} were analysed using a spectrophotometer at 360 nm. TOC was measured with a Shimadzu TOC analyser (Shimadzu Corporation, Kyoto, Japan). The glucose and lactose contents were determined using the Anthrone (9,10-dihydro-9-ketoanthracene) reaction, as described by Hodge et al. [1962]. Anions were analysed by ion chromatography and metal ions were analysed by atomic absorption spectroscopy (AAS). In the permeation experiment, the observed rejection of a solute by a membrane is calculated using Equation (3.1).

5.1.4 Streaming potential measurements

The streaming potential measurements through membrane pores were carried out in a flat-sheet flow module of polycarbonate [Pihlajamäki 1998] to suit a membrane sample area of 10.4 cm^2, fitted with reversible silver/silver chloride electrodes above and below the membrane.

The streaming potential measurements along the surface were conducted in a module where two pieces of the membrane separated by a gasket were attached into the module (height 0.001 m). The electrodes were placed close to the ends of the flow channel [Pihlajamäki 1998].

The membranes were stabilised at a constant pressure with 1 mM solution of KCl. The pH was adjusted in the range 3.4 - 7, by adding small amounts of KOH and HCl. Temperature was kept at 25 ^{o}C using a water bath. The streaming potential was measured at five different pressures, in the range 0.1 – 0.3 MPa (through the pores) and 0.02 – 0.1 MPa (along the surface).

The zeta potentials, ζ, were calculated from the Helmholtz-Smoluchowski equation [Dukhin and Derjaguin 1974]:

$$\zeta = \frac{\Delta E}{\Delta P}\frac{\mu\kappa}{\varepsilon_o \varepsilon_r} \tag{5.1}$$

where ΔE is the streaming potential, ΔP is the transmembrane pressure, μ and κ are the viscosity and the conductivity of the solution, respectively, ε_o is the permittivity of vacuum and ε_r is the dielectric constant of the medium.

5.2 Theory

For uncharged solutes the solute flow through the membrane is not dependent on electrostatic interactions and the separation is achieved through size effects. The influence of size on retention was described by Ferry [1936] in the so-called “pore model”:

$$\sigma = 1 - 2\left(1 - \frac{r_s}{r_p}\right)^2 - \left(1 - \frac{r_s}{r_p}\right)^4 \tag{5.2}$$

where σ is the reflection coefficient which represents the maximal possible retention of solute, r_s is the solute radius and r_p is the pore radius.

Zeman and Wales [1981] introduced a factor $u_{solute}/u_{water} = K_2/K_1$ (K_2 and K_1 are constants) in Equation (5.2) to represent steric hindrance during conventional transport. Based on

experimental data, they assumed that the K_2/K_1 factor can be expressed as $\exp\left(-\beta\frac{r_s^2}{r_p^2}\right)$, where β is a dimensionless constant. Equation (5.2) then becomes:

$$\sigma = 1 - \left(1 - \left(\frac{r_s}{r_p}\left(\frac{r_s}{r_p} - 2\right)\right)^2\right)\exp\left(-0.7146\frac{r_s^2}{r_p^2}\right) \tag{5.3}$$

Zeman and Wales assumed that no diffusion contribution occurs [Gekas et al. 1993].

Another modification of the "pore model" is the steric hindrance pore (SHP) model proposed by Nakao and Kimura [1982]. They introduced a "wall-correction parameter" $H_F = 1 + (16/9)(r_s/r_p)$ that represents the effect of the pore wall. According to the SHP model for a solute with known radius the reflection coefficient is determined and the pore radius can be calculated using Equation (5.4) [Wang et al. 1995]:

$$\sigma = 1 - \left(1 + \frac{16r_s^2}{9r_p^2}\right)\left(1 - \frac{r_s}{r_p}\right)^2\left[2 - \left(1 - \frac{r_s}{r_p}\right)^2\right] \tag{5.4}$$

These models all assume the nanofiltration membrane to be a network of perfectly cylindrical, parallel pores with radius r_p, in which the solvent velocity follows Poiseuille's flow with a parabolic profile. According to the models solutes are simulated to be rigid spheres with a radius r_s moving slowly inside the pores.

The quality of fit between the experimental data (R_{exp}) and the model (R_{calc}) was compared through the following least-square objective function S_y:

$$S_y = \sqrt{\frac{\sum_1^n \left(R_{\exp} - R_{calc}\right)^2}{n-1}}, \tag{5.5}$$

5.3 Results and Discussion

5.3.1 Evaluation of average pore size for nanofiltration membranes

Dilute solutions of model substances (glycine, glucose, lactose and raffinose) were used to evaluate the molar mass cut-off for NF 270 and NTR 7450 membranes. The pure water flux and the flux of model solutes varied less than 2 %. The solute concentration polarisation was negligible, thus it was assumed that the observed retentions and the real retentions for model substances were similar. Table 5.2 shows the observed retentions versus molar mass obtained for the membranes studied.

Table 5.2 Characteristics of model substances [[1]Dawson et al. 1986, [2]Longworth 1953, [3]Schaep et al. 2001, [4]Bowen et al. 1997] and their observed retention coefficients determined in this study with NTR 7450 and NF 270 membranes.

Substance	Molar mass[1], g mol^{-1}	Diffusion coefficient[2], (10^{-9} m^2 s^{-1})	Stokes radius[3,4], nm	Observed retention coefficient, R_{obs}	
				NTR 7450	NF 270
Glycine	75.1	1.06	0.24	-	0.87
Glucose	180.2	0.67	0.37	0.11	0.93
Lactose	342.3	0.57	0.49	0.32	1.00
Raffinose	504.4	0.43	0.58	0.60	-
Vitamin B12	1355	0.33	0.74	0.82	-

The observed retention coefficients (Table 5.2), determined in this study, indicate that the NTR 7450 membrane has a molar mass cut-off much higher than the NF 270 membrane, which is tighter, in opposite to the hydraulic permeability (L_p) results ($3.0 * 10^{-11}$ m s^{-1} Pa^{-1} and $5.2 * 10^{-11}$ m s^{-1} Pa^{-1}, correspondingly). The higher hydraulic permeability of the NF 270 membrane was in agreement with its superior hydrophilicity compared to the NTR 7450 membrane. The hydrophobic or hydrophilic characteristics of the membrane are usually expressed in terms of a contact angle (θ). As shown in Table 5.1 the contact angle of the NF 270 membrane was 26^o against 70^o reported for the NTR 7450 membrane, it means that the former membrane is the more hydrophilic.

A modelling of the retention coefficient as a function of pore radius was done according to the different models (Ferry, Zeman and Wales, and SHP) by fitting the Equations (5.2, 5.3 and 5.4) to the experimental data (Table 5.2) using the least-squares method (Equation 5.5). Table 5.3 gives the average pore radii calculated for each of the membrane on the basis of solute radii and their retention data presented in the previous table.

Table 5.3 Average pore radii (nm) and S_y calculated from three simple pore models, Equations (5.2-5.4).

Model	NTR 7450		NF 270	
	r_p, nm	S_y	r_p, nm	S_y
Ferry	1.2	0.132	0.37	0.103
Zeman and Wales	1.3	0.136	0.42	0.099
SHP	0.95	0.090	0.33	0.113

As shown in Table 5.3 the different models gave pore radii, which have the same order of magnitude, but are not in total agreement for the two membranes measured. The results indicate that the NTR 7450 has larger pores than the NF 270 membranes. The calculated pore radii were used to calculate the retention coefficient. The resulting retention as a function of solute radius curves for the NTR 7450 and the NF 270 membranes are presented in Figure 5.1.

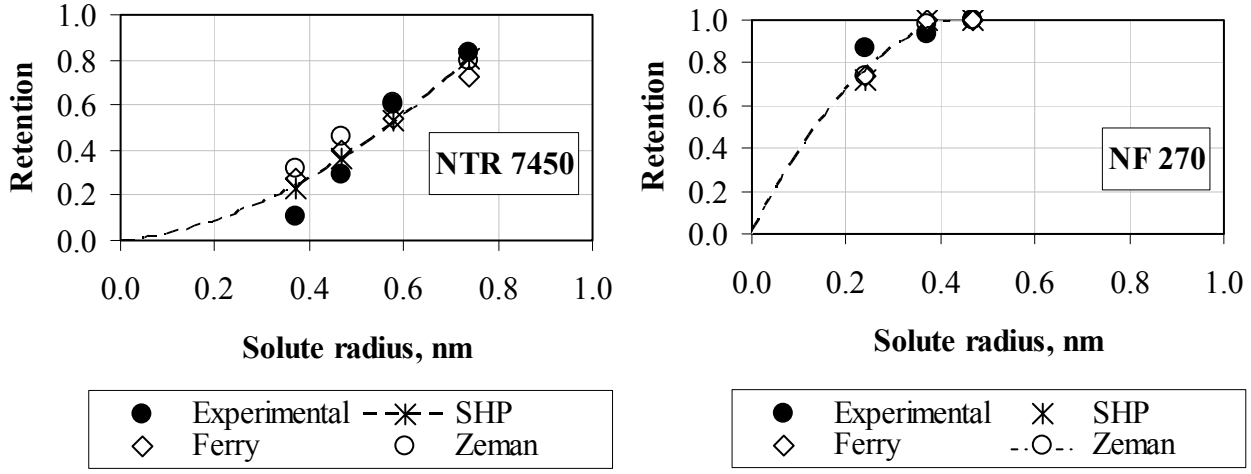


Figure 5.1. Observed and calculated retentions of neutral solutes by NTR 7450 and NF 270 membranes as a function of solute radius.

Due to the deviations of the experimental data, none of the models is obviously very good. In the case of the NF 270 membrane the SHP model gave a pore size smaller than the size of a glucose molecule (0.37 nm), according to the Ferry model the size of the pore is equal to the size of this molecule. In any case, if the membrane transport mechanism was truly convective and the pore size uniform, then a 100 % retention would be expected for this solute. This is not the case according to the experimental results (R_{obs} = 0.93). As shown in the theory section all the models are idealised, because they assume the presence of uniform pores and they are neglecting the solution-diffusion mechanism.

According to the reported previous data the average pore radius for the NTR 7450 membrane was determined as 0.7 nm [Wang et al. 1995] or 0.8 nm [Van der Bruggen 2000, Timmer 2001]. The average pore radius of the NF 270 membrane was estimated to 0.42 nm based on the Zeman Wales model, which is in agreement with data published by Nghiem et al. [2005]. The discrepancies between the pore size of the NTR 7450 membrane defined in this study and the literature data were explained by the difference in the conditions used. In our study the temperature was 37 ^{o}C, while other researchers have made their measurements at 25 ^{o}C. The temperature 37 ^{o}C was chosen because of technical reasons. The membrane cell was small and in order to get a higher flux this temperature was chosen. It was assumed that a temperature increase causes pore enlargement. Another explanation is that the retention coefficients used in the calculation of pore radius by other authors correspond to the retentions at an infinite pressure, when retention and reflection have similar values. The retention is lower at a realistic pressure due to the contribution of diffusion to the transport process, but the pressure dependence of retention is not included in the models.

