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1	Suitability of nitrogen fractions determination to assess serum protein separation
2	efficiency from a mass balance perspective during microfiltration of skim milk
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23

24 ABSTRACT

26	Efficient separation of serum from colloidal proteins in bovine milk in their native form can
27	be achieved by microfiltration (MF). This study assessed partitioning of serum proteins (SP)
28	in skim milk by a MF process using 0.1 μ m graded permeability ceramic membranes with
29	two diafiltration (DF) steps, from a mass balance and energy utilisation perspective. A mass
30	balance focused on dry matter and true protein yielded recoveries of 99.5 and 95.3%,
31	respectively. However, an accurate mass balance relative to colloidal and SP contents in the
32	retentate and permeate streams was not achieved, linked to errors surrounding quantification
33	of nitrogen fractions in processed streams using standard methodologies designed for raw
34	milk. Additionally the energy required for each MF/DF step was 13.1, 13.7 and 20.6 kW h
35	kg ⁻¹ of SP removed, respectively, demonstrating the dynamic relationship between SP
36	partition relative to diafiltrant utilisation and its impact on energy consumption.
37	
38	

39 1. Introduction

40

41 The introduction of membrane separation technology to the dairy industry can be 42 traced back to the 1970s for applications such as treatment of acid whey and standardisation 43 of milk protein concentration prior to cheese making to increase yield (Maubois & Mocquot, 44 1975). Dairy filtration processes have evolved over time and are utilised for fractionation and 45 standardisation of whole/skim milk and a variety of whey streams. Of these, microfiltration (MF) as a technology has experienced a rapid growth in the dairy industry in the past two 46 47 decades due to its potential to both retain and partition a wide range of milk components. In 48 particular, fractionation of skim milk via MF has emerged as an innovative process solution 49 for producing whey or serum protein and casein fractions in their native form (Saboyainsta & 50 Maubois, 2000). Several studies have investigated the dynamics of casein and serum protein 51 (SP) partition during MF, based on manipulation of filtration conditions and equipment 52 configurations to optimise process efficiency relative to selectivity and flux performance 53 (Nelson & Barbano, 2005; Tremblay-Marchand, Doyen, Britten, & Pouliot, 2016; Zulewska 54 & Barbano, 2014; Zulewska, Newbold, & Barbano, 2009). Ceramic MF membranes are often 55 the design of choice for dairy applications due to their long operational life and ability to 56 withstand high processing temperatures and aggressive cleaning cycles (Baker, 2004). In 57 recent years, ceramic graded permeability (GP) MF membranes, whereby the design of the 58 membrane controls the pressure drop along the membrane length, have been widely adopted 59 as a more energy efficient design compared with earlier ceramic MF systems that required 60 recirculation of the permeate to create a uniform transmembrane pressure (UTP) (Saboyainsta 61 & Maubois, 2000).

Depending on the MF equipment design, it is reported that up to 95% of the SP can be
removed from skim milk, creating a retentate stream enriched in micellar casein, and a

64 permeate stream containing the partitioned SP and other soluble components (Nelson & 65 Barbano, 2005). The study by Nelson and Barbano (2005) established a benchmark for 66 assessment of MF based protein separation efficiency, which was later applied in several 67 studies (Beckman, Zulewska, Newbold, & Barbano, 2010; Hurt, Zulewska, Newbold, & 68 Barbano, 2010; Lawrence, Kentish, O'Connor, Barber, & Stevens, 2008; Zulewska et al., 69 2009). However, the reported SP retention or depletion efficiency depends on whether the 70 permeate or retentate mass and compositional data are used for comparison with the feed 71 stream. Often studies consider soluble whey proteins or SP, measured as non-casein nitrogen 72 (NCN) by Kjeldahl, without considering the presence of soluble denatured whey proteins 73 (Brodkorb, Croguennec, Bouhallab, & Kehoe, 2016) and serum casein (Rose, 1968), which 74 may lead to inaccuracies in reported SP contents. The literature concerning SP quantification 75 post MF only considers 'whey proteins' as those measured using standard methods designed 76 for raw liquid milk. This classical definition of SP does not incorporate serum caseins that 77 contribute to the soluble proteins in the serum phase of milk (Rose, 1968; Von Hippel & 78 Waugh, 1955). Additionally, the feed milk and subsequent retentate and permeate streams are 79 often subjected to extensive thermal and mechanical treatments which denature whey 80 proteins (Hinrichs & Rademacher, 2005; Wijayanti, Bansal, & Deeth, 2014), leaving them 81 susceptible to precipitation at pH 4.6 and interpretation as casein during nitrogen fractions 82 determination.

