Production and characterisation of native whey and native whey products

Karakterisering og produksjon av nye melkebaserte ingredienser ved hjelp av filtreringsteknologi

Philosophiae Doctor (PhD) Thesis

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SUMMARY

When microfiltration is applied in order to casein-standardise cheese milk, native whey (skimmed milk microfiltration permeate) is generated. Native whey consists of minor serum components that are allowed to be transmitted through the membrane, mainly native whey proteins, lactose, and minerals. Native whey, in contrast to regular sweet whey, is free of proteolytic products from rennet activity, starter culture bacteria, has a neutral pH and contains less fat and salts. This makes native whey an excellent starting material to produce a whey protein concentrate with unique functional properties. The focus of this PhD-thesis is the production and characterisation of native whey and native whey protein concentrate. The first study of this work focused on pre-treatment of milk prior to fractionation. The hypothesis was that the composition of microfiltration fractions is influenced by initial heat treatment of skimmed milks. To study whether native whey could be obtained from other sources than pasteurised and unpasteurised skimmed milk, two native whey protein concentrates were made from microfiltration permeate of buttermilk and pasteurised skimmed milk, respectively (paper II). These native whey ingredients were compared with respect to chemical composition and functional properties. Native whey, regular cheese whey, and whey obtained from cheese making of casein-standardised milk are three different raw materials for further processing to obtain concentrated whey protein products. In paper III, the hypothesis was that casein standardisation prior to cheese making changes the composition of whey. In paper IV, the functional properties of heat-induced gels made from whey protein concentrate with an elevated content of caseinomacropeptide was investigated, to study if the actual level of influence microstructure and rheological properties.

Skimmed milk was fractionated or pasteurised (73 °C, 15 s) prior to fractionation, to study the influence of the initial heat-treatment on fractions' composition. Chemical analysis showed that both the nitrogen and mineral distributions were altered by the initial heat-treatment. The retentate from microfiltration of the unpasteurised milk contained less total protein and casein, and the permeate had higher amounts of calcium, phosphorous and native whey proteins compared to pasteurised milk's fractions. Higher amounts of caseins were found in pasteurised milks permeate (Paper I). The permeate from the pasteurised skimmed milk was concentrated by ultrafiltration and spray dried to a native whey protein concentrate powder. Industrially obtained buttermilk was skimmed prior to fractionation. The skimmed buttermilk was fractionated by microfiltration and concentrated using ultrafiltration using the same membranes and processing conditions as for the skimmed milk to

produce whey protein concentrates. The whey protein ingredients obtained from the microfiltration permeate of buttermilk were equivalent to the powder from pasteurised milk in solubility, foaming properties and chemical composition. Native whey protein concentrate obtained from buttermilk contained less TP and casein, higher amounts of fat, and had different minerals and polar lipid profiles (Paper II).

Whey from casein-standardised cheese milk had a higher total nitrogen, fat and caseinomacropeptide content than whey made from regular cheese milk (Paper III). The influence of the increase in caseinomacropeptide contents on the functional properties of whey protein ingredients was studied using native whey protein concentrate added 0-33 % caseinomacropeptide. Heat-induced gels and solubility was studied at pH 4.0, 5.5 and 7.0. No proper gels were formed at pH 5.5. The level of caseinomacropeptide influenced microstructure at pH 7.0. The gel strength of samples that did not contain caseinomacropeptide were far stronger than all other samples regardless of caseinomacropeptide level. Gel with high concentration of caseinomacropeptide had the highest G' value (Paper IV).

This work showed that the composition of native whey and native whey protein ingredients is influenced by the initial heat treatment of milk. Furthermore, native whey was obtained from buttermilk. The buttermilk native whey protein concentrate had a chemical composition and functional properties comparable to those of native whey protein concentrate made from pasteurised skimmed milk. Casein standardisation increased caseinomacropeptide content in whey. An elevated caseinomacropeptide content was detrimental to microstructure and important rheological properties of heat-induced whey protein gels.

SAMMENDRAG

Ystemelk kan kaseinstandardiseres ved hjelp av mikrofiltrering: Kaseinet konsentreres i retentatet, mens native myseproteiner og mindre melkekomponenter som mineraler og laktose, trenger gjennom membranen. Permetatet fra mikrofiltrering av skummetmelk kalles derfor nativ myse. I motsetning til ostemyse har nativ myse en nøytral pH, ingen denaturerte proteiner, spaltningsprodukter fra løpe (kasein makropeptid) eller rester av syrekultur, og har et lavere mineral- og fettinnhold. Dette permeatet, nativ myse, er et godt utgangspunkt for produksjon av myseproteiningredienser med unike funksjonelle egenskaper. Formålet med dette doktorgradsarbeidet var å fremstille melkefraksjoner og nye ingredienser ved hjelp av filtreringsteknologi, og å karakterisere disse. I den første studien i doktorgradsarbeidet ble det fokusert på behandling av melk før fraksjonering. Hypotesen var at pasteurisering påvirket sammensetningen til melkefraksjonene. Videre ble det undersøkt om nativ myse kunne fremstilles fra kjernemelk, og hvorvidt kilden til den native mysen påvirket sammensetningen og de funksjonelle egenskaper til nativt myseproteinkonsentrat produsert fra hhv. pasteurisert skummetmelk og kjernemelk. Nativ myse, myse fra ordinær ystemelk og myse fra kaseinstandardisert melk er tre ulike råstoff for å produsere myseproteinkonsentrater. I doktorgradsarbeidets tredje del ble det vist at myse fra kaseinstandardisert ystemelk inneholdt mer kasein makropeptid, natrium og fett, og mindre α_{s1}-kasein sammenlignet med myse fra en ysting av vanlig, ikke-standardisert melk. I artikkel IV ble funksjonelle egenskapene til myseproteinkonsentrat undersøkt i forhold en økning i kasein makropeptidinnholdet. Bakgrunnen til dette eksperimentet var at en økning i kasein makropeptid i myseproteinkonsentrat forventes å påvirker gelegenskapene.

Effekten av pasteurisering (73 °C i 15 sekunder) av melka før mikrofiltrering ble studert og det ble vist at varmebehandling før fraksjonering påvirket både nitrogen- og mineralbalansen i melkefraksjonene. Retentatet fra mikrofiltrering av upasteurisert melk inneholdt mindre totalprotein og kasein, sammenlignet med retnetat fra pasteurisert melk. Permeatet (nativ myse) fra mikrofiltrering av upasteurisert melk hadde et høyere innhold av native myseproteiner, kalsium og fosfat, og mindre kasein enn permeatet fra pasteurisert melk (artikkel I). Den native mysen fra pasteurisert melk ble konsertert ved hjelp av ultrafiltrering og spraytørket til et nativt myseproteinkonsentratpulver. Denne myseproteiningrediensen ble sammenlignet med et tilsvarende myseproteinkonsentratpulver, fremstilt av nativ myse fra kjernemelk: Kjernemelk fra en industriell kjerningsprosess ble skummet før fraksjoneringen og ultrafiltrering som ble gjort på samme måte som den pasteuriserte og

upasteuriserte melken i artikkel I. Myseproteinkonsentratene fra henholdsvis kjernemelk og pasteurisert melk hadde like god løselighet og skumdanningsevne. Nativt myseproteinkonsentratet fra kjernemelk hadde mindre totalprotein og kasein, mer fett og en annen sammensetning av mineraler og fosfolipid, sammenlignet med nativt myseproteinkonsentrat fra pasteurisert skummetmelk. Dette arbeidet ble beskrevet og publisert i artikkel II.

Kaseinstandardisering av ystemelk ved hjelp av mikrofiltrering resulterte i en ostemyse med mer kasein makropeptid, totalnitrogen og fett sammenlignet med ostemyse fra vanlig, ukonsentrert ystemelk (artikkel III). Effekten av et økt kasein makropeptidinnhold i myseproteinkonsentratet i forhold til funksjonelle egenskap ble undersøkt ved å tilsette 0-33 % kasein makropeptid til nativt myseproteinkonsentrat. Løseligheten og gelegenskapene til prøvene ble undersøkt ved pH 4.0, 5.5 og 7.0. Myseproteinkonsentrat- og kasein makropeptid dannet ikke gel etter varmebehandling ved pH 5.5. Mikrostrukturen ved pH 7.0 ble tydelig påvirket av et økt innhold av kasein makropeptid. Gelstyrken til prøver med nativ myse var signifikant høyere enn alle de andre prøvene, uavhengig av kasein makropeptidinnhold. Et høyt innhold av kasein makropeptid resulterte i geler med viskoelastiske egenskaper (G') (artikkel IV).

Dette arbeidet har vist at sammensetningen til nativ myse og myseproteiningredienser fremstilt fra nativ myse påvirkes av melkens varmebehandlingshistorie. Nativt myseproteinkonsentrat fremstilt vha. fraksjonering av kjernemelk hadde sammenlignbare kjemiske og funksjonelle egenskaper med nativt myseproteinkonsentrat fremstilt av pasteurisert skummetmelk. Kaseinstandardisering av ystemelk førte til en økning av kasein makropeptid in mysen. Mikrostrukturen og viktige reologiske egenskaper hos myseproteingeler ble påvirket av en økning i kasein makropeptidkonsentrasjon.

LIST OF PAPERS

Paper I — Svanborg, S., Johansen, A.-G., Abrahamsen, R. K. & Skeie, S. B. 2014. Initial pasteurisation effects on the protein fractionation of skimmed milk by microfiltration. *International Dairy Journal,* 37, 26–30.

Paper II — Svanborg, S., Johansen, A.-G., Abrahamsen, R. K. & Skeie, S. B. 2015. The composition and functional properties of whey protein concentrates produced from buttermilk are comparable with those of whey protein concentrates produced from skimmed milk. *Journal of Dairy Science, 98, 9, 5829–5840*.

Paper III — Svanborg, S., Johansen, A.-G., Abrahamsen, R. K. & Skeie, S. B. 2015. Casein-standardisation of cheese milk changes the composition of whey. Manuscript

Paper IV — Svanborg, S., Johansen, A.-G., Schüller, R. B., Abrahamsen, R. K. & Skeie, S. B. 2015. Caseinomacropeptide influences the functional properties of a whey protein concentrate. Manuscript submitted to *International Dairy Journal*.

ABBREVIATIONS

A	Area
α-LA	α-lactalbumin
β-LG	β-lactoglobulin
BM	Buttermilk
BSA	Bovine serum albumin
BTN	Buthyrophilin
C	Concentration
Ca^{2+}	Ionic calcium
$CaCl_2$	Calcium chloride
CCP	Colloidal calcium phosphate
CF	Volume concentration factor
cfu	Colony-forming unit
Cl	Chlor
CMP	Caseinomacropeptide (glycosylated and non-glycosylated)
CO_3	Carbonate
DF	Diafiltration
DWP	Demineralised whey powder
G’	Storage modulus
G’’	Loss modulus
GMP	Glycosylated caseinomacropeptide
GP	Gradient porosity
Ig	Immunoglobulin
HHP	High hydrostatic pressure
HTST	High temperature short time
i	Specific component
L	Litre
LF	Lactoferrin
MF	Microfiltration
MFGM	Milk fat globule membrane
MF-UF	Concentration by microfiltration and diafiltration
mL	Millilitre
mM	Mill mole

NF	Nanofiltration
NPN	Non-protein nitrogen
NWPC	Native whey protein concentrate
NWPI	Native whey protein isolate
P	Pressure
PAS	Periodic acid Schiff
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEF	Pulsed electric field
PI	Phosphatidylinositol
PL	Polar lipid
PO_4	Phosphate
PS	Phosphatidylserine
R_f	Retention factor
RO	Reverse osmosis
RP-HPLC	Reversed-phase high-performance liquid chromatography
s	Seconds
SEC	Size-exclusion chromatography
SEM	Scanning electron microscopy
SH	Sulfhydryl
SO_4	Sulphate
SPH	Sphingomyelin
SPM	Skimmed milk powder
TMP	Trans membrane pressure
TP	Ture protein
TS	Total solids
UF	Ultrafiltration
UF-DF	Concentration by ultrafiltration and diafiltration
V	Volume
WP(s)	Whey protein(s)
WPC(s)	Whey protein concentrate(s)
WPH	Whey protein hydrolysate
WPI(s)	Whey protein isolate(s)
XDH	Xanthine dehydrogenase/oxidase

AIMS OF THE STUDY

Concentrated whey protein (WP) products are popular ingredients because of their beneficial functional and nutritional properties. Concentrated WP products are usually obtained from whey, the by-product of cheese making. The cheese milk, and later the whey and liquid whey protein concentrate (WPC), undergoes several processes before application in final food products as powder ingredients: a certain degree of acidification and renneting of milk, heat treatment of milk and raw whey, and concentration and evaporation processes influence the composition and the final product's functionality. Developments in microfiltration (MF) technology since 1980's enable utilisation of whey directly from skimmed milk, resulting in a unique starting material for production of a new concentrated WP ingredient. The skimmed milk MF permeate, native whey is neutral and contains undenatured proteins, is free of starter bacteria, cheese fines, and proteolytic fragments from renneting. When using a membrane pore size of ≤ 0.2 µm, the resulting native whey can be considered a sterile liquid, and initial heat treatment of the permeate can be omitted. All this makes native whey a unique starting material for production of a new, concentrated WP ingredient (NWPC). Is it possible to obtain native whey from other milk sources than skimmed milk? Buttermilk (BM), the by-product from churning, has similar composition to skimmed milk's. Would the functional properties of a NWPC produced from BM be satisfactory? This study is described in Paper II.

The MF retentate, the casein-rich fraction, is widely used to produce micellar casein concentrate, or as casein-standardised cheese-milk. Reducing the volume of milk (MF retentate) to be pasteurised lowers milk treatment costs. High-temperature short-time (HTST) pasteurisation of milk is one of the most commonly applied dairy processes, even prior to MF. During HTST, heating at 73 °C for 15 s kills pathogenic bacteria in milk, without changing flavour or colour. How would an initial HTST treatment influence the MF fractions? The casein-rich fraction was characterised in Paper I with respect to initial HTST treatment, but will not be in focus in this thesis.

The fractionation of a casein-rich fraction and native whey could result in two very different types of whey: native whey and the whey generated from casein-standardised cheese milk, the latter with an

elevated CMP:TP ratio (Paper III), whereas the native whey is free of CMP. The effect of elevated CMP:TP ratio was studied concerning functional properties of WPC in Paper IV.

This PhD thesis aimed to investigate the production and characterisation of native whey, and a NWPC obtained from fractionation of dairy fluids. The four hypotheses that we tested in this study were:

- Does an initial heat treatment influence the composition of MF fractions (casein-rich retentate and native whey) obtained from fractionation of skimmed milk? (Paper I).
- Can a native WP ingredient be produced from other starting materials than skimmed milk (i.e., BM) using MF? (Paper II).
- Would an elevated casein content in cheese milk increase the CMP:TP ratio in the resulting whey during production of a Gouda-type cheese? (Paper III).
- Does the ratio of CMP:TP influence the functional properties of WPC? (Paper IV).

BACKGROUND, RESULTS AND DISCUSSION

WP is a popular ingredient in the food, feed and pharmaceutical industries due to its unique functional (Morr and Ha 1993; de Wit 1998; Ryan et al. 2013; Smithers 2015) and nutritious properties (Ha and Zemel 2003; Huth et al. 2006; Luhovyy et al. 2007; Madureira et al. 2007a; Pereira 2014; Devries and Phillips 2015). The global demand for WP products has increased strikingly the last five years, mainly driven by the strong growth in the infant formula, sport nutrition, and health foods markets (Lagrange 2015). WPs are obtained from sweet whey, the by-product from cheese making using rennet-coagulated milk. Continuously, new developments in filtration technology enable a more optimal use of milk components (Brans et al. 2004), as milk can be fractionated using MF techniques to produce a casein-rich fraction and native whey. MF is a clean, safe, and effective fractionation technology, well-suited to produce high-value food ingredients. The fractionation of skimmed milk and BM to produce native whey and NWPC has been the focus of this work. The final product, NWPC, has been evaluated with respect to chemical and functional properties. NWPC differs from WPC from cheese in that it lacks CMP. Casein standardisation by MF may eliminate the seasonal variations of casein in milk (Papadatos et al. 2003; Guinee et al. 2006), and improves the rennet-coagulation properties of milk (Neocleous et al. 2002; Guinee et al. 2006; Govindasamy-Lucey et al. 2007). However, the integration of fractionation in cheese manufacture may increase the CMP content in the whey (Outinen et al. 2008, 2010a, b, Paper III). The effect of an elevated CMP content in WPC was studied with respect to important functional properties: solubility and heat-induced gelation.

WHEY PROTEINS AND WHEY PROTEIN PRODUCTS

The term WP is defined as the proteins that are soluble in the milk serum after precipitation of casein at pH 4.6 and 20 °C (Farrell et al. 2004). In milk, the major WPs are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), immunoglobulins (Ig), lactoferrin (LF), and the proteose-peptone fraction. Sweet whey, the by-product from rennet-induced coagulation of milk and the most common source of WPs, contains other minor serum-soluble proteins and proteins from the milk fat globule membrane (MFGM) (Mather 2000) in addition to the WPs listed above. β-LG and α-LA account for 50 and 20% of the WPs in whey and native whey (Madureira et al. 2007b). CMP, the proteolytic peptide from cleavage of κ-casein (κ-CN) by chymosin (Eigel et al. 1984), is the third-most

abundant peptide component in whey. The major obstacle regarding production and utilization of WPs and concentrated WP products is their heat sensitivity. The heat stability of the major WPs differs: BSA, Ig and LF are very heat labile, whereas α-LA and β-LG are more heat stable (Considine et al. 2007). However, β-LG, α-LA, and CMP are the major contributors to the functionality of WP products, due to their high concentration in the whey. Table 1 lists some of the properties of the major WPs important to production and functionality of WP products:

Table 1. Bovine WPs and some of their properties, adapted from Farrell et al. (2004), Considine et al. (2007) and Navarro et al. (2015)

Protein	Concentration in skimmed milk (g L^{-1})	Molecular weight (Da)	Number of disulphide bonds / free thiol groups	pI	Denaturation temperature (°C)
β-LG A	2–4	18 363	2 / 1	5.13	65
β-LG B		18 277		5.13	70
α-LA	0.6–1.7	14 178	4 / 0	4.2–4.5	< 66
Bovine serum albumin	0.40	66 399	17 / 1	4.7–4.9	52–60
Immunoglobulin M	0.09	1 000 000	–	12.10	81
Immunoglobulin G1	0.3–0.6	161 000	–	5.5–6.8	62
Lactoferrin	0.02–0.1	76 110	17 / 0	8.81	90

Abbreviations: α-LA = α-lactalbumin; β-LG A/B = β-lactoglobulin genetic variant A/B; pI = isoelectric point.

WP products are applied in the food industry mainly as powders: whey protein concentrate (WPC, 25–89% protein), whey protein isolate (WPI, > 90% protein), demineralised whey powder (DWP) or whey protein hydrolysate (WPH). The application of WP products in powder form is by far most common in the food industry. However, fresh, liquid WPC could be used as an ingredient in other dairy products, if practical shelf-life challenges are overcome. The processing of sweet whey and production of commercially available WP products are described in this chapter, with focus on WPC 80-89 made from sweet whey.

Sources of whey proteins

Commercial WP products, in the following text referred to as WP products, are in general obtained from sweet whey unless other sources are identified in the text. Sweet whey from the manufacture of rennet cheese or rennet caseinate is a good starting material to produce WP products. Sweet whey contains lactose, minerals, WP, CMP, and fat. The pH of sweet whey is generally in the range of 6.2–6.4. The composition of the whey depends on the quality of milk, season, species, genetics, as well as processing variations like milk heat treatment, addition of $CaCl_2$, bacteria starter culture, type and concentration of coagulant, curd cutting, cooking and draining, pH and temperature (Morr and Ha 1993). The main component in whey is lactose, accounting for approx. 70% of the total solids (TS), and having a protein content of approx. 12% of the TS. Commercial WPC manufactured from sweet whey by ultrafiltration (UF) has a lower degree of denaturation (13–22%) than does WPC made from acid whey (56–58%) (Morr and Ha 1993). Acid whey is derived from acid coagulation of casein, production of fresh cheese, cream cheese and strained yoghurts (Walstra et al. 2006) at pH < 5.1. Whey from cottage cheese and cream cheese contained less proteins, citrate, and inorganic phosphorus and had higher concentrations of calcium and lactic acid than what is reported for sweet whey (Schmidt et al. 1984; Chandrapala et al. 2015). Some rennet is commonly applied in production of cottage cheese and quark, however, low pH is the main coagulating factor. Less amounts of rennet is used in such fresh cheese productions than in productions of rennet-coagulated cheese types, and may lead to a lower level of CMP in acid whey than in sweet whey. However, literature that reports on the content of CMP in acid whey is hard to find. In acid whey, pH may be as low as 4.5–3.5. Acid whey from Greek-style yoghurt has TS < 6% and a protein content < 0.2% due to the severe heat treatment that causes WP to aggregate to the casein micelles (Smithers 2015). In general, acid whey is a less desirable starting material for WPC production due to its acid flavour (Kosikowski 1979). The high content of mineral and lactic acid could make processing challenging. However, optimisation of processing conditions, such as pH and temperature, can manipulate the composition and surface hydrophobicity of WP, in order to reduce aggregate formation and acid whey (Chandrapala et al. 2015).

Native whey has actually nothing to do with whey. The name “native whey” is somewhat misleading, since native whey is a part of the serum phase of fractionated milk and has nothing to do with whey or cheese making. Other names have been used: *milk serum proteins*, *ideal* or *virgin whey*. Native whey contains milk serum components, has no bacteria from the starter culture, bacteriophages, cheese fines, CMP, or denatured WP aggregates. The comparison of NWPC to traditional WPC obtained from cheese whey is, however, still reasonable, since both are good sources of WP. The fat

and mineral content is lower in native whey than in sweet and acid whey. The major compositional differences between these three main sources of WP are listed in Table 2.

Table 2. Composition of sweet and acid whey from bovine milk in comparison with the composition of bovine milk, adapted from Smithers (2015) and Paper I

Components or measurements	Sweet whey [a]	Acid whey [a]	Native whey [b]	Milk [a]
pH	> 5.6	< 5.1	6.7–6.8	6.5–6.8
Total solids (%)	6.3	6.6	6.1	12.8
Total protein (%)	0.75	0.75	0.4	3.5
Casein (%)	< 0.05	< 0.05	nd	2.8
Whey protein (%)	0.7	0.7	0.4	0.7
Lactose (%)	4.9	4.9	5.0	4.9
Fat (%)	0.1	0.1	0.03	3.7
Ash (%)	0.5	0.8	0.4	0.7

[a] Adapted from Smithers (2015). [b] Paper I, native whey from fractionation of unpasteurised skimmed milk using a volume concentration factor of 2.47. Abbreviation: nd = not detected.

The starter culture used in cheese production can continue to convert lactose into lactic acid in cheese whey. The thermal treatment, membrane filtration, and/or rapid cooling prior to further processing of the whey are required to stop fermentation of lactose into lactic acid.

Production of whey protein products

Several pressure-driven membrane filtration technologies are used in production of WP product, including MF, UF, nanofiltration (NF) and reverse osmosis (RO). Concentration of whey (mainly RO and UF) and other processes for further processing of liquid WPC influence the chemical composition of WP products (Schmidt et al. 1984). Some of the process-induced denaturation and aggregation of concentrated WP products are discussed in this thesis with respect to functional properties. Figure 1 shows different membrane technologies used to produce WP powder from cheese whey or milk

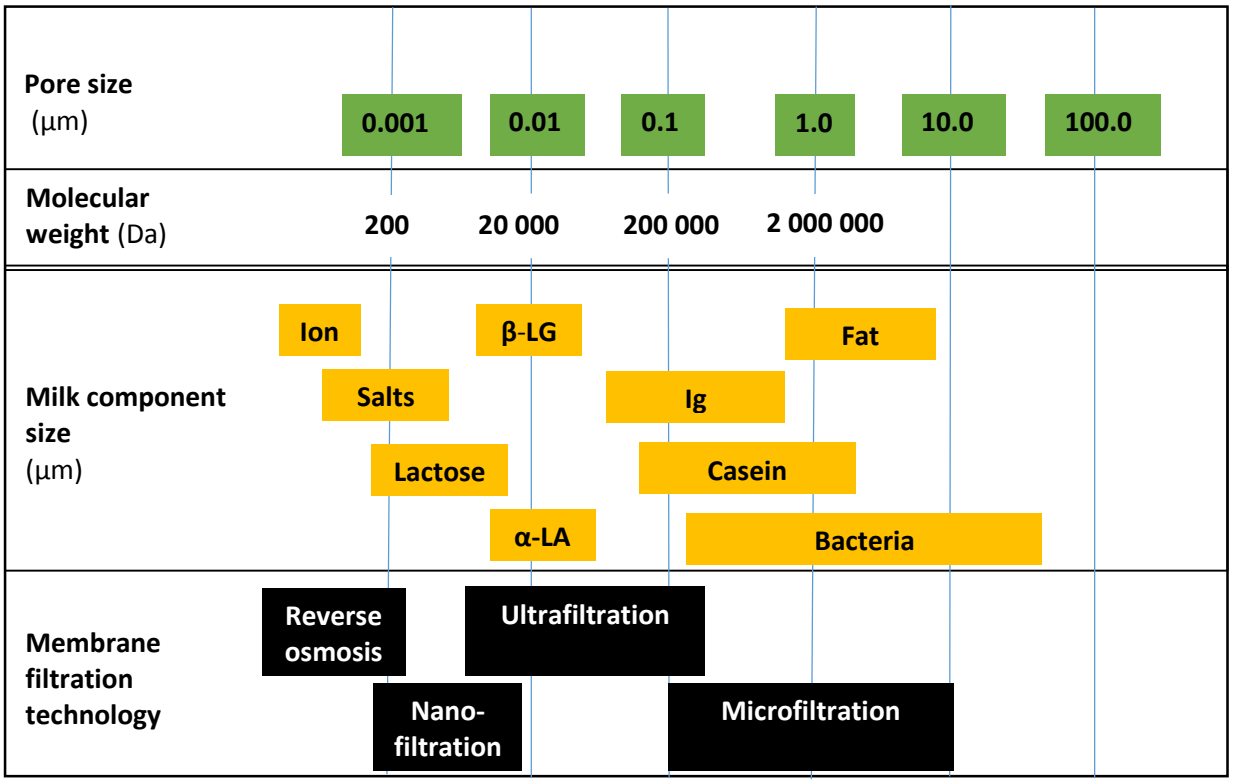

Figure 1. Membrane technologies used to produce commercial WP products (α-LA = α-lactalbumin, β-LG = β-lactoglobulin, Da = Dalton, Ig = Immunoglobulins). Adapted from Lauritzen (2015).

The composition of UF concentrate and whey powder equals the composition of solids found in the clarified and pasteurised whey: Raw or clarified whey is concentrated by RO to 50–60% (≤ 28% TS), evaporated to ~65%, and seeded with lactose crystals to induce crystallisation. After lactose crystallisation, the concentrate is then dried. When producing demineralised whey powder (DWP), WP hydrolysates (WPH), WPC, and WPI, the gross composition of the milk solids found in the final product depends on the composition of the feed, the filtration process, membrane properties, and degree of diafiltration (DF). In concentrated WP products like WPCs and WPIs, the concentration of the functional components, the proteins, is high (> 60). Different processing steps in production of concentrated WP products are illustrated by a simplified flow chart below (Figure 2):

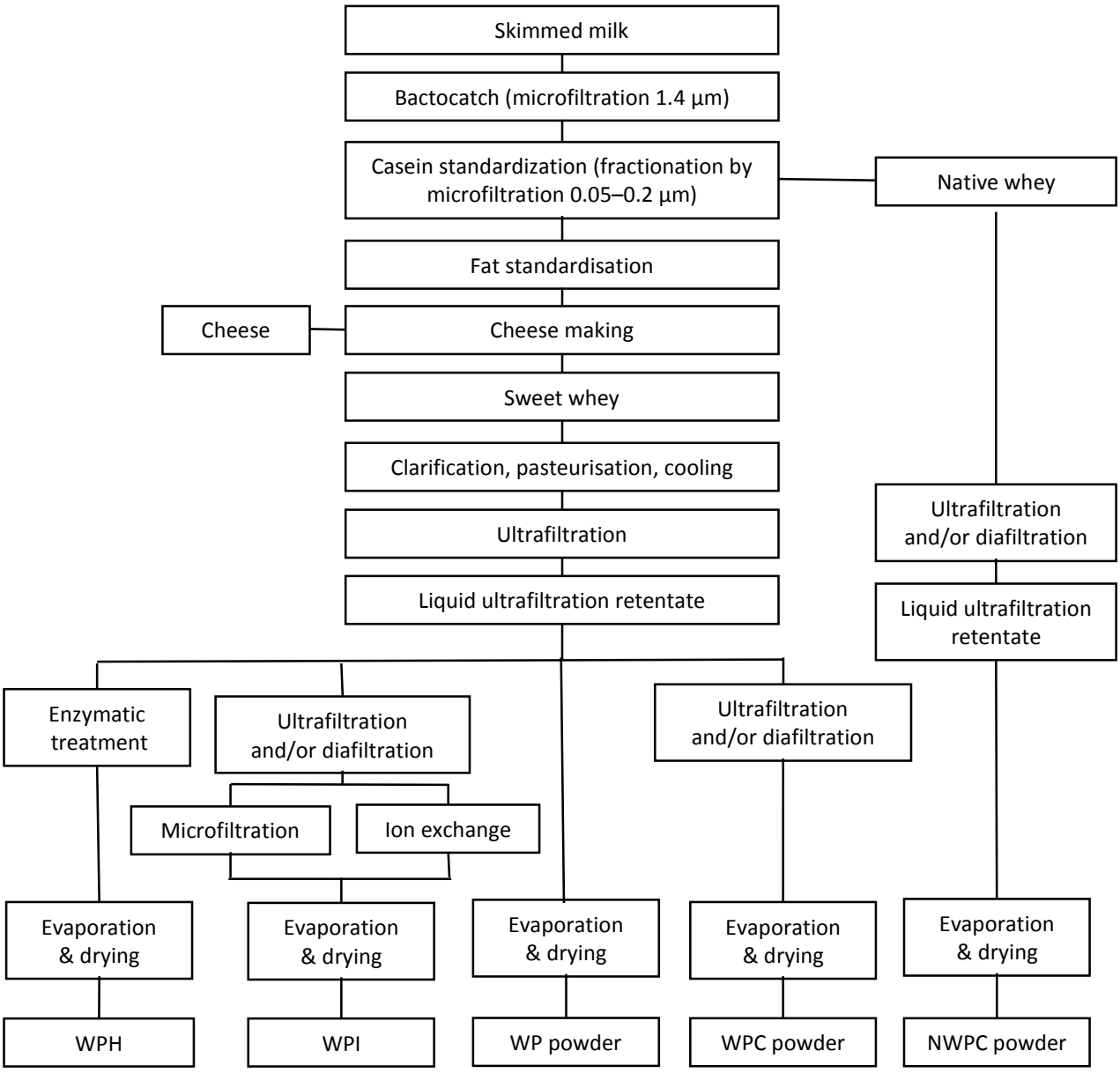

Figure 2. Production of WP products (NWPC = native whey protein concentrate, WP = whey protein, WPC = whey protein concentrate, WPI = whey protein isolate, WPH = whey protein hydrolysate)

The first step in production of concentrated WP products is the clarification of raw whey. This can be achieved either by the use of a cyclone, by centrifugation alone, or by combining centrifugation with a rotating screening, depending on the level and size of the cheese fines (Pearce 1992). Centrifugation will recover most of the whey cream. Residual lipids in whey may cause fouling during further filtration steps of whey downstream from centrifugation. Addition of $CaCl_2$ and adjustment to pH 7.5 remove

residual lipids as the first step of whey clarification. During the second step in whey clarification, a MF membrane with pore size 0.8 µm retains the flocculated calcium phosphate-lipid particles. Microfiltered cheese whey contains less fat, bacteria and cheese fines, and alternatively colour (annatto in Cheddar cheese whey), than does the unfiltered whey (Saboya and Maubois 2000). Another benefit of including MF in whey clarification is an increased permeate flux during UF (Saboya and Maubois 2000; Steinhauer et al. 2015b). After clarification, and potentially MF, the whey must be immediately pasteurised at 72–78 °C for 15–20 s for hygienic reasons (Pearce 1992). However, pasteurisation of whey or liquid WPC (McDonough et al. 1974), and potentially evaporation, could lead to some WP denaturation, which influences functional properties of the final product. After pasteurisation, the whey is cooled to 60–65 °C and held at this temperature for 30–60 min. The temperature is then lowered to 50 °C for UF (Schuck 2013). This heat-and-hold treatment serves to stabilise the calcium phosphate and thereby reduce the fouling of the membranes during UF. No clarification, defatting, or pasteurisation of native whey is needed. The native whey can be concentrated by UF or optional UF-DF. The liquid WPC from cheese or native whey is evaporated (or not, depending on the viscosity and protein content), and dried. To reduce the water content, native whey or the defatted, pasteurised whey can be concentrated by RO as a preliminary step prior to other filtration steps. The increasing viscosity in the retentate during concentration of whey by RO limits the solid content to a maximum of 20–22%. However, recent process developments enable a protein concentration of the liquid whey RO retentate up to 28% using two RO filtrations: the RO polisher permeate from the second stage serves as DF water in the first stage (Paar 2015). UF is used to increase the WP concentration, using membranes so dense that only lactose, minerals and minor non-protein nitrogen (NPN) components are transferred to the permeate. The content of soluble milk components such as NPN and lactose may protect β-LG and α-LA from unfolding during heating (Anema et al. 2006). The content of NPN and lactose is therefore important during whey processing, and for the functional properties of the final WP product. DF over the same types of membranes enables further reduction in NPN, lactose and minerals. In high-protein WPC or WPI production, a concentration by UF and DF is carried out to wash out lactose, the NPN fraction and to some degree the minerals (de la Fuente et al. 2002a). In WPI production, an ion exchange step is applied to reach protein contents > 90%. The UF retentate can be dried directly, or concentrated further by evaporation at < 68 °C before spray drying. During spray drying, liquid WPC is then atomized into a stream of hot air and the water evaporates rapidly during spray drying. This process yields a powder with 10–14% moisture. In a two-stage drying process, which is the most common industrial drying method for WP products, lactose is allowed to crystallise further before being dried to a moisture content of approx. 4% in a vibrating fluid bed (Pearce 1992).