5.3.2 Evaluation of the isoelectric point of nanofiltration membranes

The experimental results for the calculated membrane zeta potentials along the surface and through the pores are shown in Figure 5.2. Both membranes were negatively charged at a pH higher than 4. In the case of the NTR 7450 membrane, the zeta potential, calculated from the measurements made along the surface, remained negative over the pH range studied. The negative charge of the sulphonated polyethersulphone (NTR 7450) membrane is due to the

dissociation of its sulphonic acid groups. From the measurements made through the pores the isoelectric point of the NTR 7450 membrane (IEP = 3.6) was estimated.

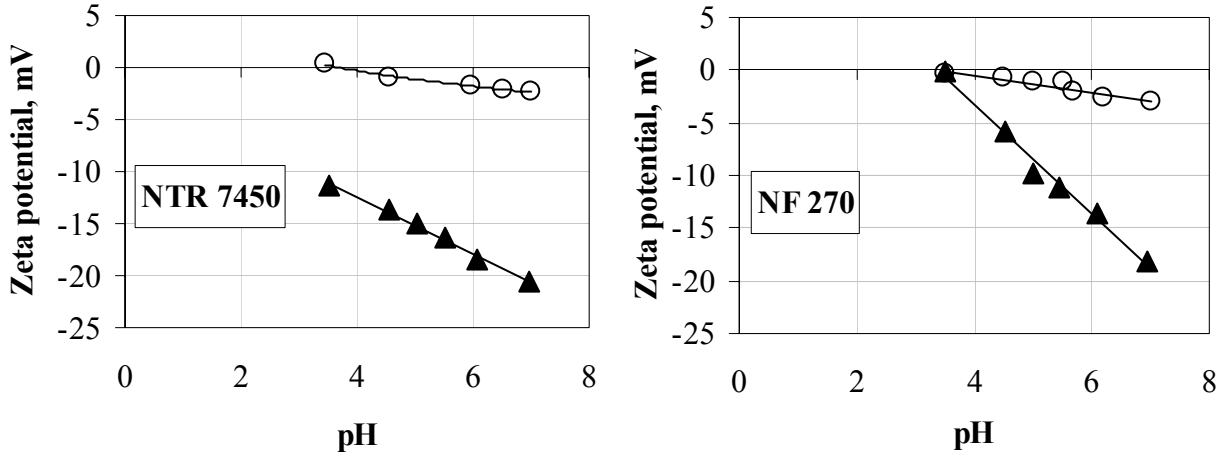


Figure 5.2 Zeta potential measurements for NTR 7450 and NF 270 membranes with 1 mM KCl: (▲) along the surface and (○) through the pores.

Both measurements (along surface and through pores) give the same isoelectric point, pH 3.7 for the NF 270 membrane. It is known that the skin layer of a piperazine-based polyamide (NF 270) membrane contains carboxylic groups, but also may contain ammonium groups. Since the NF 270 membrane shows a high negative charge, in the studied pH range, it was concluded that the surface of this membrane has a higher carboxylic than ammonium group density.

The membrane surfaces were more negatively charged than the pores for both membranes. Earlier, a similar behaviour for the polypiperazine amide (polysulphone/polyester support) NFT 50 membrane with 1 mM KCl was reported by Teixeira et al. [2005]. As shown in the previous section both membranes have narrow pores (the average radii were about 1.0 and 0.4 nm for the NTR 7450 and the NF 270 membranes, respectively). The use of 1 mM KCl as an electrolyte gives a double layer thickness of 9.4 nm that is larger than the pore sizes. Thus, it was assumed that the low zeta potential value in the measurements made through the pores was resulting from the overlapping of double layers.

5.4 Conclusion

The retention coefficients of neutral molecules were used to calculate average pore sizes for the NTR 7450 and the NF 270 membrane. Three models (Ferry's, Zeman and Wales and SHP) were applied for the calculation, and all of them gave pore radii of the same order of magnitude. The average pore radii for the NTR 7450 membrane (1.2 nm, 1.3 nm and 0.95 nm, corresponding to the Ferry, Wales and Zeman, and SHP model, respectively) were bigger than the pore radii calculated for the NF 270 membrane (0.37 nm, 0.42 nm and 0.33 nm). However, the NF 270 as a more hydrophilic membrane had a higher hydraulic permeability ($5.2 * 10^{-11}$ m s^{-1} Pa^{-1}) than the NTR 7450 membrane ($3.0 * 10^{-11}$ m s^{-1} Pa^{-1}).

The zeta potentials for the NTR 7450 and the NF 270 membranes were measured along the surface and through the pores. The decrease of the zeta potential for the measurements made through the pores was associated with double layer overlapping. Both membranes were found to be negatively charged at pH values higher than 4.

The impact of membrane pore size and electrical properties on the separation of amino acids for the NTR 7450 and NF 270 membrane is examined in the following chapter.

6 SEPARATION OF AMINO ACIDS USING NF MEMBRANES

Nanofiltration techniques have potential applications in different areas, e.g., in removing hardness and dissolved organics from water, demineralisation in the dairy industry, removal of small organic molecules from the organic synthesis membrane reactor, heavy metal ion recovery from electroplating wastewater, treatment of pulp and paper effluents, the purification of drug derivatives from concentrated saline solutions and separation of pharmaceuticals from fermentation broths [Wang and Chung 2005]. With the trend in the dairy industry towards separating milk/whey streams into their constituent components the interest in the separation of peptides and amino acids by a membrane process is increasing.

It is well known that a number of amino acids possess specific physiological properties, because of their participation in different biochemical pathways, and play a role as precursors of active metabolites. The amino acids, which are considered to be physiologically beneficial, are: arginine, glutamine, histidine, lysine, asparagine, tryptophan and tyrosine [Korhonen et al. 1998, Chalisova and Pennijajnen 2004]. Amino acids are utilised in the food industry, for example: L-aspartic acid and L-phenylalanine are raw materials in the manufacture of a dipeptide sweetener known as aspartame [Wang et al. 2002]. The molar masses of amino acids are in the range 75 to 204 g mol^{-1}. There are four classes of amino acids having: nonpolar or hydrophobic side chain groups, neutral polar side chain groups, positively charged side chain groups and negatively charged side chain groups [Lehninger 1975]. Amino acids appear to be very good model substances for studying the mechanisms of separation of nanofiltration membranes. The influence of hydrophobicity, charge and size of the solutes being separated on the performance of nanofiltration membranes may be revealed by using amino acids. These tests could provide some information, which could be used when explaining the behaviour of more complex solutions, such as whey or whey protein hydrolysates.

The aim of this work was to determine the effect of amino acid charge and double layer thickness on the separation of amino acids by polymeric membranes. Single solutions of amino acids with neutral (phenylalanine), positively charged (lysine) and negatively charged (aspartic acid) side chain groups were used as model substances. The NF 270 membrane and the NTR 7450 membrane, which differ in pore sizes, were used to reveal when steric exclusion was the dominating parameter in the separation of amino acids. In addition,

intermolecular interactions were studied using a binary solution containing positively and negatively charged amino acids.

6.1 Materials and Methods

6.1.1 Amino acids

L-phenylalanine (Phe), DL-aspartic acid (Asp) and L-lysine monohydrochloride (Lys) (Table 6.1) were used at a concentration of 0.2 kg m^{-3}. NaCl and NaOH or HCl were added to the feed solution to adjust the ionic strength and pH, respectively.

Table 6.1 Characteristics of the amino acids used in this study [[1]Lehninger 1975, [2]Dawson et al. 1986, [3]Zamyatin 1972, [4]Leo et al. 1971, [5]Longworth 1953].

Amino acid[1]	Molar mass[1], g mol^{-1}	pKa[2]	Volume[3], $Å^3$	r, nm	Hydrophobicity of side chain group[4] (-G), kJ mol^{-1}	Diffusion coefficient[5], (10^{-9} m^2 s^{-1})
Phe	165.2	2.20; 9.31	189.9	0.35	9.1	0.705
Lys	146.2	2.16; 9.06; 10.54 (ε-NH_2)	168.6	0.34	1.2	0.765
Asp	133.1	1.99; 3.90; 9.90	111.1	0.30	4.1	0.798

6.1.2 Calculation of net charge of amino acids

The net charges for the amino acids used were calculated using the degree of deprotonation for all charged groups included in their structure. The degree of deprotonation (α) of carboxylic, amino and side chain groups were estimated using the Henderson-Hasselbalch equation [Karlson 1975]:

$$\frac{[deprotonated]}{[protonated]} = 10^{pH-pK} \tag{6.1}$$

where *pK* is the dissociation constant of the corresponding group. Then,

$$\alpha = \frac{(deprotonated)}{(deprotonated + protonated)} \tag{6.2}$$

6.1.3 Analysis of amino acids

A Shimadzu high-performance liquid chromatography (HPLC) system was used to analyse the concentration of amino acids. Dabsyl derivatives of amino acids were obtained according to the procedure described by Krause et al. [1995]. Aliquots of 20 µL of amino acid were transferred into 1.8 mL screw-cap vials and diluted with 180 µL reaction buffer (0.15 M $NaHCO_3$, pH 8.6). After thorough mixing in a vortex-mixer, 200 µL of dabsyl chloride reagent was added and the vials were stoppered tightly and the solution shortly mixed again. Samples were incubated at 70 ^{o}C for 15 minutes with intermediate mixing at 1 minute and 12 minutes. The reaction was stopped by placing the vials in an ice bath for 5 minutes. Subsequent to a short centrifugation step (10 s, 10 000 g), 400 µL of the dilution buffer (mixture 50 mL acetonitrile, 25 mL ethanol and 25 mL mobile phase A) was added, followed by thorough mixing and centrifugation (5 minutes, 10 000 g). 10 µL of clear supernatant was directly injected in the column. A reversed phase column (Luna 5u C18 (2) 250×4.6 mm) supplied from Phenomenex was used. The column was thermostated at 50 ^{o}C. The mobile phase A, consisting of 9 mM sodium dihydrogen-phosphate, 4 % (w/v) dimethylformamide and 0.1 % (w/v) triethylamine was titrated to pH 6.55 with phosphoric acid. The mobile phase B was 80 % (v/v) aqueous acetonitrile. Dabsylated amino acids were eluted at a flow-rate of 16.7 µL s^{-1} using the gradient system listed in Table 6.2. The detection was at 436 nm.