83 Several studies provide data surrounding fractionation efficiency of SP in skim milk by 84 MF, focused on the composition of the permeate stream, whereby partition of SP is expressed 85 in terms of percentage removal relative to the skim milk based on determination of N 86 fractions. However, there is a lack of a standardised mass balance approach in the literature 87 relative to the separation of milk proteins by MF. This study assessed MF of skim milk with 88 respect to protein partitioning on a mass/energy consumption basis to provide an overall

89	balance of milk components for a ceramic MF/DF process at pilot plant scale. In corollary,
90	we address discrepancies reported in the literature relative to the separation efficiency of SP
91	based on quantitative analysis of N fractions by Kjeldahl, supported by qualitative analysis of
92	the protein profile to identify partition dynamics during MF.
93	
94	2. Materials and methods
95	
96 97	2.1. MF feed material
98	Pasteurised skim milk heat-treated at 73 °C for 15 s was collected from a local dairy
00	processor 24 h prior to ME trials and was stored at 4 °C. On the day of processing the skim
100	milk was heated to 50 °C prior to filtration. The trials were performed in triplicate with a
100	mink was heated to 50°C prior to intration. The trians were performed in triplicate with a
101	discrete batch of pasteurised skim milk sourced for each trial.
102	
103	2.2. Membrane system and process design
104	
105	The MF process was performed using a pilot-scale membrane plant (GEA Process
106	Technologies, Dublin, Ireland) operated in continuous mode, with the retentate and permeate
107	collected in separate tanks. The processing parameters are reported in Table 1. The feed and
108	recirculation pressures (307 kPa retentate pressure in and 100 kPa retentate pressure out,
109	respectively) were kept constant throughout the filtration run, yielding a constant trans-
110	membrane pressure (TMP). A permeate back pressure of 98 kPa was applied during the MF
111	process. Under these set operational pressure conditions, the membrane experienced an
112	overall pressure drop (ΔP) and TMP of 207 and 105.6 kPa, respectively. The plant and
113	membranes were cleaned before and after filtration according to the standard cleaning-in-

114	place procedure (see Supplementary material). The membrane plant was equipped with three
115	0.1 µm ceramic graded permeability (GP) MF membranes (Membralox® model, EP 3730,
116	0.1 μ m alumina; Pall Corp, California, CA, USA) with a total surface area of 1.05m ² . The
117	membrane plant was also equipped with a digital data logger (Endress + Hauser AG,
118	Reinach, Switzerland) that recorded performance data including, flow rates, pressures,
119	temperatures and energy consumption (each individual pump was monitored using an ABB
120	B23 direct kW h ⁻¹ meter (ABB Ltd, Zurich, Switzerland).
121	On the day of processing, pasteurised skim milk (~300 kg) was subjected to
122	microfiltration followed by two discrete diafiltration (DF) steps under a volume concentration
123	factor (VCF) of 3 at 50 °C. For the DF process, the collected MF retentate was mixed with
124	100 kg of RO water (50 $^{\circ}$ C) and re-introduced into the membrane plant as the feed material,
125	while maintaining continuous operation of the MF process. The volume reduction factor
126	(VRF) during the two DF steps was 2, i.e., 100 kg of additional permeate was collected
127	during each DF step, equivalent to the volume of added water. The entire filtration process
128	was maintained in a continuous steady state at a VCF of 3 across all three stages. The mass of
129	all streams was recorded including the dead/residual volume of the membrane plant, which
130	was collected as a final flush post processing to minimise solids loss, ensuring an accurate
131	mass balance. The final MF permeate was concentrated by reverse osmosis (RO) with the
132	same membrane plant used for the MF process. For RO concentration the membrane plant
133	was equipped with two spiral-wound composite polyamide RO membranes (Dairy
134	AF3838C30, David Kellet & Partners Ltd, Hereford, UK) connected in series with a total
135	surface area of 14 m ² , whereby MF permeates were concentrated to a VCF of 5 at ~49 $^{\circ}$ C.
136	
137	2.3. Sample preparation and compositional analysis