Different WPC and WPI are categorised according to their protein concentration: WPCs containing 25–40% protein (WPC25–40) are classified as low-protein WPCs. WPC34 is made from whey UF concentrate, and serves as a cheap replacement for skimmed milk powder (SPM) in several applications or in demineralised form in infant formula production. In the medium range, WPC45-60 contains 45–60% protein. UF without DF can achieve up to 60% protein in WPC (WPC60), which is a popular ingredient in pastry and baked products. Including DF, WPCs containing 80–89% protein can be produced. In the production of demineralised and highly concentrated WP products, UF is combined with NF, electrodialysis and/or ion exchange. Nanofiltration can concentrate whey or liquid WPC to 25–35% demineralised whey powders (DWP25–35). Nanofiltration is a monovalent demineralisation process that alters the mineral composition of the final product in addition to reducing the content. Electrodialysis permits removal of 50–90% of the minerals (DWP50–90), whereas ion exchange is mainly used to produce DWP90 high grades and serves as a polisher after NF, RO or electrodialysis (Nejedly 2015).

Concentrated whey protein products

Popular uses for high-protein WP ingredients are protein-enhanced food products, as an egg replacement, or other applications based on their functionally unique solubility, gelling, foaming, and blend flavour (El-Salam et al. 2009). WPC80-89 are high-protein WPCs manufactured using UF/DF. Different commercial WPC80 varies in chemical composition and application depending on the whey source and choice of production technology (Morr and Ha 1993; Evans et al. 2010). WPI contain > 90% protein and are often used in sport nutrition and nutraceuticals. For example, α-LA and LF isolates are now available commercially, as ingredients in nutritional beverages and infant formulas. α-LA and β-LG can be fractionated from milk, whey or rehydrated concentrated WP powders by combining membrane filtration technology (Kamau et al. 2010) with precipitation (Eugenia Lucena et al. 2007), hydrophobic interaction/anion-exchange chromatography (Santos et al. 2011) or UF (Baldasso et al. 2011) in combination with acid precipitation (Akpinar-Bayizit et al. 2009). In this thesis, WPI refers to WP products with a protein content > 90% and all the major WPs. WPI contains less fat, polar lipids (PL), minerals, and lactose than does WPC80 (Morr and Ha 1993; Wang and Lucey 2003; Lorenzen and Schrader 2006). WPC80 had higher amounts of sialic acid, indicating higher amounts of CMP than WPI (Nakano and Ozimek 1999; Lorenzen and Schrader 2006). Wang and Lucey (2003) found that WPI made using ion-exchange chromatography contained less CMP than did WPI made by membrane

filtration processes. Lorenzen and Schrader (2006) studied the gelation properties of WPC80 and WPI, and found that WPI made stronger and more elastic gels, which was attributed to the lower fat, lactose and phospholipid contents of WPI.

CMP, the C-terminal (f 106-169) end of κ-casein that is released by chymosin during cheese making, is present in most commercial WP products obtained from sweet whey. This peptide accounts for about 20–25% of the protein nitrogen in whey (Thoma-Worringer et al. 2006) and WPC (Farias et al. 2010), and is important to functional and nutritional properties of high protein WP products. Several genetic variants of CMP have been identified, although variants A and B are the most frequent forms from bovine milk's whey (Smith et al. 2002). All post-translational modifications of κ-casein occur in the CMP segment (Huppertz 2013), which is classified into two major fractions due to its glycosylation: the glycosylated and phosphorylated glycomacropeptide (GMP), and the phosphorylated but non-glycosylated aglyco-peptide (Kreuss et al. 2009). These two classes of CMP are distributed evenly in whey (Furlanetti and Prata 2003; Molle and Leonil 2005). Jensen et al. (2015) found no correlation between the various CMP isoforms and the distinct genetic variant of κ-CN in individual cow's milk. However, the κ-CN glycosylated isoforms had a lower reaction rate of CMP release compared with that of non-glycosylated CMP. Glycosylation can show a high grade of heterogeneity by having variable binding sites and several kinds of carbohydrates attached. One of the sugars most frequently observed in GMP, N-acetyl-neuraminic acid, is used to determine total CMP (Fernando and Woonton 2010), although this method is associated with a high number of interferences and false positive and negative results (Sharma et al. 2013). The numerous variants of CMP make analytical work challenging. Several detection methods for CMP in milk and whey have been proposed (Picard et al. 1994; Molle and Leonil 1995; Vanriel and Olieman 1995; Elgar et al. 2000; Tran et al. 2001; Fukuda et al. 2004; Molle and Leonil 2005; Bremer et al. 2008; Fernando and Woonton 2010; Hernandez-Hernandez et al. 2010). Today, reversed-phase high-performance liquid chromatography (RP-HPLC) and size-exclusion chromatography (SEC) are the most used methods for CMP detection. SEC has reported false positive CMP results caused by Pseudomonas proteases producing pseudo-CMP (Campos Motta et al. 2014). RP-HPLC and SEC methods are unable to distinguish between pseudo-CMP (caused by *Pseudomonas* proteases) and CMP because these two peptides differ from each other by only one amino acid (Campos Motta et al. 2014). Recently, Campos Motta et al. (2014) differentiated and subsequently quantified CMP and pseudo-CMP in milk samples using liquid chromatography coupled to mass spectrometry with electrospray ionization. The implication of pseudo-CMP's effect on the functional properties of the WP product remains to be elucidated.

The absence or presence of CMP is the most distinct difference between functional properties of NWPC and WPC. During processing, CMP is retained by the UF membrane and it accounts for 20–25 (w/w) % of the protein nitrogen in cheese whey (Thomä-Worringer et al. 2006), depending on the particular whey source (Martin-Diana et al. 2006). Even higher amounts of CMP have been found in whey from MF retentate (Outinen et al. 2008, 2010b, Paper III). Regular milk typically coagulates when approx. 90% of the κ-caseins are hydrolysed, whereas a lower degree of hydrolysis is necessary to increase curd firmness to a similar level during cheese making using protein-enriched milk (Brandsma and Rizvi 2001). Because of the higher casein concentration, a reduced amount of rennet is one of the adjustments to consider when changing the cheese-making protocol from regular to concentrated milk. Using the same amount of rennet in protein-enriched cheese milk would probably release high amounts of CMP. Another option is to apply a lower temperature during renneting to slow down rennet activity and CMP release.

Functional properties of concentrated whey protein products

The functional properties of proteins can be classified into four main groups according to Kresic et al. (2006), as shown in Figure 3 derived from Jeewanthi et al. (2015).

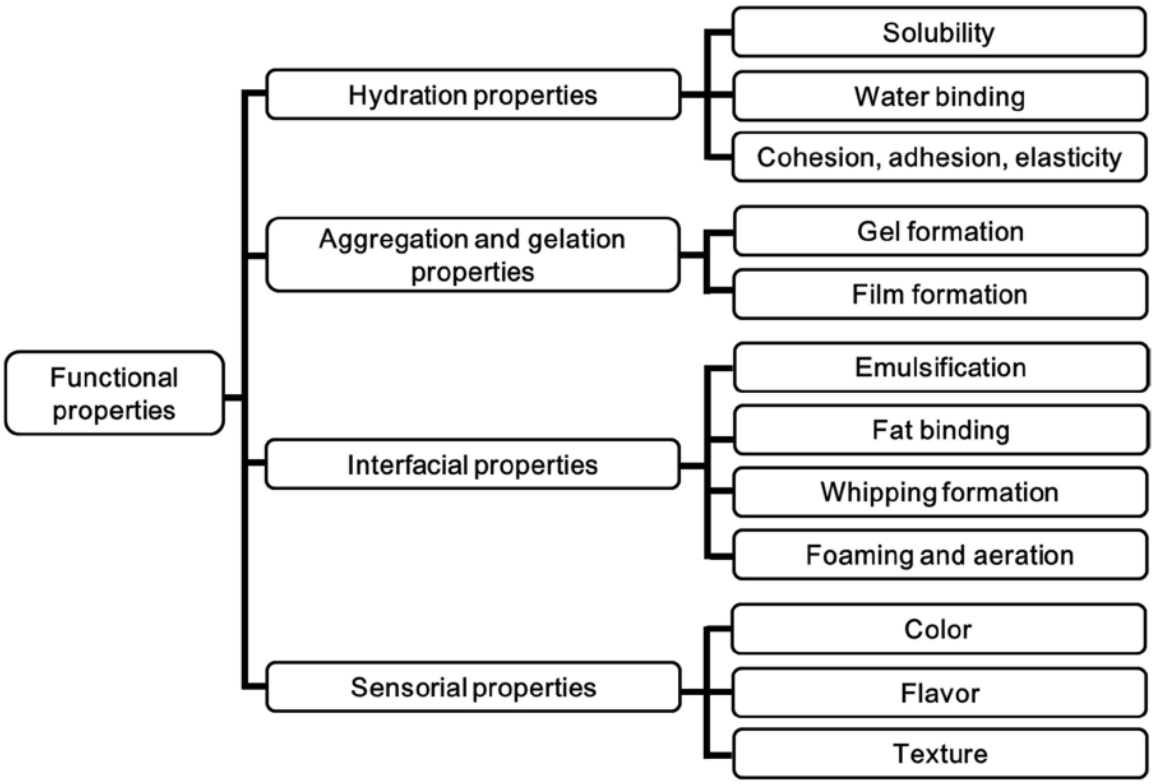

Figure 3. Classification of functional properties of proteins (Jeewanthi et al. 2015)

Solubility

WPC and WPI have good solubility even at low pH, a unique property that allows the products to function in acidic foods and beverages (Pelegrine and Gasparetto 2005), as well as in neutral and high-pH applications. Solubility defines other functional properties such as emulsifying, gelling, and foaming. In beverages and other applications where clarity is important, solubility is the primary factor to determine the final product's quality (Mangino 1992). Solubility of proteins relates to surface hydrophobic (protein-protein) and hydrophilic (protein-solvent) interactions, and is a function of the state of the protein (native, partly unfolded or denatured) and solvent conditions like pH, ionic strength and temperature (Pelegrine and Gasparetto 2005). Proteins are usually less soluble at their isoelectric points (pI) because the electrostatic repulsion between the molecules is minimal, and the protein molecules will under the right conditions aggregate and precipitate (Zayas 1997). At pH far

from pI or high ionic strength, protein has a net charge, and water can interact with the protein charges. WPs in their native states have a high solubility at pH 3 to 8, according to Zayas (1997). Hydrophobic and hydrophilic regions of proteins affect the solubility, as protein-protein (hydrophobic) interactions decrease solubility, whereas hydrophilic interactions promote water binding. During heat-induced denaturation, the globular conformation of WP is irreversibly disordered to structure that favours hydrophobic interactions, which leads to aggregation with other protein molecules and/or precipitation. Because undenatured WPs are soluble at pH 4.6, solubility at this pH reflects the degree of denaturation in WP products.

Heat-induced aggregation and gelation of concentrated whey protein products

β-LG is assumed to be the driver of protein aggregation in WPC solutions due to its abundance and relatively low heat stability. Under heat treatment at neutral pH, the native β-LG dimers dissociate to monomers, which makes previously hidden SH groups in the globular molecule available to interact with other proteins via disulphide-bond and/or hydrophobic interaction (Havea et al. 2004). The unfolding of globular proteins is followed by an endothermic effect (heat uptake) (de Wit 1990). The irreversible denaturation (i.e., the aggregation) starts at approx. 60% unfolding of β-LG (de Wit 1990). Unfolded β-LG may form aggregates with other β-LG molecules, caseins (for example κ-casein) (Donato and Guyomarc'H 2009), BSA, α-LA (Dalgleish et al. 1997) or proteins from the MFGM (Ye et al. 2004). The aggregation of WPs may be interrupted by the presence of chaperone proteins, lactose, or NPN components. Chaperone proteins assist the covalent folding/unfolding and the assembly/disassembly of other proteins and prevent irreversible aggregation induced by thermal, as well as non-thermal, stress by providing their hydrophobic surfaces to the other unfolding proteins (Ellis and van der Vies 1991). A chaperone behaviour of caseins (O'Kennedy and Mounsey 2006; Guyomarc'h et al. 2009) and CMP (Croguennec et al. 2014) have been reported: CMP increased the rate of β-LG denaturation at pH 3.0-6.7.

A gel is an intermediate structure between solid and liquid. The gelling ability of proteins provides textural and water-holding properties in many foods (e.g., puddings, pates, and yoghurts). Heat-induced gelation is the most commonly applied method to make WP gels, and has been the focus of this work. However, other gelation methods to form WP gels are reported: cold gelation that includes salt-induced and acid-induced gelation (Bryant and McClements 1998; Alting et al. 2000; Alting et al. 2003), and gelation induced by high hydrostatic pressure (HHP) (Funtenberger et al. 1995; Lopez-Fandino 2006), or by enzymatic crosslinking (Famelart et al. 1998). In general, heat-induced gels are

stronger and more adhesive than cold-set gels induced by acid, salt or protease treatment (Ju and Kilara 1998). Heat-induced protein gels are formed in a multi-step process in which all steps occur simultaneously. Unfolding and aggregation initiates the gelation. The aggregation rate of native β-LG is exponential with increasing temperature, and the aggregates increase in size during heating until no native proteins are left. At sufficiently high protein concentrations, the aggregates grow into a gel network through formation of linked strings. The size and structure of these aggregates depend on type of protein, protein concentration, heating regime, ionic strength and other external conditions like presence of sugar (Bordenave-Juchereau et al. 2005; Nicolai et al. 2011). The presence of Ca^{2+} enhances heat-induced aggregation of WPI by decreasing the aggregation temperature and increasing the aggregation rate (Xiong 1992). However, excess calcium ions may have an inhibitory effect on the protein aggregation (Sherwin and Foegeding 1997). At pH values far from the pI of WPs, the net charge causes electrostatic repulsion that stabilises monomers or small oligomers of WPs in aqueous solutions. Heating or pressurizing causes interaction between monomers/dimers/small oligomers through hydrophobic interaction or formation of hydrogen bonds. At pH 4–5, the gelation properties of WPI and WP are negligible due to a low net charge and a low number of possible protein-protein interactions. However, gels formed at high ionic strength (low electrostatic interaction) have an opaque appearance with a coarse and particulate structure (Verheul and Roefs 1998). In general, WP gels are stabilised by covalent disulphide bridges at pH 7–8. Smaller aggregates are formed at these pH values, and the gel appears translucent. At pH 6, the aggregates grow in size and gel strength increases due to the additional stabilisation by non-covalent bonds (Lorenzen and Schrader 2006). At low pH, the heat-induced gel network is stabilised mainly by hydrophobic bonds. A high net charge of proteins at low pH makes the network fine-stranded and translucent, similar to what occurs at high pH and high negative charge (de la Fuente et al. 2002b; Singh and Havea 2003; Resch et al. 2005; Lorenzen and Schrader 2006).

CMP had a negative effect on the gel strength of heat-induced WPC and WPI gels (Britten and Pouliot 1996; Veith and Reynolds 2004; Lorenzen and Schrader 2006; Heino et al. 2007). The addition of CMP had the same effect on gel strength whether the CMP was replacing some of the WP in the model system, or whether the protein content was kept constant (Xianghe et al. 2012). When CMP was present, the gel strength was equally low, regardless of the CMP concentration (Paper IV). CMP influenced the storage modulus G' of the gels with 33% CMP at pH 7.0, in accordance with Martinez et al. (2010), who reported a synergetic interaction between CMP and β-LG heated at pH 7.0. In mixed systems (CMP to β-LG ratio 25:75), the β-LG was allowed to gel, and the presence of CMP enhanced

high G' values. However, gels containing CMP were softer than pure β-LG gels and the mixed gels with a CMP to β-LG ratio of 50:50 and 75:25 showed much lower G' than did pure β-LG gels.

Using microscopy techniques (SEM, transmission electron microscopy, light microscopy, and confocal laser scanning microscopy), the structure of the food gel network can be characterised by aggregate size, pore size, and volume distribution, all of which can correlate to other functional properties (Langton and Hermansson 1996). Image analysis may give other information than that provided by other analysis measurements, for instance by various rheological parameters. In Paper IV, CMP influenced the microstructure of heat-induced WPC gels at pH 7.0. As the CMP:TP ratio increased, the microstructure became more swollen and coarse with larger pores as shown in the SEM micrographs in Figure 4 adapted from Paper IV. In contrast, the microstructure of all gels was very similar at pH 4.0 (not shown), which probably can be related to the strength of the CMP-β-LG interaction.

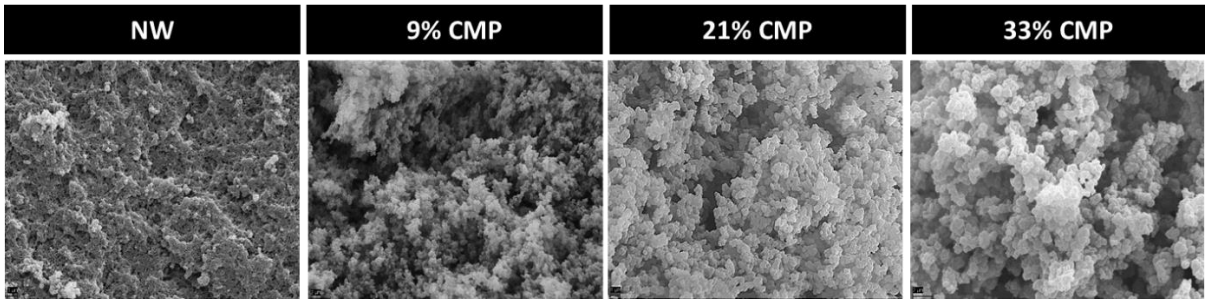

Figure 4. Microstructure of heat-induced 12% NWPC gels with 0, 9, 21 and 33% CMP of TP at pH 7 (Paper IV)

Xianghe et al. (2012) observed a large effect of CMP on microstructure of heat-induced WPC gels at neutral conditions using SEM, similar to the observations reported in Paper IV. The network became swollen, had larger pores and a more particulate feature with increasing CMP:TP ratios. The chaperone function of CMP, as suggested by Croguennec et al. (2014), may account for a neutralisation of charges on the β-LG molecules. The neutralisation facilitated a non-covalent association of large aggregates, being more pronounced at high CMP concentrations.

Interfacial properties: foaming and emulsifying

WPs are efficient foaming and emulsifying agents, making concentrated WP products useful ingredients in several food applications. Functionality of WP products mainly depends on the behaviour of β-LG, and on the solubility and flexibility of the proteins, as well as on other non-protein components. Foam consists of air bubbles separated by a thin, continuous liquid layer (Zayas 1997). Whipped cream, cappuccino milk foams, meringue, the head of an English ale, bread and mousses are all foams. Soluble proteins form the most stable foams because they can interact and form viscous films: the proteins diffuse to the air/water interface, concentrate, and reduce the surface tension. When unfolded, the hydrophobic and hydrophilic parts of the polypeptide are exposed at the aqueous/non-aqueous phase and form a film through further interactions (Zayas 1997). Foaming properties of WPC are commonly evaluated by overrun (maximum foam expansion) and foam stability (Phillips et al. 1987). Several factors affect these parameters, like pH and protein concentration of the solution, whipping aids and foam-inhibiting substances (lipids). An increased flexibility through a partial denaturation induced by heat treatment of WPC (Nicorescu et al. 2011) and WPI (Zhu and Damodaran 1994b) has been shown to improve foam stability, whereas a more severe heat treatment would have an opposite effect. In general, WP foams are not very heat-stable (Gauthier et al. 1993). High contents of fat and denatured proteins in concentrated WPC will suppress foaming. As long as the whey has been defatted (which also keeps the degree of denaturation low) (Gauthier et al. 1993), the foaming properties are good. Commercial WPC manufactured using UF contains 3.3–7.4% total lipids and 0.8–1.5% PL (Morr and Foegeding 1990). However, NWPC has less total fat than does WPC made from cheese whey (Heino et al. 2007; Evans et al. 2009, 2010; Luck et al. 2013, Paper II, Paper IV).

Emulsion-type products, for example, coffee whiteners, dietary formulas, liquid nutritional products and nutraceuticals, are an important application of WPs in the food industry (Singh 2011). Food emulsions, a heterogeneous mixture of fat globules, have a droplet size of 0.5–50 μm (Zayas 1997). Proteins are the dominant component in two kinds of food emulsions, that is, in emulsions that are the oil-in-water type or the water-in-oil type. The emulsifying properties of WPCs are influenced by lipids, minerals and sulfhydryl, and are related to denaturation and loss of solubility caused by different processing conditions (Zayas 1997). However, WPs are not as effective emulsifiers as caseins are (Gauthier et al. 1993) because of an unbalanced distribution of hydrophobic and hydrophilic groups (Yamauchi et al. 1980).

Sensorial properties

The sensorial properties of concentrated WP products are important to consider because they directly affect the final product's quality. Ideally, the flavour of WPC and WPI should be bland to avoid carryover off-flavours. Unfortunately, the flavour of WP is a concern for the dairy industry (Drake et al. 2009; Wright et al. 2009). Astringency and off-flavours from WPC or WPI in both acidic and neutral beverage applications reduce consumer acceptance (Evans et al. 2009, 2010). Volatile lipid oxidation products are primarily responsible for off-flavours (Carunchia Whetstine et al. 2003; Wright et al. 2009). Evans et al. (2009) found lipid- and protein-oxidation products in WPC34 and native WPC34 obtained from the same milk. The sensorial properties of the two types of WPC34s were good, with a bland flavour compared to that of commercially manufactured WPC products of similar composition, but having a higher degree of lipid oxidation.

Commercial WPC varies in flavour, and is influenced by cheese type, storage conditions of the product (Drake et al. 2009; Wright et al. 2009), processing time (Tomaino et al. 2004), and bleaching agent (Croissant et al. 2009) and other additions (Park et al. 2014). Whey, liquid WPC and rehydrated WP products have a turbid, greyish appearance, due to their aggregate size and possibly use of additives during cheese making (e.g., colour in Cheddar manufacture). In contrast, native whey is clear and yellowish (depending on the casein content) with a slightly brownish tint, although still a clear liquid concentrate or rehydrated powder. Recently, studies have demonstrated that WPI processing steps, including acidification (White et al. 2013; Park et al. 2014) enhance off-flavour production from WP in clear acidic beverages. Strategies to overcome formation of visual aggregates formed by CMP during storage of acidic whey beverages have recently been reported (Wang and Ismail 2012; Villumsen et al. 2015a; Villumsen et al. 2015b). Storage-induced aggregation was lowered by decreasing pH to 2.5, and was prevented by heat treatment at 120 °C for 20 s instead of 95 °C for 3 min (Villumsen et al. 2015a; Villumsen et al. 2015b). Using dextran and partially glycosylated WP, Wang and Ismail (2012) controlled and limited the Maillard-induced glycosylation in acidic whey drinks.

MICROFILTRATION TECHNOLOGY APPLIED IN THE DAIRY INDUSTRY

Basic principles of cross-flow filtration

Filtration processes in the dairy industry are pressure driven and use semi-permeable membranes. (Figure 1) shows all the filtration technologies commonly applied in the dairy industry. The size of the membrane pores defines the retention and permeability of the feed. Reverse osmosis, NF, UF and MF all involve cross-flow filtration. In cross-flow filtration, the retentate and feed flow parallel to the membrane surface, unlike traditional dead-end (static) filtration where the feed moves towards the membrane perpendicularly. Some of the particles in the feed are small enough to be transmitted through the membrane pores and to end up in the permeate, the liquid passing thorough the membrane. The particles larger than the pore size will be rejected by the membrane, and are concentrated in the retentate. Pore size and retention indicate the membrane's ability to retain molecules of a certain size. However, several factors influence the composition of the final retentate, and pore size alone does not define the composition of the fraction.

Retention, or rejection, describes the membrane's ability to retain feed solids, focusing on the retentate fraction. The retention factor (R_f) is equal to one minus the ratio of the concentrations (C) of a component (i) in the permeate and the retentate, as shown in Equation 1 (Koros et al. 1996):

Equation 1 $$R_f = 1 - \frac{C_{i\,permeate}}{C_{i\,retentate}}$$

In a similar manner, permeability is used as a measurement of membrane selectivity or performance with focus on the permeate fraction. To evaluate the filtration process, a mass balance showing the composition of both retentate and permeate can be calculated using Equation 2 where V is the flow (volume) of the different streams and C defines the concentration:

Equation 2 $$Vfeed \times C\ feed = (V\ retentate\ \times C\ retentate\ ) + (V\ peremate \times C\ permeate)$$

The concept of cross-flow filtration allows a continuous transmission of particles and water from the feed to the permeate. The feed moved parallel to the membrane surface preventing concentration

polarisation and clogging of pores ("caking") by the shear stress created by the turbulence on the membrane surface (Figure 5):

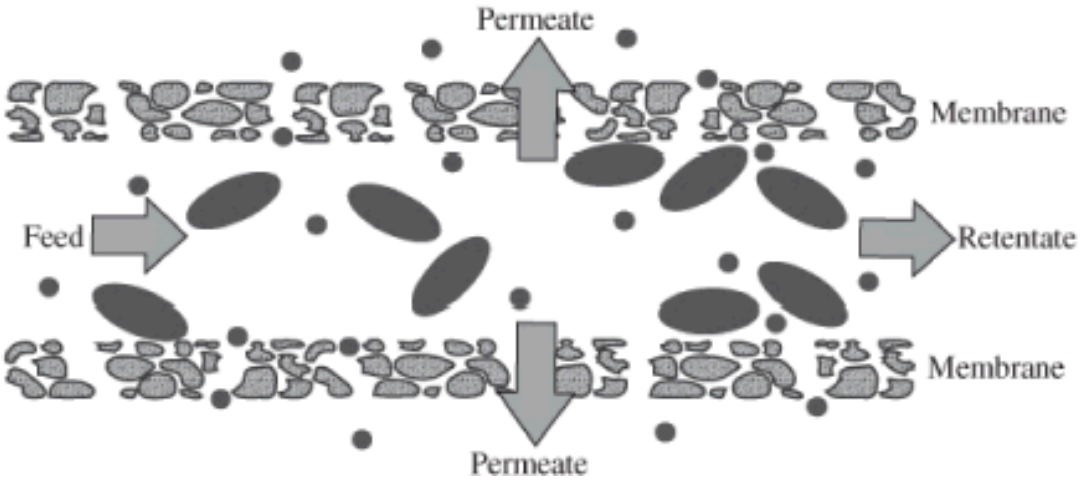

Figure 5. Cross-flow filtration, adapted from Smith (2013b)

The driving force in cross-flow filtration is the pressure across the membrane, from the feed side (inlet) to the permeate side (outlet). Due to the flow, there will be a pressure drop along the membrane – this pressure drop implies that the outlet (P_r) pressure is lower than the inlet pressure (P_f) (Piry et al. 2008). The pressure drop depends on flow resistance (flow velocity, flow spacer design, channels and fouling) (Hausmann et al. 2013). Figure 6 adapted from Lauritzen (2015) illustrates the pressure drop along the membrane:

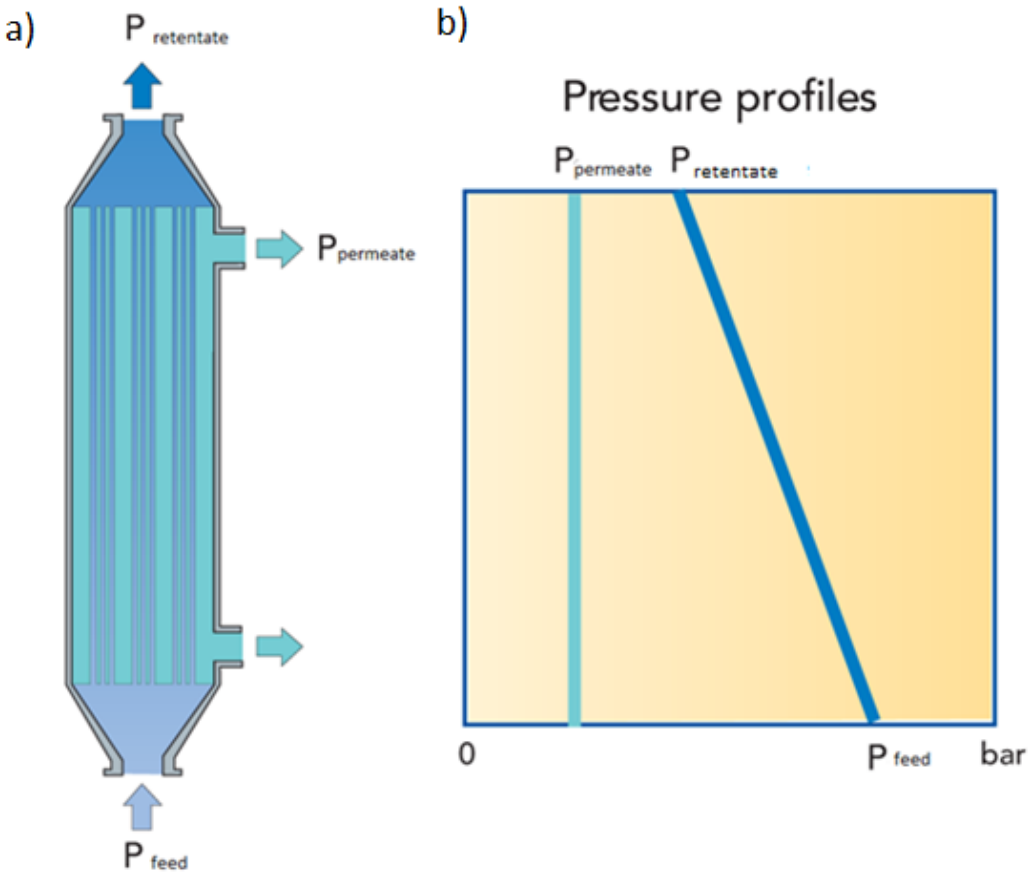

Figure 6. A pressure relation along the membrane during cross-flow filtration (Lauritzen 2015); a) is a membrane module and b) is the pressure profile, showing the pressure drop over the membrane.

In cross-flow filtration, the average pressure applied over the membrane, named trans-membrane pressure (TMP), is calculated as the average pressure over the length of the membrane module (*Equation 3*), whereas P_{feed} is the inlet pressure, $P_{retentate}$ is the outlet pressure, and $P_{permeate}$ is the permeate pressure. The permeate pressure is almost constant, and is lower than the retentate pressure.

Equation 3 $$TMP = \left(\frac{P_{feed}+P_{retentate}}{2}\right) - P_{peremeate}$$

Different concepts to maintain a steady TMP during filtration are developed (uniform TMP, gradient porosity, spiral wound, and isoflux) and will be discussed later in this chapter. Permeate flux is a

function of TMP and will be discussed in relation to critical TMP, the TMP corresponding to the critical flux where fouling is introduced.

Fouling and concentration polarisation reduce the process efficiency with respect to permeate flux and selectivity. Fouling, defined as "the process in loss of performance of a membrane due to the deposition of suspended or dissolved substances on its external surfaces, at its pore openings, or within its pores" (Koros et al. 1996) must be kept to a minimum to maintain optimal filtration performance. Concentration polarisation is the accumulation of rejected particles on the membrane surface due to a slower diffusion back to the retentate as an effect of the increased viscosity (James et al. 2003) in MF filtration, and is not fouling, strictly speaking. The polarisation layer consists of retained bacteria, fat, caseins or aggregated WP concentrated on the membrane surface, and causes the transmission of smaller molecules to decrease. Fouling, the build-ups of feed solids on membranes have different expressions. Pore narrowing consists of particles attached to the interior surface of the pores. Pore blocking occurs when the particles of the feed become stuck in the membrane's pores. On the membrane surface, formation of a gel or cake layer takes place when feed solids that are larger than the pore sizes of the membrane accumulates. Fouling and the polarisation layer depend on the composition of the feed, operating parameters such as feed velocity and filtration temperature, as well as the membrane design. A good strategy to maintain high flux is to avoid fouling as long as possible. High-frequency back pulsing of ceramic membranes is a physical cleaning method that removes reversible fouling effectively. However, only chemical cleaning can remove irreversible fouling. Irrespective of fouling-minimizing techniques, the membranes must be cleaned regularly (daily) to restore flux and ensure dairy product quality. Disinfection of the clean membrane surface prior to filtration is essential.

Membrane filtration is all about making a high throughput of permeate. Separation efficiency is measured as selectivity (Equations 1 and 2), or as permeate flux. Permeate flux is a good measure of the filtration performance and process economy and is determined by the permeate volume (Δ V) per time unit (Δ t) per membrane area (A), measured at standard operating temperature and pressure for a particular feed. The permeate volume flux is normally calculated using Equation 4 (Koros et al. 1996):

Equation 4 $$J = \frac{\Delta V / \Delta t}{A}$$

The permeate flux is a useful tool to monitor fouling because the flux decreases when fouling increases. Regardless of operational mode, a high flux is preferred during processing, as a membrane with low permeates flux demands a larger surface area to process the same volume of feed within the same time than does a membrane with high permeate flux. A high porosity (pore density, the part of the membrane occupied by pores) (Hausmann et al. 2013), provides a high throughput of permeate, and is dependent on the pore size. When comparing membranes with the same pore size, high porosity is important to achieve an economic process. Operational conditions during filtration affect process capacity. The permeate flux should be kept close to the critical flux, or at least just below, to be able to maintain filtration for a long time. In cross-flow filtration, critical flux can be described as the permeate flux where fouling is introduced. At flux values lower than the critical value, little or no fouling takes place, and increases sharply above the critical flux (Blake et al. 1992; Bacchin et al. 1995; Howell 1995; Chen et al. 1997). Critical flux is dependent on wall shear stress (τ_w) (i.e., velocity), the feed characteristic, temperature and membrane design, and composition (Grandison et al. 2000). Figure 7 explains the relationship between flux and TMP.