Table 6.2 Scheme of elution gradient

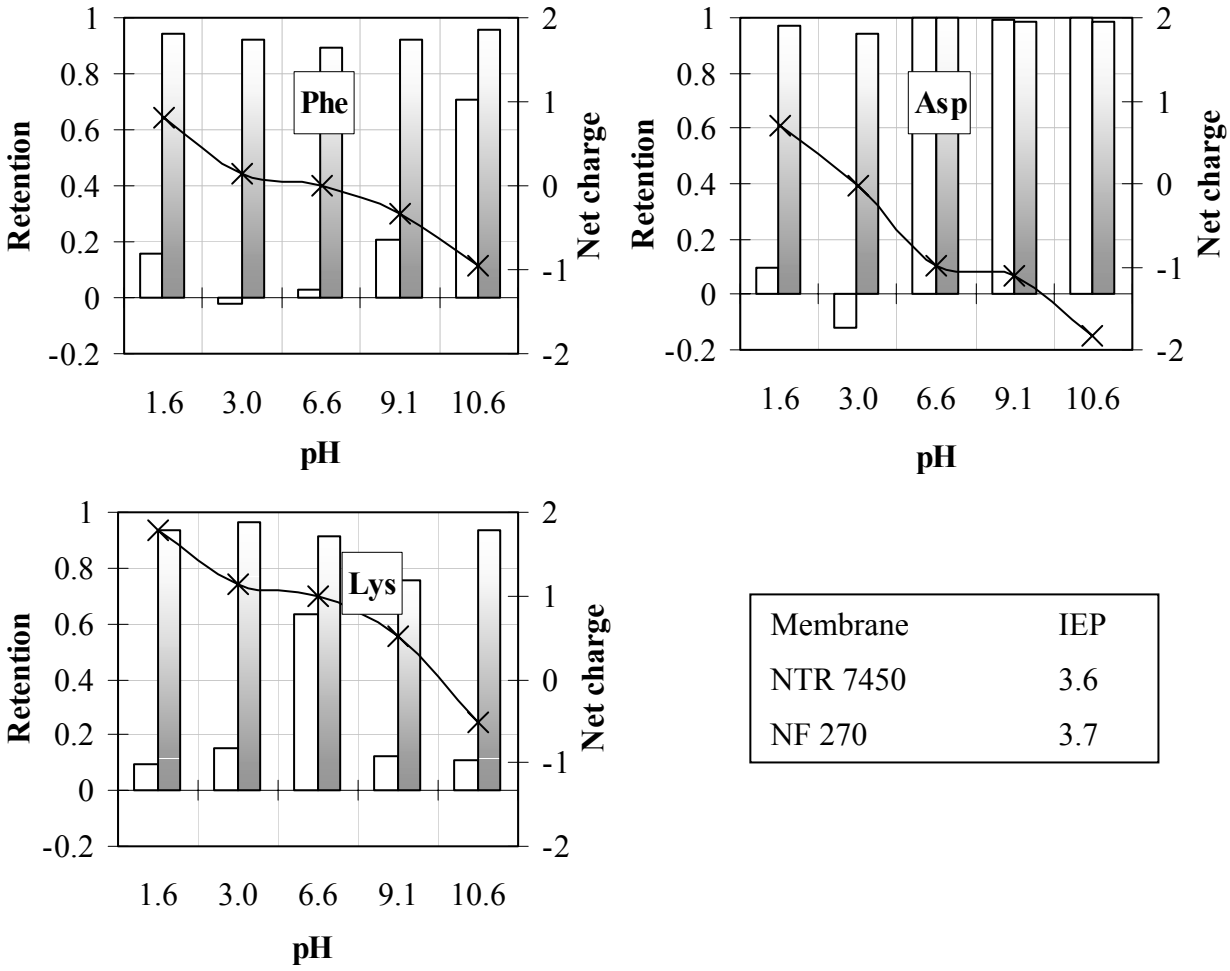
Time, min	0.0	2.0	7.0	35.0	45.0	66.0	71.0	77.0	77.5	90.0
Solvent B, % (v/v)	8.0	8.0	20.0	35.0	50.0	100.0	100.0	100.0	8.0	8.0

The concentration of a single amino acid was determined by the ninhydrine colorimetric method [Friedman 2004, Diamond 1973].

6.2 Results and Discussion

6.2.1 Effect of pH on retention of single amino acids

Since both membranes are charged due to fixed charged groups on the surface layer, the pH is expected to have a strong effect on the retention of the charged species (amino acids). Experimental retentions obtained at different pH for single solutions of neutral (phenylalanine), acidic (aspartic acid) and basic (lysine) amino acids are reported in Figure 6.1.



Membrane	IEP
NTR 7450	3.6
NF 270	3.7

Figure 6.1 Retention of Phe, Asp and Lys as a function of pH: white bars correspond to the NTR 7450 membrane, grey bars correspond to the NF 270 membrane, and (-x-) corresponds to the net charge of the amino acid at the given pH. Operating conditions: 1 mM amino acid dissolved in 0.5 mM NaCl, pressure 0.35 MPa, temperature 37 ^{o}C and cross-flow velocity 2 m s^{-1}.

The retention of the amino acids by the NTR 7450 membrane was strongly affected by repulsive interactions. For phenylalanine (IEP = 5.76) and aspartic acid (IEP = 2.85), which have the same sign as the membrane at pH values higher than their isoelectric points, retention increased with increasing the alkalinity of the feed solution. Minimal retention for both amino acids was found at pH 3.0, and thus it was assumed that this pH could probably correspond to the isoelectric point of the NTR 7450 membrane. The retention of lysine was highest (about 0.6) at neutral pH, where the attraction between positively charged lysine (net charge of +1) and negatively charged membrane (– SO_3^-) is supposed to be significant. It is worth to

notice that the normalised flux expressed as the ratio of solute flux (1 mM lysine in 0.5 mM NaCl) to pure water flux (J_v/J_w) at this pH was equal to 0.60 and was constantly decreasing with time. No significant differences between the permeate fluxes of the other amino acids and the pure water fluxes were found. Therefore, it was concluded that the high retention of positively charged lysine was a result of its adsorption on the negatively charged membrane surface and in the pores.

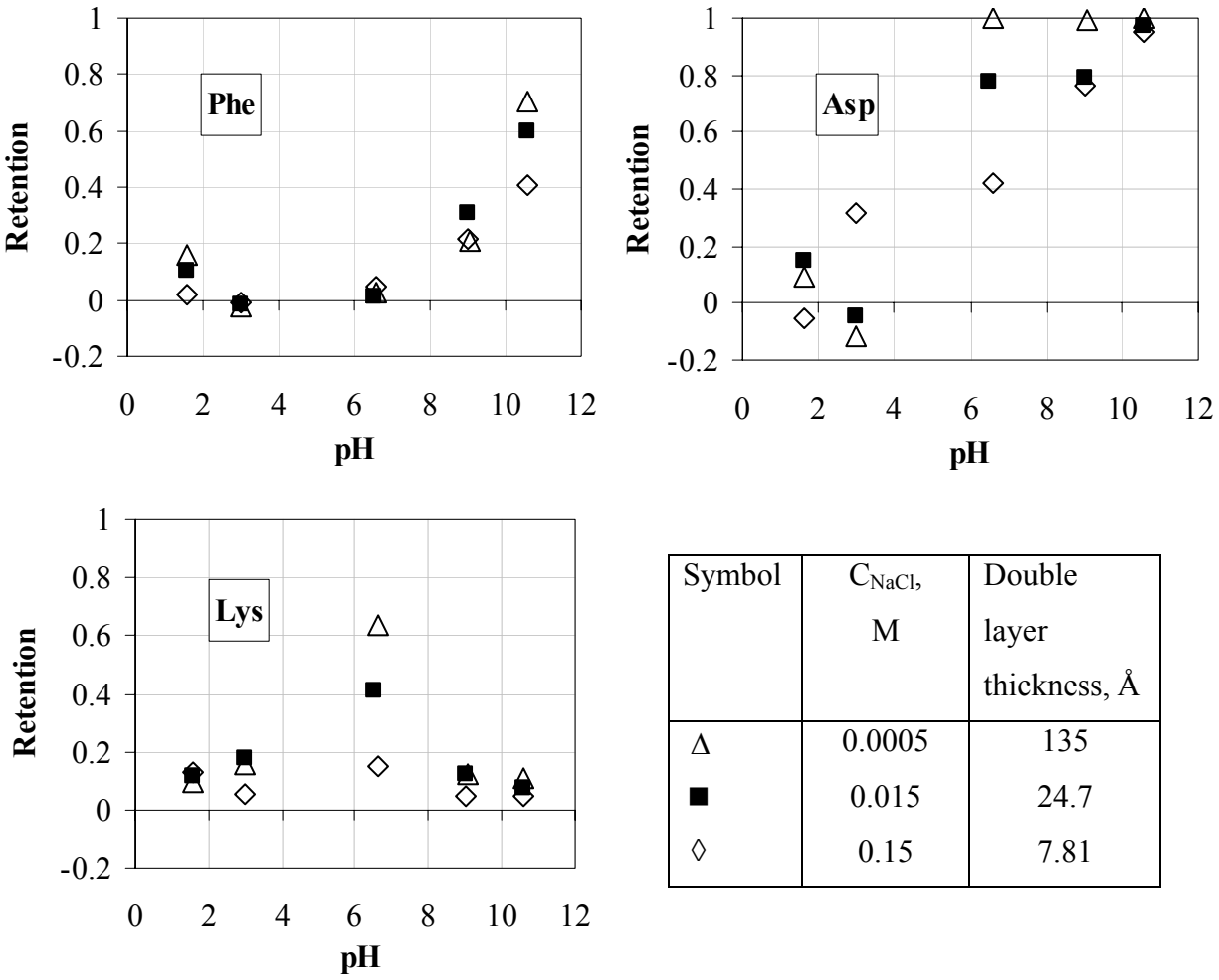
The retention of the amino acids by the tight NF 270 membrane was high in the whole pH range, showing that steric exclusion is the dominating factor for a membrane with a pore size (Table 5.3) close to the size of the separated molecule (Table 6.1). However, the retention of each amino acid was slightly lower near its isoelectric point.

6.2.2 Effect of ionic strength on retention of single amino acids

Ionic strength can influence electrostatic interactions between the membrane functional groups and the amino acids. To examine this effect, retention experiments were conducted in 0.0005 M, 0.015 M and 0.15 M solutions of sodium chloride.

NTR 7450 membrane

As seen in Figure 6.2 ionic strength affects the retention of charged species, mostly by decreasing it. Significant decreases in retentions were found for aspartic acid and lysine, at pH 6.6. The retention of aspartic acid by the NTR 7450 membrane decreases from 1.0 to 0.4, while the retention of lysine decreases from 0.6 to 0.2 with an increase of sodium chloride concentration from 0.0005 M to 0.15 M. This can be explained by charge shielding and double layer compression at enhanced salt concentration, which decreases the electrostatic interactions between the negatively charged membrane surface and the charged amino acids. In the case of negatively charged Asp, it is the repelled ion at low salt concentration, but owing to the lower amount of free effective negative charges at higher NaCl concentration, it will permeate more easily.