139	The skim milk feed was sampled prior to MF, while retentates and permeates were
140	sampled after MF, MF/DF1, MF/DF2 steps and post RO of MF permeates All samples were
141	stored at 4 $^{\circ}$ C post sampling. The final MF/DF2 permeate sample was only used for dry
142	matter (DM) determination to support the mass balance calculations. The remaining
143	compositional analysis pertaining to the MF/DF2 permeate was performed on the RO
144	retentate, to limit the analytical error associated with determination of N fractions in low DM
145	content permeates. The composition of the RO retentate was used to calculate the
146	composition of the MF permeate relative to the dry matter therein. Analysis of DM and
147	nitrogen fractions was performed within 48 h, while for other analyses samples were stored
148	for two weeks at 4 °C with a preservative added (Broad Spectrum Microtabs® II, D & F
149	Control Systems, Inc., CA, USA).
150	DM was measured by oven drying (AOAC International method 925.23; AOAC,
151	2000). Total nitrogen (TN), non-protein nitrogen (NPN) and non-casein nitrogen (NCN) were
152	determined by the Kjeldahl method (methods IDF 20-1:2014, IDF 20-4:2016 and IDF 29-
153	1:2004; IDF, 2014, IDF, 2016 and IDF, 2004, respectively). To ensure further accuracy in
154	NCN analysis and minimise overestimation of NCN, the pH was monitored during acetic acid
155	and sodium acetate addition according to the procedure of Zhang and Metzger (2011).
156	Calculation of crude protein (CP), true protein (TP), casein (CN) and serum protein (SP)
157	contents were determined from N fraction analysis using a conversion factor of 6.38. Total
158	CP was calculated as TN \times 6.38, TP content was calculated as (TN – NPN) x 6.38, CN
159	content was calculated as $(TP - NCN) \times 6.38$ and SP (where SP is considered as non-
160	sedimentable true protein) was calculated as $(NCN - NPN) \times 6.38$.
161	
162	2.4. Separation of serum and colloidal proteins

164	Separation of serum phase from colloidal proteins in skim milk and MF retentate
165	samples was achieved by ultracentrifugation at 100,000 \times g for 1 h at 25 °C (Sorvall
166	Discovery 90SE ultracentrifuge, Kendro Laboratory Products, Asheville, NC, USA). Prior to
167	ultracentrifugation, skim milk and retentate samples were heated to 50 °C.

168

169 2.5. Protein profile analysis by SDS-PAGE

170

171 The protein profiles of skim milk, RO and MF retentates, skim milk and MF retentate 172 ultracentrifugates and filtrates from NCN analysis were determined using pre-cast sodium 173 dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Novex Technologies, 174 ThermoFischer Scientific, Dublin, Ireland) using the method described by Buggy, McManus, 175 Brodkorb, McCarthy, and Fenelon (2017). All samples were dissolved in a lithium dodecyl sulphate (LDS) buffer under reducing conditions at pH 8.4, with 10 µL of the sample loaded 176 177 to the wells in a 12% Bis-Tris Gel. The electrophoresis was performed using an X-Cell 178 Surelock electrophoresis unit (Novex Technologies). The samples were not standardised 179 relative to their protein content with dilution factors and loading volumes (10 μ L) kept 180 constant for all samples. Post electrophoresis, the samples were stained in instant blue stain 181 (Expedeon, Cambridge, UK) and gels were de-stained in MilliQ® water until a clear 182 background was achieved. Proteins were identified using a standard molecular mass kit 183 (PageRulerTM Unstained Low Range Protein Ladder, Thermo Fisher Scientific, Dublin. 184 Ireland).