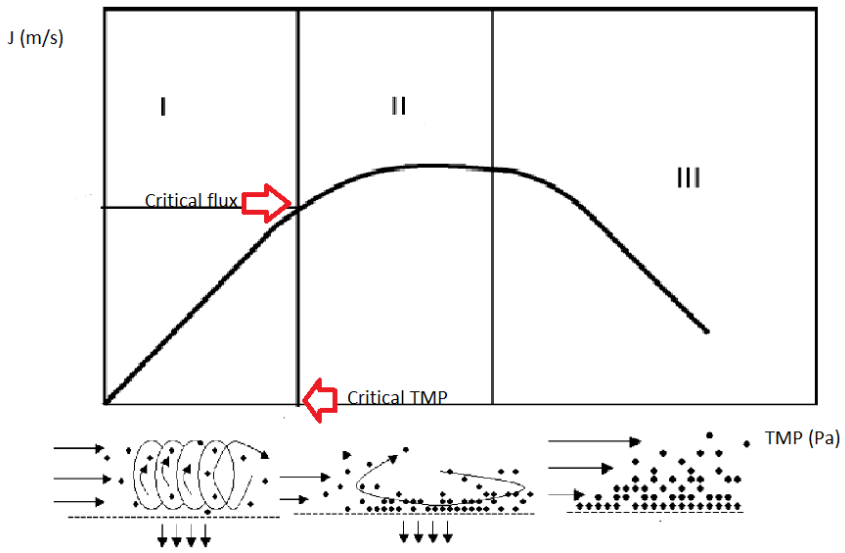

Figure 7. Critical flux regimes and flux dependency on TMP. (I) Sub-critical operation without flux-dependent fouling. Flux is linear with TMP and the suspended particles in the feed move with a turbulent flow. (II) TMP is above the critical pressure as indicated by arrows. The particles in the flow move back to the feed, and there is a dynamic cake layer on top of the membrane. (III) TMP is far above the critical pressure and the flux will decline, in time, and cause a severe cake formation (adapted from Brans et al. (2004)).

At values below the critical flux, the membrane's selectivity is better. Then again, operating at flux values too far below critical flux will reduce the process capacity. However, to evaluate the process economically, permeation/retention of the specific component(s) of interest should be considered in addition to permeate flux, optimally at an operation mode close to critical flux.

The relationship between concentration factor (CF) 1–5 and flux decline is linear, as showed by Kersten (2001). The specific permeation and mass transfer for α-LA, β-LG, and BSA differ (Kersten 2001), and because of these differences, permeates obtained at different CFs may differ in protein distribution.

CF describes the volume reduction defined as the volume (V) of feed divided by the volume of the retentate as shown in Equation 5 (Smith 2013b):

Equation 5 $$CF = \frac{V_{feed}}{V_{retentate}}$$

In skimmed milk fractionation, the degree of casein concentration is approx. the degree of volume concentration. A high CF increases viscosity and TMP. To achieve high purification of the MF and UF retentates, DF can be applied. In DF, the viscosity of the feed is reduced by adding water, and more of the permeable material can be transferred through the membrane (Smith 2013a). Water is added to UF-DF and MF-DF retentates to wash out small molecules like those of lactose and minerals. Optionally, WP can be removed during casein standardisation without depletion of minerals and lactose (which could be important to the cheese-making property of the retentate) using UF permeates as DF water (Lauritzen 2015). Furthermore, acidified DF water can reduce the buffering capacity of MF retentate (Aaltonen 2012a).

Microfiltration of milk and whey

In general, MF is applied for three purposes in the dairy industry: defatting as a part of whey clarification, to reduce bacteria prior to cheese making or for other dairy processes, and protein fractionation. About two thirds of the membrane area installed in the dairy industry is used for the treatment of whey, and about one third for milk (Saxena et al. 2009). In fractionation of skimmed milk (or potentially other raw materials such as BM) micellar casein is separated from WP using membranes with pore size 0.05–0.2 µm. Defatting and bacteria reduction of whey are carried out using membranes with a pore size of 0.45–0.8 µm, an intermediate cut-off those commonly used for bacteria reduction (0.8–1.4 µm) and fractionation. Polymeric and ceramic membranes are applied for these purposes. In addition, MF prolongs the life of brine used in cheese production by removing bacteria. Membranes with pore size 0.8 µm are applied in the production of milk having extended shelf life (Lindquist 2002). MF may replace bactofugation and severe heat treatment in the production of dairy products having extended shelf life, up to 21 days stored under refrigerated conditions (Garcia et al. 2013). Extended-shelf-life milk processed in this way combines the flavour and natural taste of HTST milk with the advantage of the longer shelf life of ultrahigh-temperature-treated milk. Sever heat treatment of cheese milk in order to kill spores causes WP denaturation, which prolongs the renneting time and impairs coagulation (Banks 1990; Kelly et al. 2008). Bactocatch, a Tetra Pak patent (Holm et al. 1986), uses ceramic membranes with a cut-off of 1.4 µm at 50 °C to reduce the bacteria count in milk prior to cheese making using. The retentate from Bactocatch (approx. 5% of the total milk volume) filtration must be heat-treated at a higher temperature to ensure bacteria and spore elimination.

Using MF membranes with pore sizes in the range of 0.05–0.2 µm enables milk fractionation: a selective separation of skimmed milk proteins into a permeate and a casein-rich retentate (Brans et al. 2004). The selectivity of polymeric membranes is somewhat lower than that of ceramic membranes because of a broader nominal pore-size distribution (Saboya and Maubois 2000). However, several studies on production of native whey have used polymeric SW membranes because of the lower capital cost. MF technology enables fractionation of native fat globules in milk, which has been shown to enhance dairy product quality (Goudedranche et al. 2000; Michalski et al. 2003; Michalski et al. 2006).

Permeate flux and filtration of milk and whey

The following Equation 6 describes the overall flux reduction. Concentration polarisation and fouling are considered as resistance added to the resistance of the membrane (Hausmann et al. 2013), where J is the permeate flux, TMP is the trans-membrane pressure, μ is the viscosity of the retentate stream, and R_m is the resistance of the clean membrane. R_m accounts for polarisation and fouling effects, and equals the sum of resistance caused by irreversible (R_{ir}) and reversible (R_r) fouling (Field et al. 1995):

Equation 6

$$J = \frac{TMP}{\mu\,(R_m + R_f)} = \frac{TMP}{\mu\,(R_m + R_{ir} + R_r)}$$

Reversible fouling is removed after filtration by flushing with water. Irreversible fouling is removed only by chemical cleaning. Irreversible fouling can be calculated as the change in water flux on a clean membrane. For MF and UF, fouling is directly influenced by viscosity (Hausmann et al. 2013): Increasing the CF leads to a higher viscosity and reducing the filtration temperature has the same effect. The main foulants in both milk and whey are protein and calcium phosphate. Some authors have concluded that casein is the main fouling protein in skimmed milk filtration (Le Berre and Daufin 1998; Brans et al. 2004; Jimenez-Lopez et al. 2008), whereas others claim WP to be the main contributor in membrane fouling (Madaeni et al. 2011; Tan et al. 2014). The solubility of calcium is reduced with increasing temperature and pH (Gaucheron 2005). Calcium contributes to fouling through precipitation of calcium phosphate (Tan et al. 2014) and/or through crosslinking of proteins. The effect of temperature is not linear due to the calcium phosphate groups of casein micelles, and the fact that calcium phosphate is less soluble at high temperatures. The filtration temperature influences the feed viscosity and the accumulation of bacteria (biofouling) during MF of whey and milk. To avoid growth of mesophilic bacteria and to generate an optimal flux, filtration temperatures ≥ 50 °C are common.

Either high (≥ 50 °C) or low (< 15 °C) filtration temperatures can be selected to avoid microbial spoilage in fractionation of native WP and denatured WP aggregates in cheese whey (Merin 1986; Steinhauer et al. 2015a). Madaeni et al. (2011) found that an increase in temperature (10–50 °C) decreased viscosity of the retentate, and therefore increased the flux during fractionation of skimmed milk. Filtration capacity differs at such temperatures (Steinhauer et al. 2015a) with a much lower flux during cold filtration. Regardless of the filtration temperature, WP and particularly β-LG are the main fouling

components during skimmed milk fractionation or defatting of whey (Mourouzidis-Mourouzis and Karabelas 2006; Steinhauer et al. 2015a; Steinhauer et al. 2015b). Proteins in the deposit layer on the membrane may crosslink with calcium ions in the feed/retentate stream as salt bridges and/or as precipitation of calcium phosphate (Marshall et al. 2003; Steinhauer et al. 2015a), and thereby make the fouling layer even more compact.

Lactose is not expected to foul MF membranes (Adams et al. 2015), but lipids may affect membrane fouling when concentrations are high. Defatting of whey reduces the membrane fouling caused by fat, and milk is skimmed prior to fractionation for the same reason.

Strategies to avoid fouling during milk and whey microfiltration

Flux decline due to fouling is prevented by operational strategies based on feed type, TMP, and flow rate in the feed channel. The composition of and concentration of solids in milk and whey prior to filtration are mostly given. One operational strategy to avoid fouling is to have a low TMP at the starting point, and gently increase the TMP to avoid irreversible fouling and to keep the total fouling low (Field et al. 1995; Howell 1995). During cross-flow filtration, particles in the feed stream move with a laminar flow, meaning that the feed or retentate in the middle of the membrane channel flows faster than at the surface of the membrane. The feed flow in direct contact with the membrane surface induces shear wall stress. Shear wall stress influences lift and drag forces of the particles in the feed (i.e., the removal of particles > 100 nm from the membrane surface), preventing a polarisation layer from forming and preventing fouling formation (Piry et al. 2008). Increasing cross-flow velocity will in general increase the turbulence that reduces fouling formation. However, a high TMP increases the demand for higher pumping energy (Hausmann et al. 2013). At high wall shear, forces (high cross-flow velocity over the membrane) large particles are removed from the membrane surface at a higher rate than the smaller molecules or particles are which results in a deposit layer consisting of more small particles. This fouling layer is compact and less porous. The small particles in this type of fouling layer reduce the nominal pore size of the membrane, which in turn may reduce the permeation (LeBerre and Daufin 1996).

Different concepts to optimize fractionation by microfiltration

Fouling is unevenly distributed along the ceramic membrane, due to the pressure drop between the inlet and the outlet of the membrane module (Piry et al. 2008; Hurt et al. 2010; Piry et al. 2012). Piry et al. (2008) investigated the effect of uneven fouling along the membrane: Heavier fouling at the inlet

of the membrane resulted in a transmission of β-LG 38% at the inlet and 87% at the outlet during skimmed milk. Developments like UTP, gradient porosity and isoflux membranes have solved the problems with uneven fouling and pressure drop along the membrane.

There are two major membrane types developed to optimize the fractionation of milk by MF in terms of cross-flow efficiency: ceramic and polymeric membranes. Membrane material and design significantly affects the composition of the final fractions produced. (Zulewska and Barbano 2013). Polymeric membranes made of polyvinylidene fluoride, polysulfone, polyether sulfone, polyarilonitril or polyethylene are applied in UF and MF. Spiral wound (SW) is the most popular design for polymeric membranes. In a polymeric SW membrane, a spacer placed between the membrane's sheets induces an unsteady flow. The cross-flow velocity of the feed is lower in polymeric membranes compared to ceramics. The lower cross flow influences the foaling formation, which is driven by concentration polarisation in polymeric filtration. (James et al. 2003). Fractionation of casein and WP with SW polymeric membranes depended on the combination of membrane properties and the fouling layer, and less WP was removed than was removed by the ceramic membranes (Zulewska and Barbano 2013). The nominal pore-size distribution of polymer membranes is broader than that of ceramic membranes, which also results in poorer selectivity. The advantages of SW membranes are primarily related to their lower capital cost compared to that of ceramic membranes; there is no demand for permeate circulation pumps (meaning no extra running cost) and the membranes are cheaper, and the SW membranes possess a large membrane area because of the membrane's compact packing. In addition, the retention volumes of polymeric SW membranes are low (Smith 2013a). However, polymeric membranes are less resistant to chemicals and high temperature, thus more complicated to clean than are ceramic membranes that are resistant to high and low pH. The SW membranes cannot be sanitised using hot steam as most other dairy process equipment can be, meaning that the initial bacteria count in milk must be low prior to fractionation. The life span of SW membranes is only about two years, compared to the five-fold longer life span of ceramic membranes.

Uniform TMP (UTP or UTMP) was invented to maintain a high flux by using an even TMP across a tubular ceramic membrane (Sandblöm 1978). This principle enabled efficient ceramic MF on an industrial scale. The principle of UTP is that the circulation of permeate concurrently at high speed creates a pressure drop inside the membrane. A pump (triangle) as illustrated in Figure 8b drives the permeate circulation.

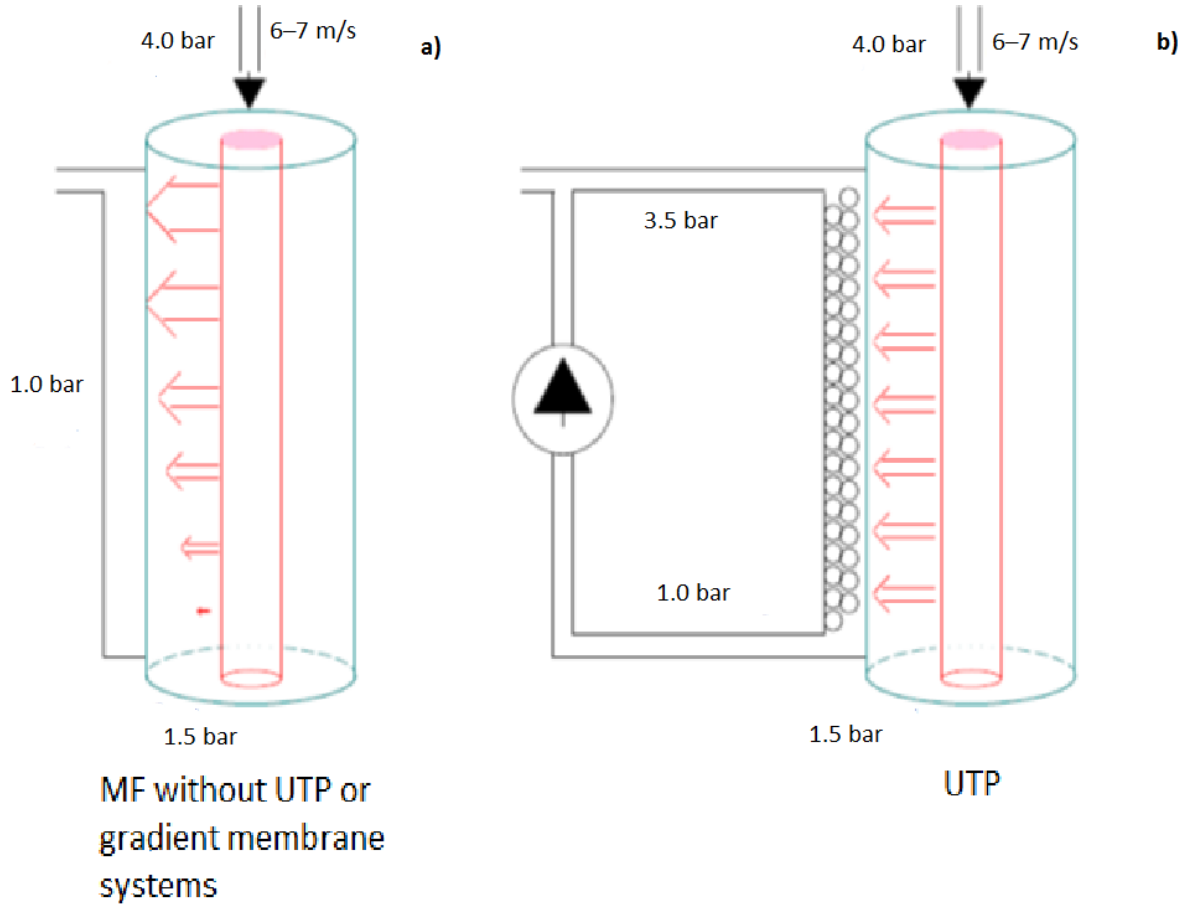

Figure 8. Membrane modules and typical pressure profiles in a) ceramic MF without UTP and b) with a UTP system invented by Sandblöm (1978). The figure is adapted from Hoffmann (2013).

The membranes used in UTP systems are tubular ceramic membranes like those used before this invention (Sandblöm 1978). However, ceramic membranes differ in surface-layer construction, channel number, pore size, and geometry to fit the purpose(s) of the filtration process. To reduce the pressure loss over the membrane without the need for an additional permeate loop and pump (as in the UTP), two different ceramic membrane designs were developed: gradient porosity (GP, Membranlox® GP) membranes and isoflux membranes (ISOFLUX™, Tami). Gradient porosity membranes have a gradient pore shaping which works as an in-membrane UTP (Garcera and Toujas 2003). Isoflux membranes have a separating layer which decreases in thickness from the inlet to the outlet, in order to maintain a constant ratio of pressure to separating-layer thickness (Grangeon et al. 2002).

The composition of the MF fraction is dependent on the membrane design, which influences the selectivity. By comparison, fractionation of milk by ceramic (UTP) membranes has accounted for a higher removal of WP using 0.1 µm than has fractionation by polymeric SW membranes (Zulewska et al. 2009). In general, UTP membranes showed the best WP removal, followed by GP and ISOFLUX™ membranes (Zulewska et al. 2009; Hurt et al. 2010; Adams and Barbano 2013). No report has been found comparing the selectivity and efficiency between ISOFLUX™, GP, and ceramics for UTP on equal terms (i.e., comparing membranes with the same pore size, type of feed, and energy requirement).

Recent developments in fractionation of milk by microfiltration

Several dynamic filtration units have been used as an alternative to UTP in milk fractionation, for instance vibrating membranes, rotating disc systems with a disc rotating above a fixed membrane, or rotating discs for MF and UF (Espina et al. 2008; Espina et al. 2009b; Espina et al. 2010; Meyer et al. 2015). Today the dynamic filtration systems are expensive alternatives compared to the alternatives (UTP, SW, GP, and ISOFLUX™). However, the advantage of dynamic filtration is the high shear rate created at a low TMP (Espina et al. 2009a). Another option is to apply electrical-charge wide-pore UF technology to allow separation of α-LA and β-LG (Bhushan and Etzel 2009; Arunkumar and Etzel 2013b; Arunkumar and Etzel 2013a; Arunkumar and Etzel 2014), with the electrical charge working as an additional driving force. Microsieve membranes have a higher selectivity and permeability than conventional MF membranes. The pore-size distribution is narrow, and the surface of inert silicon is smooth and has a low resistance (Saxena et al. 2009) and therefore microsieve membranes have suggested as an alternative to currently used MF membranes (Brito-de la Fuente et al. 2010). The main advantage of microsieve is the large flux that allows operation using extremely low TMP, which hence, reduces the operational cost. Verwijst et al. (2015) recently described a new filtration method using rotating microsieves.

Continuous development of processes through small-scale studies, processes not yet implemented industrially, show yield, purification, and product quality comparable with those of existing processes in the dairy industry. Further improvements will determine whether such innovations can provide better throughput, higher purity, or higher concentrations, and become competitive in terms of investment and maintenance costs.

FRACTIONATION OF MILK TO OBTAIN NATIVE WHEY AND CHARACTERISATION OF NATIVE WHEY PROTEIN INGREDIENTS

Fractionation of milk commonly targets the production of casein-standardised milk that increases capacity during cheese making, to produce micellar casein concentrates, or other products where an increased casein concentration in milk is beneficial. However, the permeate fraction and the NWPC and NWPI obtained therefrom deserve more attention due to their unique chemical and physical properties. The proteins in native whey are in their native state, as the membrane retains any aggregated proteins. Furthermore, native whey is clear, sterile, free of bacteriophages (Saboya and Maubois 2000), has no off-flavour and contains less fat (Maubois 2002; Marcelo and Rizvi 2006; Heino et al. 2007; Evans et al. 2009; Luck et al. 2013, Paper II, Paper IV) and minerals (Evans et al. 2009, Paper IV) than does cheese-derived WPC. The lower mineral content and the absence of CMP make NWPC and NWPI suitable as infant formula ingredients. Whereas WPC quality depends on several factors such as milk quality, cheese-making protocol, heat treatment of whey, defatting etc., NWPC and NWPI properties depends mainly on the source of WP (skimmed milk, buttermilk, milk from other animals than cow) and, if any, initial treatment(s) of the milk.

Effect of pre-treatment of milk on microfiltration performance and fraction composition

Heat treatment

During fractionation of skimmed milk, targeting to optimise the casein-rich fraction or the production of native whey, a maximum removal of WP from the milk is advantageous. Different strategies to increase selectivity have already been discussed with respect to membranes and operational matters. It is also shown that pre-treatment of milk affects the composition of the MF fractions (Hurt and Barbano 2010, Paper I). Heat treatment is compulsory in most dairy processes to ensure food safety. However, in addition to ensure food safety, heat treatment changes the physio-chemical properties of milk: Hydrophobic interactions between WP and caseins are enhanced during heat treatment, the solubility of calcium and phosphate is decreased, and more calcium phosphate moves to the casein micelles (Pouliot et al. 1989; Gaucheron 2005). Moreover, heat treatment of milk and whey induces

reactions between lactose and proteins, resulting in lactocylated WP. The hydrophobicity, and hence, the solubility, of α-LA and β-LG changes upon lactocylation, and may result in browning and a cooked flavour (Thomas et al. 2004). Figure 9 shows phase shift changes occurring in milk under heating, cooling, acidification, and addition of salts or chelatants.

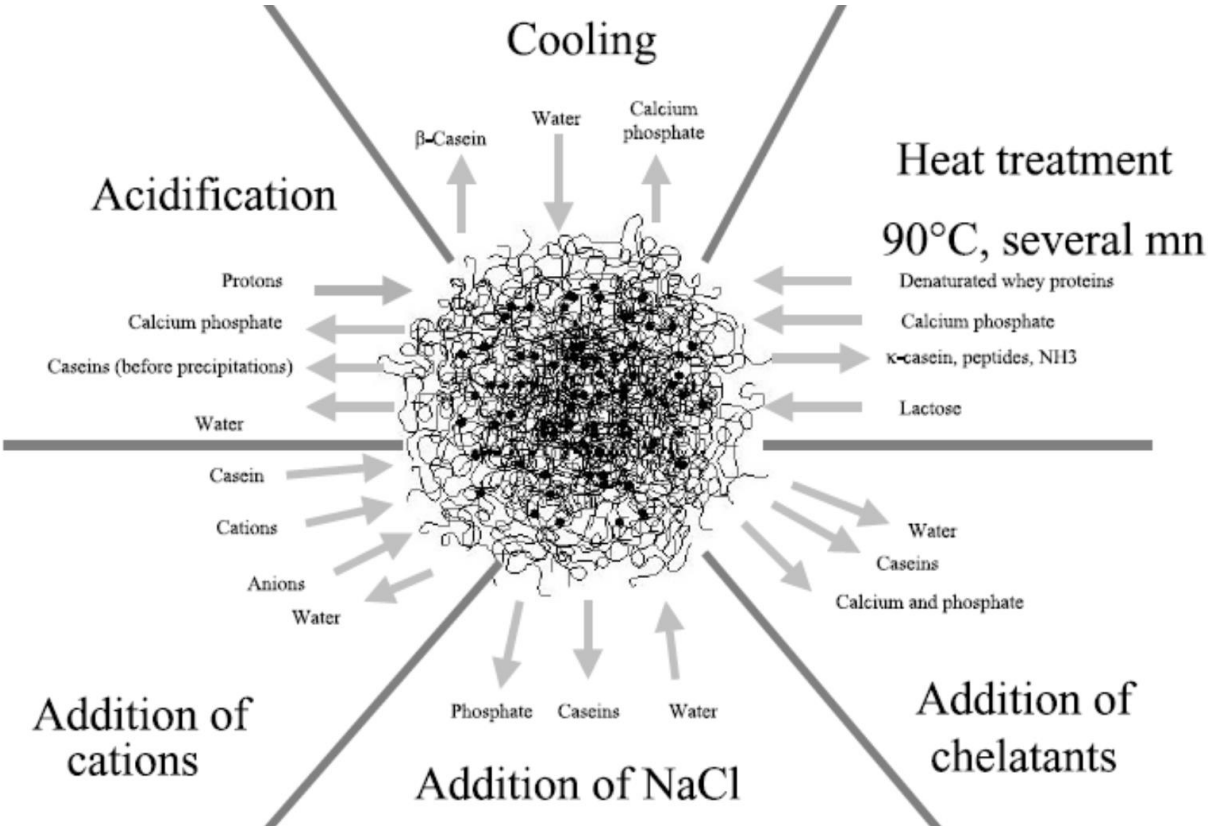

Figure 9. Changes in the casein micelle stability, mineral and nitrogen balance during acidification, cooling, heat treatment and addition of salts or chelatants (adapted from Gaucheron (2005))

Unfolding β-LG molecules expose free and previously buried thiol groups during HTST treatment (Anema and Li 2003) and starts to form complexes with starts to form complexes with micellar or free κ-casein (Donato and Guyomarc'H 2009) or αS2-caseins. The interactions between β-LG molecules, or β-LG and caseins results in aggregates in the size range of 30 to 100 nm. The interaction between β-LG and micellar κ-casein complexes causes an increase in casein micelle size, and an incorrect increase in casein measured by Kjeldahl's method (Rowland 1937). α-LA has a lower transition temperature (66 °C) than β-LG (73 °C) (Dalgleish et al. 1997). The molten globular conformation of α-LA differs from

that of β-LG (Fang and Dalgleish 1998), and α-LA renatures on cooling after heat treatment (Ruegg et al. 1977) due to the lack of a free thiol group. Calcium ions stabilise α-LA and influence the transition temperature: apo- α-LA, that contains no Ca^{2+}, starts to unfold at ~35 °C (Relkin 1996), whereas the Ca^{2+}-containing holo-form of α-LA has a transition temperature of 66 °C (Relkin et al. 1993). Heat treatment at temperatures ≥ 90 °C induces irreversible denaturation (Fang and Dalgleish 1998). However, denaturation of α-LA appears to interact with the casein micelle only after aggregation with β-LG (Anema 2008). Dissociation of caseins from the micelle is dependent on pH and temperature, and κ-casein is removed more easily then α_s-and β-caseins (Donato and Dalgleish 2006; Kethireddipalli and Hill 2015).

Heat treatment of milk changes the mineral balance between the undissolved and dissolved state. The casein micelle contains colloidal calcium, though the casein micelle includes other minerals and salts such as potassium, sodium, magnesium, and citrate. The colloidal calcium phosphate (CCP) is a mix of calcium caseinate and calcium phosphate: calcium caseinate are caseins directly bound to phosphoserines (organic phosphate) in the casein molecule, and calcium phosphates are composed of calcium associated to the inorganic phosphate in the micelle (Gaucheron 2005). Both types of colloidal calcium are exchangeable with the calcium in milk serum; however, not all of the calcium bound to organic phosphate is exchangeable. The soluble salts present in the milk serum affect various milk properties, such as WP stability. Table 3 adapted from Walstra et al. (2006) shows the distribution of the major minerals in milk.

Table 3 Salt / mineral composition and distribution in raw milk (Walstra et al. 2006)

Milk component	Soluble (%)	Colloidal (%)	mg $^{-1}$ 100 g milk
Na	95	5	48
K	94	6	143
Ca	32	68	117
Mg	66	34	11
Cl	100	0	110
CO_3	~100	0	10
SO_4	100	0	10
PO_4	53	47	47
Citrate	92	8	175

The amount of soluble calcium is important to functional properties like gelling and foaming, and could be critical in membrane fouling. The amount of Ca2+ is critical to aggregation of para-casein micelles during renneting, and it may impair coagulation due to heat-induced precipitation of Ca^{2+} on the micelle as CCP. However, during the first stage of rennet-induced aggregation, the surface properties of the casein micelle and the amount of Ca^{2+} in milk serum of the casein micelle are more important. Later, the interiors of the casein micelle and its CCP become increasingly important (Dalgleish and Corredig 2012) and could induce changes in gel microstructure, a decreased G' and loss modulus (G'') values, and reduced syneresis rate (Choi et al. 2007, 2015).

Initial HTST treatment of skimmed milk influenced the mineral and nitrogen balance of the MF fractions (Paper I). The permeate from unpasteurised skimmed milk contained less casein, calcium, magnesium, and phosphorous, and higher levels of sodium than did native whey produced from pasteurised skimmed milk. However, the HTST treatment was confounded with a higher content of these minerals in raw milk, with the exception of sodium. The MF permeates arising from milk with different heat treatments described in Paper I were concentrated by UF-DF (as described in Paper II), and the composition of the liquid NWPCs is shown in Table 4. The data from unpasteurised milk's NWPC is previously unpublished.

Table 4. Composition of liquid NWPC derived by UF-DF of the MF permeates from unpasteurised skimmed milk and pasteurised skimmed milk. Except for total solids, the values are given on a dry matter basis as means and their standard deviations (SD), n = 4.

	Liquid NWPC from unpasteurised skimmed milk		Liquid NWPC from pasteurised skimmed milk [a]	
Component (g 100 g^{-1} sample)	Mean	SD	Mean	SD
Total solids (g)	7.46	(0.41)	7.45	(0.53)
Component per 100 g^{-1} total solids				
True protein (g)	92.1	(0.3)	90.0	(1.7)
Whey protein (g)	82.4	(1.1)	75.9	(0.4)
Casein (g)	7.6	(1.1)	12.0	(2.0)
κ-casein (% of true protein)	2	(0)	3	(0)
α_{s1}-casein (% of true protein)	4	(1)	7	(0)
β-casein (% of true protein)	9	(1)	12	(0)
α-LA (% of true protein)	30	(1	30	(1)
β-LG (% of true protein)	54	(1)	48	(1)
Total minerals (g)	1.3	(0.1)	1.6	(0)
Lactose (g)	1	(0.4)	2.5	(0.6)
Fat (g)	ND		ND	

[a] From Paper II. Abbreviations: α-LA = α-lactalbumin, β-LG = β-lactoglobulin, ND = not detected (below the threshold of the analysis).

The difference in amount of casein between the two NWPCs was higher than expected; however, using membranes with pore size 0.2 µm enables transmission of free caseins and small casein micelles. Reorganisation of the casein micelle due to HTST treatment and cooling to filtration temperature changed the casein equilibrium between the serum and colloidal phases: heating and cooling influenced the rearrangement of casein micelles by favouring self-aggregation and formation of small micelles with increasing temperatures (Mikheeva et al. 2003; Liu et al. 2013). The initial pasteurisation resulted in a liquid NWPC higher in αS1- and β-casein, slightly higher in κ-casein, and with less β-LG. The results shown in table 4 demonstrated that even a moderate heat treatment would affect the nitrogen and mineral distribution of a final NWPC powder. A casein concentration accounting for 10% of the proteins in NWPC would surely affect the solubility of the concentrate, as caseins precipitate at pH 4.6. Furthermore, caseins are known to possess chaperone functions. O'Kennedy and Mounsey

(2006) observed that αs1- and β-casein and micellar casein prevented WPs from undergoing aggregation during heat treatment, although they had a different protective behaviour. Micellar casein, present in casein/WPI mixtures dispersed in simulated milk ultrafiltrate, was able to control aggregation even at pH 5.4 when heated at 85 °C for 10 min, although denaturation was not prevented. Hurt and Barbano (2010) studied how an increase in heat treatment from 72.9 °C to 85.2 °C prior to fractionation effected WP removal during MF, and found that the increased heat-treatment temperature increased the retention of casein-bound WP and WP aggregates. These results showed that heat treatment decreases yield of WP in NWPI, since less WP could be transmitted through the membrane, and is consistent to results reported in Paper I.

Cold filtration

MF at low temperatures (4–10 °C) reduces the risk of thermophilic bacteria growth in raw milk and microbial fouling on membranes with pore size 1.4 µm (Fritsch and Moraru 2008). The flux is lower during cold filtration of skimmed milk than it is during filtration at regular temperatures ≥ 50 °C (Steinhauer et al. 2015a). The higher viscosity of milk during cold filtration is a plausible reason for this lower flux. Due to the increased viscosity, the large membrane areas of polymeric SW membranes are more appropriate to this application than ceramic membranes are. By employing cold filtration temperatures, the WP to β-casein ratio of 20:80 in bovine milk can be changed to about 40:60 as in human milk (de Wit 1998), and the skim milk retentate would become more suitable as an ingredient for infant formula manufacture. At low temperatures, β-casein can dissociate from the micelle into the serum phase of milk, and the concentration of caseins in the milk serum increases with holding time at low temperatures (Creamer et al. 1977). Amount and distribution of caseins in permeate depends on membrane pore size. Crowley et al. (2015) reported α-LA, β-LG, αs1-casein, β-casein and κ-casein in permeates using 0.45 µm and 1000 kDa membranes at 4 °C. LF was also found in the permeate from the having 0.45 µm pore size. Using membranes with 0.1 µm pore size only allowed β-casein and the two major WPs in the permeate. Fractionation of skimmed milk at either 7 or 23 °C resulted in NWPCs containing 22 and 1% casein, respectively (Coppola et al. 2014). The same authors found that NWPC enriched with β-casein made less turbid solutions, foams with higher overruns and stability, and had different volatile and sensory profiles than those of the NWPC with a low β-casein content. Moreover, MF retentate from cold filtration increased yield of pizza cheese (Govindasamy-Lucey et al. 2011). However, native whey and NWPC obtained at normal filtration temperatures may also contain higher amounts of casein than does WPC obtained from cheese production. The amount

of casein found in the native whey is dependent on the membrane cut-off and previous heat treatment used in milk fractionation: Evans et al. (2010) found less than 4% casein in NWPC80 produced at 50 °C using a membrane with a smaller pore size (0.1 µm). MF of unpasteurised skimmed milk with a membrane having 0.14 µm pore size resulted in a NWPC powder with a casein content of more than 5% (Paper IV). Cold storage induces phase shift in the mineral balance of milk. The minerals of milk are more soluble at low temperature, and cold filtration may reduce the content of minerals in the casein-rich fraction, and hence alter the buffering capacity of the MF retentate, as more CCP would be transferred into the permeate. However, information is limited concerning the influence of cold filtration on the mineral content in the MF fractions.