Symbol	C_{NaCl}, M	Double layer thickness, Å
Δ	0.0005	135
■	0.015	24.7
◊	0.15	7.81

Figure 6.2 Retention of Phe, Asp and Lys by the NTR 7450 membrane at three different ionic strengths as a function of pH. Operating conditions: 1 mM amino acid dissolved in 0.0005 M, 0.015 M and 0.15 M NaCl, pressure 0.35 MPa, temperature 37 ^{o}C and cross-flow velocity 2 m s^{-1}.

NF 270 membrane

Figure 6.3 shows the retention of the three amino acids by the NF 270 membrane over the pH range 1.6 to 10.6. Because the retention of the amino acids by the tight NF 270 membrane was complete or near complete at all applied pH, the steric exclusion was thought to be the dominant separation mechanism. The theoretical retentions for Phe, Asp and Lys calculated according to the size exclusion model from Zeman and Wales [1981] are 0.98, 0.89 and 0.96, respectively.

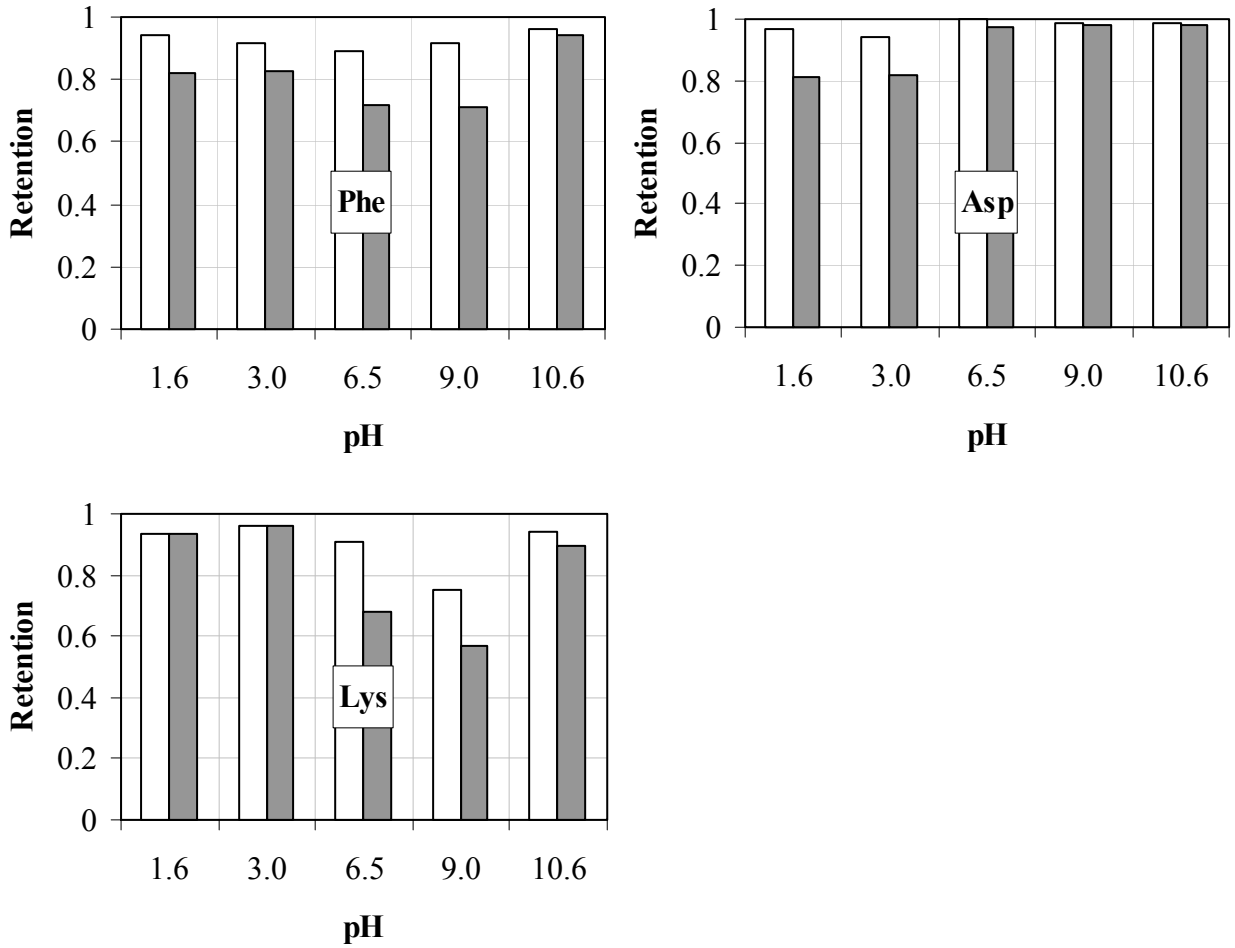


Figure 6.3 Retention of Phe, Asp and Lys by the NF 270 membrane as a function of pH: white bars correspond to 0.5 mM NaCl; dark bars correspond to 0.15 M NaCl. Operating conditions: 1 mM amino acid, pressure 0.35 MPa, temperature 37 ^{o}C and cross-flow velocity 2 m s^{-1}.

The minimum of Lys retention was found at pH 9.0, where this amino acid is still carrying a positive charge and can be adsorbed on the negatively charged NF 270 membrane. When the ionic strength of the solution has been increased from 0.5 mM to 0.15 M, the retention of the amino acids decreases. As was previously mentioned, the increase of ionic strength results in a decrease of electrostatic interactions between charged species (membrane-solute and solute-solute). Moreover, an increased salt concentration would encourage amino acids to be adsorbed on the membrane due to the "salting-out" effect. This results in an enhanced leakage of amino acid, which is in agreement with the experimental results. Salting-out depends primarily on the ionic strength and not as much on the pH [Freger et al. 2000]. As can be seen

from Figure 6.3 the retention vs. pH profiles for all amino acids studied do not change much with pH and move downwards with the increase of ionic strength.

6.2.3 Effect of ionic strength on permeate fluxes in single amino acid filtration experiments

NTR 7450 membrane

The permeate fluxes of Asp and Phe for the NTR 7450 membrane were found to be close to the pure water flux and indifferent to an increase of salt concentration. However, for lysine a significant flux decline was found at low ionic strength. Flux enhanced with increased ionic strength, and no flux decline was found during lysine filtration at pH 6.6 with a 0.15 M solution of sodium chloride for the NTR 7450 membrane. Figure 6.4 shows the normalised flux as a function of double layer thickness, which is a reverse value of salt concentration.

The assumption that the high retention of lysine at neutral pH was associated with fouling caused by adsorption of the positively charged amino acid on the membrane with a fixed negative charge seems to be valid.

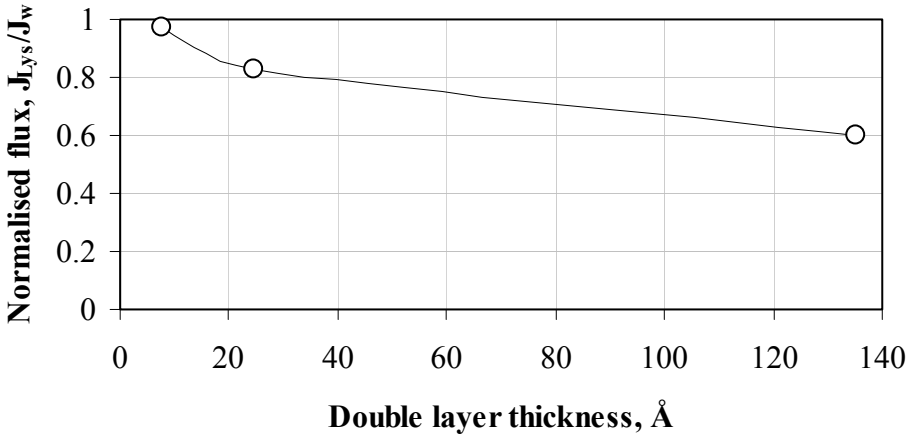


Figure 6.4 Normalised flux of lysine solution at pH 6.6 with the NTR 7450 membrane as a function of double layer thickness. Operating conditions: 1 mM lysine dissolved in 0.0005 M, 0.015 M and 0.15 M NaCl, pressure 0.35 MPa, temperature 37 ^{o}C and cross-flow velocity 2 m s^{-1}.

NF 270 membrane

The permeate fluxes obtained with the NF 270 membrane show a different behaviour. At low salt concentration (0.5 mM NaCl) the fluxes of all the tested amino acids (Table 6.3) were close to the pure water flux. A significant flux decline with an increase of sodium chloride concentration was found in the case of the NF 270 membrane independent of amino acid being filtered.

Table 6.3 Fluxes of amino acids at different concentration of NaCl measured at pH 6.5 for the NF 270 membrane.

C_{NaCl}, M	Permeate flux, μm s^{-1}		
	Asp	Phe	Lys
0.0005	17.4	18.8	14.7
0.15	9.94	10.3	8.98

The osmotic pressure was the major factor affecting the permeate flux of amino acid solution through the NF 270 membrane. According to the osmotic pressure model the deviation of solute flux (J_v) from pure water flux occurs due to the osmotic pressure, which reduces the effective transmembrane pressure:

$$J_v = L_p\,(\Delta P - \sigma\Delta\pi) \tag{6.3}$$

where L_p is the permeability of the membrane, ΔP is the transmembrane pressure difference, σ is the reflection coefficient, and $\Delta\pi$ is the osmotic pressure difference.

The effect of salt concentration on permeate flux of the NF 270 membrane was studied using 0.0005 M, 0.015 M and 0.15 M solutions of sodium chloride without amino acids (Figure 6.5). The increase in salt concentration results in a high osmotic pressure difference $\Delta\pi_{(b\text{-}p)}$ and thus, in a low efficient transmembrane pressure and consequently low permeate flux.

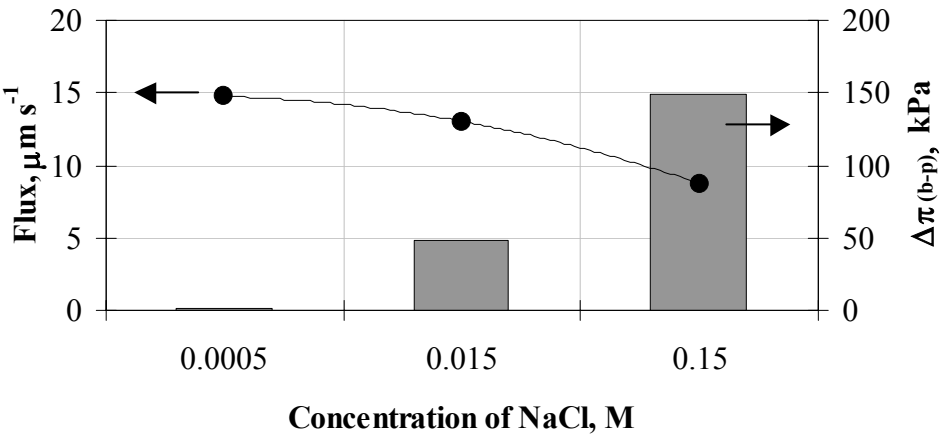


Figure 6.5 Flux and osmotic pressure difference as a function of salt concentration. Operating conditions: transmembrane pressure 0.35 MPa, cross-flow 2 m s^{-1}, T = 37 ^{o}C.