185

186 2.6. Reverse-phase high pressure liquid chromatography for casein and whey protein
187 profiles

189	The RP-HPLC analysis for casein and whey protein profiles in skim milk, MF
190	retentates (and ultracentrifugates thereof) and RO retentates was performed as described by
191	McCarthy, Wijayanti, Crowley, O'Mahony, and Fenelon (2017). Samples were pre-treated in
192	7 M urea buffer and 2-mercaptoethanol followed by a 1 h incubation at room temperature
193	before being filtered (0.2 μ m PES syringe filters, Agilent Technologies, Dublin, Ireland). A 5
194	μL aliquot of filtered sample was injected twice into a Poroshell 300SB-C18 (Size: 2.1×7.5
195	mm, 5 210 μ m; Agilent Technologies) column. The HPLC system (Agilent 1200 Series,
196	Agilent Technologies) was equipped with a UV-vis detector (61365D MWD Agilent
197	Technologies 1200 series) with detection at an absorbance of 214 nm. Identification and
198	quantification of caseins and whey proteins was carried out using α -lactalbumin/ β -
199	lactoglobulin and κ -, $\alpha_{S1/S2}$ - and β -casein standards (Sigma, St. Louis, MO, USA).
200	
201	2.7. Mass and energy balance for serum protein removal
202	
203	To assess the separation performance of the MF process, mass balance calculations
204	were performed. The total mass (kg) of all microfiltration streams was recorded and the
205	corresponding mass (kg) of DM and N fractions (CP, TP, CN and SP) therein was calculated
206	to assess component partition dynamics.
207	After converting DM and protein contents into kg (Table 3), % recoveries for DM, CP,
208	TP, CN and SP were calculated as follows (equations 1 to 5):
209	1) % TS Recovery = $\frac{(\text{kg of TS in permeate}) + (\text{kg of TS in retentate})}{\text{kg of TS in skim milk}} \times 100$
210	2) % CP Recovery = $\frac{(\text{kg of CP in permeate}) + (\text{kg of CP in retentate})}{\text{kg of CP in skim milk}} \times 100$
211	3) % TP Recovery = $\frac{(\text{kg of TP in permeate}) + (\text{kg of TP in retentate})}{\text{kg of TP in skim milk}} \times 100$

212
 4) % CN Recovery =
$$\frac{(\text{kg of CN in permeate)} + (\text{kg of CN in retentate})}{\text{kg of SP in retentate})} \times 100$$

 213
 5) % SP Recovery = $\frac{(\text{kg of SP in permeate)} + (\text{kg of SP in retentate})}{\text{kg of SP in skim milk}} \times 100$

 214
 215
 SP removal efficiency was calculated using either MF permeate or retentate composition

 216
 (equations 6 and 7):
 217

 217
 6) % SP Removal (permeate composition) = $\frac{\text{kg of SP in permeate}}{\text{kg of SP in skim milk}} \times 100$

 218
 7) % SP Removal (retentate composition) = $\frac{(\text{kg of SP in skim milk}) - (\text{kg of SP in retentate + flush)}}{\text{kg of SP in skim milk}} \times 100$

 219
 2.8. Energy consumption

 210
 2.8. Energy consumption

 211
 7) % SP nemoved (Eqn 8) and kW h kg⁻¹ of SP removed (Eqn 9) for the MF and DF

 219
 permeate produced (Eqn 8) and kW h kg⁻¹ of SP removed (Eqn 9) for the MF and DF

 219
 processes:

 220
 8) Energy kW h lg⁻¹ TP= $\frac{(\text{feed pump energy in kWh + recirculation pump energy in kWh)}{Permeate flow rate in L h-1}$

 221
 The energy usage was calculated relative to kilowatt-hour (kWh) consumed by the

 222
 feed pump and recirculation pump. Energy consumption was calculated relative to kW hL⁻¹

 224
 of permeate produced (Eqn 8) and kW h kg⁻¹ of SP removed (Eqn 9) for the MF and DF

 225<

- ANOVA and significant differences were tested with Tukey's honest significant difference (HSD) test at p < 0.05.
- 235
- 236 **3.** Results and discussion
- 237