Acidification, addition of salts and carboxylation

Milk protein concentration by UF or MF increases the content of casein-micelle-associated minerals (Brandsma and Rizvi 1999; Schreier et al. 2010), and the retentate's buffering capacity is increased, resulting in a slower pH reduction than in traditional cheese making from regular milk (Salaün et al. 2005). To overcome this challenge, pre-acidification of milk or casein-standardised milk prior to MF or UF is an effective method. During acidification, the buffering capacity of the casein-rich fraction is reduced by lowering the calcium content (St-Gelais et al. 1992; Aaltonen 2012b). Acidification of milk (pH 6.5, 6.2, 5.9) has been shown to decrease flux during MF (Kuhnl et al. 2010). A reduced repulsion between casein micelles probably caused a more compact deposit layer on the membrane surface than was caused by the filtration of milk at neutral conditions (pH 6.8). However, greater amounts of soluble calcium phosphate could participate in cross-linking of unfolded WP and caseins in the fouling layer during MF of acidified milk.

Carbon dioxide (CO_2) inhibits the growth of psycrotrophs in milk (Hotchkiss et al. 2006), and is applied as a pre-treatment of cheese milk (Kelly et al. 2008). However, in the case of cheese making, CO_2 is simply lost during further processing and the acidifying effect of CO_2 is not affecting the casein micelle stability. Carboxylation of UF or MF retentate has been suggested as a way to decrease milk's buffering capacity as incorporation of CO_2 decreases the pH, which leads to solubilisation of CCP (de la Fuente 1998). The effect of protein concentration and composition on functional properties of MF/UF concentrate, which was injected with CO2, has been investigated (Ma and Barbano 2003). However, information is lacking on the effect of CO_2 treatment prior to fractionation.

The stability of the casein micelle is important in most cheese-making applications, and is dependent on the mineral distribution between the casein micelle and the milk serum. Addition of citrate, phosphate salts and $CaCl_2$ to milk induces changes in the milk's buffering capacity. The casein micelle size decreases with the addition of citrate and increases with the addition of calcium and phosphate (Le Berre and Daufin 1998). When calcium is added to milk, the casein micelles become saturated in calcium phosphate, and the concentration of Ca^{2+} increases in the serum phase. Because of this, $CaCl_2$ accelerates rennet coagulation and increases curd strength by formation of additional bonds in the casein network (Salaün et al. 2005). Inorganic phosphate is added to raw, heated, concentrated and/or recombined milk as a common practice in the dairy industry (Gaucher et al. 2007) to increase milk stability prior to or after heating. Citrates have a strong chelating effect on calcium cations and are thus able to destabilize the casein micelle (Le Berre and Daufin 1998). One possible pre-treatment method prior to MF is addition of salts (Hernandez and Harte 2009). Hernandez and Harte (2009) added different salts ($CaCl_2$, sodium phosphate and potassium citrate) to milk prior to MF to study the effect on the milk fraction composition. The addition of sodium phosphate increased the casein concentration in the MF permeate, due to the formation of insoluble calcium phosphate and a destabilisation effect on the casein micelle (Gaucher et al. 2007). As expected, addition of citrate resulted in a low casein recovery in the MF permeate, whereas the strengthening effect of $CaCl_2$ addition on casein micelle stability (less casein in the permeate) was observed up to 50 mM.

Other pre-treatments of microfiltration feed

Ultrasonic treatment of whey increased flux (Muthukumaran et al. 2005). Recently Koh et al. (2014) found that sonication alone, prior to UF of liquid WPC, relieved membrane fouling to a small extent. Heat treatment followed by sonication reduced membrane pore blockage and cake growth, without causing any change in the permeate composition. Fractionation of ultrasonic-treated skimmed milk prior to fractionation by 0.2 µm membranes increased flux, and a higher flux was obtained at a lower pressure than was used in fractionation of untreated skimmed milk (Mirzaie and Mohammadi 2012). However, the influence of ultrasonic treatment on the composition of the fractions was not reported.

Combination of pulsed electric field (PEF) and MF (1.4 µm) was found more effective than conventional HTST treatment to reduce bacteria count in skimmed milk (Walkling-Ribeiro et al. 2011).

However, no results on the influence of PEF on composition of the MF fraction are reported. Conformal changes of α-LA and β-LG induced by HHP are different from those caused by thermal treatment (Considine et al. 2007): denaturation and aggregation caused by heat treatment of milk involves rupture of non-covalent bonds followed by subsequent reformation of intra- and intermolecular bonds. In contrast, HHP of WP-model systems results in formation of mainly non-native monomers of α-LA and β-LG. High hydrostatic pressure treatment of milk could induce a phase shift of the minerals that is different from that induced by heat treatment (López-Fandiño et al. 1998), as HHP increases contents of Ca^{2+} in the milk serum. The effect of initial HHP or PEF treatment of milk on the composition of the MF fractions would be an interesting phenomenon to investigate. However, to the author's knowledge, no studies have been reported on these issues.

Characterisation of high-protein native whey protein products

Presently, concentrated native WP products are used as ingredients mainly for human nutritious products, for instance in weight-management and instant formulas to reduce the risk of hyperthreoninemia due to the absence of CMP, formulas which are rich in the amino acid threonine (Rigo et al. 2001). Native WPC produced by UF concentration of native whey has superior gelling, foaming, solubility and sensorial properties (Maubois 2002; Marcelo and Rizvi 2006; Heino et al. 2007; Evans et al. 2009; Luck et al. 2013, Paper II, Paper IV) compared to those of traditional WPC.

NWPC has been compared to its equivalents made from sweet whey regarding TP content: Evans et al. (2009) studied the composition, volatile compounds, and sensorial properties of NWPC34 and WPC34 made from the same milk, and a commercial WPC34. They concluded that NWPC and WPC manufactured under controlled conditions from the same milk, and using the same UF process, had few sensorial but distinct compositional and physical differences. Heino et al. (2007) studied similar products and using a similar experimental design to that of Evans (2009): NWPC and WPC with 24–37% total protein were made from cheese whey from the same milk (50% Gouda and 50% Emmental whey), and compared to a commercial WPC. The undenatured WP:TP ratio was higher in all NWPCs, despite a somewhat higher TP in the cheese-derived WPCs. However, NPN contents in the different WPCs were not reported. Because of the absence of CMP in native whey, less NPN was found in NWPC than in commercial WPC (Paper IV). The influence of membrane design and operational conditions on the specific mass transfer has been discussed earlier in this thesis. The amount of the specific WP in

WPC depends on the pre-treatment of milk (Outinen et al. 2010c) and treatment of cheese whey (Outinen et al. 2010b). Even a mild initial heat treatment alters the protein profile of NWPC (Paper I).

The difference in mineral content and distribution between cheese-derived WPCs and NWPCs relates to the lower pH in cheese than in milk /MF permeate, allowing solubilised calcium to migrate from the cheese curd into the cheese whey (Walstra et al. 2006). The presence and level of minerals affects gelation properties of WP products (Morr and Ha 1993; Havea et al. 2002; Wijayanti et al. 2014). Heino et al. (2007) did not find any differences in ash content between NWPCs and WPCs produced from the same milk without DF. NWPC and WPC made from the same milk, using the same equipment and process resulted in a NWPC with less calcium (Evans et al. 2009, 2010), phosphorous and sodium (Evans et al. 2010).

Foams made from NWPC have high overrun (Heino et al. 2007; Coppola et al. 2014, Paper II). WPI and egg-white protein are often used as foaming agents, and Punidadas and Rizvi (1998) demonstrated that the foaming properties of a pH 7.0, dialyzed 5% native WP product were comparable to egg-white foams, and better than products made from cheese whey WPC produced by UF. The latter contained foam-suppressing fat and high-molecular-weight protein complexes. When these aggregates are removed by clarification/defatting, regular WPC still contains more proteins that are denatured than does NWPC- this means less native proteins able to participate in the foam network. The source of native whey influenced foaming properties (Paper II): foam made of NWPC from BM was far less stable than foam from pasteurised milk's NWPC, probably due to the difference in fat content. However, the overruns were equally high. The mineral composition of WPC and WPI may also be important. Zhu and Damodaran (1994a) showed that divalent cations (Ca^{2+} and Mg^{2+}) improved the foaming properties of WPI, suggesting that the cations linked two negatively charged proteins and caused aggregation during film formation.

The fact that WPs are soluble even at low pH makes them an interesting ingredient for protein fortification of acidic or carbonated clear beverages. Native WP powder remains clear after recombination in water, contains no CMP that could induce aggregates at low pH, and is a highly soluble ingredient that would be a good choice for these types of applications. To use NWPC in acid applications, the casein content in it must kept to a minimum. Native WP concentrates and isolates usually have higher solubility than do cheese-derived WPC and WPI. This higher solubility could be explained by several factors as native WP ingredients contains less denatured proteins (Britten et al.

2008), less fat (Heino et al. 2007; Marcelo and Rizvi 2008; Luck et al. 2013) and no CMP (Veith and Reynolds 2004, Paper III). Despite a higher fat content (approx. 1.5%) in NWPC made of BM, the solubility was just as high as for the NWPC made from pasteurised skimmed milk at pH 4.6 and 7 (Paper II).

Native whey from other sources than skimmed milk

Skimmed milk is an obvious starting material to produce native whey. It is of interest to investigate if native WP could be derived from foremilk or another milk stream than skimmed milk. BM is an interesting raw material with respect to fractionation because of its similarity to skimmed milk in solid composition (Corredig et al. 2003), with a high ratio of casein to WP. BM is a rich source of MFGM material (Dewettinck et al. 2008), which has a composition and properties completely different from those of other milk solids. Native MFGM acts as a natural emulsifying agent, preventing flocculation and coalescence of fat globules in milk, and protecting the triglycerides within the milk fat globule against enzymatic (lipase and esterase) action. Proteins account for 25–60% of the total mass of the MFGM, and these proteins constitute about 1–2% of the total protein in milk (Mather 2000). The main MFGM proteins are xanthine dehydrogenase/oxidase (XDH), buthyrophilin (BTN), lactadherin (also known as periodic acid Schiff (PAS) 6/7), and PASIII. BTN accounts for 40% of the MFGM proteins (Martin et al. 2012). The major MFGM proteins separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) are mucin 1, XDH, periodic PASIII, cluster of differentiation 36, BTN, lactadherin, adipophilin, proteose peptone 3, and fatty acid binding protein (Mather 2000). However, the MFGM contains far more proteins, especially proteins of low abundance that are typically not observed in SDS-PAGE patterns. These proteins can be detected by mass spectrometry (Le et al. 2013). The MFGM proteins, as well as PLs, are suggested to be bioactive (Spitsberg 2005; Dewettinck et al. 2008). The main PL classes found in bovine MFGM are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS); lysophosphatidylcholine (LPC); lipo phosphatidylethanolamine (LPE) and a sphingolipid, sphingomyelin (SPH). Phosphatidylethanolamine, PC and SPM account for more than 20 wt. % of the total PL in milk (Lopez 2011). The emulsifying property of the MFGM material is good because the MFGM-specific proteins are amphiphilic molecules that can act as surface-active compounds (Singh 2011). In addition, PL is amphiphilic and has surfactant properties (Corredig and Dalgleish 1997; Sachdeva and Buchheim 1997; Kanno et al. 1998; Roesch et al. 2004; Dewettinck et al. 2008). These features makes MFGM-rich products like BM powder (dried BM) useful in baking and applications

where wetting properties are favoured (Vanderghem et al. 2010). Sodini et al. (2006) and Phan et al. (2014) made good emulsions from a MFGM material with a high PL:TP ratio. However, whether proteins or polar lipids are responsible for the emulsifying activity is still not completely understood.

BM powders had much lower foaming capacity than skim milk powder or whey powder (Wong and Kitts 2003; Sodini et al. 2006). However, the PL content in BM powder found by Sodini et al. (2006) was far higher than observed in BM powder studied in Paper II (1.2–1.8% of TS compared to 0.2% of TS, respectively). Polar lipids can supress overrun because they easily displace protein from the surface by means of the surfactant. The low foam stability of NWPC suspensions made from BM makes this new ingredient attractive for some industrial applications or processes where foaming is undesirable. MFGM is evidently affected by milk processing treatments, for example, heating, cooling, homogenization, and evaporation, and by spray-drying treatments such as heating, cooling, and mechanical agitation during processing (McPherson and Kitchen 1983; Walstra et al. 2006). β-LG could react with MFGM-specific proteins during heat treatment of milk (Kim and Jimenez-Flores 1994), and XDH and BTN formed large protein complexes at 60 °C, whereas lactadherin was more heat stable. The functional properties of dairy products are partly affected by the state of the MFGM components after such treatments (Keenan et al. 1983; Houlihan et al. 1992a; Houlihan et al. 1992b; Morin et al. 2008). During fractionation, the MF membrane retains caseins, aggregates, and large MFGM fragments consisting of MFGM proteins and WP.

Several studies have focused on the separation and isolation of MFGM proteins and/or PLs from BM using MF (Sachdeva and Buchheim 1997; Corredig et al. 2003; Morin et al. 2006; Morin et al. 2007a; Morin et al. 2007b). An industrially obtained BM (TINE Sandnessjøen) was fractionated using a membrane with 0.2 µm pore size, filtration temperature 56 °C and CF 2.34. Table 5 (previously unpublished data) shows the composition of the BM fractions. All these fractions had much higher content of PL than did the fractions from skimmed BM described in Paper II. The fractionation and all chemical analysis were carried out as described in Paper II, with the exception of soluble calcium that was determined by complexiometric titration (Visser 1976),

Table 5. Mass balances from MF fractionation of BM. Values are means and standard deviation (SD) from four replicates.

	BM		MF retentate of BM		MF permeate of BM	
Mass (kg)	900		365		535	
	Mean	SD	Mean	SD	Mean	SD
Milk component:						
Total solids (kg)	73	(1.6)	46	(1.4)	27	(0.3)
True protein (kg)	22.9	(1)	20.8	(0.3)	1.5	(0.3)
Whey protein (kg)	3.1	(0.3)	2.3	(1.3)	1.3	(0.3)
Casein (kg)	18.2	(0.9)	17.9	(0.15)	ND	
PE (kg)	1.76	(0.09)	1.51	(0.15)	0.27	(0.05)
PS (kg)	0.17	(0.08)	0.13	(0.03)	0.11	(0.01)
PC (kg)	0.42	(0.11)	0.44	(0.07)	0.03	(0)
SPH (kg)	0.18	(0.04)	0.19	(0.02)	ND	
LPC (kg)	0.15	(0)	0.02	(0.01)	0.01	(0)
Total phospholipids (kg)	2.58	(0.36)	2.29	(0.25)	0.41	(0.05)
Soluble calcium* (kg)	0.88	(0.05)	0.60	(0.19)	0.37	(0.28)

Abbreviations: LPC = lysophosphatidylcholine; LPE = lipo phosphatidylethanolamine, ND = not detected (below the threshold of the analysis), PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; SPH = sphingomyelin.
*Calcium ions analysed by titration (Visser 1976).

The cow's diet influences the composition of MFGM. A diet rich in polyunsaturated fatty acids affects the lipid composition of the MFGM (Lopez et al. 2008). No significant differences in the PL profile were found between MFGM extracted from small and large milk-fat-globule fractions (Fauquant et al. 2007). Disagreement in PL distribution and variation in transmission between BM and skimmed BM, probably related to the size of the MFGM fragments in the BM. The amount of PL in the MF permeate of BM was almost twice (1.5% of TS) as high as the amount in permeate from skimmed BM (0.7% of TS) reported in Paper II. The permeate from fractionation of BM had a PL to TS ratio similar to that reported for BM powder (Sodini et al. 2006), but had a different protein distribution. The functional properties of a NWPC like this, enriched with MFGM components, may differ from those of regular NWPC. For instance, the addition of BM powder has been shown to increase aggregation yields and to decrease water-holding capacity of the aggregated proteins in mixtures with liquid WPC (Saffon et al. 2011). A permeate with higher content of PL, as shown in Table 5, could result in a WP ingredient

with altered and interesting functional and nutritional properties. Addition of PL-enriched material had a heat stabilising effect to milk-based emulsion (Kasinos et al. 2014).

NWPC produced from pasteurised skimmed milk and skimmed BM (Paper II) had similar mineral contents as NWPC (Paper IV) which was also was produced from unpasteurised milk. However, the source of native whey influenced the mineral composition of NWPC: NWPC made from BM had higher amounts of iron and magnesium, and lower content of calcium than did NWPC from skimmed milk (Paper II). These differences were probably related to the combination of an initial higher fat content in native whey from BM, a slightly lower pH in BM than in skimmed milk, and the severe heat treatment of butter cream. Literature that reports on the influence of native whey mineral composition on functional properties is scarce.

Fractionation using MF enables utilisation of milk solids in by-products and fore milk, which could lead to new ingredient products. In addition to fresh skimmed milk or BM, colostrum (the first milk of postpartum), can be fractionated to produce native whey and a casein-rich fraction. Colostrum contains bioactive components. Sero-colostrum, the permeate from MF-DF fractionation of skimmed bovine, caprine and equine colostrum (Piot et al. 2004), was produced using 0.1 µm membranes. The sero-colostrum was free of blood and somatic cells and had a high bacteriological quality (<10 cfu·mL^{-1}) and could be characterised as a native whey, and is an excellent starting material for further purification of IgG and other minor colostrum components, or to feed newborn animals.

Concluding remarks and further perspectives

This study has shown that high-protein native WP ingredients could be produced from MF permeates of skimmed milk and alternatively skimmed BM, and that the functional properties of these NWPCs were comparable. Furthermore, even conventional HTST pasteurisation prior to fractionation changed the protein profile of the MF fractions as more β-LG was rejected, and more casein was transferred to the permeate during fractionation of pasteurised skimmed milk than during fractionation of skimmed milk that was not heat-treated. NWPC foams had large overruns and high solubility, however, the solubility was lower than expected at pH 4.6 due to the casein content. Heat-induced NWPC gels had a denser microstructure than gels made from WPC from an industrial Gouda-type cheese production. An increased CMP:TP ratio in WPC influenced the rheological properties and the microstructure of heat-induced WP gels in a pH-dependent manner. The effect of CMP level on gel strength seemed to pH dependent, as the gels without any CMP added had a far higher gel strength at pH 4.0 than at 7.0, whereas gels containing CMP had an equally low gel strength regardless of pH. The microstructure of NWPC-CMP mixtures were influenced by the CMP level at pH 7.0, whereas all gels had a dense structure at pH 4.0. Gel strain was far higher in samples not containing CMP, and gels with 33% CMP had higher G' than did gels with less CMP. Furthermore, casein standardisation of cheese milk increased the concentration of CMP and sodium, and decreased the content of α_{S1}-casein in the resulting cheese whey.

Based on the findings in this PhD project, and to increase knowledge so that the use of milk fractions can be optimised, researchers should continue to explore the functional properties of native WP. Of particular interest is the possible effect of altering the mineral content and balance of native whey on the properties of the final NWPC or NWPI. It is of both industrial and academic interest to investigate the influence of MFGM components on functional properties in WP systems. Furthermore, the mechanisms in CMP-WP interaction are not yet fully understood and needs to be investigated with focus on the influence from pH and minerals. In addition, more knowledge is needed to understand the effects of CMP glycosylation on WP functionality.

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APPENDICES

Paper I

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Initial pasteurisation effects on the protein fractionation of skimmed milk by microfiltration

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ABSTRACT

Microfiltration using 0.1–0.2 μm membranes enables protein fractionation of skimmed milk into a casein-rich fraction and a permeate that contains native whey proteins. High-temperature, short-time pasteurisation is often one of the first treatments in many dairy processes. The objective of this study was to investigate whether initial pasteurisation would affect the fractions that are obtained from the one-stage microfiltration of skimmed milk. Pasteurised and unpasteurised skimmed milk were fractionated at 55–58 °C using 0.2 μm ceramic membranes. Chemical analysis of the microfiltration fractions showed that both the nitrogen and mineral distributions were altered by the initial pasteurisation. The permeate that was obtained from microfiltration of the unpasteurised milk contained higher amounts of calcium, phosphorous and native whey proteins, in addition to a lower amount of casein fragments passing through the membrane. The retentate that was obtained from microfiltration of the unpasteurised milk contained less total protein and casein.

1. Introduction

Microfiltration (MF) using membranes with pore sizes of 0.8–1.4 μm is widely used in the dairy industry to remove bacteria and spores from milk prior to further processing. Using ceramic membranes with pore sizes in the range of 0.05–0.2 μm, however, enables milk fractionation: a selective separation of skimmed milk into a permeate and a casein-rich retentate (Brans, Schroen, van der Sman, & Boom, 2004). The permeate obtained from MF of raw milk after the use of such dense membranes may be considered a sterile solution of lactose, native whey proteins (NWP) and minerals. The MF retentate may be treated at higher temperatures because of the reduction of heat-sensitive NWP. Moreover, the energy cost of milk treatment can be lowered by reducing the volume of milk (retentate) to be pasteurised, assuming that the downstream equipment can prevent contamination of the permeate. In the cheese-making process, the concentration of casein in the retentate achieved by MF fractionation may improve the rennet coagulability of the retentate (Saboya & Maubois, 2000).

In a high-temperature, short-time pasteurisation (HTST, defined as 72 °C for 15 s), the main whey proteins begin to unfold (de Wit, 2009) and, some whey protein/κ-casein complexes may be formed when caseins are present (Donato & Guyomarc'H, 2009). Aggregated whey proteins, mainly β-lactoglobulin (β-LG), may form complexes with micellar or serum κ- and α_{S2}-caseins, resulting in particles in the size range of 30–100 nm (Anema & Li, 2003). However, α-lactalbumin (α-LA) is more heat-stable than β-LG despite its lower transition temperature (Dalgleish, Senaratne, & Francois, 1997).

In milk, minerals are found in association with casein, whey protein and fat globule membranes. Furthermore, the minerals in the serum phase of milk are present as free ions, salts, or in association with whey proteins. These minerals may pass through the MF membrane, and their distribution after MF fractionation will influence the functional properties of the fractions. For example, the treatment of milk with either ultrafiltration (UF) or MF will increase the calcium (Ca) content and the buffering capacity of the retentate, therefore delaying the pH reduction during traditional cheese making (McMahon, Paulson, & Oberg, 2005). The cold storage of milk causes a shift in the mineral and casein balance between the casein micelles and milk serum, which could be reversed by pasteurisation. More severe heat treatments than HTST, however, result in a transfer of soluble Ca, magnesium (Mg), and phosphorous (P) to an insoluble micellar state (Kelly, Huppertz, & Sheehan, 2008). After heat treatment, if the milk is maintained at room temperature, the minerals will re-equilibrate very slowly

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(Walstra, Wouters, & Geurts, 2006). Thus, an initial HTST pasteurisation prior to MF could have an impact on the mineral distribution in the resulting fractions. Recently, Kaombe, Du, and Lewis (2012) found that dialysates and permeates from the UF of milk pasteurised at temperatures between 75 °C and 80 °C differed in Ca content from those processed at 20 °C.

From an industry point of view, exploiting the use of both fractions from MF processing with rather dense membranes would be economically interesting; thus, processing steps prior to MF should be optimised. Even relatively mild heat treatments, such as HTST, will most likely affect the composition and protein yield of the MF permeate. Moreover, heat denaturation of whey proteins may also change the performance of the membranes during MF. The objective of this study was to investigate the effects of initial HTST pasteurisation on the chemical composition of the fractions obtained from one-stage MF fractionation of skimmed milk with 0.2 μm ceramic membranes.

2. Materials and methods

2.1. Experimental design

This study was performed at a pilot scale MF plant at The Norwegian University of Life Sciences (NMBU), Department of Chemistry, Biotechnology and Food Science (IKBM) in Ås, Norway. Raw milk (maximum age of 3 days) was obtained from the university herd (Norwegian red cattle) during a test period of 4 months. The unpasteurised skimmed milk was either heated to MF temperature (~50 °C) or pasteurised at 73 °C for 15 s before adjusting the temperature to 50 °C for MF. This study was part of a larger project also focussing on the use of the MF retentate from pasteurised skimmed milk. Thus, the MF of the pasteurised skimmed milk was performed over a concentrated period of 2 weeks. For technical reasons, it was not possible to conduct MF of both the unpasteurised and pasteurised skimmed milk simultaneously; therefore, this part of the study was performed over the ensuing 3 months. For this reason, the described combinations of milk treatment and filtration were replicated eight times to minimise the contribution of natural variation in the raw milk composition.

2.2. Initial milk treatment

The raw milk, 900 L per day, was separated (SA 1-01-175, Westfalia Separator AG, Oelde, Germany) into approximately 750 L of skimmed milk at 55 °C. The pasteurised skimmed milk was heat treated at 73 °C for 15 s using a plate heat exchanger (A3-HRB, Alfa Laval, Lund, Sweden) prior to MF. The treated skimmed milk was then kept at 50 °C until MF, with a maximum holding time of 3 h.

2.3. Microfiltration

The milk was fractionated in an MF pilot plant (APV UF/MF pilot MCC RV 00109921 RKA 01118340, APV, Silkeborg, Denmark) using a Uniform trans membrane pressure (UTP) system with a target volume concentration factor (CF) of at least 2.5 to achieve high yields of permeates and an operating time of the MF system of approximately 5 h. The average CF of all of the filtrations was 2.47. The filtration temperature was varied from 55 to 58 °C, with an average of 56.3 °C. Filtration was performed using 0.2-μm ceramic membranes with a total area of 1.38 m^2 (OD25-19033-1016, Jiuwu, Nanjing, Jiangsu, China) and a transmembrane pressure (TMP) of 0.4 bar. The MF pilot plant had a permeate cooling system, which prolonged the operation time, as described in the Norwegian patent no. 330181 (Hoffmann, 2011).

2.4. Methods of analysis

The total solids were analysed according to IDF (1987). The total nitrogen (TN), casein nitrogen (CN), non-casein nitrogen (NCN), and non-protein nitrogen (NPN) were analysed according to IDF (2001a), IDF (2001b), IDF (2001c), and IDF (2004), respectively.

The analysis of fat was performed according to Röse-Gottlieb (Richmond, 1927), whereas α-LA, β-LG, and α_{S1}-, β-, and κ-casein were analysed by reversed phase-high pressure liquid chromatography (RP-HPLC) according to the method described by Hinz, Huppertz, and Kelly (2012). Sample preparation for the quantitative determination of native α-LA and the sum of β-LG A and B, expressed as β-LG, by RP-HPLC was performed according to a modification of the method described by Beyer (1990). All of the samples were diluted with milliQ water and adjusted to pH 4.6 with 0.05 M HCl to precipitate the denatured proteins. The mixture was then left at ambient temperature for 1 h before filtering (Blue ribbon 589/3, Whatman GmbH, Dassel, Germany). Prior to analysis, the samples were diluted with trifluoroacetic acid (TFA, 0.1%, v/v) buffer (99% pure TFA; Sigma–Aldrich, St. Louis, MO, USA) and filtered through 0.2-μm filters (25 mm Syringe Filter, VWR, West Chester, PA, USA). From each sample, 10 μL was injected into the column (Zorbax 300SB-C_{18}, 4.6 × 150 mm, 5 μm with a pre-column Zorbax 300SB-C_{18} 4.6 × 12.5 mm 5-Micron, Agilent, Santa Clara, CA, USA) in an HPLC system (Perkin Elmer Series 200, Santa Clara, CA, USA) with a UV/VIS detector (226 nm). TotalChrom Workstation software version 6.2.1 (Perkin Elmer, Shelton, CT, USA) was used for the data analysis. The proteins eluted at a flow rate of 0.5 mL min^{-1} at 25 °C using gradient A (0.1% TFA) and gradient B (80% acetonitrile and 0.1% TFA), both from Merck KGa (Darmstadt, Germany). The system was stabilised at 40% gradient B for 3 min and then raised to 50% gradient B over 2 min. Gradient B was further increased to 54% over 12 min, then to 60% over 3 min and finally maintained at 60% for 8 min. The column clean-up and equilibration required 20 min.

The standards used for the whey proteins were α-LA L6010, β-LG A L7880, and β-LG B L8005 (Sigma Aldrich). The calibration was prepared by diluting individual standard solutions of α-LA and β-LG in gradient A, followed by filtering through 0.2-μm filters and injecting into the HPLC. Organic acids and carbohydrates were analysed by using HPLC, as described by Moe, Porcellato, and Skeie (2013). Minerals in the milk and milk fractions were decomposed by 65% concentrated HNO_3 at 250 °C in a Milestone Ultrawave UltraClave III (Milestone, Sorisole, Italy) and diluted to 10% HNO_3 before analysis (De La Fuente, Carazo, & Juárez, 1997) via Inductively Coupled Plasma Optical Emission Spectrometry (Perkin Elmer Optima 5300 DV). The amount of ash collected was calculated as the sum of all of the minerals analysed.

2.5. Statistical analysis

As the natural variation of the initial total solid content of the milk differed prior to heat treatment, the results were adjusted according to the total solids of each sample to balance the data in Table 2. The data from each MF fraction were analysed using one-way analysis of variance (ANOVA) by R statistical software (version 2.13.1 2011-07-08, R Development Core Team; http://www.r-project.org). The experimental factor was the pre-treatment of the milk.

3. Results

3.1. Performance of microfiltration of pasteurised and unpasteurised skimmed milk

Microfiltration of the unpasteurised milk resulted in lower flux values and a 2 °C lower permeate temperature than that of the

Table 1
Mass balance of milk components (from 100 g of milk) during microfiltration of pasteurised and unpasteurised skimmed milk.[a]

Parameter	U milk	P milk	UR	PR	UP	PP
Mass (g)	100	100	40	40	60	60
Total solids (g)	9.34 ± 0.19***	9.05 ± 0.03	5.40 ± 0.21**	5.14 ± 0.08	3.76 ± 0.02	3.68 ± 0.06
Lactose (g)	4.9 ± 0.1**	5.0 ± 0.1	1.8 ± 0.1**	1.9 ± 0.0	3.0 ± 0.1*	3.1 ± 01
Total protein (g)	3.22 ± 0.11	3.14 ± 0.10	2.83 ± 0.12	2.76 ± 0.05	0.25 ± 0.02*	0.23 ± 0.01
Casein (g)	2.47 ± 0.11	2.46 ± 0.10	2.42 ± 0.11	2.37 ± 0.06	ND	ND
Native whey protein (g)	0.57 ± 0.01	0.49 ± 0.02	0.35 ± 0.02***	0.32 ± 0.01	0.24 ± 0.02***	0.19 ± 0.02
Fat (g)	0.12 ± 0.05*	0.04 ± 0.00	0.67 ± 0.58*	0.09 ± 0.00	0.02 ± 0.01	0.01 ± 0.01
Ca (mg)	116.78 ± 1.34*	115.62 ± 0.51	95.39 ± 2.11*	92.25 ± 2.53	19.38 ± 0.32**	20.00 ± 0.42
Ash (mg)	431.3 ± 3.3	413.0 ± 31.3	170.3 ± 1.2	169.0 ± 3.0	257.3 ± 4.1	248.4 ± 3.9

[a] Abbreviations are: P, pasteurised milk; U, unpasteurised milk; PP, microfiltration permeate from pasteurised milk; PR, microfiltration retentate from pasteurised milk; UP, microfiltration permeate from unpasteurised milk; UR, microfiltration retentate from unpasteurised milk; ND, not detected. Ash is the sum of minerals analysed. Values are means ± standard deviation, $n = 8$; asterisks in the U milk, UR and UP columns represent significant differences between the pasteurised and unpasteurised samples in the same row within the same fraction (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

pasteurised milk (80.3 ± 2.4 L h^{-1} m^{-2}, 48.4 ± 0.7 °C and 82.9 ± 4.64 L h^{-1} m^{-2}, 50.4 ± 1.8 °C for unpasteurised and pasteurised milk, respectively). The filtration temperature was standardised during the two different treatments (56.3 ± 1.0 °C). Initial heat treatment had no influence on the transmembrane pressure (TMP) of 0.4 bar.