The osmotic pressure difference prevailing on the membrane surface is higher than the osmotic pressure calculated using the salt concentration determined in the retentate, due to the increased salt concentration near the surface. Sutzkover et al. [2000] suggested that the magnitude of the osmotic pressure, π_m, on the membrane surface could be determined from measurements of the fluxes of pure water and saline solution, respectively:

$$\pi_m - \pi_p = \Delta P\left[1 - \frac{(J_v)_{salt}}{J_w}\right] \tag{6.4}$$

where π_p is the osmotic pressure on the permeate side of the membrane, ΔP is the transmembrane pressure difference, $(J_v)_{salt}$ and (J_w) are the permeate fluxes measured for salt and water, respectively. The difference between $(\pi_m - \pi_p)$ and $(\pi_b - \pi_p)$ was estimated to decrease with increasing of salt concentration (from 20 to 6 kPa).

6.2.4 Amino acid mixture

For a selective separation of amino acids the NTR 7450 membrane is more suitable than the NF 270 membrane due to the domination of charge effects. The retention of the NTR 7450 membrane was examined using a mixed solution of lysine and aspartic acid at pH 9.0 and pH 10.6 in 0.5 mM sodium chloride. The results are presented in Table 6.4. As shown above the charge plays a more dominant role than the size for this membrane.

Table 6.4 Observed retention coefficients of amino acids in single solutions or in mixture at pH 9.0 and 10.6. Transmembrane pressure = 0.35 MPa, cross-flow = 2 m s^{-1}, T = 37 ^{o}C.

Amino acid	Observed retention coefficient, R_{obs}			
	pH 9.0		pH 10.6	
	single	mixture	single	mixture
Aspartic acid	1.00	0.92	1.00	1.00
Lysine	0.20	0.36	0.20	0.28

Table 6.4 shows that the observed retention coefficients for amino acids in single and binary solutions vary. The retention of aspartic acid decreased in a mixture compared to single solution at pH 9.0. However, no difference in the retention of this amino acid between single solution and mixture was observed at pH 10.6. The retention of lysine increased in binary mixtures compared to single solutions and it was more pronounced at pH 9.0. At pH 9.0 the net charge of lysine is + 0.51 (degree of protonation for the α-amino group is 0.53, and for the ε-amino group it is 0.97), it means that two lysine species ($^{+}H_3N$-$(CH_2)_4$-CH(-NH_3^{+})-COO^{-} and $^{+}H_3N$-$(CH_2)_4$-CH(-NH_2)-COO^{-}) coexist in solution. These positively charged ions can interact with the negatively charged surface of the NTR 7450 membrane, which results in flux decline. The permeate flux was 0.80 of the water flux at the same conditions and it decreased steeply with time. If lysine adsorption is taken into account, then the enhanced transmission of negatively charged aspartic acid at pH 9.0 can be explained by a decrease of the repulsive interaction between this acid and the membrane. On the other hand an increase in lysine retention in mixtures may be explained by heteroaggregate formation, aspartic acid has two carboxylic groups within the structure (COO^{-}-CH_2-CH(-NH_3^{+})COO^{-}), which can interact with the positively charged lysine.

The amount of lysine molecules with protonated amino groups decrease with increasing pH (0.03 α-amino groups and 0.47 ε-amino groups at pH 10.6). The net charge of the lysine molecule is negative (-0.51) at pH 10.6. Thus, the electrostatic attraction between lysine and the negatively charged membrane surface and fouling diminish. As a consequence no flux decline was observed at the given pH. Due to the minimal lysine adsorption the sulphonic groups of the NTR 7450 membrane are unoccupied and the electrostatic repulsion between them and aspartic acid molecules lead to the complete retention of the latter. Heteroaggregate formation could also be a cause because about 50 % of the ε-amino groups of lysine are still remaining protonated and can interact with the carboxylic groups of aspartic acid that lead to complete retention of the latter. Heteroaggregate formation is assumed to be the reason for the higher lysine retention in mixtures compared to single solution.

The selectivity, ψ, is defined as the ratio $(1 - R_{obs}(Lys))/(1 - R_{obs}(Asp))$. At pH 9.0 the selectivity of the membrane to lysine was 8, while at pH 10.6 because no transmission of aspartic acid was observed the selectivity values between lysine and aspartic acid tend to infinite values.

6.3 Conclusion

The results of the amino acid filtration showed that for the NTR 7450 membrane charge plays a more important role than for the NF 270 membrane, where steric exclusion is more important. Minimal retention for the amino acids was found near their IEP. For the NTR 7450 membrane the retention coefficients increased with an increase of electrostatic repulsion (Asp and Phe) or because of electrostatic attraction (Lys). The effect of ionic strength was pronounced for the charged amino acid species, an enhanced salt concentration resulted in lower retention coefficients. However, in the case of the NTR 7450 membrane the effect of ionic strength on retention was explained in terms of charge shielding, for the NF 270 membrane the salting-out effect was assumed to be a more appropriate explanation.

The permeate fluxes of aspartic acid and phenylalanine, obtained at pH 6.5 in the experiments with NTR 7450 membrane, were stable independent of ionic strength. However, the permeate flux of lysine increased when the salt concentration was increased and for 0.15 M sodium

chloride it reached the value of the pure water flux. It means that adsorption caused by electrostatic interactions between the positively charged lysine and the negatively charged NTR 7450 membrane was minimised at high salt concentration.

In the case of the NF 270 membrane, the increased salt concentration was found to decrease significantly the permeate fluxes for all amino acids studied. The study on the effect of salt concentration on permeate flux of pure sodium chloride solution revealed the significance of osmotic pressure.

The filtration of an amino acid mixture containing Asp and Lys was conducted at pH 9.0 and pH 10.6 using the NTR 7450 membrane. The selectivity in the amino acid fractionation was very sensitive to pH. At pH 9.0 the selectivity of the membrane to lysine was 8, while at pH 10.6, because no transmission of aspartic acid was observed, the selectivity values between lysine and aspartic acid tended to infinite values.

7 NANOFILTRATION OF WHEY ULTRAFILTRATION PERMEATES

As was shown in the previous section the NTR 7450 membrane can be used for the purification of positively charged amino acids from binary mixtures. Low molar mass positively charged peptides formed after hydrolysis of bovine milk casein by heating and chymosin treatment at neutral pH have been reported to possess antimicrobial properties [Liepke 1996]. The molar mass cut-off of the NTR 7450 membrane allows to assume that this membrane could be used for the concentration of low molar mass peptides and for the separation from other low molar mass compounds, such as lactose or mineral salts. The lactose crystallisation process would also benefit from the removal of peptide additives, which are usually present in whey ultrafiltrates.

The objective of this chapter was to study the effect of environmental conditions on permeate flux and retention of components from whey ultrafiltration permeates after nanofiltration using NTR 7450 membrane. The effect of calcium ions on fouling and retention of the NTR 7450 membrane was studied using model solutions of lactose in phosphate buffer at three different pH values.

7.1 Materials and Methods

7.1.1 Whey UF permeates

The UF permeates from the sweet whey filtration (Chapter 4) were further nanofiltered through NTR 7450 membrane. The average composition of the whey UF permeates is shown in Table 7.1.

Table 7.1 Average composition of the UF-whey permeates used in this study.

Component	Concentration, kg m^{-3}
Peptides (AAN)	1.00
Lactose	51.0
Sodium	0.41
Potassium	1.67
Magnesium	0.09
Calcium	0.24
Chloride	0.94
Citrate	1.62
Phosphate	0.82
Sulphate	0.20

7.1.2 Filtration procedure

The laboratory-scale, cross-flow membrane filtration unit described in Chapter 3 was used in this study. The effective membrane surface area was 21.6 cm^2. Prior to each experiment, the membrane was stabilised at 0.4 MPa using water (Millipore grade) for at least 1 hour until the permeate flux attained a constant value at 40 ^oC. The feed reservoir temperature was kept at 40±1 ^oC throughout all experiments. After stabilising the membrane, the water permeability was determined at a cross-flow velocity of 2 m s^{-1} under the working conditions (T = 40 ^oC, υ = 2 m s^{-1}) in the pressure range 0.1-0.4 MPa. All filtration runs were conducted at an applied pressure of 0.35 MPa and a cross-flow velocity of 2 m s^{-1}. Before nanofiltration the whey UF permeates were diluted four times by addition of 0.005 M (0.29 kg m^{-3}) sodium chloride in order to keep the ionic strength of the solution constant. Diluted whey UF permeate samples were filtered at two pH values: 3.0 and 9.5. NaOH or HCl were added to the feed solution to adjust the pH.

7.1.3 Estimation of fouling

A resistance in series method was applied to estimate the fouling during nanofiltration. The resistance of the virgin membrane (after 1 hour of compression) was evaluated using

Equation (2.1) from the pure water flux value at a pressure of 0.35 MPa, and a temperature of 40 °C corresponding to a viscosity of 0.66 mPa s. The hydraulic resistances of the NTR 7450 membranes applied in the experiments at pH 3 and pH 9.5 were the same ($R_m = 5.91 \times 10^{13}$ m^{-1}). The fouling resistance can be evaluated from the pure water flux measurements of the fouled membrane using Equation (2.2).

7.1.4 Analysis of whey components

The concentration of α-amino nitrogen was determined by the ninhydrine colorimetric method [Friedman 2004, Diamond 1973]. The lactose content was determined using the Anthrone (9,10-dihydro-9-ketoanthracene) reaction, as described by Hodge et al. [1962]. Anions were analysed by ion chromatography and metal ions were analysed by atomic absorption chromatography (AAS).

7.1.5 Size exclusion chromatography of whey NF samples

The molar mass distribution for NF samples from permeate and concentrate of whey was studied by size exclusion chromatography on a Superose 12 (10 x 300 mm) column (GE Healthcare). Before injection in the column the pH of the samples was adjusted to neutral in order to prevent interaction with the column material. The fractions were eluted with 10 mM ammonium acetate at a pH of 6.0 and a flow rate of 8.3 μL s^{-1}. The elution was monitored at 220 and 280 nm. The molar mass markers were dextran blue (2000 kg mol^{-1}), lactalbumin (14 kg mol^{-1}), aprotinin (6.5 kg mol^{-1}) and L-pro-leu-gly (280 g mol^{-1}).