3.2. Chemical composition of skimmed milk and the MF retentate and permeate

3.2.1. Total solids and nitrogen distribution

Total solids were significantly higher ($P < 0.001$) in the unpasteurised milk than in the pasteurised milk, with the same differences found in their MF fractions (Table 1). The differences in the milk composition can be explained by natural variations in the milk. As shown in Table 1, the initial content of total protein (TP) in the unpasteurised and pasteurised milk was not significantly different. However, the amount of TP was significantly higher in the MF permeate from the unpasteurised milk compared with the MF permeate from the pasteurised milk. The casein content, on a total solids basis, differed significantly between the two types of MF retentate, with higher content in the retentate from the pasteurised milk than in the retentate from the unpasteurised milk (Table 2). Using the Kjeldahl analysis, casein could not be detected in the MF permeate; however, a small amount was found in the MF permeate by using RP-HPLC (Fig. 1). The NWP content (as measured by Kjeldahl) was initially higher in the unpasteurised milk than in the pasteurised milk (Table 1). Additionally, when adjusted for the differences in total solids, the NWP was significantly higher ($P < 0.001$) in the unpasteurised milk and its permeate ($P < 0.01$) than in the pasteurised milk and its permeate (Table 2). A significantly higher concentration of β-LG was found in the unpasteurised milk ($P < 0.05$) and its permeate ($P < 0.001$) than in the pasteurised milk and its permeate. Pasteurisation did not, however, influence the α-LA concentration in the milk or permeate. Initial heat treatment had no significant influence on the relative ratio between the casein components and the individual whey proteins (α-LA and β-LG) found in the retentate (Fig. 1). However, α-LA was significantly higher ($P < 0.05$), quantitatively, in the retentate from the unpasteurised milk than from the pasteurised milk (Table 2). A higher relative ratio of casein components was found in the permeate from the pasteurised milk than from the unpasteurised milk (Fig. 1). The α_{S1}- and β-casein ratios ($P < 0.001$ and $P < 0.01$, respectively) of the total peak area were higher in the permeate from the pasteurised milk than in the permeate from the unpasteurised milk. The relative κ-casein ratio was significantly higher in the pasteurised milk than in the unpasteurised milk ($P < 0.001$) before fractionation (Fig. 1). This result was also found for the permeates obtained after fractionation, though the difference was not significant.

3.2.2. Milk minerals, lactose and citric acid

When adjusted for the total solids (Table 2), the Ca concentration was significantly higher ($P < 0.01$) in the pasteurised milk and its permeate than in the unpasteurised milk and its permeate. The concentration of phosphorous (P) followed the same distribution as Ca (Table 2), with a significantly higher concentration in

Table 2
Amount of total solids and components of milk, retentate and permeate after microfiltration of pasteurised and unpasteurised skimmed milk calculated on a total solids basis.[a]

Milk components	U milk	P milk	UR	PR	UP	PP
Total solids (g)	9.3 ± 0.2***	9.1 ± 0.0	13.6 ± 0.5***	12.8 ± 0.2	6.3 ± 0.0	6.1 ± 0.1
Total protein (g)	34.4 ± 1.1	34.7 ± 1.2	52.5 ± 1.7*	53.7 ± 0.5	6.6 ± 0.5	6.3 ± 0.5
Casein (g)	26.4 ± 1.1	27.2 ± 1.1	44.8 ± 1.5*	46.1 ± 0.6	ND	ND
Native whey protein (g)	6.1 ± 0.2***	5.4 ± 0.2	6.4 ± 0.3	6.2 ± 0.3	6.3 ± 0.6**	5.2 ± 0.5
α-LA (mg)	111 ± 29	96 ± 15	85 ± 15*	80 ± 5	130 ± 43	93 ± 39
β-LG (mg)	534 ± 98**	401 ± 62	434 ± 83**	340 ± 51	676 ± 166***	453 ± 70
Lactose (g)	52.3 ± 1.6*	55.5 ± 0.8	32.7 ± 2.1*	36.4 ± 0.8	80.2 ± 3.3*	85.0 ± 1.6
Citric acid (g)	2.2 ± 0.4**	2.4 ± 0.0	1.5 ± 0.1*	1.6 ± 0.0	3.3 ± 0.2*	3.4 ± 0.1
Fat (g)	1.3 ± 0.6*	0.3 ± 0.2	4.8 ± 3.9*	0.7 ± 0.0	0.2 ± 0.1	0.2 ± 0.2
Ca (mg)	1250.0 ± 22.5**	1276.9 ± 4.2	1767.9 ± 58.6	1794.3 ± 28.5	515.2 ± 8.1**	542.9 ± 11.8
K (mg)	1838.2 ± 48.5	1845.1 ± 20.7	1339.7 ± 54.7	1335.2 ± 27.2	2642.0 ± 23.0	2639.3 ± 49.0
Na (mg)	411.8 ± 7.6	407.2 ± 5.4	297.0 ± 8.9**	295.5 ± 6.4	595.8 ± 16.7***	586.1 ± 14.8
Mg (mg)	130.7 ± 1.6**	133.0 ± 0.5	126.2 ± 3.1	127.2 ± 1.6	141.7 ± 2.0	143.7 ± 2.5
P (mg)	986.3 ± 30.9**	1029.0 ± 11.6	1238.3 ± 52.1	1278.8 ± 12.0	631.4 ± 20.2*	675.8 ± 16.0
Ash (mg)	461.7 ± 9.9	469.1 ± 3.1	476.9 ± 16.8***	483.1 ± 3.6	452. 6 ± 2.6	458.8 ± 8.1

[a] Abbreviations are: P, pasteurised milk; U, unpasteurised milk; PP, microfiltration permeate from pasteurised milk; PR, microfiltration retentate from pasteurised milk; UP, microfiltration permeate from unpasteurised milk; UR, microfiltration retentate from unpasteurised milk; α-LA, α-lactalbumin; β-LG, lactoglobulin; ND, not detected. Ash is the sum of minerals analysed. With the exception of Total Solids that is given as per 100 g^{-1} sample, values are given as per 100 g^{-1} TS; all values are means ± standard deviation, $n = 8$. Asterisks in the U milk, UR and UP columns represent significant differences between the pasteurised and unpasteurised samples in the same row within the same fraction (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

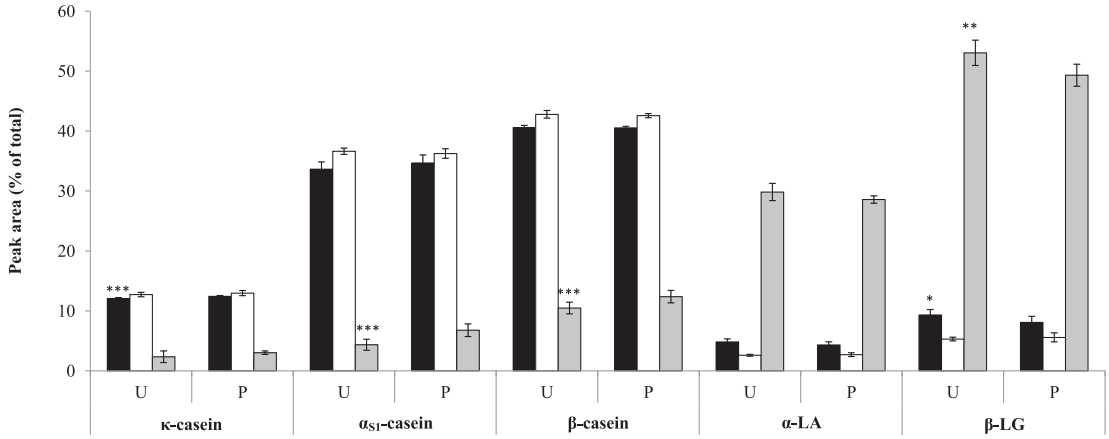

Fig. 1. Effects of pasteurisation on protein composition of skimmed milk and the fractions obtained by microfiltration (MF) of the milk. Protein composition was analysed by reverse phase-high performance liquid chromatography (RP-HPLC) as content of κ-, α_{S1}- and β-casein, α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) in relative distribution as a percentage of total peak area. Skimmed milk (black bars), retentate (white bars) and permeate (grey bars). Error bars represent the standard deviation (SD), $n = 8$. Asterisks above the U bars indicate significant differences between the corresponding P and U columns ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

the pasteurised milk and its permeate. The concentration of sodium (Na) was higher in the permeate from the unpasteurised milk than in the permeate from the pasteurised milk ($P < 0.001$). The concentration of magnesium (Mg) was significantly higher in the pasteurised milk than in the unpasteurised milk ($P < 0.01$) before fractionation, while no significant differences were found in the MF fractions. The concentration of ash, in terms of total minerals calculated on total solids basis, was significantly lower ($P < 0.001$) in the retentate from the unpasteurised milk despite only minor differences detected in the milk and permeates. The amounts of lactose and citric acid were higher in the pasteurised milk and its fractions compared to the unpasteurised milk and its fractions (Tables 1 and 2).

4. Discussion

This study showed that MF resulted in higher levels of native β-LG in the permeate fractions obtained from the unpasteurised milk than from the pasteurised milk. Pasteurisation did not alter the amount of native α-LA found in the skimmed milk; however, it did influence the retention of α-LA during MF. A lower content of total NWP (as measured by Kjeldahl fractionation) in the permeate from the pasteurised milk could be a result of induced protein denaturation during the pasteurisation, followed by formation of β-LG/κ-casein complexes, serum aggregates with other β-LG molecules, or formation of other aggregates with soluble or micellar casein. These aggregates would be retained in the retentate during MF and analysed as casein by the Kjeldahl method.

Pasteurisation (HTST) of skimmed milk affected the nitrogen distribution of the MF fractions. Lau, Barbano, and Rasmussen (1990) reported a 5% heat denaturation of WP by heat treatment at 63 °C for 30 min Low temperature long time (LTLT) and found a higher degree of WP denaturation in HTST-treated milk when comparing LTLT with commercial HTST treatment of milk. This observation is in accordance with the present results, whereby more NWP were found in the unpasteurised milk and its permeate than in the pasteurised milk and its permeate. We revealed that small amounts of casein were present in the permeates from both the pasteurised and unpasteurised milk, while the content of whey proteins was highest in the permeates from the unpasteurised milk. The origin of the κ-, α_{S1}-, and β-casein found in the permeate could be casein micelles small enough to pass through the membrane pores, soluble caseins in a non-micellar state, or a mixture of micellar and soluble casein. The range of micelle diameter in milk is from less than 0.1 μm to greater than 0.3 μm (Horne & Dalgleish, 1985). The major casein dissociation from the micelle during cold storage has generally been understood to be linked to β-casein leakage (Creamer, Berry, & Mills, 1977; Reimerdes & Klostermeyer, 1976; Rose, 1968). Casein released from the micelles during cold storage has been shown to be completely reversible after 18 h at 20 °C (Davies & Law, 1983). The relative amounts of both β- and α_{S1}-casein were higher in the permeate from the pasteurised milk than in the permeate from the unpasteurised milk, with the same tendency was observed for κ-casein. Raw milk was stored cold (4 °C) before processing, and the highest temperature obtained by the unpasteurised milk during processing was during cream separation (55–58 °C). Regardless of the prior treatment, the milk was held at approximately 50 °C for up to 3 h prior to MF. At 50 °C, the diameter of the casein micelles were reduced compared with 6 and 20 °C in the UF permeate (Beliciu & Moraru, 2009), which can be explained by an increased hydrophobic interaction. This effect may have influenced the casein transmission during fractioning. Holding milk at 50 °C may reverse the β-casein solubilisation obtained during cold storage of raw milk. Liu, Weeks, Dunstan, and Martin (2013) showed that the amount of soluble casein decreased when the temperature was increased between 10 and 40 °C, leading to the formation of an increasing number of small casein micelles. Liu et al. (2013) also showed that the rearrangement of the casein micelle could be different during warming than during cooling. Mikheeva, Grinberg, Grinberg, Khokhlov, and de Kruif (2003) studied the self-aggregation of soluble, non-micellar casein (mainly β-CN), which suggested an increase in self-aggregation of soluble β-CN with increasing temperatures. Because heat treatment affects the casein equilibrium between the micelle-orientated and the soluble casein states, the results of the present study are most likely a consequence of this equilibrium in the serum phases of the pasteurised and unpasteurised milk. Fractionation of unpasteurised milk resulted in a higher yield of NWP and a lower contribution of casein to the total protein in the permeate after MF.

After adjusting for total solids, the content of Ca and P was higher in the pasteurised milk and its permeate than in the unpasteurised milk and its permeate, while no differences were found in the retentate. During heating, the solubility of the diffusible Ca and P in milk serum decreases, causing these minerals to associate with the casein micelle. This shift in the Ca balance at temperatures less than 80 °C would be reversed by further cold storage, though very slowly (Walstra et al., 2006). The initial heat treatment of the milk did not influence the content of Ca and P on a

total solids basis in the retentate because Ca and P were mainly associated with the casein. Ca and Mg found in the permeate could be linked to serine phosphate of (non-micellar) caseins, WP or micellar colloidal P, such as $CaPO_4^-$, HPO_4^{2-}, or $H_2PO_4^-$. Pasteurisation seemed to cause a lesser change in the shift in the mineral balance than in the nitrogen balance. This observation is in agreement with Liu et al. (2013) who found that the dynamic shift of the milk mineral balance induced by changes in temperature appeared almost instantaneous, whereas re-equilibration of the casein micelles was slower.

The permeate temperature during MF of the unpasteurised milk was 2 °C lower than that obtained during MF of the pasteurised milk. This difference may have influenced permeate flux but is most likely not directly related to the initial heat treatment of the milk. The lower flux of the unpasteurised milk could also be related to the higher total solid (TS) content of this milk, compared with the pasteurised milk. Espina, Jaffrin, Paullier, and Ding (2010) found that during MF under similar conditions and using rotating ceramic membranes, the permeate flux was higher for UHT milk than for pasteurised milk (HTST). This observation was explained by differences in viscosity as a result of different contents of NWP, which were lower in the UHT milk. This finding supports our results and may be explained by a higher content of NWP in the unpasteurised milk, which may have affected the viscosity of the skimmed milk and hence the permeate flux. The filtration temperature used was slightly higher (55 °C), which is a normal filtration temperature (50–55 °C) for MF with ceramic membranes, and does not normally cause increased fouling. An increased filtration temperature, optimally between 56 °C and 60 °C, would be favourable to optimise flow; however, the temperature would need to be thoroughly controlled to avoid WP denaturation and the subsequent fouling that may occur. The patented permeate cooling system used during this MF experiment is based on the idea that cooling of the permeate reduces the temperature on the membrane surface, which will then act as a cooling surface during processing. Therefore, by preventing fouling caused by calcium phosphate precipitation and whey protein denaturation associated with high filtration temperatures, the processing time may be prolonged using this technique.

5. Conclusion

Pasteurisation of skimmed milk at 73 °C for 15 s prior to MF resulted in a permeate containing a lower NWP content than the MF permeate obtained from the unpasteurised skimmed milk at the given processing conditions. Initial pasteurisation reduced the content of native β-LG in skimmed milk, therefore MF of the unpasteurised skimmed milk resulted in a higher yield of native β-LG in the permeates. Interestingly, the pasteurisation of skimmed milk prior to MF also increased the ratio of casein compounds passing through the membrane. Any heat denaturation of whey proteins caused by the initial pasteurisation did not change the performance of the membranes during MF.

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Paper II

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The composition and functional properties of whey protein concentrates produced from buttermilk are comparable with those of whey protein concentrates produced from skimmed milk

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ABSTRACT

The demand for whey protein is increasing in the food industry. Traditionally, whey protein concentrates (WPC) and isolates are produced from cheese whey. At present, microfiltration (MF) enables the utilization of whey from skim milk (SM) through milk protein fractionation. This study demonstrates that buttermilk (BM) can be a potential source for the production of a WPC with a comparable composition and functional properties to a WPC obtained by MF of SM. Through the production of WPC powder and a casein- and phospholipid (PL)-rich fraction by the MF of BM, sweet BM may be used in a more optimal and economical way. Sweet cream BM from industrial churning was skimmed before MF with 0.2-μm ceramic membranes at 55 to 58°C. The fractionations of BM and SM were performed under the same conditions using the same process, and the whey protein fractions from BM and SM were concentrated by ultrafiltration and diafiltration. The ultrafiltration and diafiltration was performed at 50°C using pasteurized tap water and a membrane with a 20-kDa cut-off to retain as little lactose as possible in the final WPC powders. The ultrafiltrates were subsequently spray dried, and their functional properties and chemical compositions were compared. The amounts of whey protein and PL in the WPC powder from BM (BMWPC) were comparable to the amounts found in the WPC from SM (SMWPC); however, the composition of the PL classes differed. The BMWPC contained less total protein, casein, and lactose compared with SMWPC, as well as higher contents of fat and citric acid. No difference in protein solubility was observed at pH values of 4.6 and 7.0, and the overrun was the same for BMWPC and SMWPC; however, the BMWPC made less stable foam than SMWPC.

Key words: buttermilk, microfiltration, whey protein concentrate, phospholipid, milk fat globule membrane

INTRODUCTION

Over the past several years, the interest in high-protein ingredients such as whey protein concentrate (**WPC**) or isolate (**WPI**) has increased. Both WPC and WPI are usually produced from cheese whey. However, WPC can also be derived from the fractionation of skim milk (**SM**) by microfiltration. Skim milk WPC contains less minerals and has no caseinomacropeptides compared with traditional WPC from cheese whey. The functional properties of WPC from SM fractionation have recently been compared with those of cheese whey WPC by Coppola et al. (2014) and Evans et al. (2010). The WPC from SM fractionation have unique functionalities, such as excellent solubility, gelling after heat treatment, and foaming properties (Bacher and Køningsfeldt, 2000; Heino et al., 2007).

Buttermilk (**BM**), the by-product from the churning of butter, has a CN and whey protein ratio similar to that of SM (Corredig and Dalgleish, 1997), and spray-dried BM has a comparable nutritional value to that of SM powder (Morin et al., 2004). Govindasamy-Lucey et al. (2006) studied the use of BM in pizza cheese, and the solubility, foaming, and emulsifying properties of BM powders have been described in Sodini et al. (2006). However, a high fat content in BM may lead to sticky powder as well as increased risk of off-flavors from oxidation products. The fractionation of BM into a CN- and milk fat globule membrane (**MFGM**)-rich fraction and a whey protein fraction may increase the value of this by-product. Most of the BM produced today is used as animal feed (liquid) or for the production of BM powder, but it can also be exploited in a more optimal way to produce WPC, as the global demand for whey protein is increasing (Lafougère, 2014). Buttermilk is higher in MFGM protein and phospholipids (**PL**) than SM, which might alter the functional properties of its WPC.

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Buttermilk is a rich source of MFGM components, such as proteins and PL (Rombaut et al., 2005), because parts of the MFGM are released from the fat globule into the serum phase during butter churning. Membrane technology is frequently used in the dairy industry to concentrate milk constituents, and by using microfiltration (**MF**), CN and MFGM can be separated from whey proteins. Several studies have focused on the separation and isolation of MFGM proteins or PL from BM by MF (Sachdeva and Buchheim, 1997; Corredig et al., 2003; Morin et al., 2006, 2007a,b) for the further purification of the PL (Astaire et al., 2003). Few studies, if any, have focused on the utilization of the whey protein fraction obtained from BM.

The high ratio of CN to total protein in BM (Corredig et al., 2003) gives a high CN recovery during MF. The retentate from the MF of sweet BM could be used for further extraction of the PL. Achieving high-purity PL from the MF of BM is difficult, as CN micelles and MFGM fragments have similar size distributions (Rombaut et al., 2006; Singh, 2006). However, a MF retentate from BM, which is rich in CN and MFGM components, can be used as a starting material for further separation and purification of PL or as a supplement in other dairy processes. However, several studies have reported the transmission of PL and MFGM proteins through the MF membranes (Astaire et al., 2003; Morin et al., 2007a), thus affecting the composition and functional properties of the products used from these permeates. Whey proteins and PL are applied in the food industry as ingredients for baking, as well as in pharmaceuticals and in cosmetics (Vanderghem et al., 2010) due to protein solubility, foaming, and emulsifying properties.

The major whey proteins, which are most important for the functional properties in most food applications, are β-LG and α-LA. Heat treatment does influence the functional properties of the whey proteins (Abd El-Salam et al., 2009) or the sizes of their aggregates (Schokker et al., 2000; De La Fuente et al., 2002). At approximately 67.5 to 78°C, the β-LG starts to unfold, and at higher temperatures of 78 to 82.5°C aggregation occurs (Sava et al., 2005). Denatured β-LG may aggregate with other β-LG molecules, CN (Donato and Guyomarc'h, 2009), α-LA (Dalgleish et al., 1997), or MFGM proteins (Ye et al., 2004). α-Lactalbumin has no free thiol group and contributes less to the aggregation caused by denaturation (Calvo et al., 1993).

The objective of the current study was to compare the final WPC powders obtained by MF fractionation and further UF and diafilitration (**DF**) concentration of BM and SM MF permeates. The BM used was of industrial origin with varying cream pasteurization histories, whereas the SM used was obtained from controlled pasteurization (73°C/15 s).

MATERIALS AND METHODS

Experimental Design

The study was carried out at a pilot-scale dairy processing plant at the Norwegian University of Life Sciences (**NMBU**), Department of Chemistry, Biotechnology and Food Science (Ås, Norway). The fractionation, concentration, and spray drying were carried out for each of 4 batches of BM and SM. To yield enough MF permeate to produce 1 batch of approximately 3 kg of WPC powder, the skimmed BM or SM of each powder production was MF on 2 consecutive days. The MF permeate from the first day was kept at 4°C and pooled with the MF permeate from the second day before further concentration by UF/DF and the final spray drying. The 2 pooled MF permeates originated from the same raw material batch (BM or SM) and were, therefore, treated as a single MF replicate block during further analysis. The UF and powder production were repeated 4 times (4 replicate blocks).

BM and Cream Treatment

Four batches of fresh sweet BM were collected from TINE Sandnessjøen (Sandnessjøen, Norway). The cream used for churning originated from different dairy plants in northern Norway. As the cream was obtained from different dairy plants, the cream had undergone different pasteurization routines prior transportation to TINE (Sandnessjøen, Norway). The cream was pasteurized upon arrival at TINE. Therefore, the heat treatment of the cream might have been repeated 2 to 3 times using the following conditions: 74.2°C/20 s (approximately 40% of the cream) or 86°C/5 s (approximately 60%) based on different routines at the supplying dairies. Each batch of BM was pasteurized once (76°C for 20 s) before transportation to the NMBU pilot plant. Including transportation, the BM was stored cold (4°C) for up to 5 d before MF. Upon arrival at the NMBU pilot-scale dairy processing plant, the BM was skimmed by a cream separator to 0.3% fat at 55°C (SA 1–01–175, Westfalia Separator AG, Oelde, Germany) to minimize the differences in the residual fat in the 4 batches investigated. The TS content of the BM after skimming was 7.50 ± 0.56 g/100 g, on average, and the pH was 6.84 ± 0.19. After skimming, the MF feed was kept at approximately 50°C for a maximum of 4 h before fractionation.

MF of BM and SM

The MF of skimmed BM was carried out using pilot plant equipment (APV UF/MF pilot MCC RV

00109921 RKA 01118340, APV, Silkeborg, Denmark) with a target volume concentration factor of 2.2 to 2.4 to achieve high yields of the permeate. A module containing 7 ceramic membranes with a pore size of 0.2 μm and total membrane surface area of 1.38 m^2 (OD25–19033–1016, Jiuwu, Nanjing, Jiangsu, China) was used. The processing conditions were the same as for the fractionation of pasteurized SM as described by Svanborg et al. (2014). The filtration temperature (55–58°C), permeate and retentate flow, and pressure differences on the permeate and retentate inlet and outlet were monitored to control fouling during fractionation. The target concentration factor was 2.2 to 2.5. The SM was pasteurized (73°C/15 s) before MF as described by Svanborg et al. (2014). The MF filtration of 650 L of SM was achieved in 3 h and 40 min, whereas the MF filtration of 800 L of BM took 4 h and 50 min (half the batch of the 1,600 L of skimmed BM).

UF and DF of BM Permeate and Pasteurized SM Permeate

The MF permeates from BM and SM were UF at 50°C using a pilot plant unit (UFS-4/6 Alfa Laval, Silkeborg, Denmark) with a single UF-membrane (cut-off value of 20 kDa, GR 62–6338/48P, Alfa Laval). Diafiltration was performed using pasteurized tap water until no remaining lactose was detected by a brix measurement (Atago N1 refractometer, Tokyo, Japan) of the UF concentrate. The amount of DF water used during UF of the different MF permeates was 350 to 500 and 650 to 1,075 L for SM and BM, respectively. The inlet and outlet pressures were held at 2.1 and 0.9 bar, respectively, for both types of permeates.

Production of WPC Powder from UF/DF Concentrates

The UF/DF concentrates of the MF permeates from of BM and SM were spray-dried (Niro Atomizer, Gea Niro, Søborg, Denmark) directly after concentration was finished. An inlet air temperature of 185 to 190°C and a constant outlet air temperature of 85°C were used. The spray drier rotary atomizer had a drying rate of 15 L of liquid/h.

Methods of Analysis

Total solids were analyzed according to IDF 21B:1987 (IDF, 1987b). Total nitrogen, casein nitrogen, noncasein nitrogen, and NPN were analyzed according to IDF 20–1:2001 (IDF, 2001b), IDF 20–4:2001 (IDF, 2001c), 20–5:2004 (IDF, 2001a), and 29–2:2004 (IDF, 2004), respectively. Organic acids and carbohydrates were analyzed using HPLC as described by Moe et al. (2013). The fat contents of skimmed BM, MF fractions, and UF/DF concentrates were analyzed according to the Gerber method (IDF, 2008) as an average of 2 measurements. The analysis of the fat contents in the powders was performed according to Röse-Gottlieb (IDF, 1987a). Sodium dodecyl sulfate-PAGE and sample preparation of 8% (wt/wt) protein WPC solutions followed the procedure described by Devle et al. (2014). Seven microliters of samples were applied to respective wells on 12% polyacrylamide separating gel (Mini-Protean TGX Precast Gel, Bio-Rad, Hercules, CA). In addition, 5 μL of a SDS-PAGE standard (Low range, Bio-Rad) was applied to one well. To identify some of the proteins visualized in the SDS-PAGE gels, the bands were cut and in-gel digested according to Devle et al. (2014), followed by identification by ultra-performance liquid chromatography and Q-extractive mass spectrometry as described by Islam (2014). The resulting peak list files were searched against the *Bos taurus* UniProt database by an in-house Mascot server (2014, version 2.4.1; Matrix Science, London, UK), containing a total of 24,205 protein sequences. Evaluation was carried out using the Exponentially Modified Protein Abundance Index (emPAI; Ishihama et al., 2005), with a protein threshold of 99.9% and a peptide threshold of 95% with a minimum of 5 peptides. Minerals in the milk fractions and powders were analyzed according to De La Fuente et al. (1997). The samples were digested by 65% concentrated HNO_3 at 250°C in a Milestone Ultra wave UltraClave III (Milestone, Sorisole, Italy) and were diluted to 10% HNO_3 before determination of the minerals by inductive coupled plasma optical emission spectrometry (Perkin Elmer Optima 5300 DV, Perkin Elmer, Shelton, CT). A commercial laboratory, Vitas AS (Oslo, Norway), analyzed phospholipids and α-tocopherol. The quantification of α-tocopherol was carried out using HPLC-fluorescence detection system as described by Berhe et al. (2007) with the following modifications: the separation was performed on a Zorbax SB-C18 50 × 4.6-particle size 1.8 μm column from Agilent (Santa Clara, CA) using methanol and water as the mobile phase. Identification of the unknowns was performed against standards from Alfa Aesar (Ward Hill, MA). For the PL analysis, the detection was carried out using a normal phase-HPLC with an evaporative light scattering detector. The samples were accurately weighed, dissolved in isopropanol, shaken, and centrifuged (4,000 × *g*, 10°C, 10 min). Then, the supernatants were transferred to new vials. The pellets were washed with isopropanol, centrifuged (4,000 × *g*, 10°C, 10 min), and the resulting supernatants were pooled with the first ones. The supernatants were evaporated to dryness and dissolved

in buffer A (hexane and isopropanol) before analysis. Separations were performed on a normal phase polyvinyl alcohol functionalized silica 250- × 4.6-mm HPLC column (YMC, Dinslaken, Germany) with buffers A and B (hexane, isopropanol, and water) as the mobile phase. The system used a linear gradient changing from 25 to 100% buffer B over 12 min, and then it was held for 15 min at 100% B before returning to the initial conditions. The total run time was 22 min. Analyses were calibrated against known standards from Lipoid GmbH (Lipoid GmbH, Köln, Germany) and Larodan (Larodan, Malmö, Sweden).

Functionality Tests of the Protein Solutions

The overrun was tested by preparing 200 mL of protein solutions (8% wt/vol protein) of WPC made from SMWPC or BMWPC and hydrated in deionized water by stirring for at least 30 min at ambient temperature. The solutions were kept overnight at 4°C for complete hydration and were adjusted to room temperature for 30 min before whipping with a Bosch MFQ35 hand mixer (Bosch, Gerlingen, Germany) at the maximum speed for 2 min the next day. The preparation of the solution and whipping was replicated at least 2 times for each batch of powder. Foam overrun was measured according to the methods of Phillips et al. (1987). Immediately after whipping, the foam was gently scooped into standard laboratory beakers and levelled off at 100 mL. The average of the foam weights of 10 measurements per whipping and the average initial weight of the protein solution, were used for the calculation of the overrun as follows:

$$\text{Overrun (\%)} = (\text{weight of 100 mL of solution} - \text{weight of 100 mL of foam/weight of 100 mL of foam}) \times 100\%.$$

The foam stability was measured as the time required to reduce the initial foam volume by 50% using a 250-mL graded cylinder, generating foam the same way as described above.

The suspension stability was measured in 1% (wt/wt) protein solutions of the powders as described by Sikand et al. (2011). Samples were adjusted to pH values of 7.0 and 4.6 using 5.0 or 1.0 *M* NaOH and 2.0 or 0.1 *M* HCl. The samples were centrifuged at 12,000 × *g* for 15 min at 4°C. The amount of soluble nitrogen in the samples was measured before centrifugation and in the corresponding supernatant using the Kjeldahl method (IDF, 2001b) taking the dilution effect due to pH adjustment into account in the calculation of the suspension stability.

Statistics

Due to differences in the TS in the UF concentrates, the results were balanced according to their TS contents before statistical analysis. One-way ANOVA was performed by using R statistical software (version 2.13.1 2011–07–08, R Development Core Team; http://www.r-project.org). The experimental factor for the different parts of the experiment (UF concentrates and powders) was the raw material (BM and SM) used for MF.

RESULTS

Fractionation of BM by MF

The microfiltration of SM has been described previously (Svanborg et al., 2014). During the microfiltration of BM, process observations were recorded 3 times per hour, and the average values from 8 filtration days are shown in Table 1. The initial batches of BM (1,600 L divided for use on 2 consecutive days) were larger than the batches of SM (650 L/MF); therefore, the flux was kept at a lower level to minimize time-dependent fouling when fractioning the BM. The fractionation of BM was comparable to the fractionation of pasteurized SM in terms of development of the transmembrane pressure (**TMP**). The TMP increased toward the end of filtration of both types of feed. Despite a higher concentration factor ($P < 0.05$) and permeate flux ($P < 0.001$), no significant difference in the TMP was observed between the 2 raw materials.

The mass balance of the 4 batches of BM (in total 8 filtrations) was calculated and is shown in Table 2. Approximately 62% of the TS were retained by the membrane, and around one-third of the whey proteins passed through the membrane. The majority of the minerals, fat, and almost all of the trace metals (Fe and Cu) were retained in the retentate. Most of the PL were retained by the membrane, except lysophosphatidylcholine (**LPC**), which was found in equal amounts in both fractions. Calcium and phosphorus followed the same

Table 1. Processing conditions during the microfiltration of skimmed buttermilk and skim milk given as means and their SD (n = 8)

	Skimmed buttermilk		Skim milk[1]	
Item	Mean	SD	Mean	SD
Volume concentration factor	2.43	0.06*	2.52	0.52
Flux (L × $h^{-1}min^{-2}$)	76.8	2.8***	82.9	4.6
Filtration temperature (°C)	56.2	0.6	56.2	1.2
Transmembrane pressure (kPa)	43	8	43	8

[1]Results from Svanborg et al. (2014).

*$P < 0.05$; ***$P < 0.001$.

Table 2. Mass balance after microfiltration (MF) of 1,000 kg of skimmed buttermilk (BM); values are expressed as means and their SD (n = 8)

	BM (1,000 kg)		MF retentate from BM (411 kg)		MF permeate from BM (589 kg)	
Milk component[1]	Mean	SD	Mean	SD	Mean	SD
TS (kg)	74.95	5.65	46.43	4.44	28.87	1.46
TN (kg)	3.51	0.40	3.22	0.32	0.12	0.03
NCN (kg)	0.76	0.07	0.46	0.05	0.40	0.09
NPN (kg)	0.29	0.05	0.11	0.01	0.16	0.01
TP (kg)	20.54	2.78	19.83	2.10	ND[2]	
Casein (kg)	15.67	3.10	16.57	2.21	ND	
Whey protein (kg)	3.05	0.46	2.37	0.19	1.55	0.57
Lactose (kg)	39.54	2.02	15.24	0.74	24.48	1.46
Fat (kg)	3.00	0.58	3.75	1.88	ND	
Ca (kg)	0.74	0.08	0.61	0.06	0.14	0.02
Cu (g)	0.02	0.04	0.60	0.10	0.00	0.00
Fe (g)	0.65	0.15	0.57	0.07	0.07	0.02
K (kg)	1.32	0.08	0.57	0.03	0.75	0.05
Mg (kg	0.09	0.01	0.05	0.03	0.04	0.00
Na (kg)	0.33	0.01	0.14	0.03	0.19	0.01
P (kg)	0.69	0.06	0.50	0.41	0.19	0.02
α-Tocopherol (mL)	40.7	7.4	58.5	31.0	ND	
PE (kg)	0.26	0.04	0.34	0.05	ND	
LPE (kg)	ND		ND		ND	
PC (kg)	0.47	0.05	0.44	0.05	0.03	0.00
PS + PI (kg)	0.36	0.07	0.56	0.08	ND	
SPM (kg)	0.05	0.00	0.07	0.02	ND	
LPC (kg)	0.30	0.03	0.17	0.04	0.17	0.02
Total PL (kg)	1.44	0.13	1.59	0.20	0.21	0.00

[1]NCN = noncasein nitrogen; LPC = lysophosphatidylcholine; LPE = lipophosphatidylethanolamine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PL = polar lipid (phospholipids); PS = phosphatidylserine; SPM = sphingomyelin; TN = total nitrogen; TP = total protein, (TN − NPN) × 6.38; whey protein = (NCN − NPN) × 6.38; casein = (TN − NCN − NPN) × 6.38.

[2]ND = not detected (below the threshold of the analysis).

trend as CN and fat during the fractionation of BM, whereas potassium and sodium were found in higher amounts in the permeate compared with the other minerals and trace metals. However, some discrepancy was noted between the somewhat low amounts of PL and α-tocopherol in the initial BM compared with the much higher summed amounts detected in the MF fractions of BM (Table 2).

UF Concentrate

The liquid WPC produced by UF/DF of MF permeates of BM and SM were compared on a TS basis to balance the data according to the initial difference in the TS in Table 3. In the liquid WPC from the BM, the fat content was rather high, whereas fat was not detected in the liquid WPC originating from SM. In addition, less total protein and lactose were observed in the BM liquid WPC compared with in the SM liquid WPC. The amounts of undenatured whey proteins were similar in the 2 concentrates. Meanwhile substantial amounts of CN were found in the liquid SM WPC, and no CN was detected in the liquid BM WPC. The concentrates also differed in their mineral compositions ($P < 0.01$), with less calcium and higher amounts of magnesium and iron in the liquid BM WPC than in the SM WPC. Only the BM WPC was analyzed for PL, and only phosphatidylcholine (0.18 ± 0.03 g/100 g) and PL (0.35 ± 0.08 g/100 g) were detected.

Composition and Functionality of WPC Powder

Results from the chemical analysis of BMWPC and SMWPC powders are shown in Table 4. As in the liquid WPC, the amounts of native whey proteins were similar in BMWPC and SMWPC. The BMWPC contained less total protein than SMWPC, although small amounts of caseins were also found in BMWPC. The samples of SMWPC powder showed higher CN contents compared with those of BMWPC, which was confirmed by SDS-PAGE as giving stronger bands in the area where CN is normally found (Figure 1). The BMWPC samples showed a strong band in the high molecular weight area of the SDS-PAGE gel (>97 kDa). This band was identified to be FA synthase by liquid chromatography-mass spectrometry, but it also contained an embryo-specific fibronecitin 1 transcript variant and complement C3. The same band was present in lanes loaded with

Table 3. Amounts of TS and components in the ultrafiltration/diafiltration (UF/DF) concentrates of the MF permeates from skimmed buttermilk (BM) and skim milk (SM) calculated on a DM basis[1]

	UF/DF concentrate of MF permeate from BM		UF/DF concentrate of MF permeate from SM	
Milk component[2]	Mean	SD	Mean	SD
TS (g)	5.51	0.49**	7.45	0.53
TN (g)	13.64	0.16**	14.43	0.30
NPN (g)	0.93	0.23	0.32	0.04
NCN (g)	12.88	0.19***	12.22	0.05
Total protein (g)	81.10	0.71***	90.00	1.74
CN (g)		0***	12.03	1.96
Whey protein (g)	76.23	1.44	75.89	0.40
Fat (g)	17.66	1.74*	ND[3]	
Ca (g)	0.591	0.045**	0.703	0.018
Cu (mg)	0.524	0.262	0.431	0.114
Fe (mg)	1.870	0.114**	1.071	0.287
K (g)	0.281	0.099	0.342	0.021
Mg (g)	0.078	0.005**	0.068	0.001
Na (g)	0.193	0.033	0.164	0.005
P (g)	0.303	0.029	0.315	0.007
Citric acid (g)	0.51	0.37	0.15	0.04
Lactose (g)	0.96	0.83*	2.48	0.56

[1]With the exception of TS given as per 100 g/sample, values are given as per 100 g/TS. Values are given as means and their SD (n = 4).

[2]NCN = noncasein nitrogen; TN = total nitrogen; TP = total protein (TN − NPN) × 6.38; whey protein = (NCN − NPN) × 6.38; CN = (TN − NCN − NPN) × 6.38.

[3]ND = not detected (below the threshold of the analysis).

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

SMWPC, but it was weaker. Bands identified as xanthine dehydrogenase (**XDH**; ~97 kDa) were stronger in the BMWPC samples compared with in the SMWPC samples. The BMWPC bands between 14 and 22 kDa were not very well defined, but were identified to be calcineurin B homologous protein and glycosylation-dependent protein 1. Lactadherin (molecular weight 48 kDa) was identified from the BMWPC samples at approximately 45 kDa. Perilipin 2 and vitamin D-binding protein were identified in bands between 45 and 66 kDa, respectively, and were more pronounced in the SMWPC samples than in the BMWPC samples, whereas lactadherin, which appeared in the same area, was not apparent in the SMWPC samples. Bovine serum albumin was detected in all of the bands analyzed by liquid chromatography-mass spectrometry, and traces of CN, α-LA, and β-LG were detected in several of the other proteins.

Despite the higher fat content of BMWPC, the 2 powders had similar amounts of PL but differed in their PL composition (Table 4). Lysophosphatidylcholine was found in a smaller amount in BMWPC ($P < 0.01$) compared with in SMWPC and accounted for the majority of the PL in SMWPC. Sphingomyelin was only detected in BMWPC ($P = 0.0534$), whereas phosphatidylcholine was the dominating PL in BMWPC and was found in higher amounts compared with in SMWPC ($P < 0.05$). However, the somewhat higher amounts of phosphatidylethanolamine (**PE**), phosphatidylserine (**PS**), and phosphatidylinositol (**PI**) found in BMWPC were not significantly different from the levels found in SMWPC.

The functional properties of dispersions made from whey protein concentrates (BMWPC and SMWPC) are shown in Table 5. High overruns were observed for both powders tested: 835 ± 150 and 884 ± 230% for BMWPC and SMWPC, respectively. The SMWPC foam was, however, far more stable than the BMWPC foam; the foam of the BMWPC solution completely decomposed after only 5 min, whereas the SMWPC foam was stable for hours. The BMWPC foam had larger bubbles than the SMWPC foam (results not shown), whereas the SMWPC foam had a denser appearance than the BMWPC foam. Both powders showed high nitrogen solubility even at low pH values. At a pH of 7.0, the suspension stability was much higher than at a pH of 4.6, whereas some variation was observed in the suspension solubility between the powder batches at a pH of 4.6.

DISCUSSION

Filtration of BM can be challenging due to residual fat and MFGM fragments of various particle sizes. The largest particles (butter fragments and large fat globules) can be removed from BM using a cream separator

Table 4. Composition of powder made from whey protein concentrate from permeate after the microfiltration of buttermilk (BMWPC) and skim milk (SMWPC)[1]

	BMWPC		SMWPC	
Milk component[2]	Mean	SD	Mean	SD
TS (g)	94.50	1.29	94.50	0.58
Total protein (g)	82.12	0.98**	88.45	2.23
CN (g)	4.27	2.32**	10.90	2.03
Native whey protein (g)	72.73	0.70	75.15	3.80
Lactose (g)	0.22	0.21**	1.72	0.45
Citric acid (g)	0.62	0.30*	0.08	0.03
Fat (g)	1.48	0.22***	0.13	0.04
Ca (mg)	578.3	51.6*	662.5	13.0
K (mg)	272.8	95.6	318.8	16.1
Mg (mg)	188.8	35.2***	63.5	0.6
Na (mg)	76.0	5.8***	152.8	2.6
P (mg)	294.3	27.8	295.5	5.9
Cu (mg)	0.6	0.3	0.4	0.1
Fe (mg)	2.0	0.0***	1.1	0.2
α-Tocopherol (μg/mL)		ND		ND[3]
PE (mg)	47.2	11.6	4.9	0.6
LPE (mg)			9.8	2.3
PC (mg)	86.5	44.3*	12.0	0.6
PS+PI (mg)	58.1	6.1	12.4	0.5
SPM (mg)	8.8	2.1		ND
LPC (mg)	47.0	36.9**	227.9	58.7
Total PL (mg)	220.0	121.0	268.9	56.2

[1]All values are means given as per 100 g/sample. Values are given as means and their SD (n = 4).

[2]LPC = lysophosphatidylcholine; LPE = lipophosphatidylcholine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PL = polar lipid (phospholipids); PS = phosphatidylserine; SPM = sphingomyelin; TP = total protein (TN − NPN) × 6.38; whey protein = (NCN − NPN) × 6.38; CN = (TN − NCN − NPN) × 6.38.

[3]ND = not detected (below the threshold of the analysis).

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

(Fauquant et al., 2014); thereby, a decreased fouling during fractionation by MF may be obtained. Due to the larger batch volumes of BM, precaution during the MF of the skimmed BM was taken and the flux was lowered compared with SM fractionation. However, no difference in fouling in terms of increased TMP (above that expected from the time-dependent fouling) was observed between the MF of SM and BM. The CN-rich MF retentate contained the majority of the PL obtained, and the fractionation of BM could be carried out under similar conditions to those of the fractionation of SM. The BM was skimmed before fractionation to reduce some of the possible effects of fat content variations between the 4 batches of BM received. In spite of this, the MF permeate from BM was higher in fat than that from SM, and further concentration of the MF permeate resulted in a BMWPC that also contained higher amounts of fat than the SMWPC. A more severe skimming may have equalized the differences in the initial fat contents of BM and SM (0.3 and 0.04 g/100 g, respectively). Alternatively, a membrane with a smaller pore size would be able to retain more MFGM fragments and some of the fat globules. Although the MF membrane retained most of the fat during the MF of both the BM and SM, BMWPC contained higher amounts compared with SMWPC. One possible explanation is that the skimmed BM contained more fat initially compared with SM. Another possible explanation could be that the fat remaining in the skimmed BM had a higher content of free fat (Mulder and Walstra, 1974). Therefore, more fat was transferred through the membrane compared with in the fractionation of SM. Free fat cannot be separated from SM or BM by regular milk separators, and most of the triglyceride molecules are probably too small to be retained by the MF membranes. However, neither the fat globule size distribution nor the contents of the free fat were observed in the experiment reported here. The mass balance (Table 2) showed that the majority of the PL were retained in the retentate during MF. The origin of the PL obtained in the retentate would be the MFGM from intact fat globules, fragments of MFGM from disrupted fat globules, or complexes consisting of MFGM proteins and whey proteins or CN micelles, which were too large to pass through the membrane.

The temperatures during the filtrations in this study were all above 50°C. The filtration temperature is known to influence the transmission of PL over the

Table 5. Functional properties of dispersions made from the whey protein concentrates of microfiltered buttermilk (BMWPC) and microfiltered skim milk (SMWPC) given as means and their SD (n = 4)[1]

	BMWPC		SMWPC	
Functional property	Mean	SD	Mean	SD
SS (%) pH 7.0	98.00	2.39	98.09	2.22
SS (%) pH 4.6	79.11	9.01	79.44	4.86
TN (%) pH 7.0	99.50	2.18	98.26	2.14
TN (%) pH 4.6	85.73	2.93	84.59	5.28
Foam stability (min)	<2		>300	
Overrun (%)	884	230	835	150

[1]SS = suspension stability, TS in supernatant of 1% powder solution after centrifugation as a percentage of the TS in the sample; TN = soluble protein, total nitrogen in the supernatant of the sample solution as a percentage of the total nitrogen in sample; Overrun = [(wt protein solution − wt foam)/wt foam) × 100]. Foam stability is the time required to reduce the foam volume of an 8% protein solution by 50%.

MF membrane, and Morin et al. (2004) found that transmissions of the PL and total lipids were higher at high and intermediate temperatures of 50 and 25°C, respectively, than at 7°C. However, Morin et al. (2007b) found that the previous history of heat treatment of the BM and cream was the most important technological factor affecting the isolation of MFGM components by filtration technology. For this reason, the isolation of MFGM components from BM using membrane filtration could be improved if the heat denaturation of whey proteins, and possibly components in the MBGM membrane, was reduced by use of less harsh pasteurization conditions of the cream in the industry. Due to the transportation of cream between different dairy plants, the heating regimen of the butter cream in this experiment was representative for industrially obtained BM in Norway. From an industrial point of view, the diversity in cream treatment and BM handling would lead to a higher range in composition and functional properties of BMWPC compared with cheese WPC or SMWPC. To optimize the utilization of the whey protein fraction from BM, cream treatment and BM handling must be standardized between creameries, and the heat treatment controlled and documented. Severe heat treatment of BM could lead to formation of aggregates consisting of MFGM fragments and other milk

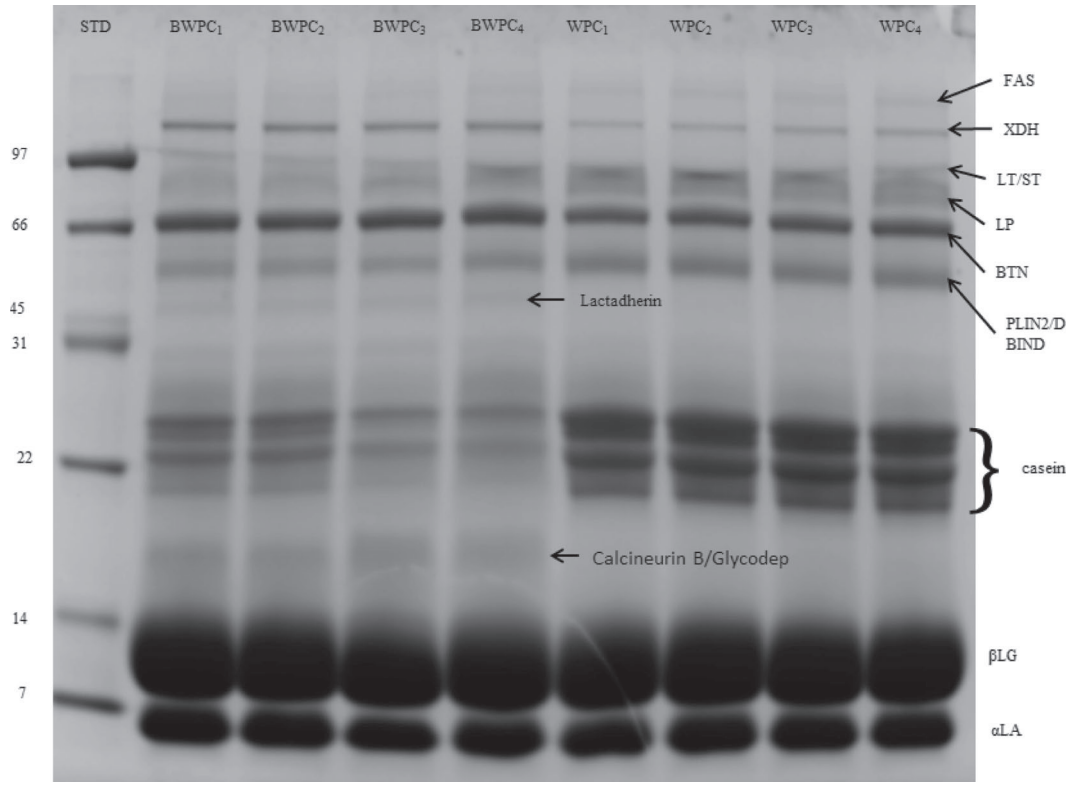

Figure 1. Sodium dodecyl sulfate-PAGE patterns of reconstituted whey protein concentrates (WPC) of buttermilk (BWPC) and skim milk. Subscripts indicate replicates. Lane STD was loaded with 5 μL of low-molecular-weight standard. Numbers to the left of lane STD indicate molecular weights (kDa). Lanes $BWPC_{1-4}$ and WPC_{1-4} were loaded with 7 μL of sample solution containing 8% protein (wt/wt). Identification of milk fat globule membrane and whey proteins by MS are indicated on the right side of the figure: BTN = butyrophilin; DBIND = vitamin D-binding protein; FAS = fatty acid synthase; Glycodep = glycosylation-dependent protein 1; LP = lactoperoxidase; LT = lactotransferrin; PLIN2 = perilipin 2; ST = serotransferrin; and XDH = xanthine dehydrogenase.

proteins (Ye et al., 2002), whey protein-CN interactions (Donato and Guyomarc'h, 2009), or aggregates of major whey proteins. The MF membrane retained the largest aggregates. However, some of the aggregates could be transmitted through the membrane, as the identified SDS-PAGE gel bands revealed aggregates containing casein, α-LA, and β-LG in both the BMWPC and the SMWPC samples.

Due to the analytical threshold, some PL classes could not be detected in the MF permeate. However, they may be identified in the more concentrated BMWPC powder, just as LPC was detected in the liquid WPC from BM. Variations in the sphingomyelin (**SPM**) content in milk have been reported, and the variation is mainly related to the size distribution of fat globules in the milk and thus to the amount of the MFGM available (Lopez et al., 2011). Sphingomyelin molecules, which are loosely bound to the outside of the MFGM membrane (Deeth, 1997), were not detected in the SMWPC. This is probably due to a lower initial fat content in the SM and to the fact that SPM tended to follow the fat fraction during separation (Spitsberg, 2005). The majority of SPM in the MF permeate from the fractionation of SM would be derived from intact fat globules that were small enough to be transported through the membrane. Phospholipids that originate from the smaller MFGM fragments and free fat in the BM are able to pass through the membrane (Astaire et al., 2003; Morin et al., 2004, 2006, 2007a). The PL distributions in BMWPC and SMWPC did not reflect the PL distribution usually found in the native MFGM present in milk, where phosphatidylcholine, phosphatidylethanolamine, and SPM are expected in higher quantities than the other varieties of PL (Vanderghem et al., 2010). Morin et al. (2007a) showed that the PL distribution was only slightly affected by MF and suggested that mainly whole MFGM fragments rather than dissociated PL were transmitted through the MF membrane during filtration. In milk, phosphatidylcholine, PI, and PS are buried in the inner layer of the MFGM (Danthine et al., 2000), preventing their interaction with other milk solids. When MFGM are no longer intact, both the inner and outer layers of the MFGM are exposed to the serum components of the BM. Morin et al. (2007b) showed that the intensity of heat treatment of the butter cream affected the MFGM protein aggregation and the solubility of BM solids more than the churning process itself. However, they found that spray drying of BM reduced the amount of phosphatidylethanolamine and to a much smaller extent the amounts of PI and PS. The functional properties of dairy products are partly affected by the state of the MFGM components after heat treatment (Keenan et al., 1983; Houlihan et al., 1992 a,b; Morin et al., 2008). In our study, protein denaturation caused by various and repeated cream pasteurization procedures did not significantly influence the soluble solids or the soluble total N in the final BMWPC compared with in the final SMWPC. However, a higher free fat content may reduce the solubility in the BMWPC (Schuck et al., 2012). Due to its high concentration of PL, BM may be easily oxidized (O'Connell and Fox, 2000). α-Tocopherol, a potential antioxidant to protect the polyunsaturated fat from oxidation (Nagachinta and Akoh, 2013), followed the CN, PL, and fat-rich fraction during MF and was therefore not detected in the MF permeates or in the final WPC powders. Therefore, the oxidative stability of BMWPC should be further investigated.

Whey proteins are known for their surface-active functionality. Undenatured β-LG and α-LA are recognized to have excellent foaming capacities and stabilities (Abd El-Salam et al., 2009). The large difference in foam stability observed between BMWPC and SMWPC was probably due to the difference in fat content, as the presence of lipids is known to depress the foaming properties of WPC (Morr, 1985). In an application where the WPC would have an emulsifying function, a higher lipid content would be favored; however, in most other applications, a fat reduction would be beneficial. A further improvement of the process to reduce the fat content prior membrane fractionation by a more intensive skimming or by using membranes with denser pore sizes (<0.2 μm) than used during MF in our study might enhance the foaming stability of BMWPC. No significant differences in the overrun between the WPC were observed despite the differences in the fat contents as well as in the PL and protein compositions. The total amounts of PL were similar in the 2 WPC, although higher levels of PL in the BMWPC was expected because of the PL following the MFGM fractions during churning.

The main difference in the nitrogen contents of BMWPC and SMWPC was the somewhat higher CN content in SMWPC and a corresponding higher content of whey proteins in the BMWPC samples. The reason for this is probably the low content of CN found in skimmed BM before MF. The BMWPC had a similar protein solubility to that of SMWPC at neutral and acidic pH values. The difference in the protein solubility in the WPC at a pH of 7.0 compared with at a pH of 4.6 may be explained by the acid precipitation of CN or aggregates of CN, denatured whey proteins, and MFGM proteins. The repeated cream pasteurization (74.2°C/20 s and 86°C/5 s) did not seem to influence the suspension stability or the protein solubility of the BMWPC, although we observed some variation in the suspension solubility under acidic conditions between the powder batches. This may possibly reflect the

variation in cream pasteurization procedures between the different BM batches.

The MFGM proteins, as well as the PL and sphingolipids in milk may have beneficial nutritional (Spitsberg, 2005; Britten et al., 2008; Dewettinck et al., 2008) and functional properties (Corredig and Dalgleish, 1997; Goudedranche et al., 2000). Traces of a wide spectrum of proteins and peptides were identified in the BMWPC and SMWPC, indicating the formation of aggregates during heat treatment of SM, BM, and cream before churning. Proteins account for 25 to 60% of the total mass of the MFGM, and membrane-specific proteins comprise approximately 1 to 2% of the total protein in milk (Mather, 2000). The MFGM proteins are known to react with β-LG during the heat treatment of milk (Kim and Jimenez-Flores, 1995). Ye et al. (2002) found that heating whole milk above 60°C resulted in larger protein complexes of XDH and butyrophilin (**BTN**), whereas lactadherin was more heat stable. In our study, the aggregates in the WPC consisted of MFGM-proteins (XDH, lactadherin, or BTN), CN, β-LG, and α-LA. The same compounds were detected in both BMWPC and SMWPC, with the exception of lactadherin, which was only identified in BMWPC. The XDH and BTN were, as expected, more pronounced in the BMWPC samples. Mucin 1, generally regarded as a major MFGM protein (Mather, 2000), was not detected in any of the samples. According to Le et al. (2012), a high resistance against trypsin and pepsin hydrolysis may influence the detection of mucin 1 during sample preparation due to its heavy glycosylation. Glycosylation-dependent cell adhesion molecule 1 was identified together with calcineurin B homologous protein, tetranectin, and Ras-related protein Rab-18. The latter protein was recently identified in BM by Le et al. (2013), who also found calcineurin B homologous protein associated with the MFGM fractions. However, a potential higher content of MFGM proteins in the BMWPC compared with in the SMWPC did not seem to influence the functional properties of the WPC, most likely due to the low levels of MFGM present in the final powders.

The level of lactose was lower in BMWPC compared with in the SMWPC. The reason for this is probably that during UF of the MF permeate from BM more water was used than during the UF/DF of the MF permeate from SM. The reason for the difference in volumes of DF water (350–500 and 650–1,075 L for the UF/DF of the SM and BM permeates, respectively) was the initial differences in the MF permeates produced. During the MF of SM, Ca and P tended to follow the CN fraction. The initial mineral composition of the feed influenced the mineral contents of the MF fractions. Buttermilk and its MF fractions contained less Ca compared with SM and SM fractions, most likely due to an initial lower CN content in the BM. Approximately 70% of the Ca in milk is associated with the CN micelle (Walstra et al., 2006). The higher amount of DF water used during UF/DF may have also influenced the mineral content of BMWPC. The transmission of serum-soluble minerals during the fractionation of BM resulted in a BMWPC richer in trace metals (Fe, Mg, and Cu) compared with the SMWPC. Evers (2004) reported a transfer of Cu from milk serum to MFGM due to heat treatment. Approximately 10% of Cu and nearly 50% of Fe is associated with the MFGM in milk (Walstra et al., 2006). The higher contents of Cu and Fe in BMWPC compared with in SMWPC are most likely a result of the higher initial fat content in BM and due to the storage of BM at 4°C for several days. Copper and Fe are present in approx. 0.3 and 0.01 mg/100 g of fat globules, respectively; but during cold storage, Cu may migrate irreversibly into the milk serum (Walstra et al., 2006). Conversely, the heating of cream above 60°C may cause considerable migration of Cu from the milk serum to the MFGM (Mulder and Walstra, 1974).

Iron is present in the MFGM of milk fat. Differences in the mineral composition can affect the functional properties of WPC and WPI (Lorenzen and Schrader, 2006). The BMWPC had a higher content of Mg but a lower content of Ca compared with SMWPC. Zhu and Damodaran (1994) showed that divalent cations (Ca^{2+} and Mg^{2+}) improved the foaming properties of WPI. The cations can link 2 negatively charged proteins and cause aggregation.

CONCLUSIONS

This study showed that a WPC powder, which has interesting composition (PL profile and protein composition) and solubility properties, could be produced from BM fractionation. The BMWPC was equivalent to SMWPC in its solubility, foaming properties, and chemical composition. The protein profiles of BMWPC and SMWPC were comparable, but SMWPC had a higher content of CN. Despite the comparable compositions of the WPC, WPC from BM showed a lower foam stability, probably due to its higher fat content. The SMWPC and BMWPC contained comparable amounts of total PL, but the compositions of the PL differed. All PL classes investigated, except for LPC, were found in significantly higher amounts in BMWPC than in SMWPC.

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Paper III

Microfiltration of cheese milk for casein standardisation affects whey protein composition

Casein standardisation of cheese milk affects whey composition

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Abstract

The effect of casein standardisation of cheese milk on whey composition was studied by comparing whey from regular cheese milk with whey made from microfiltration retentate. The same raw milk was used for both types of cheese milk. The nitrogen composition of the samples was tested using Kjeldahl digestion and RP-HPLC and minerals were decomposed and detected by Inductive Coupled Plasma Optical Emission Spectrometry. Whey made from casein-standardised cheese milk contained higher amounts of total solids, total nitrogen, fat and sodium, and less α_{S1}-casein compared to whey made from regular cheese milk. The elevated nitrogen content in whey made from casein-standardised milk was due to an increase in the levels of caseinomacropeptide.

Keywords: Whey, microfiltration, caseinomacropeptide, minerals, casein standardisation.

1 Introduction

Microfiltration (MF) is applied in the dairy industry for casein standardisation of cheese milk, to produce micellar casein concentrates, or for the production of other products with increased protein content such as Greek yoghurt. Casein standardisation by MF may eliminate the seasonal variations of casein in milk (Guinee et al. 2006; Papadatos et al. 2003), and improve the rennet coagulation properties of milk (Govindasamy-Lucey et al. 2007; Guinee et al. 2006; Neocleous et al. 2002). The protein content of cheese milk is increased with ultrafiltration (UF) or MF, which increases the yield from the cheese vat and hence, increases the capacity of the cheese-making equipment (Neocleous et al. 2002; Govindasamy-Lucey et al. 2005). Casein standardisation of cheese milk by MF also generates native whey, a novel starting material for the production a high value whey protein concentrate or isolate (Saboya and Maubois 2000; Maubois 1997; Heino et al. 2007). Only a few studies have aimed to investigate the composition of whey derived from cheese making of the casein-enriched MF retentate (Outinen et al. 2008, 2010a, b). The studies presented here have focused mainly on the protein profile in cheese milk and whey, particularly with respect to the content of caseinomacropeptide (CMP), which increases in whey generated from the rennet coagulation of MF-retentate.

During MF and UF, the colloidal minerals, i.e. minerals associated with the casein micelle, will be retained in the retentate (Brandsma and Rizvi 1999; Schreier et al. 2010), whereas diffusible minerals in the serum phase are distributed in the permeate. The salts dissolved in the milk serum influence various milk properties, such as protein stability (de la Fuente 1998). Micellar α_{s1}-, α_{s2}- and β-casein are stabilised by calcium phosphate, which can coordinate with phosphoserine residues (Gaucheron 2005). Moreover, the positive charge of calcium or other cations neutralise the negative sites of the surface of casein micelles, which also contains other minerals and salts such as K, Na and citrate. To the authors' best knowledge, only a few studies

(Outinen et al. 2010b) have reported minerals other than calcium in whey made from MF retentate. The objective of this work was to investigate how casein standardisation of cheese milk by MF influences the protein content, protein composition and the mineral distribution of the resulting whey.

2 Materials and methods

2.1 Experimental design

Two batches of regular cheese milk and two batches of casein-standardised cheese milk were produced from the same raw milk. Differences in the chemical composition of the cheese milks and of the whey produced as a by-product of cheese making from these milks were investigated.

2.2 Milk treatment and whey production

Approximately 2400 L of raw milk was obtained from the Norwegian University of Life Sciences farm. The raw milk was allowed to separate and the cream (approximately 300 L) was removed. The skimmed milk was then pasteurised at 73 °C for 15 s and the separated cream was pasteurised at 80 °C for 15 s. Regular cheese milk (approximately 600 L) was prepared by adding pasteurised cream to the pasteurised skimmed milk to a final concentration of 2.7 % (v/v) fat. 300 L of the regular cheese milk was used in each of two cheese vats to produce cheese and whey according to a regular full-fat Dutch cheese protocol (Porcellato et al. 2013).

To generate the casein-standardised cheese milk, the remaining 1500 L of skimmed milk was held at 50 °C and microfiltered. Microfiltration was performed with a concentration factor (CF) of 1.45 as described by Svanborg et al. (2014) using 0.14 µm membranes (Inside Céram, Tami

Industries, Nyonce Cedex, France) to generate 800 L of retentate. The retentate was stored at 4 °C overnight. The next morning the retentate was pasteurised at 73 °C for 15 s and pasteurised cream was added to obtain 2.7 % (v/v) fat. 600 L casein-standardised milk was then used to produce two vats of cheese and whey using the same protocol as with the regular milk.

A MilkoScan FT1 (Foss ProcessScan™ FT, Foss, Hillerød, Denmark) Fourier Transform Infrared Spectroscopy (FTIR) was used to control the protein content of the skimmed milk, retentate, and cream during fractionation and cheese making. Cheese making was initiated by a 1% (v/v) inoculation with a Probat Visbyvac 505 (Danisco, Copenhagen, Denmark) starter culture. Rennet (ChyMax Plus, Chr. Hansen, Hørsholm, Denmark) was added at a concentration of 6.9 $mL \cdot kg^{-1}$ protein to catalyse curdling. The coagulum was cut according to an evaluation of the firmness by an experienced cheese maker (coagulation time for the regular milk and for the casein-standardised milk were 45 and 22 min, respectively). The whey from the first whey drainage (20 min after cutting, before water addition) from the four cheese vats were collected and were filtered through grade one (11 µm) filter paper to remove cheese fines. All filtered whey samples were frozen at -20 °C until chemical analysis.

2.3 Methods of analysis

A sample of whey from each of the four cheese vats and a sample of each cheese milk (pooled replicates) were analysed: total solids (TS), total nitrogen (TN), casein nitrogen (CN), non-casein nitrogen (NCN), and non-protein nitrogen (NPN) were analysed according to IDF 21B:1987 (IDF 1987), IDF 20-1:2001 (IDF 2001b), IDF 20-4:2001 (IDF 2001c), IDF 20-5:2001 (IDF 2001a), and IDF 29-2:2004 (IDF 2004), respectively. Lactose were analysed using HPLC as described by Moe et al. (2013). Fat content was analysed by MilkoScan FT1 FTIR. Minerals in the milk fractions and powders were analysed by Inductive Coupled Plasma Optical

Emission Spectrometry according to a method described by de La Fuente et al. (1997) with modifications as described by Svanborg et al. (2014). The protein profile, including CMP from whey protein concentrates (WPCs) and fractions from the cheese making, were analysed using reverse phase HPLC (RP-HPLC) according to a method based on Hinz et al. (2012) and Visser et al. (1991). Data analysis was done with Chromeleon software version 7.1 (ThermoFisher Scientific). The amount of each protein was determined by dividing the corresponding peak area (obtained by RP-HPLC) for that particular protein by the sum of all the peak areas for the predominant peaks (CMP, α_{S1}-casein, β-casein, α-lactalbumin and β-lactoglobulin). α_{S2}- and κ-casein were not detected by this method.

Finally, the % of each protein was converted to a concentration (g L^{-1}) using equation (1):

$$(1)\ Protein\ x\ (g\ L^{-1}) = \frac{\%\ protein\ X}{100} \times protein\ content\ (g\ L^{-1})$$

One-way analysis of variance (ANOVA) was carried out to test the effect of casein standardisation of cheese milk on the chemical composition of the whey.

3 Results and discussion

3.1 Nitrogen distribution

Casein standardisation increased the TP:WP ratio by 30% (Table 1). As expected, the casein-standardised cheese milk had a higher TP (4.6% vs 3.4% for regular cheese milk) and casein levels (4.0% versus 2.9% for regular cheese milk). In contrast, the regular cheese milk contained higher amounts of β-lactoglobulin compared to the cheese milk made from MF retentate; this is likely due to the removal of milk serum from casein-standardised milk by MF. Cheese making from casein-standardised milk resulted in a whey that had a higher content of both TN and TS ($P < 0.05$) than whey from regular milk did (Table 2). This is probably due to

the small, but significant increase ($P < 0.05$), in the CMP content from 39.7% in regular milks' whey to 42% in the whey from MF retentate. However, the two types of whey did not have different ($P > 0.05$) TP- or NPN-contents. The solubility of CMP in trichloroacetic acid (included in Kjeldahl analysis IDF 29-2:2004) varies due to variation in carbohydrate composition (Lieske and Konrad 1996), and could be the reason for the insignificant difference in NPN. The increase in the CMP:TP ratio in the from casein-standardised milk's whey in this study is probably related to the increase in TP:WP ratio to 12.1 in the retentate. A TP:WP ratio of 7.5 in Emmental cheese milk resulted in whey containing 38 % CMP (Outinen et al. 2008) In the present study, the CMP content in whey from both milks were higher than previously reported (Outinen et al. 2008, 2010b). Typical concentrations reported for CMP are ≤ 25 % of the total protein in regular cheese whey (Thomä-Worringer et al. 2006; Tolkach and Kulozik 2006). However, the quantification of CMP can be challenging due to the large variability in glycosylation and genetic variants (Neelima et al. 2013; Campos Motta et al. 2014).