7.1.6 HPLC analysis of whey peptide fractions

Reversed phase chromatography analyses were performed on a Jupiter 5u C18 300 Å (150 x 4.6 mm) (Phenomenex) column using a Shimadzu HPLC system. The analyses were performed at 35 °C with a flow rate of 16.7 μL s^{-1}, using a linear acetonitrile gradient, starting with 94 % (v/v) of buffer A (Milli-Q water, 0.1 % (v/v) trifluoroacetic acid (TFA)) ending at

60 % (v/v) of buffer B (acetonitrile, 0.1 % (v/v) TFA) in 54 minutes. Then the 60 % (v/v) of buffer B was kept for 5 minutes. After analysis the column was equilibrated for 10 minutes with buffer A. The column was calibrated with the amino acid standards: glycine, phenylalanine and tryptophan (Table 7.2). The signals were monitored at 257 and 280 nm.

Table 7.2 Properties of amino acid standards and retention times (R_t) determined in this study using Jupiter 5u C18 300 Å (150 x 4.6 mm) [[1]Lehninger 1975, [2]Leo 1971].

Amino acid	Molar mass[1], g mol^{-1}	Hydrophobicity of side chain group[2] (-G), kJ mol^{-1}	R_t, ks
Gly	75.1	0	0.22
Phe	165	9.12	0.37
Trp	204	11.3	0.42

Retention coefficients for fractions eluted on SEC and HPLC chromatograms were calculated using the ratio of their peak areas in the permeate and retentate samples.

7.2 Results and Discussion

7.2.1 Effect of pH on permeate flux and fouling in nanofiltration of whey ultrafiltrates

The nanofiltration permeate flux and fouling resistance ($R_f = R_i + R_c$) at pH 3.0 and 9.5 in the filtration of whey ultrafiltration permeate solutions using the NTR 7450 membrane are shown in Figure 7.1a and 7.1b.

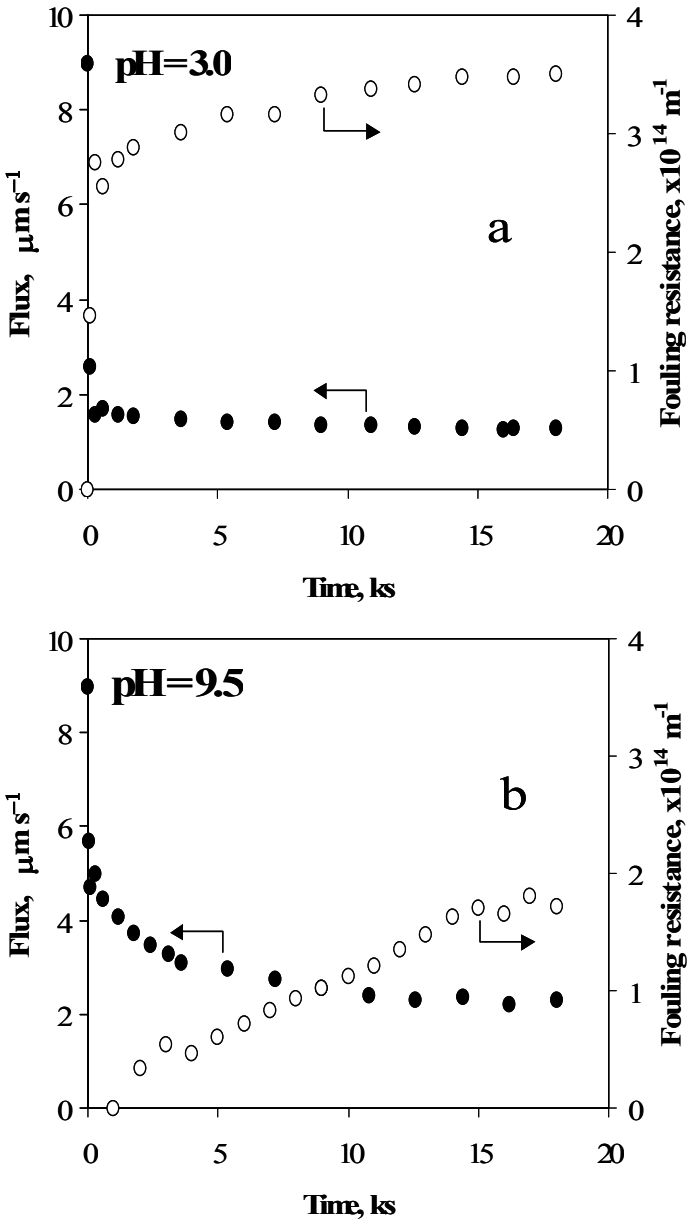


Figure 7.1 Permeate flux and fouling resistance (R_i + R_c) versus time in NF of UF permeates from whey filtration at P = 0.35 MPa, T = 40 °C and υ = 2 m s^{-1} at pH 3.0 (a) and at pH 9.5 (b) using NTR 7450 membrane.

Fouling and consequently flux decline were found at both pH, but the flux profiles were found to change with pH. As has been mentioned before, fouling is a complex phenomenon, which depends on the experimental operating conditions and the characteristics of the membrane and the feed solution. A temperature of 40 °C, a cross-flow velocity of 2 m s^{-1} and a pressure of 0.35 MPa were the operating conditions used in all experiments. Therefore, the differences in the fouling profiles must arise from the properties of the NTR 7450 membrane and the whey solutions at acidic and basic pH. To check the pH dependence of membrane permeability, the

model buffer solution (5 mM phosphate buffer) was filtered at the same operating conditions as in the actual experiments. The flux of the model buffer solution at pH 9.5 was 8.8 μm s^{-1}, while at pH 3.0 it was 8.0 μm s^{-1}. Above the isoelectric point (IEP = 3.6) the sulphonic groups of the membrane are ionised, and the water shell around the charged groups, like in the diffuse double layer, is larger than for the neutral state. A pore enlargement is believed to be responsible for the slight increase in the permeability of the NTR 7450 membrane at basic pH compared to acidic conditions.

The analysis of the resistances of the fouling layer showed that an increase in resistance owing to the irreversible part of fouling was higher at acidic pH ($R_i = 1.5 \times 10^{14}$ m^{-1} and $R_i = 0.6 \times 10^{14}$ m^{-1}, corresponding to pH 3.0 and pH 9.5, respectively). Hydrophobic interactions between the almost neutral membrane at pH 3.0 and the whey components (such as hydrophobic peptides, lactose) have been suggested to take place, in contrast to the electrostatic repulsion between membrane and peptides carrying negative charges at pH 9.5.

The molar masses of the peptide fractions contained in the feed solutions were shown in Table 4.4 (Chapter 4). Peptides with molar masses of 1000 g mol^{-1} and higher, which are in the range of the cut-off value of the NTR 7450 membranes, were thought to block the pores. Earlier, Wang et al. [2002] showed that amino acids with molar masses close to the cut-off value of the nanofiltration membrane ES 20 (aromatic polyamide) were totally retained irrespective of the pH value of the solution.

Calcium ions contained in whey solution (Table 7.1) could also trigger fouling at alkaline pH. The fouling resistance (Figure 7.1b) increases gradually with time at pH 9.5. In this case the calcium ions may lower the electrostatic repulsion between membrane and peptides/amino acids through specific binding of negatively charged species [Waite 2005].

A slight increase in turbidity of the feed solution, which was observed in the process of pH adjustment to 9.5, could be explained by calcium precipitation. The observed sieving coefficient for calcium was 0.26 and 0.04 at pH 3.0 and pH 9.5, respectively. The higher retention of calcium found at an alkaline pH could be explained by the participation of calcium ions in the formation of an increasingly impermeable fouling layer at this pH. Most of

the fouling layer was loosely bound to the membrane surface and, therefore, it was removed by flushing with pure water.

7.2.2 Retention of whey components by NTR 7450 membrane

The difference between the permeate compositions obtained at different pH is shown in Table 7.3. Here the concentrations of components are given in NF permeate from UF whey permeate after nanofiltration through the NTR 7450 membrane at P = 0.35 MPa, T = 40 °C, and υ = 2 m s^{-1}. There was no significant difference between the peptide/amino acid (AAN) contents in the NF permeates for pH 3.0 and pH 9.5. However, the lactose content was almost 9 times higher in the NF permeate obtained at pH 9.5 than at pH 3.0. The effect of pH on lactose retention will be reviewed in the next section.

The higher content of chloride at pH 3.0 and of sodium at pH 9.5 in both retentate and permeate samples was due to the fact that hydrochloric acid and sodium hydroxide were applied to adjust the pH of the solution. Monovalent cations (Na^+ and K^+), divalent cations (Ca^{2+} and Mg^{2+}) and anions (citrate and phosphate) were more retained at pH 9.5. Chloride and partly sulphate ions had higher retention coefficients at pH 3.0. It is obvious that a correlation exists between the high retention of calcium and phosphate ions at pH 9.5. At this pH calcium phosphate precipitates can form that could cause a reduced concentration of these ions in the permeate and in the retentate samples.

Table 7.3 Concentration of components of NF permeate (p) and retentate (r) samples of UF whey permeates and their retention coefficients. Nanofiltration was carried out at P = 0.35 MPa, T = 40 °C and υ = 2 m s^{-1}.

Component	pH 3.0			pH 9.5		
	C_p, g m^{-3}	C_r, g m^{-3}	R_{obs}	C_p, g m^{-3}	C_r, g m^{-3}	R_{obs}
Peptides (AAN)	60	158	0.62	65	149	0.56
Lactose	814	14600	0.94	7170	13400	0.47
Sodium	95	170	0.44	118	269	0.56
Potassium	260	462	0.44	173	416	0.58
Magnesium	1.22	23.5	0.95	0.75	18.5	0.96
Calcium	8.56	32.8	0.74	0.99	27.0	0.96
Chloride	374	725	0.49	292	385	0.24
Citrate	33.7	318	0.89	7.00	477	0.99
Phosphate	26.1	219	0.88	13.2	141	0.91
Sulphate	-	34.6	1.00	2.19	32.0	0.93

7.2.3 Effect of pH and calcium on the retention of lactose by NTR 7450 membrane

The impact of pH and calcium ions on lactose retention was studied using a model solution containing 5 mM sodium phosphate and 5 kg m^{-3} lactose with and without the addition of 1 mM $CaCl_2$. The experiments were made at operating conditions similar to the conditions of whey nanofiltration (P = 0.35 MPa, T = 40 °C and υ = 2 m s^{-1}). Figure 7.2 shows that the observed retention coefficients of lactose at different pH in the presence of calcium were different from the retentions obtained without calcium.