Rennet coagulation occurred faster in the casein-standardised cheese milk because of the higher casein content. Despite the adjustment of rennet according to casein content, the actual coagulation time could influence the release of CMP. Milk typically coagulates when approximately 90 % of κ-casein is hydrolysed, whereas a lower degree of hydrolysis in protein enriched milk is necessary to obtain a curd with firmness similar to that obtained from regular milk (Brandsma and Rizvi 2001). The cutting process was closely monitored so that cutting could be initiated when the gel was sufficiently soft, and in order to avoid excessive firmness of the gel. The fat:casein ratio was not adjusted in the casein-standardised milk in this study. Despite this, the increased fat content of the whey from casein-standardised milk may be an indicator that the curd was cut when it was too firm (Guineea et al. 1994) compared to the cutting of the gel-like curd derived from regular milk. Another explanation is that a lower degree of hydrolysed κ-casein in the casein-standardised in formed a network as less able to

retain fat compared to the curd of regular milk. In contrast to our result, casein standardisation of cheese milk with a constant fat:casein ratio has previously been reported to reduce loss of fat to the whey (Outinen et al. 2010a). However, all whey samples from the cheese makings were filtered prior to analysis, and therefore they do not report the true values of fat and casein concentration in the crude whey, but rather reflect the contents found in WPCs, which are usually clarified prior to evaporation and spray drying.

During cheese making, casein-standardised cheese milk resulted in a stronger coagulum than regular cheese milk; in addition, the casein-standardised cheese milk generated a less turbid whey with less cheese fines than in regular cheese milk's whey (results not shown). Casein was not detected in any of the whey samples by using Kjeldahl analysis (IDF 20-4:2001), and only small amounts of α_{S1}-casein, but not β- or κ-casein, were detected by RP-HPLC. Here we report a lower ($P < 0.05$) content of α_{S1}-casein in whey from casein-standardised cheese milk than in whey from regular cheese milk, which, to the authors' knowledge, has not been previously reported. α_{S1}-casein is tightly bound to the micelle structure, and has a lower transition barrier from the casein micelles in the curd to the whey. Therefore, a lower content of α_{S1}-casein could be related to the more stable coagulum and less cheese fines from cheese making of the MF retentate. The heat treatments of the MF retentate (all milk before MF and the retentate before cheese making) may have altered the rearrangement of the casein micelle upon cooling, as Law (1996) found less α_{S1}-casein in milk serum after heat treatment and storage at 30 °C compared to the serum of raw milk stored at comparable conditions.

The mineral composition of whey is critical to the functional properties of the resulting whey protein ingredients (Havea et al. 2002; Riou et al. 2011). The calcium, phosphorous and

168 magnesium content of cheese milk increased with casein standardisation, whereas iron, copper,
169 potassium and sodium levels were unaffected (Table 1). However, the mineral content and
170 composition of whey from casein-standardised and regular cheese milk was similar, with the
171 exception of sodium, which was found in higher ($P < 0.05$) amounts in whey from casein-
172 standardised cheese milk (Table 2). Only about 5 % of the sodium and potassium in milk is
173 colloidal (Walstra et al. 2006; Gaucheron 2005). Our results may indicate that less sodium was
174 associated with the caseins in the curd from the MF retentate compared to caseins in the curd
175 from regular milk. The casein micelles of the MF retentate are suspended in a smaller volume
176 of milk serum than in regular milk. The MF retentate is depleted in WP, lactose and minerals,
177 but the serum phase has still a high ionic strength. Although most of the potassium and sodium
178 are considered as diffusible salts, it has been suggested that these minerals could bind to
179 proteins in the whey fraction (de la Fuente et al. 1996). Outinen et al. (2010b) found no change
180 in mineral composition of the resulting whey from milk that was casein-standardised using MF
181 and diafiltration and a high CF. However, in order to avoid depletion of minerals and lactose,
182 diafiltration water was obtained from ultrafiltration of the MF permeate from the previous
183 casein standardisation in the experiment by Outinen.

184

185 4 Conclusion

186 Casein standardisation of cheese milk by MF resulted in a whey with an elevated CMP:TP ratio
187 that contained less α_{S1}-casein compared to whey from regular cheese milk. The mineral
188 composition of the whey did not change due to casein standardisation, with the exception of
189 sodium, which was higher in whey made from milk with increased casein content than in whey
190 from regular milk.

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298 **Table 1.** Composition of regular and casein-standardised cheese milk. Values are from the
299 analysis of pooled samples from two cheese vats for each milk type

Analysis method	Milk component	Regular milk	Casein standardised milk
	Total solids (g 100 g^{-1})	11.65	13.09
Kjeldahl digestion (g·100 g^{-1})	Total nitrogen	0.56	0.75
	Total protein	3.42	4.61
	Whey protein	0.36	0.38
	Casein	2.89	4.04
	Total protein/whey protein	9.50	12.13
HPLC [a] (g·100 g^{-1} TP)	Caseinomacropeptide	0.00	0.00
	α_{S1}-casein	38.2	40.0
	β-casein	45.9	47.5
	α-lactalbumin	4.3	3.5
	β-lactobglobulin A	4.1	3.2
	β-lactobglobulin B	7.5	5.9
	Lactose	5.0	4.9
FTIR (g·100 g^{-1})	Fat	2.7	2.7
Inductive Coupled Plasma Optical Emission Spectrometry (mg·100 g^{-1})	Ca (mg 100 g^{-1})	119.0	158.83
	Cu	0.01	0.01
	Fe	0.02	0.03
	K	166.2	169.8
	Mg	12.6	14.1
	Na	37.4	38.4
	P	99.8	123.7

300 [a] HPLC-analysis: percentage of total peak area. Abbreviations: FTIR = Fourier Transform
301 Infrared Spectroscopy.

Table 2. Composition of whey resulting from cheese making with regular and casein-standardised cheese milk. Values represent the average of two replicates. Standard deviations are provided in parentheses. Asterisks indicate a significant difference between the two whey samples (* $P < 0.05$).

Analysis method	Whey component	Milk source			
		Regular whey		Casein-standardised whey	
(g 100 g^{-1})	Total solids	6.95	(0.01) *	7.02	(0.00)
Kjeldahl digestion (g·100 g^{-1})	Total nitrogen	0.14	(0.00)*	0.17	(0.00)
	Non-protein nitrogen	0.04	(0.00)	0.05	(0.02)
	True protein	0.63	(0.03)	0.79	(0.09)
	Whey protein	0.62	(0.03)	0.55	(0.25)
HPLC [a] (g·100 g^{-1} TP)	Caseinomacropeptide	39.7	(0.7)*	42.0	(0.2)
	α_{S1}-casein	3.9	(0.6)*	1.2	(0.1)
	α-lactalbumin	15.1	(0.3)	15.7	(0.1)
	β-lactobglobulin A	14.5	(0.3)	14.6	(0.0)
	β-lactobglobulin B	26.7	(0.6)	26.5	(0.0)
	Lactose	5.4	(0.0)	5.3	(0.0)
FTIR (g·100 g^{-1})	Fat	0.2	(0.0)*	0.3	(0.0)
Inductive Coupled Plasma Optical Emission Spectrometry (mg·100 g^{-1})	Ca	43.0	(0.4)	42.2	(0.5)
	Cu	0.002	(0.0)	0.003	(0.0)
	Fe	0.013	(0.0)	0.015	(0.0)
	K	166.5	(0.1)	172.13	(1.6)
	Mg	9.64	(0.1)	9.55	(0.1)
	Na	39.42	(0.5)*	41.68	(0.6)
	P	50.89	(0.9)	50.80	(0.5)

[a] HPLC-analysis: percentage of total peak area. Abbreviations: FTIR = Fourier Transform Infrared Spectroscopy.

Paper IV

Caseinomacropeptide influences the functional properties of a whey protein concentrate

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ABSTRACT

Casein-standardisation changed the protein profile in whey during cheese making and in the resulting whey protein products (Paper III). To investigate the effect of the caseinomacropeptide to true protein ratio on the functional properties of a whey protein concentrate powder, heat-induced gels with four different concentrations of caseinomacropeptide (0, 9, 21 and 33 %) at three different pH levels (4.0, 5.5, and 7.0) were made from a native whey protein concentrate and a commercial caseinomacropeptide concentrate powder. The microstructure, rheology, and solubility of the whey protein-caseinomacropeptide mixes were evaluated. The microstructures of the gels became porous and coarse as the caseinomacropeptide to concentration increased. Gels without caseinomacropeptide were more resistant to stress ($\tau_{LVR,3\%}$) and strain ($\gamma_{LVR,3\%}$), with a 3 % decrease in the linear viscoelastic region of G', than gels containing 9 - 33 % caseinomacropeptide. Solubility was influenced by pH, but not by the protein composition.

1. Introduction

One strategy to reduce the seasonal variations in milk is to increase the casein content in cheese milk using microfiltration (MF) (Guinee, O'Kennedy, & Kelly, 2006; Papadatos, Neocleous, Berger, & Barbano, 2003). Casein standardisation of cheese milk has been shown to improve the coagulation properties of rennet (Rodríguez, Requena, Fontecha, Goudédranche, & Juárez, 1999; St-Gelais, Roy, & Audet, 1998) and increase the fat and protein recovery (Heino & Uusi-Rauva, 2010; Neocleous, Barbano, & Rudan, 2002). When MF is used to casein-standardise cheese milk, native whey (the skimmed milk's MF permeate) is generated. The resulting whey from cheese making using casein-standardised milk may have a higher caseinomacropeptide (CMP) to true protein (TP) ratio (Outinen, Heino, & Uusi-Rauva, 2008, 2010). Native whey, regular cheese whey, and the whey made from cheese making of casein-standardised milk are three different raw materials for further processing to obtain concentrated whey protein products, as they have different chemical compositions. The major difference between the whey protein concentrate (WPC) made from native whey protein concentrate

(NWPC) compared with the WPC from cheese whey is the lack of CMP in the former. Additionally, the NWPC may contain less fat and denatured proteins, depending on the process conditions and type of membrane filtration used during manufacture of the cheese-derived WPCs.

The degree of whey protein denaturation and the fat content influence the functional properties of the WPCs (Morr & Ha, 1993). Undenatured whey proteins are soluble at a wide range of pH values, whereas denatured and/or aggregated whey proteins are insoluble. The loss of solubility indicates that the whey proteins are denatured. The degree of denaturation of the whey proteins is commonly expressed as the degree of solubility at pH 4.6. Very low amounts of denatured proteins are expected in the NWPC, and if found, they are most likely induced during the evaporation (before spray drying). Due to heat treatment of milk and whey, and possibly during evaporation, commercial WPCs will have a significantly higher denatured whey protein content compared with NWPC. The solubility of globular proteins is strongly correlated to their gel properties (Zayas, 1997a). Gelation is an important functional property of protein ingredients, such as WPCs. The heat-induced gelation mechanism of globular proteins, such as β-lactoglobulin (β-LG), the dominant protein in whey products, is a 3-step process (Sawyer, 1968). The gelation steps are characterised by (1) the unfolding of the protein molecules, exposing free and previously buried sulfhydryl and hydrophobic groups; (2) the aggregation of denatured protein molecules; and (3), if the protein concentration is sufficiently high, the formation of a three dimensional network that traps water. According to Lorenzen and Schrader (2006), the network of WPC gel at pH values far from the pI of β-LG consists of linear strands (“fine stranded”) because electrostatic repulsion stabilises the proteins. Such gels are translucent, rubbery, and deformable. At pH values close to the isoelectric point (pI) (5.1 for β-LG), the net charge of the proteins equals zero, and due to the low electrostatic repulsion, hydrogen bonds and hydrophobic interactions may lead to aggregation. In this situation, gelation may be poor due to a small net charge and a low number of intermolecular bonds, but, when formed, it is described as an intermediate particulate gel (Cornacchia, de la Fortelle, & Venema, 2014). The same authors showed that the net charge of the proteins was reduced when increasing the ionic strength either by adding salts or shifting the pH toward the pI. This

resulted in a gel composed of a random association of particulate aggregates ("particulate gels"). The concentration of ions can alter the net charge on the protein molecules and, hence, the types of interactions and aggregates that are involved in the formation of the overall gel network. Havea, Singh, and Creamer (2002) found that the differences in the level and types of salt between different commercial WPCs affected the aggregation of the proteins during heat-induced gelation.

Caseinomacropeptide or glycomacropeptide, the C-terminal (f 106-169) end of κ-casein that is released by chymosin during cheese making, is present in most commercial whey protein products obtained from sweet whey. The composition of CMP is variable, depending on the particular whey source (Martin-Diana, Gomez-Guillen, Montero, & Fontecha, 2006). Caseinomacropeptide is the third most abundant peptide fraction found in WPC, accounting for 20 - 25 (w/w) % of the nitrogen fraction in these products (Thomä-Worringer, Sorensen, & Lopez-Findino, 2006). Caseinomacropeptide has two major genetic variants, A and B (Jensen, et al., 2015), and is classified into two major groups with respect to post-translational modifications: the glycosylated and non-glycosylated forms which are evenly distributed in whey. Throughout this paper, CMP is referred to as a mixture of non-glycosylated and glycosylated CMP. The pI of CMP varies from 4.15 for non-glycosylated CMP to 3.15 for glycosylated CMP, due to the presence/absence of sialic acid in the glycan moieties (Kreuss, Strixner, & Kulozik, 2009). The presence of CMP has been found to have a negative effect on the gel strength of heat-induced WPC or WPI gels (Britten & Pouliot, 1996; Heino, Uusi-Rauva, Rantamäki, & Tossavainen, 2007; Lorenzen & Schrader, 2006; Veith & Reynolds, 2004). However, when comparing functional properties of NWPC with the WPC's , the lack of CMP in NWPC is confounded by the lower concentrations of denatured proteins (Britten, Lamothe, & Robitaille, 2008), lower contents of minerals (Evans, Zulewska, Newbold, Drake, & Barbano, 2009, 2010) and lesser amounts of fat (Heino, et al., 2007; Luck, et al., 2013; Marcelo & Rizvi, 2008). Recently, Xianghe, Pan, Peilong, Ismail, and van de Voort (2012) studied the effect of increasing the amount of CMP on WPC gels. The WPC produced from acidified (pH 4.6) skimmed milk was filtered, concentrated, neutralised by diafiltration and ultrafiltration, and spray dried. Gels with a fixed protein concentration of 12 % were made from neutralised WPC and a

CMP concentrate using CMP:TP ratios of 0, 5, 15, 25, and 100. They found that the gel strength decreased as the CMP:WP ratio increased. Even when CMP did not replace any of the whey proteins but was added to a WPC solution containing 12 % protein, the gel strength was lost at a similar rate, indicating that the CMP has an important contribution in diminishing the strength of the gels network. However, Xianghe et al. (2012) only studied heat-induced gelation at neutral conditions and not at a low pH.

The objective of this study was to investigate how increasing the CMP:TP ratio from 0 - 33 % influenced the microstructure, solubility and rheology of heat-induced WPC gels at pH 4.0, 5.5 and 7.0.

2. Materials and methods

2.1. Experimental design

Four different CMP:TP ratios were tested at three different pH values to investigate the influence of the CMP on the functional properties of the WPC. The mixtures of the NWPC and a commercial CMP concentrate contained 0 % (NW0), 9 % (NW9), 21 % (NW21), and 33 % (NW33) CMP and were tested at pH 4.0, 5.5, and 7.0. A commercial WPC (WPC80) made from the whey of Dutch type cheese containing 31 % CMP was used as a reference for the NWPC-CMP mixture with the highest concentration of CMP (NW33).

2.2. Protein concentrates

A commercial CMP concentrate powder (Lacprodan® C-GMP 10, Arla Foods, Aarhus, Denmark) with 36 % TP was used to adjust the NWPC dispersions to 0 - 33 % CMP:TP. In the CMP powder, 85% of the TP was CMP and the powder contained 1200 mg kg^{-1} calcium. The NWPC powder was produced from an unpasteurised skimmed milk MF permeate as described by Svanborg, Johansen, Abrahamsen, and Skeie (2014). The MF permeate was further concentrated and diafiltered by UF at 50 °C in a pilot

plant unit with a 25 kDa cut-off membrane (GR60PP-6338/48, Alfa Laval, Nakskov, Denmark), as previously described by Svanborg, Johansen, Abrahamsen, and Skeie (2015). The UF concentrates from two NWPC products were stored at -20 °C until they were freeze-dried. The frozen UF/DF concentrate was then brought to 4 °C overnight before batch-wise freeze-drying. The commercial WPC80 (TINE Verdal, Oslo, Norway) contained 69 % TP, of which 31 % was CMP, and served as a standard for comparisons with the NWPC-CMP containing 33 % CMP in the TP (NW33).

2.3. Chemical analysis

The total solids were analysed according to IDF 21B:1987 (IDF, 1987). The total nitrogen (TN), casein nitrogen (CN), non-casein nitrogen (NCN), and non-protein nitrogen (NPN) levels were analysed according to IDF 20-1:2001 (IDF, 2001c), IDF 20-4:2001 (IDF, 2001b), IDF 20-5:2001(IDF, 2001a), and IDF 29-2:2004 (IDF, 2004), respectively. Lactose were analysed using HPLC as described by Moe, Porcellato, and Skeie (2013). The analysis of the fat contents of the powders was performed according to IDF 124-3:2005 (IDF, 2005). The minerals in the milk fractions and powders were analysed using the method described by de La Fuente, Carazo, and Juárez (1997). In short, the samples were digested with 65% concentrated HNO_3 at 250 °C in a Milestone Ultra wave UltraClave III (Milestone, Sorisole, Italy) and diluted to 10 % HNO_3 before the mineral composition was determined by Inductive Coupled Plasma Optical Emission Spectrometry (Perkin Elmer Optima 5300 DV, Perkin Elmer, Shelton, CT, USA). The protein profile, including the CMP of the WPCs and the cheese making fractions, were analysed using reverse phase HPLC (RP-HPLC), based on the methods described by Hinz, Huppertz, and Kelly (2012) and Visser, Slangen, and Rollema (1991). The data were analysed with the Chromeleon software version 7.1 (ThermoFisher Scientific).

The amount of each protein was determined by dividing the corresponding peak area (obtained by RP-HPLC) for that particular protein by the sum of all the peak areas for the predominant peaks (CMP, α_{S1}-casein, β-casein, α-lactalbumin and β-lactoglobulin). α_{S2}- and κ-casein were not detected by this method. Finally, the % of each protein was converted to a concentration (g L^{-1}) using equation (1):

141 $$(1)\ Protein\ x\ (g\ L^{-1}) = \frac{\%\ protein\ X}{100} \times protein\ content\ (g\ L^{-1})$$

142 LC-UV-MS (high-performance liquid chromatography (LC) coupled to diode array UV detection and
143 mass spectrometry (MS)) was performed to evaluate the RP-HPLC peaks for CMP at retention times
144 of 6.9 and 6.7 min for CMP A in the CMP concentrate and the NWPC, respectively, and 8.6 min for
145 CMP B. Prior to the LC-UV-MS analysis, the WPC, NWPC and CMP samples were dissolved in a
146 reducing buffer containing Bis-Tris, urea, trisodium citrate and dithiothreitol (DTT) at pH 7.0. The
147 sample was stirred at room temperature for 60 min, after which it was diluted further by the addition of
148 4 volumes of a urea solution adjusted to pH 2.5. The samples were filtered through a 0.22-µm filter
149 before being applied to the LC-UV-MS column (Phenomenex, Utrecht, The Netherlands). The MS was
150 comprised of a triple quadrupole equipped with an electro spray ionisation (ESI) source, and the
151 analyses were performed in the positive detection mode. The MS experiments were performed by
152 scanning the parent ions of the proteins in the range from 500 – 1500 m/z. The data were analysed with
153 Xcalibur software version 2.2 (ThermoFisher). The semi-quantitative analysis was performed using
154 standards for the caseins and whey-proteins.

155

156 *2.4. Rheological analysis*

157 Samples containing 12 % protein consisting of NWPC powder, CMP concentrate powder, and MilliQ
158 water were mixed to obtain a CMP:TP ratio of 0 - 33 %. The solutions were stirred for 30 min, left at 4
159 °C overnight and then adjusted to a pH of 4.0, 5.5 or 7.0 shortly before analysis using a Physica
160 MCR301 rheometer (Anton Paar GmbH, Graz, Austria) with a smooth parallel plate-plate system
161 (PP50, Anton Paar). The temperature of the bottom plate was controlled with a Peltier system and
162 brought to 4 °C before an approx. 3 mL sample was added and the top plate was lowered to the
163 measurement position. A guard ring was used to prevent the sample from evaporating and sunflower
164 oil was added to the exposed surfaces for the same reason. The gap between the plates was
165 approximately 1 mm, and, due to swelling, the height of the probe was adjusted by applying a normal
166 force of 0.1 N, allowing the gap to change during the measurement period. Each sample was heated

from 4 to 90 °C at a rate of 17 °C min^{-1}, held at 90 °C for 10 min, and cooled to 20 °C at the same rate as for the temperature increase. The gels were then incubated at 20 °C for 10 min prior to the amplitude sweep. The amplitude sweeps used an angular frequency of 10 rad s^{-1}, with the strain increasing from 0.01 % to 100 %. A normal force of 0.1 N was applied to the sample, and the temperature was kept constant at 20 °C. G' and G'' (storage modulus and loss modulus, respectively) were continuously recorded at a strain level of 0.02 % and an angular frequency of 10 rad s^{-1}. The data were collected from a strain of 0.1 % at 20 °C in the linear viscoelastic region (LVR) prior to the amplitude sweep and at the amplitude sweep where the LVR ended (G' < 3% of the plateau value of $\tau_{LVR,3\%}$, $\gamma_{LVR,3\%}$ and $G'_{LVR,\,3\%}$, respectively) (Rheoplus/32 V3.40 software, Anton Paar GmbH) as measures of the final gel strength (resistance to stress, $\tau_{LVR,3\%}$), strain ($\gamma_{LVR,3\%}$) and viscoelastic properties ($G'_{LVR,\,3\%}$). The protein solutions were stored on ice, and at least three replicates per sample were analysed.

2.5. Sample preparation and scanning electron microscopy

Dispersions of the NWPC, the CMP concentrate and MilliQ water containing 0 - 33 % CMP with a total of 12 % TP were rehydrated overnight and the pH was adjusted immediately prior heat-induced gelation. Thirty mL samples of aqueous WPC solutions in glass beakers were incubated at 90 °C for 20 min in a water bath and cooled rapidly to 4 °C prior to preparation for the microstructure study. The gels were stored and stabilised at 4 °C for at least 3 hours before they were cut in cubes of approx. 0.5 × 0.5 × 0.5 cm and prepared as described by Penna, Subbarao, and Barbosa-Cánovas (2007), with the exception that osmium tetroxide was omitted during fixation. The dry sections were fractured and the fragments were mounted on aluminium stubs and coated with gold-palladium under a vacuum using a Polaron SC 7640 sputter device (Quorum Technologies Ltd, Laughton, UK). The microstructures of the whey protein gels with CMP:TP ratios of 0 - 33 % and of WPC80 at pH 4.0 and 7.0 were examined using a Zeiss EVO-50–EP (Carl Zeiss SMT Ltd., Cambridge, UK) scanning electron microscope (SEM) operating at 20 kV.

2.6. Differential scanning calorimetry

The influence of the CMP:TP ratio on the thermal transition of the protein solutions that had been adjusted to pH 7.0 was studied using a differential scanning calorimeter (DSC) (Mettler Toledo DSC823e/500/335, Mettler Toledo, Greifensee, Switzerland) with Stare software version 8 (Mettler Toledo). The instrument was calibrated against Indium prior to use. Approx. 25 µL of the 12 % (wt/wt) protein solutions were accurately weighed into 40 µL standard aluminium pans (Mettler Toledo) and sealed with a perforated aluminium lid (ME 511408232, Mettler Toledo). The DSC scanning rate was 10 °C min^{-1} from 20 to 90 °C and the sample was incubated at 90 °C for two min. An empty pan of equal weight was used as a reference. Each sample solution was measured five times. The temperature at the maximum endothermic peak height (denaturation) and the onset and end-set temperatures were recorded.

2.7. Solubility and particle distribution

Solubility was investigated by measuring the nitrogen solubility of 1 % protein dispersions with 0 - 33 % CMP that had been adjusted to pH values of 4.0, 4.6, 5.5, and 7.0 using 1.0 M NaOH and 1.0 or 0.1 M HCl. The samples were centrifuged at 20 000 *g* for 25 min at 4 °C. The supernatants were filtered through grade 1 (11 µm) filter paper. The amount of soluble nitrogen in the supernatant after centrifugation was calculated as percentage of the TN content in the corresponding sample prior to centrifugation. The total nitrogen level was determined by the Kjeldahl method, IDF 20-1:2001. The particle size distribution in the NWPC and WPC80 dry powder samples was measured using a Malvern Master sizer 3000 (Malvern Instruments Ltd., Malvern, Great Britain), with an Aero S Optic cell and Aero S dry disperser and Mastersizer software version 2.20 (Malvern Instruments Ltd., Great Britain). Particle distribution was expressed as D_{V10} (the value of the volume diameter where 10 % of the powder particles have powder diameters below this value), D_{V50} (the volume median diameter), and D_{v90} (the value of volume diameter where 90 % of the powder particles have powder diameters below this value). The analysis was repeated eight times for each powder.

2.7. Data analysis

To test the effect of the whey source on the composition and the functional properties of commercial WPC80 and NW33 (which had a similar CMP:TP ratio) one-way analysis of variance (ANOVA) was performed using the whey source as the variable. Multi-way ANOVA was performed to test the effect of (1) increasing the CMP:TP ratio, (2) the pH, and (3) the CMP:TP ratio × pH interaction on the functional properties of NWPC samples with 0 – 33 % CMP. No proper gels were formed at pH 5.5; therefore, the results obtained at pH 5.5 were omitted from the data prior to further statistical analysis. The differences between the four CMP:TP ratios were analysed by Tukey's pairwise comparison (95 % confidence). All statistical analyses were performed using R statistics (version 3.1.1, 2014-07-10, R Development Core Team; http://www.r-project.org).

3. Results

Table 1 shows the chemical composition of the NWPC and commercial WPC80. The most obvious difference between the powders was the much higher TP content per 100 g^{-1} in the NWPC compared with the WPC80 ($P < 0.001$), and the presence of CMP in WPC80 compared with its absence in the NWPC. When adjusted for the difference in TP, NWPC contained less TN, whey protein and NPN per 100 g^{-1} TP than WPC80 ($P < 0.001$). The mineral concentrations were lower in the NWPC ($P < 0.01$), despite the higher ($P < 0.001$) content of casein compared with WPC80. Despite that NWPC contained less whey protein per 100 g^{-1} TP; the α-LA:TP ratio was higher ($P < 0.05$) in the NWPC than in WPC80, and the β-LG:TP ratios were similarly distributed in the two powders. The semi-quantitative RP-HPLC analysis detected peaks at the CMP retention time area in both the CMP concentrate powder and WPC80. These peaks had a similar profile (fig. 1), although the peak height was far lower in the WPC80 sample. Fig. 1 also shows that the NWPC has a peak in the area of non-glycosylated CMP (genetic variant A) at a retention time of 6.7 min. However, the profile of the peak did not match the

corresponding peak-profile of the CMP concentrate or WPC80 (fig. 1). Therefore, the compounds in the NWPC samples that eluted in the peak at the CMP retention time were investigated further by LC-UV-MS, and this analysis showed that the profile of the NWPC peak (fig. 2b) did not match the profile of the non-glycosylated CMP genetic variant A (CMP A) observed in the commercial CMP concentrate (fig. 2a). Due to the mismatched profiles of the peaks in the area of CMP A in the NWPC sample by LC-UV-MS, the content of the peaks were regarded to be peptides other than CMP.

The two powders had a similar particle size distribution (Table 2), despite the different drying methods used. The WPC80 powder was manufactured using an industrial scale spray drier that included an agglomeration step, whereas the NWPC powder was freeze dried at the pilot scale, with manual milling after drying. Denaturation during heat treatment is crucial for the gelling properties of the dispersions. The solubility of the two WPCs was equally high (approx. 85 %) at pH 4.6. The denaturation of whey proteins can also be measured by DSC because the enthalpy changes associated with protein unfolding are proportional to the extent of denaturation (de Wit, 1990). One well-defined endothermic peak was observed for the NWPC and WPC solutions (Table 3). The onset, endset and peak temperature of the NWPC solutions was influenced ($P < 0.001$) by the CMP:TP ratio, but was not affected by the whey source when comparing WPC80 (31 % CMP) and the NWPC sample with 33 % CMP (NW33). The CMP:TP ratio influenced the onset and end-set temperatures ($P < 0.001$). The sample with no CMP added, NW0, had the lowest peak and end-set temperature; however, NW33 had the lowest onset temperature. Compared with WPC80, NW33 had a higher endset temperature ($P < 0.001$). The transition enthalpy was not influenced by the whey source or the CMP:TP ratio, and showed a somewhat high variation between parallels compared with the other DSC data. WPC80 had the lowest transition enthalpy compared with the samples made from the NWPC and NWPC-CMP concentrate mixtures.

The rheological analysis revealed a significant effect of the CMP:TP ratio on important gel properties, including gel strain ($\gamma_{LVR,3\%}$) ($P < 0.001$), gel strength ($\tau_{LVR,3\%}$) ($P < 0.001$) and $G'_{LVR,\ 3\%}$ ($P <$

0.05) (table 4), while the pH had a significant impact on the gel strength ($P < 0.05$) only. In addition, gel strength was influenced by an effect of the interaction between the pH and the CMP:TP: NW0 had a gel strength at pH 4.0 that was higher ($P < 0.05$) than any of the other samples. In general, all gels had a low gel strain, regardless of the pH and CMP:TP ratio, however, NW0 gels were more resistant to strain than the other gels. The sample containing the highest CMP concentration, NW33, had a more than 10 times higher G' value ($P < 0.05$) than NW21 (NWPC with 21 % CMP of the TP).

The viscosity of the WPC dispersions was increased at pH 5.5; however, they did not form proper gels. Nevertheless, by evaluating the NWPC gels at 20 °C (in the LVR prior to the amplitude sweep), the results showed that the heat-induced protein dispersions at pH 5.5 had a significantly higher phase angle (δ) (10.5°) and damping factor (0.186) than the gels formed at pH 7.0, but was not different ($P > 0.05$) from the gels formed at pH 4.0. Due to the lack of gelling properties at pH 5.5, the gels formed at pH 5.5 were excluded from the further statistical analysis, and the results from pH 5.5 are not shown in Table 4. When comparing the gels formed at pH 4.0 and 7.0, it was shown that the pH did not influence the phase angle and damping factor, but significant interaction effects were found between the CMP:TP ratio and pH (Table 4). However, an interaction effect of CMP:TP × pH ($P < 0.05$) was found and showed that the gels formed from NW33 at pH 7.0 had the lowest damping factor and phase angle (0.13 and 7.4, respectively) and those of NW9 at pH 7.0 had the highest damping factor and phase angle (0.18 and 10.6, respectively). The gels with all other CMP:TP ratios and pH combinations were insignificantly different.

Whey source influenced the gel properties: WPC80 gels obtained a higher damping factor ($P < 0.05$) and phase angle ($P < 0.05$) than the NWPC gels containing 33 % CMP. The WPC80 gels were resistant to higher strain than the NW33 gels ($P < 0.05$), but whey source did not influence the gel strength. The solubility of the 1% NW0 - NW33 samples was influenced by pH, but not by the CMP:TP ratio (fig. 3a), with a higher mean solubility at pH 7.0 ($P < 0.05$) compared with the mean solubility at pH 4.0 and 5.5, which had an equally low solubility. Commercial WPC80 was found to have a lower solubility ($P < 0.05$) compared with samples with a similar CMP:TP ratio (NW33) (fig. 3b). The solubility of the

298 WPC80 samples at pH 4.0 was significantly lower than the WPC80 samples at pH 5.5 and 7.0, and
299 lower than the NWPC samples at any pH.

300

301 The gels studied by SEM were made in a similar manner as the gels formed in the rheometer, except
302 that they had a longer heating time (20 min instead of 10 min) at pH 4.0 and 7.0. After heating, samples
303 adjusted to pH 5.5 were pourable and some of the samples showed extensive precipitation. Because of
304 this, only the gels formed at pH 4.0 and 7.0 were prepared for SEM analysis. The NW0 gel at pH 7.0
305 was almost translucent compared with the opaque gels made from WPC80 and NWPC with 9 - 33 %
306 CMP. The gels formed at pH 7.0 obtained stronger syneresis after three days at 4 °C compared with the
307 pH 4.0 gels. At pH 4.0, the NW0 gel became slightly more opaque, but none of the NWPC gels became
308 as white as the WPC80 gel. All gels formed at pH 4.0 were generally very stiff and brittle, and cracked
309 during the 4 °C storage prior to fixation. The SEM micrographs (fig. 4) show an obvious effect of the
310 increasing CMP:TP ratio in the samples, as the structure became more open with larger pores at pH 7.0.
311 This tendency was more pronounced for the gels at pH 7.0 than those at pH 4.0 because the structures
312 of all samples at pH 4.0 were dense and less porous. The NW0 gels had a compact and somewhat less
313 coarse structure at both pH levels compared with the gels with a higher CMP:TP ratio. The differences
314 in microstructure between NW0 and NW33 gels were more pronounced compared with the differences
315 between the NW9 and NW21 gels, which appeared more similar. NW9 was comparable to the reference
316 (WPC80) gel in structure (fig. 4 and 5).