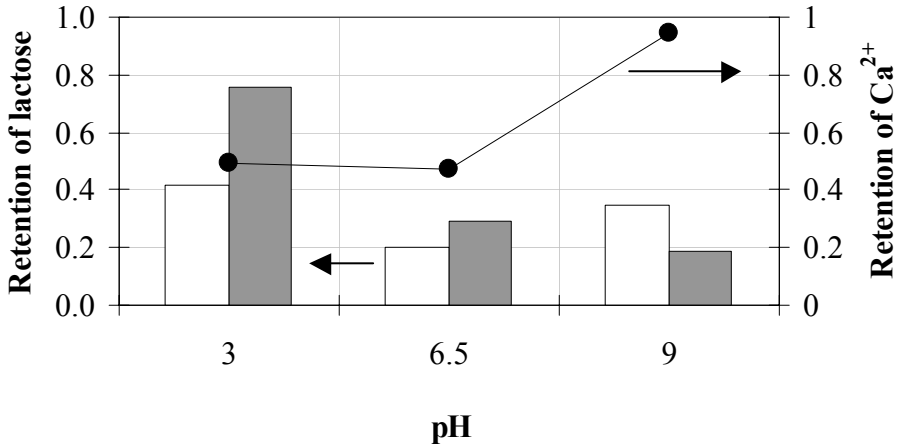


Figure 7.2 Observed retention coefficients for lactose and calcium ions as a function of pH in NF of lactose in phosphate buffer: white bars correspond to lactose retention with no $CaCl_2$ added, dark bars are lactose retention in the presence of $CaCl_2$, line with black dots corresponds to Ca^{2+}. Operating conditions: P = 0.35 MPa, T = 40 °C and $\upsilon = 2$ m s^{-1}.

At acidic conditions the retention of lactose was highest and it doubled when calcium chloride was added to the feed. As has been mentioned above, the higher retention of lactose at pH 3.0 is the result from interaction with the hydrophobic uncharged membrane surface ($IEP_{NTR\ 7450}$ = 3.6). A significant flux decline was observed in the filtrations conducted at acidic pH with and without an addition of Ca^{2+} (Figure 7.3). However, the membrane flux decline at acidic pH with Ca was faster than that during filtration of lactose solution alone. In the absence of Ca^{2+} ions a minimal retention of lactose was found at neutral pH.

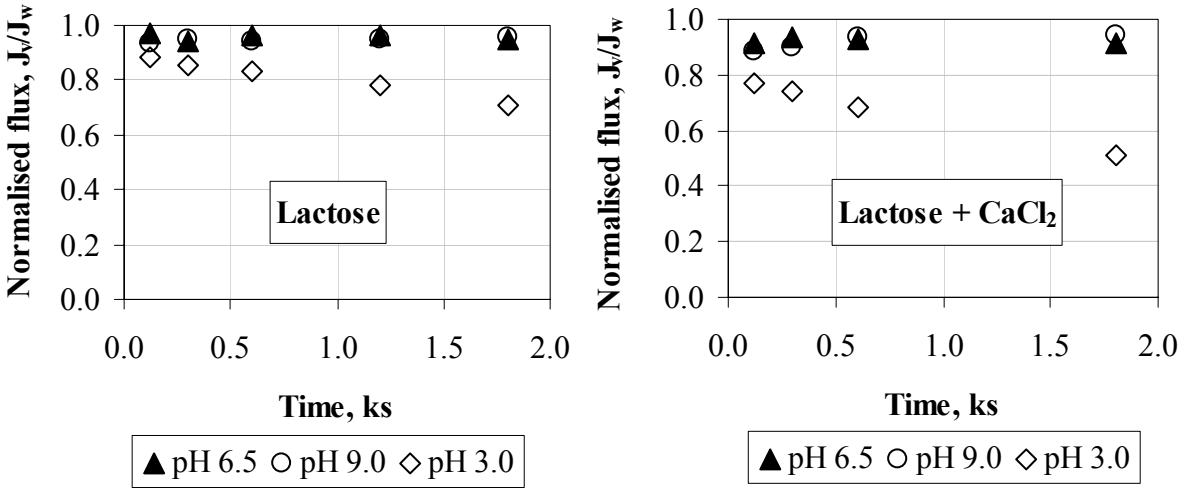


Figure 7.3 Flux of 5 kg m^{-3} lactose solution in phosphate buffer at different pH values as a function of time without $CaCl_2$ and with 1 mM $CaCl_2$ added. (P = 0.35 MPa, T = 40 °C and υ = 2 m s^{-1}).

At alkaline pH the retention of lactose was higher in the absence of calcium, which also could be associated with fouling due to the interaction of lactose with the membrane. An addition of calcium leads to a decrease in retention, which becomes similar to the retention at neutral pH without Ca^{2+} ions. The high retention of calcium at alkaline pH was associated with the precipitation of calcium phosphate because an increase in turbidity was observed in the process of pH adjustment to pH 9.0. Particles larger than the pores may deposit as a cake, with the porosity depending on a variety of factors including particle size distribution, aggregate structure and compaction effects. The filter cake formation involves crystalline particles formed in the bulk of the solution that are deposited onto the membrane to create a usually porous, not very coherent, soft layer [Schäfer et al. 2005]. Figure 7.3 shows that the line corresponding to normalised flux versus time at pH 9.0 is steady in the time interval of the experiment and deviates from unity only slightly. This cake may prevent the direct interaction of lactose with the membrane surface.

7.2.4 Distribution of peptides between NF permeate and concentrate

The UF-whey solutions were filtered at two pH values: pH 3.0 and pH 9.5. These pH values were chosen in order to study the separation of peptides under conditions when they are carrying different charges. Based on their dissociation constants, most of the amino acids carry a net positive charge at pH 3, while at pH 9.5 most of them have net negative charges [Dawson et al. 1986].

Permeate and retentate samples of UF-whey permeate collected after nanofiltration were analysed on the same size exclusion chromatography column that was used for ultrafiltration permeate under identical conditions (Figure 7.4). Three peptide fractions were eluted from the NF permeate and the retentate with retention times corresponding to the retention times of fractions 1, 2 and 3 of the ultrafiltration permeate.

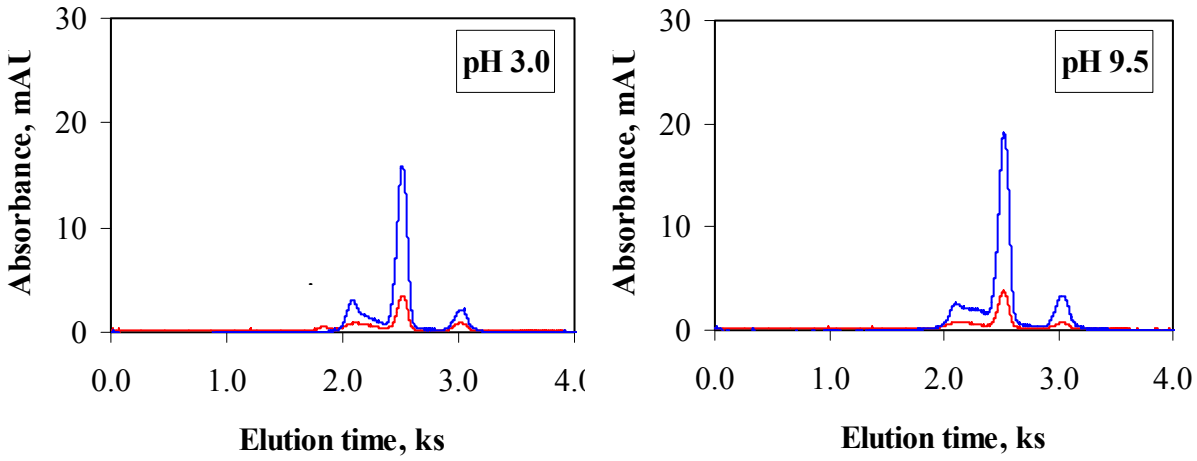


Figure 7.4 Chromatographic profiles of NF permeate and retentate samples from UF-whey permeate filtration eluted from a Superose 12 (10 x 300 mm) column using 10 mM ammonium acetate with a pH of 6.0 at a flow rate of 8.3 μL s^{-1}. UV-detection was monitored at 280 nm. The blue line corresponds to retentate and the red line to permeate.

Retention coefficients for these fractions were calculated using the ratio of their peak areas in the permeate and the retentate samples. The first fraction (elution time 2.1 ks) was found to be more permeable, retention coefficients being 0.63 at pH 3.0 and 0.58 at pH 9.5. The second fraction (elution time 2.5 ks) had a high retention coefficient, about 0.8. The observed

retention coefficient of the third fraction (elution time 3.0 ks) was found to be affected by pH more than the others; the retention coefficient was increased with an increase in pH (0.63 and 0.82 at pH 3.0 and 9.5, respectively).

As known, the separation mechanism of reversed phase chromatography is based on the difference in hydrophobicity of the molecules being separated. A reversed-phase C_{18} column was used to study the hydrophobic characteristics of the peptides from the samples obtained after nanofiltration at pH 3.0 and 9.5. The resolved fractions are presented in Figure 7.5.

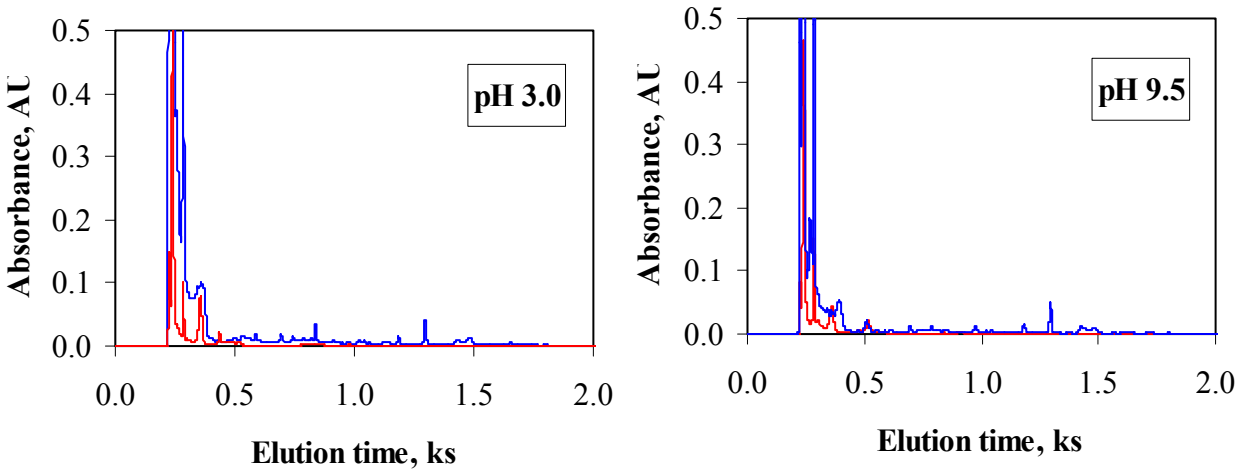


Figure 7.5 Chromatographic profiles of NF samples eluted from a Jupiter C_{18} (4.6 x 150 mm) column using a linear acetonitrile gradient, starting with 94 % (v/v) of buffer A (Milli-Q water, 0.1 % (v/v) TFA) ending with 60 % (v/v) of buffer B (acetonitrile, 0.1 % (v/v) TFA) in 54 minutes. The analyses were performed at 35 °C with a flow rate of 16.7 µL s^{-1}. UV-detection was monitored at 280 nm. The blue line corresponds to retentate and the red line to permeate.

As shown in Figure 7.5 the highest detected signals were observed in the initial part of the chromatograms both at pH 3.0 and at pH 9.5. The intensity of the signals usually depends on the concentration of the substance eluted. Thus, it was assumed that most of the peptides and amino acids were eluted in the time interval between 0.2 to 0.4 ks. The retention times of different fractions eluted from the C_{18} column and their retention coefficients calculated using the ratio between the peak areas in the permeate and retentate are shown in Table 7.4. Only the fractions with a peak area larger than 100 mAU s were considered for analysis.

Table 7.4 Elution times, retention coefficients, and relative hydrophobicity of peptides eluted from NF samples of UF-whey permeates.

Elution time, ks	Retention = 1 – $(A_p)/(A_r)$		Hydrophobicity, (-G), kJ/mol
	pH 3.0	pH 9.5	
0.23	0.87	0.91	0.47
0.24	0.67	0.76	1.05
0.28	0.92	0.83	3.35
0.30	0.84	0.83	4.50
0.36	0.66	0.58	7.95
0.80	1.00	1.00	33.2
1.30	1.00	1.00	44.7

The retention coefficients of the hydrophilic fractions, containing peptides and amino acids, and eluted in the time interval 0.23 to 0.3 ks were slightly different for NF samples collected at pH 3.0 and pH 9.5. The peaks eluted at 0.23 ks and 0.24 ks had a higher retention for NF permeate samples collected at alkaline pH, while for the following peaks, 0.28 ks and 0.3 ks, the retention coefficients were found to be higher for the NF permeates obtained at acidic conditions. The fractions eluted with a retention time of 0.36 ks, which corresponded to phenylalanine (amino acid standard) were found to have lower retention coefficients independent of the pH value applied in nanofiltration. The signals corresponding to the other fractions eluted after 0.36 ks, which were assumed to be more hydrophobic, were detected only in the NF retentate samples at both pH values. It should be mentioned that the intensity of the signals found at 0.8 ks and 1.3 ks in the NF retentates was much lower than for earlier peaks, thus the concentration of peptides in these peaks were lower. Accordingly, even if a small part of these peptides could penetrate the NTR 7450 membrane, their detection in the NF permeate samples was not possible.

Analysis of retention coefficients of different peptide/amino acid fractions obtained from ninhydrin reaction (α-amino nitrogen), SEC and RP-HPLC chromatograms showed that pH was not so important as in the case of lactose distribution. In the case of lactose its content in the NF permeate at pH 9.5 was twofold of its amount at pH 3.0. However, the retention coefficients of the peptide/amino acid fractions are summary values and might vary for the individual peptides or amino acids contained in it.

The sieving selectivity, ψ, of the NTR 7450 membranes of lactose (S_{obs}(lactose)) compared to peptides/amino acids (S_{obs}(AAN)) was evaluated to be 1.2 (ψ = 0.53/0.44) and 0.16 (ψ = 0.06/0.38) at pH 9.5 and pH 3.0, respectively.

7.3 Conclusion

Nanofiltration of whey ultrafiltration permeates at alkaline pH (around 9.5) was more feasible than at a low pH. At this pH the membrane resistance associated with irreversible fouling was lower compared to the acidic pH. Most of the fouling layer at pH 9.5 was reversible. The calcium phosphate precipitates that are favourably formed at alkaline pH were assumed to form the major part of this fouling layer. At acidic pH the hydrophobic NTR 7450 membrane was very susceptible to the irreversible fouling caused by adsorption of organic substances (such as lactose and peptides/amino acids), and it was enhanced by the presence of calcium ions.

Fouling of the nanofiltration membrane altered the membrane retention characteristics. The content of lactose in the NF permeate at pH 9.5 was twofold of its amount at pH 3.0. Only small deviations between retention coefficients for the peptide fractions studied with SEC and RP-HPLC were found at these pH values. The calculated sieving lactose/peptides (AAN) selectivity was higher (1.2) at pH 9.5, than at pH 3.0 (0.16).

8 CONCLUSIONS

This thesis has focused on the study of the impact of physico-chemical conditions and operating parameters on flux and retention of components of sweet whey in ultrafiltration and nanofiltration fractionation. The feed material properties, membrane material characteristics and chemical engineering aspects were considered.

The retention characteristics for ultrafiltration membranes were evaluated by using single polyethylene glycol solutions. At a given module configuration (d_h = 0.002 m) and cross-flow velocity (2 m s^{-1}) providing turbulent flow, the flux (11.1 μm s^{-1}) was selected such that concentration polarisation was at a minimum. In all cases the measured molar mass cut-off values were lower than the nominal molar mass cut-offs given in manufacturer specification sheets.

Sweet whey (pH 6.6 ± 0.1) was concentrated by a factor 2 using a hydrophilic regenerated cellulose C 10F membrane under subcritical conditions. No flux alterations were found. The RP-HPLC, size exclusion chromatography and MALDI-TOF mass spectrometry were used to study the protein/peptide fractions in the retentate and the permeate samples. Analyses of retentate and permeate samples showed that the used ultrafiltration membrane was selective to the major whey proteins, but not to the low molar mass components such as lactose, peptides/amino acids and minerals. The peptides could thus be selectively separated in the permeate.

The retention of the chosen nanofiltration membranes (NTR 7450 and NF 270) were optimised for use to fractionate peptides from sugar and salt. The retention of neutral model molecules (e.g., sugars, vitamin B_{12} and glycine) by these nanofiltration membranes was affected by steric exclusion mechanisms. All the three simple steric models (Ferry's, Zeman and Wales and SHP) gave similar values for the pore radii calculated in this thesis.

Amino acids were chosen as model substances to study the effect of charge on separation using nanofiltration membranes. The two nanofiltration membranes (NTR 7450 and NF 270) used in this study were characterised to be negatively charged at pH values higher than 4. The electrostatic interactions in accordance with the DLVO theory were found to influence retention of the amino acids by the NTR 7450 membrane. On the opposite, the steric

exclusion mechanism and the osmotic pressure effects were found to be responsible for the retention of amino acids by the tight NF 270 membrane. It was found possible to purify positively charged amino acids from a mixture at alkaline pH.

The nanofiltration of whey ultrafiltration permeates containing peptides, sugars and salt at alkaline pH (around 9.5) was more feasible than at acidic pH, because of the reversibility of the fouling layer at this pH, and the superior sieving lactose/peptides (AAN) selectivity (1.2 at pH 9.5 versus 0.16 at pH 3.0). Differences were found between the retention coefficients of the whey components in model solutions (e.g. lactose in phosphate buffer) and in sweet whey UF permeates. The purest peptides could thus be obtained at pH 9.5 in the retentate.

The results presented in this study indicate the importance of all factors (feed material properties, membrane material characteristics, and chemical engineering aspects) on selective fractionation of sweet whey components. Due to the complexity of the natural whey solution the studies on the behaviour of individual components of whey or mixtures of components cannot be directly utilised when dealing with whey. However, the model solutions provide useful information on selectivity and fouling susceptibility of membrane materials to certain whey components and also help in finding the correct operating conditions. From this point of view, the application of whey ultrafiltration permeates, as model solutions to investigate the possibility of the selective fractionation of peptides from mixtures seems to be useful.

The methods used in this thesis enabled the isolation and identification of some peptides present in whey. In the future more studies using optimal nanofiltration membranes can make it possible to purify new bioactive peptides using ultrafiltration and nanofiltration membranes.

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APPENDIX I
REPRODUCIBILITY IN DIFFERENT EXPERIMENTS

First, it was decided to use only membrane pieces from the same batch in the experiments, which permeabilities were within a 10 % range. To prevent a systematic error, screening for different pieces of the same membrane batch was made, by means of pure water flux measurements. Usually, membrane pieces having the same permeability (relative standard deviation, RSD < 10 %) showed the same retention profile.

In the case of characterisation of ultrafiltration membranes (Chapter 3) all PEG solutions were filtered three times and relative standard deviations between runs were less than 2 %. At least two pieces of the same membrane were characterised in this manner. The reproducibility (three runs for each membrane piece, n = 6 - 12) of retention coefficients for PEG molecules filtered through the different membrane pieces was high (0.16 % < RSD < 3.70 %).

Newly prepared whey was ultrafiltered two times with a following nanofiltration at two different pH values. Each time the new piece of the C 10F membrane and new pieces of the NTR 7450 membrane were used. The average composition of sweet whey prepared in two separated experiments was shown in Table 4.1 (Chapter 4). All nanofiltration experiments were made in duplicate (Chapter 5, 6 and 7).

For all analytical tests, such as the Lowry test for proteins, the ninhydrin test for amino acids and peptides, and the Anthrone (9,10-dihydro-9-ketoanthracene) reaction for sugars, three aliquots of the same sample were analysed. The relative standard deviations for replicate measurements were less than 2 %.

In Table 1 some of the performance characteristics of the separation of the proteins in whey samples using RP-HPLC (Chapter 4) are presented along with the precision on retention times, taken from the mid-peak. There was a linear response between protein concentration and UV absorbance at 280 nm for all standards over the calibration range studied (20 to 400 g m^{-3}).

Table 1. Precision (reproducibility) of retention times of the major whey proteins in the whey samples

N	Standards	Reproducibility[a]
		Retention time RSD, %
1	Lysozyme	0.20
2	BSA	1.03
3	Lactoferrin	0.40
4	α-lactalbumin	0.14
5	β-lactoglobulin	0.10 0.11

[a]Three replicate injections of each six samples (n = 18).

The reproducibility of retention times for the three peaks found on the size exclusion chromatogram was evaluated for 10 separate analyses (Chapter 4 and Chapter 7). RSD of the retention time taken from the middle of the peak were equal 0.80, 0.46 and 0.91 %, for the first, second and the third peak, respectively. On a short-term basis, for 20 replicate injections of the same sample (whey permeate after UF collected for the lyophilisation preceding to mass-spectrometry, Chapter 4), an RSD of 0.10 % was observed.

Quantification of the amino acids in mixture was made using RP-HPLC (Chapter 6). Calibration curves (plot peak areas versus amount analysed) of the aspartic acid and lysine were linear ($R^2 > 0.99$) in the studied range 2 to 50 nmol. Reproducibility of peak retention times was estimated from 16 separate samples (for each sample three injections have been made, n = 48). Relative standard deviations for the retention times, taken from the middle of the peak were 0.99 % and 0.35 % for aspartic acid and lysine, correspondingly. The three injections of the same sample give the relative standard deviations (0.93 and 1.30 %) for the peak area of aspartic acid and lysine, respectively.

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