317

318 The pH influenced the microstructure of the NWPC and WPC gels, which was reflected in the higher
319 gel strength at pH 4.0. The network structures of the gels formed at pH 7.0 were more swollen and had
320 large cauliflower-like aggregates compared with the denser and less porous networks obtained in the
321 gels formed at pH 4.0. The differences between pH 4.0 and 7.0 were not as pronounced for the NW0
322 gels compared with the differences obtained for the gels with higher CMP concentrations.

323

4. Discussion

WPC80 had a somewhat different chemical composition than NWPC that might have influenced the properties of the WPC gels in this study. Some of the compositional differences between the whey sources were the mineral and fat contents. The calcium content in the WPC80 samples was almost twice as high as found in NWPC. The initial mineral content in WPC is critical to gel network structure because ionic calcium is known to contribute to the aggregation and gelation of the whey protein products (Riou, Havea, McCarthy, Watkinson, & Singh, 2011). However, in this study, the dissimilarity in microstructure of WPC80 and NW33 gels, that had a similar CMP content, could be related to the higher minerals content in WPC80. The most obvious difference in the chemical composition of the NWPC and commercial WPC80 is the difference in TP. When adjusting for this difference, the NWPC had a higher casein concentration and a lower NPN concentration, as expected. The CMP:TP ratio in WPC80 was 31 %, which was a little higher than had been reported as typical values for cheese whey (Thomä-Worringer, et al., 2006). At the point when CMP normally elutes, the chromatographic profile of the eluted compounds in the samples of the NWPC and CMP concentrate differed. The co-eluting peptides that caused a false positive CMP identification may origin from proteolytic activity during NWPC processing, such as activity from heat-resistant proteolytic enzymes from psychrotrophic bacteria. For instance, the composition of pseudo-CMP, which is caused by *Pseudomonas* proteases, differs from CMP in only one amino acid. Moreover, RP-HPLC methods are unable to distinguish between these two varieties of CMP (Campos Motta, et al., 2014). The indigenous CMP found in raw milk has been reported to be approximately ten-fold lower than the amount of CMP in cheese whey (Furlanetti & Prata, 2003). Therefore, these ingenious CMPs were not expected to be present in detectable quantities in the NWPC.

For various applications, the solubility is the most important functional property of WPC and is known to have a strong correlation to gelling and foaming (Zayas, 1997b). Solubility at pH 4.6 and the transition enthalpy are closely related to protein denaturation (de Wit & Kessel, 1996; Li-Chan, 1983). However, neither the solubility at pH 4.6 nor the transition enthalpy differed significantly between the

two WPCs studied, although the transition enthalpy tended to be higher in the NWPC-CMP concentrate mixtures compared with WPC80. The somewhat low solubility of NWPC at pH 4.6 is likely related to the precipitated casein, (which accounted for more than 6 % of the proteins in these samples) rather than denatured whey proteins. The whey protein concentrate made from spay-dried industrially obtained cheese whey normally contains 20 - 50 % denatured whey proteins (Kohnhorst & Mangino, 1985), whereas the NWPC usually has a very low level of denatured proteins. Only a modest increase in the onset temperature was observed as the CMP content increased up to 21 %; however, NW33 had the lowest onset temperature of all of the tested samples. Additionally, NW33 also had the highest end-set temperature. The broader transition boundaries of these samples compared with the samples with a lower CMP content may indicate that the protein aggregation step was somewhat interrupted. In this study the peak temperature at pH 7.0 was slightly increased with increasing CMP:TP ratio, in contrast to Martinez, Carrera Sanchez, Rodriguez Patino, and Pilosof (2009) who found an decrease in peak temperature of CMP - β-LG mixes containing 25 - 75 % CMP compared to pure β-LG samples. No difference in the transition enthalpy (ΔH) with respect to the CMP concentration or the whey origin was detected in this sudy. However, Croguennec, et al. (2014) found that increasing the CMP:β-LG ratio increased the rate of β-LG unfolding, regardless of the pH and heating temperature. CMP is known not to have a defined transition temperature (Xianghe, et al., 2012) because it lacks tertiary structure (Kreuss, et al., 2009). Xianghe, et al. (2012) found that WPCs containing as much as 25 % CMP did not have the typical denaturation transition observed for β-LG and α-LA, and at this concentration, the solution behaved more like a CMP concentrate.

The gelling point defines the point at which a fluid is converted to a solid. Reliable gelling points, the G'-G''-cross over measurements, were not obtained from the rheological analysis, due to a rapid temperature increase that led to a steep G' curve. As shown in the DSC analysis, the tendency of interruption during the gelling of samples with a high CMP:TP ratio was obvious, as the increase in G' and G'' were slower. The G' curve was not smooth during the temperature rising from 4 to 90 °C (data not shown), as also was reported by Martinez, Farias, and Pilosof (2010) when heating CMP - β-LG mixes with a ratios of 75:25. In our study, the broader transition temperature range for the samples with

high CMP:TP ratios observed by DSC, and the slower increase in G' and G'' during heating, indicates that the CMP interrupted the heat-induced gelation of WPCs. DSC studies of β-LG-CMP mixes showed that the formation of β-LG-CMP aggregates was driven by electrostatic interactions and/ or by hydrogen bonding (Martinez, et al., 2009)

The NW0 gel was more than three times stronger (higher $\tau_{LVR,3\%}$) at pH 4 compared with any other gel, regardless of pH. The negative influence of the CMP on the gel strength is in accord with several other studies (Heino, et al., 2007; Rantamaki, et al., 2000; Veith & Reynolds, 2004). Most comparisons of the gel strength between the native whey protein ingredients and WPCs containing CMP have been performed at neutral pH. The shape and size of the aggregates formed from β-LG and CMP is crucial to the properties of the gel network. DSC studies of β-LG - CMP mixes at neutral pH showed that the formation of β-LG - CMP aggregates was driven by electrostatic interactions and/ or by hydrogen bonding (Martinez, et al., 2009). At neutral pH, both β-LG and CMP are negatively charged, which may cause a weaker interaction between the CMP and unfolded whey proteins. Croguennec, et al. (2014) suggested that CMP possesses a similar chaperone-like behaviour as some caseins (Guyomarc'h, Nono, Nicolai, & Durand, 2009; O'Kennedy & Mounsey, 2006), thus stabilising the β-LG at neutral conditions. Croguennec, et al. (2014) suggested that the aggregates in the β-LG - CMP-systems consisted of β-LG surrounded by CMP monomers. The molecular interactions between β-LG and CMP would likely be stronger at pH 4.0 than at pH 7.0, due to the differences in electrical charge. Below the pI (pH 5.1), the net charge of β-LG is positive and that of CMP is negative. In our study, the effect of the CMP:TP ratio on the microstructure seemed to be pH-dependent, as the CMP:TP ratio had a different effect on the gel structure at pH 4.0 and 7.0. The influence of CMP:TP ratio seemed to be pH-dependent with respect to gel strength, as NW0 gels had a far higher gel strength at pH 4.0 than at pH 7.0, whereas the gels containing CMP had an equally low gel strength, regardless of pH. The equally reduced gel strength for NW9-33 may indicate that the presence, rather than the actual level of CMP, is crucial for this gel property. However, the increase in the CMP:TP ratio seemed to influence the microstructure more than the rheological parameters, as the structure became dramatically more porous

405 and swollen as the CMP:TP ratio increased. A large difference in structural appearance was observed
406 between NW0 and the other samples at pH 7.0. The network became more swollen and the aggregates
407 and pores larger as the CMP:TP ratio increased at pH 7.0. These findings is consistent with the findings
408 of Xianghe, et al. (2012). A network with large pores would have a lower water holding capacity than
409 gels with a denser network. The water holding capacity of the gels was not measured in this study, but
410 based on visual observations, the gels formed at pH 7.0 had more syneresis compared with the gels
411 formed at pH 4.0. The effect of increasing CMP:TP ratio was less pronounced in the microstructure of
412 gels formed at pH 4.0, most probably due to the difference in β-LG and CMP charge. The gels formed
413 at pH 4.0 appeared more rigid and cracked more easily compared with the gels formed at pH 7.0. These
414 visual observations were in accord with the rheological results, as the NW0 gels formed at pH 4.0 were
415 significantly stronger and had a higher G' value than the pH 7.0 gels. However, this is in contradiction
416 to the findings of Verheul and Roefs (1998), who reported that whey protein gels at pH > pI are stronger
417 and more elastic, whereas gels formed at pH < pI are weak and brittle.

418

419 5. Conclusions

420 The addition of 9 – 33 % CMP to NWPC did not influence the solubility of the NWPC; however, the
421 solubility was influenced by pH and was higher at pH 7.0 than at pH 4.0 and 5.5. The effect of the
422 CMP:TP ratio on the microstructure and gel strength of the heat-induced gels seemed to be pH-
423 dependent: At pH 7.0, the NWPC gels without CMP had a less porous and more swollen network, with
424 an increasing aggregates size and as the CMP % increased. The gels without CMP had a far higher gel
425 strength at pH 4.0 than at pH 7.0, whereas gels containing CMP had an equally low gel strength,
426 unrelatedly of pH. However, the effect of the CMP:TP ratio was not as pronounced at pH 4.0, where all
427 samples had a similarly dense structure. The gel strain was far higher in the samples that did not contain
428 CMP, and gels with 33 % CMP had higher G' values than the gels with less CMP.

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Tables and figures

Table 1. Composition of native whey protein concentrate (NWPC) powder made from microfiltration permeate of skimmed milk and commercial whey protein concentrate (WPC80) powder. Mean values from two batches of NWC and from one batch of WPC80 are presented. Asterisks in the NWPC row indicate significant difference between the two whey protein concentrates (* $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$).

	NWPC	**WPC80**
Component per 100 g^{-1} sample		
Total solids (g)	95.9	97.37
True protein (g)	88.0***	68.94
Components per 100 g^{-1} true protein		
Total nitrogen(g)	16.10***	18.03
Non-protein nitrogen (g)	0.42***	2.36
Non-casein nitrogen (g)	15.12***	17.56
Casein[a] (g)	6.24***	2.99
Whey protein (g)	93.76**	97.01
β-lactoglobulin variant A (g)	21.6	21.7
β-lactoglobulin variant B (g)	41.8	29.5
α-lactalbumin (g)	21.3*	15.1
Caseinomacropeptide (g)	0[b]	31.0
α_{S1}-casein (g)	1.2	1.2
β-casein (g)	1.9	1.8
Lactose (g)	0.0	0.06
Fat (g)	0.00**	0.07
Sodium (mg)	192	2.90
Magnesium (mg)	65*	115
Phosphorous (mg)	182***	638
Potassium (mg)	366**	1124
Calcium (mg)	486*	885
Iron (mg)	15	11
Zink (mg)	7	12
Cupper (mg)	7	2
Total minerals (mg)	1291**	3153

[a] Casein concentration calculated as true protein minus whey protein. [b] Compounds eluting in the CMP genetic variant A peak area accounted for 12 % of the total peak area; using mass spectrometry, it was confirmed that these compounds were not CMP (Figure 2).

Table 2. Particle size distribution and nitrogen solubility of native whey protein concentrate (NWPC) powder made from microfiltration permeate of skimmed milk and commercial whey protein concentrate (WPC80) powder. Mean values with standard deviation (in brackets) from two batches of NWC and from one batch of WPC80 are presented. Asterisks in the NWPC row indicate significant difference between the two whey protein concentrates from one-way ANOVA using whey source as factor (* $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$).

Measurement	WPC80	NWPC
D_{v10} (μm)	26 (0)*	11 (4)
D_{v50} (μm)	82 (1)	46 (16)
D_{v90} (μm)	185 (21)	172 (36)
Solubility at pH 4.6	84.8 (2.6)	86.5 (8)

Abbreviations: D_{V10} is the value of volume diameter such that 10 % of the powder particles have powder diameters below this value, D_{V50} is the median volume diameter, and D_{v90} is the value of volume diameter such that 90 % of the powder particles have powder diameters below this value.

Table 3. Effect of caseinomacropeptide (CMP) content of true protein (TP), CMP:TP ratio and whey source on onset and end of transition temperature, transition enthalpy (ΔH) and peak temperature measured by differential scanning calorimetry (DSC) of 12 % native whey protein dispersions with 0 % (NW0), 9 % (NWC9), 21 % (NW21), 33 % (NW33) CMP of TP at pH 7 and WPC80 (with 31 % CMP of TP). Mean values with standard deviation (in brackets) from five replicates and *p*-values from one-way ANOVA for the effect of CMP:TP ratio and whey source (NW33 vs WPC80) (* $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$).

	Sample id.										*p*-values	
	NW0		NW9		NW21		NW33		WPC80		Effect of CMP % of TP	Effect of whey source
Onset (°C)	58.24	(0.21)	63.88	(2.13)	65.85	(1.38)	48.04	(6.46)	55.14	(3.28)	< 0.001	< 0.01
Peak (°C)	70.34	(0.52)	72.97	(0.23)	73.04	(0.19)	71.87	(0.56)	73.28	(0.48)	< 0.001	n.s.
End (°C)	78.81	(1.67)	80.53	(0.82)	81.25	(0.86)	86.57	(1.08)	84.28	(1.43)	< 0.001	< 0.001
ΔH (J g^{-1}_{sample})	-0.76	(0.25)	-0.82	(0.25)	-0.77	(0.31)	-0.79	(0.03)	-0.55	(0.13)	n.s.	n.s.

Abbreviations: n.s. = not significant

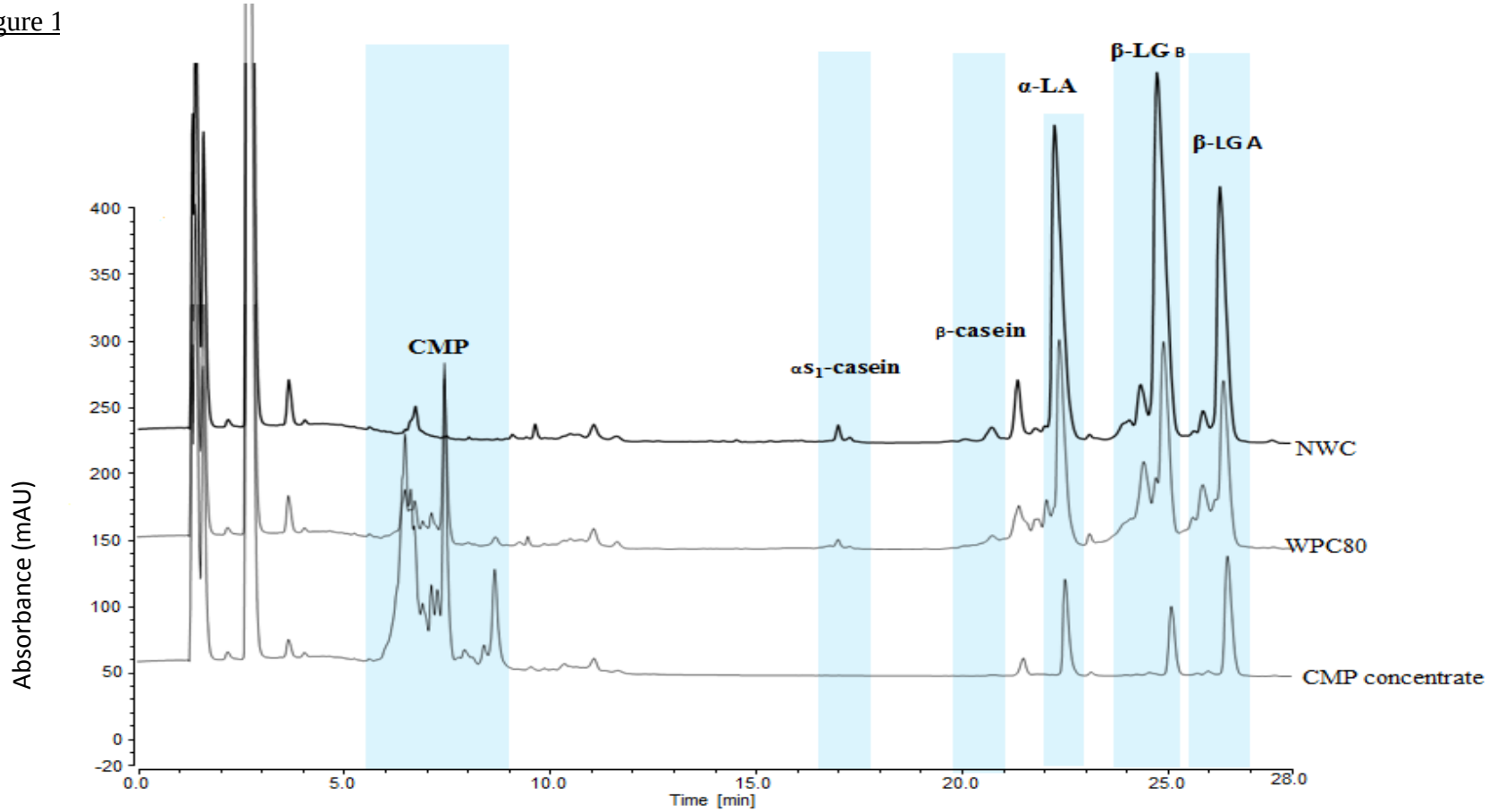
Table 4. Influence of caseinomacropeptide (CMP) content of true protein (TP), CMP:TP ratio, pH and the interaction effect CMP:TP ratio × pH on rheological properties of heat induced whey protein gels containing 0% (NW0); 9 % (NW9); 21 % (NW21); 31 % (WPC80) and 33 % (NW33) CMP at pH 4 and 7 on gel strength ($\tau_{LVR,3\%}$), elastic modulus (G' $_{LVR,3\%}$) and gel strain ($\gamma_{LVR,3\%}$) at 3 % decrease from linear viscoelastic range (LVR) of G', and the damping factor and phase angel (°) in the LVR. p-values from multi-way ANOVA for the effect of CMP:TP ratio, pH, the interaction effect CMP:TP × pH, and the effect of whey source (NW33 vs WPC80) are shown in the last four rows of the table. Small case letters in superscripts of pH 4 values indicate differences between samples with 0 – 33 % CMP by Tukey's pairwise comparison for 95 % level in the column for each response.

Sample id	pH	$\gamma_{LVR,3\%}$ (%)	$\tau_{LVR,3\%}$ (Pa)	G' $_{LVR.3\%}$ (Pa)	Damping factor	Phase angel (°)
NW0	4	4[b]	1 571[b]	44 977[a]	0.17	9.59
	7	2	523	29 003	0.17	9.60
NW9	4	1[a]	210[a]	112 006[a]	0.15	8.79
	7	1	239	20 740	0.18	10.57
NW21	4	0[a]	249[a]	192 038[a]	0.17	9.90
	7	1	162	32 358	0.17	9.73
NW33	4	0[a]	241[a]	166 360[b]	0.17	9.76
	7	0	231	280 100	0.13	7.43
WPC80	4	1	242	22 787	0.18	10.22
	7	2	187	13 737	0.19	10.48
Effect of CMP:TP ratio[c]		< 0.001	< 0.001	< 0.05	n.s.	n.s.
Effect of pH[c]		n.s.	< 0.05	n.s.	n.s	n.s.
Interaction effect CMP:TP ratio ×pH[c]		n.s.	< 0.005	n.s.	< 0.01	< 0.01
Effect of whey source (NW33 vs WPC80)[d]		< 0.05	n.s.	< 0.05	< 0.05	< 0.05

Abbreviations: n.s = not significant.

[c] p-values, results from multi-way ANOVA of native whey protein concentrate samples with CMP:TP ratio, pH and the interaction CMP:TP ratio × pH as classification factors. [d] p-values, results from multi-way ANOVA using whey source as classification factor when comparing commercial WPC80 (31 % CMP) and NW33.

Figure 1



1. Reversed-phase high-performance liquid chromatography (RP-HPLC) chromatograms of native whey protein concentrate (NWC), reference WPC80 and caseinomacropeptide (CMP) concentrate showing the major proteins found in whey (α-LA = α-lactalbumin, β-LG A/B = β-lactoglobulin var. A/B).

Figure 2

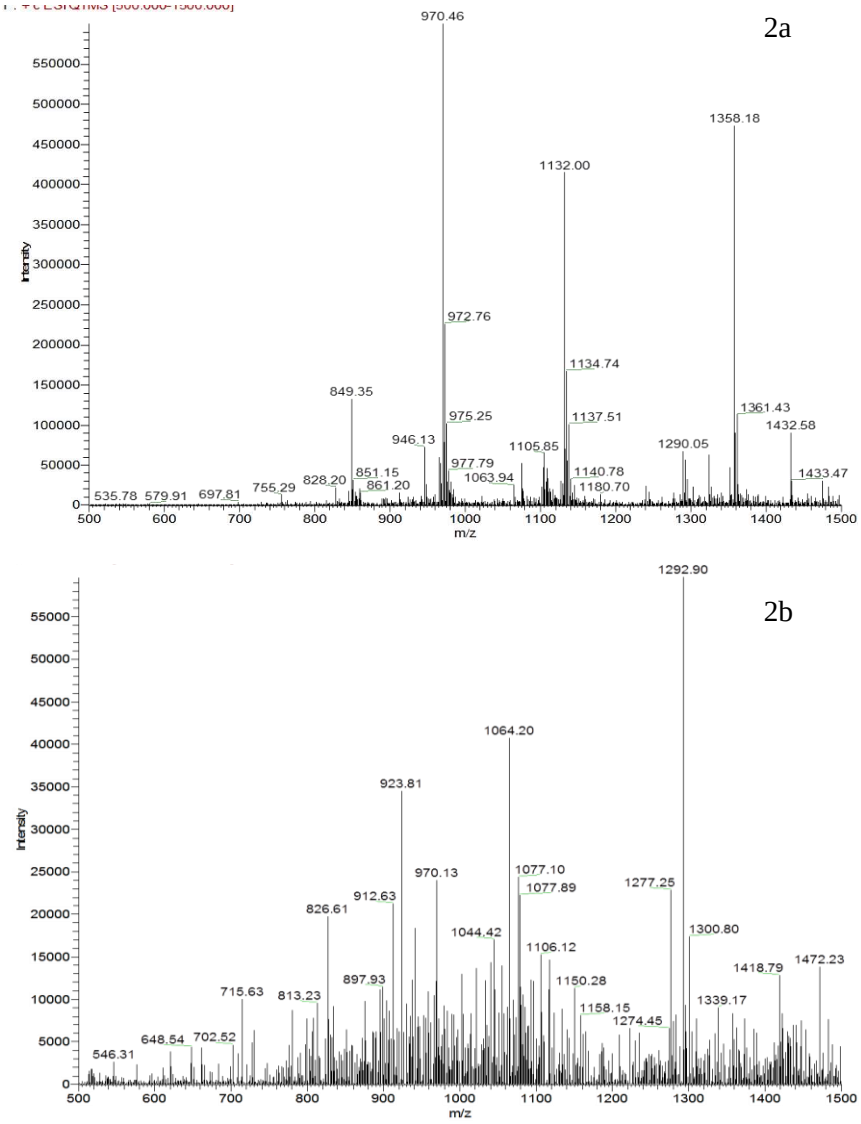


Figure 2. Identification of peaks in the elution range of caseinomacropeptide (CMP) genetic variant A (retention time 6.9 min.) by reversed-phase high-performance liquid chromatography (RP-HPLC) with detection by diode array ultraviolet spectroscopy and mass spectrometry (LC-UV-MS). Representative profiles of peaks obtained from a CMP concentrate (2a) and a native whey protein concentrate (NWPC) (2b).

Figure 3

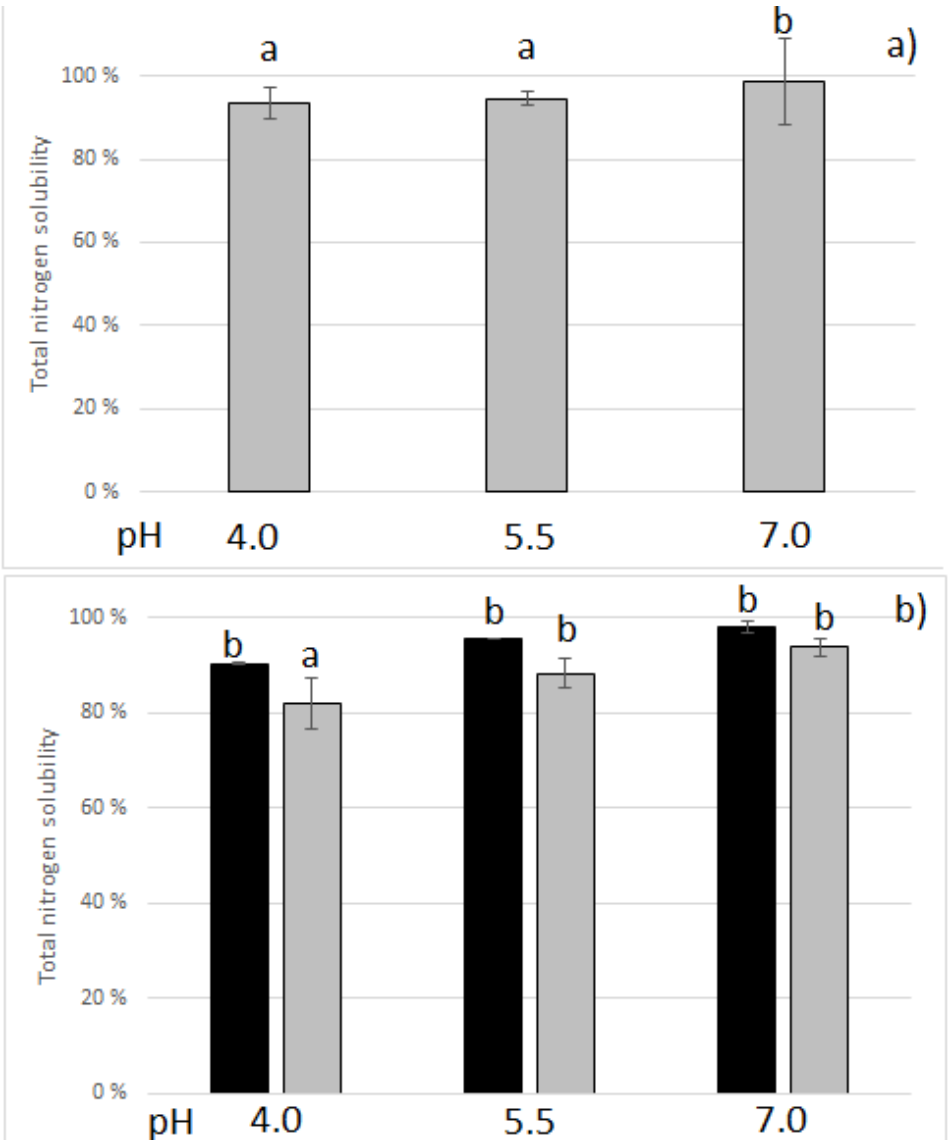


Figure 3. (a) The effect of pH on solubility. Comparison of the average percentage of total nitrogen in the supernatants of native whey protein concentrate (NWPC) samples containing 0-33 % caseinomacropeptide (CMP) and the pH-adjusted sample prior to centrifugation. The letters above the bars indicate a significant effect of pH on solubility. **(b)** The effect of the source of whey on solubility. Comparison of the percentage of total nitrogen in the supernatants of NWPC containing 33 % CMP (NW33, black bar) and WPC80 (31 % CMP, grey bar) at pH levels of 4.0, 5.5 and 7.0, and the pH-adjusted sample prior to centrifugation. The letters above the NW33 and WPC80 bars indicate a significant effect of the source of whey.

Figure 4

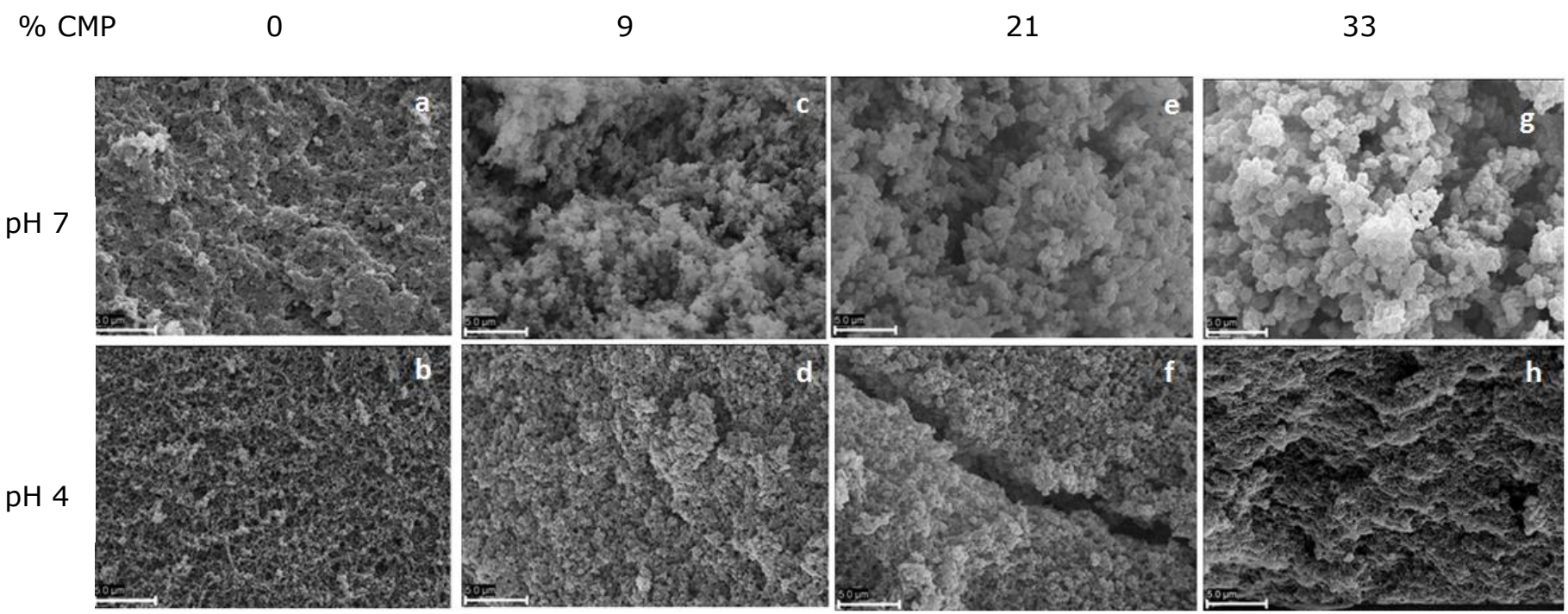


Figure 4. Scanning electron microscopy (SEM) micrographs of heat-set WPC gels made from native whey concentrate with various ratios of caseinomacropeptide to true protein at pH 7.0 [0 % (a); 9 % (c); 21 % (e); 33 % (g)] and at pH 4.0 [0 % (b); 9 % (d); 21 % (f) and 33 % (h)]. Scale bars represent lengths of 5 µm

Figure 5

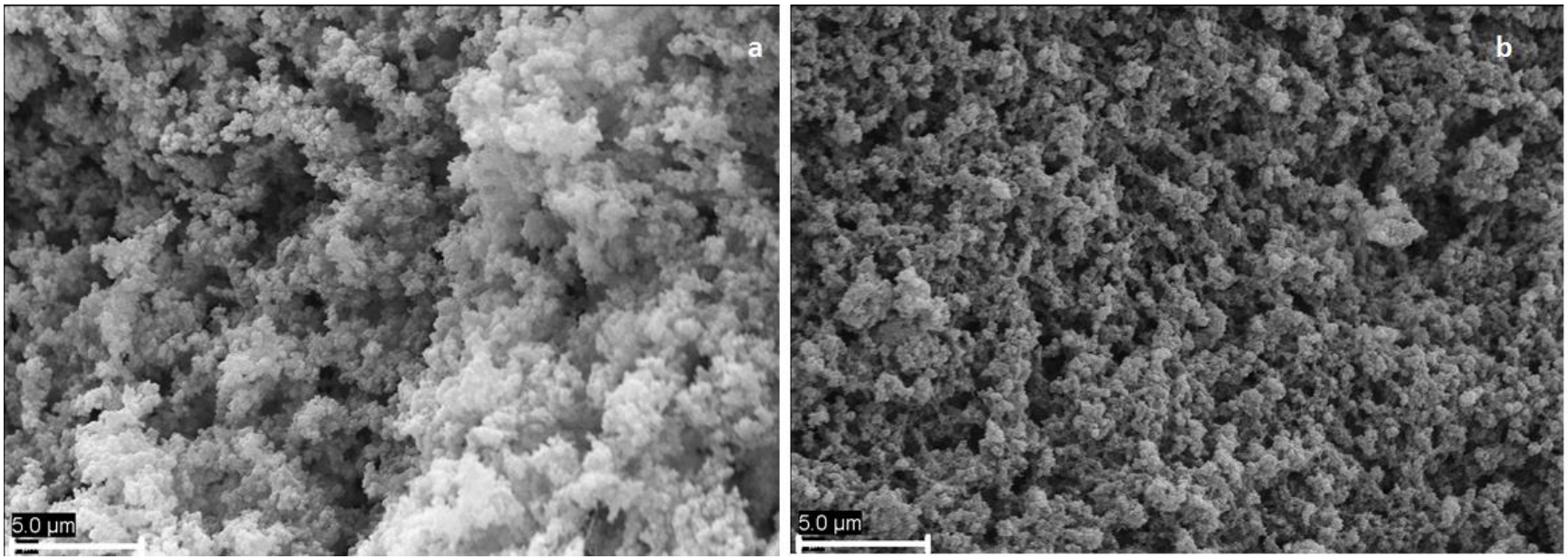


Figure 5. Scanning electron microscopy (SEM) micrographs of heat-set WPC gels made from commercial WPC80 at (a) pH 7.0 and (b) pH 4.0. Scale bars represent lengths of 5 µm.