238 The total processing time for the ceramic MF process was on average 8 h 20 min. The 239 flux and processing time for individual processing stages of the MF process are shown in Fig. 240 1. The mean running temperature of the process was 50.1 °C and a TMP of ~105.6 kPa and 241 VCF3 were maintained throughout the production cycle. The recommended TMP range for 242 the GP membrane is 100 to 150 kPa. A value close to the lower end of this range was chosen 243 to minimise the potential for fouling accumulation throughout the processing cycle. The average flux for the initial VCF 3 MF stage was 45.5 L m⁻² h⁻¹, which is considerably lower 244 than reported by Zulewska & Barbano (2014) and Zulewska et al. (2009) and at 71.8 and 72.5 245 L m⁻² h⁻¹, respectively, for continuous MF processes. Tremblay-Marchand et al. (2016) also 246 observed a higher flux of 79–90 L m⁻² h⁻¹ using a similar GP MF membrane, although the 247 248 TMP was higher at 152 kPa. As their study involved a batch process, where the end of the filtration process was achieved once a VCF3 was reached, it is difficult to predict the flux 249 250 evolution throughout a typical production cycle at higher TMP. In the current study, an initial flux of 326 L m⁻² h⁻¹ decreased to \sim 76 L m⁻² h⁻¹ in the first 13 min of production, after which 251 252 the plant entered a steady state with a more gradual flux decline thereafter (Fig. 1). The rapid 253 initial flux decline may be attributed to accumulation of foulants on the membrane surface 254 increasing resistance (Carić, Milanović, Krstić, & Tekić, 2000; Savello, Caric, & Mahmoud, 255 1997), coupled with changes in DM content and viscosity as the plant stabilises and initial water within the recirculation loop is expelled. The addition of diafiltrant caused a slight 256 increase in flux at 46.1 and 46.5 L m⁻² h⁻¹ for MF/DF1 and MF/DF2 stages respectively 257

258 (Table 1), which is significantly lower than the increases reported by Zulewska and Barbano 259 (2014) and Tremblay-Marchand et al. (2016) for a similar MF/DF process albeit with half the 260 diafiltrant on a volume basis used in the current study. The design of the pilot MF process in 261 this study more closely reflects commercial operations in terms of process cycle duration and 262 maintenance of a continuous steady state during production, as opposed to the batch 263 processes more commonly reported in the literature. The sequential addition of lower 264 volumes of DF water directly into the feed stream while maintaining steady state conditions within the plant simulates commercial practice. The processing cycles were not extended 265 266 beyond 8 h 20 min, which could have been achieved by increasing the skim milk feed volume, due to concerns for the microbial quality of the MF retentate when operating at 50 267 268 °C for an extended time.

269

270 3.1. Determination of mass and SP partition efficiency during MF

271

272 The compositional analysis for DM and N fractions and associated mass balance for 273 the skim feed and MF/DF retentates/permeates at each stage of the filtration process are 274 presented in Tables 2 and 3. The overall mass balance focused on directly comparing the 275 skim feed with the final MF/DF2 retentate and permeate relative to DM contents and the N 276 fractions therein. The MF/DF2 retentate flush was also collected to limit any loss of milk 277 solids, minimising errors associated with determination of component recovery. The 278 composition of the DM within the MF retentate flush was equivalent to that of the MF/DF2 279 retentate. Recoveries were calculated for DM (99.52%), CP (94.87%) and TP (95.33%) 280 according to equations 1, 2 and 3 respectively. The high DM recovery indicates minimal loss of milk solids, while the lower recoveries for CP/TP identified the standard methodologies 281 282 for N determination as a potential source of error.

283	Based on the MF permeate mass/compositional data, the total SP removal decreased as
284	the solvent phase was diluted by successive diafiltrant addition from 0.69 for MF, 0.34 (1.03-
285	0.69) for DF1 and 0.25 (1.28–1.03) kg for DF2 (Table 3). Relative to the skim milk feed this
286	equates to 48.6, 23.9 and 17.6% SP removal (Eqn 6) for each individual filtration step. These
287	results are lower than those reported by Zulewska and Barbano (2014) who reported SP
288	removal efficiencies of 56, 26.6 and 13.9%. respectively. with a cumulative value of 96.5%,
289	for their 3 step ceramic MF/DF process. Zulewska et al. (2009) reported a SP removal
290	efficiency of 61% for a single stage MF process. Similar to the current study a SP removal
291	rate of 47% was reported by Tremblay-Marchand et al. (2016) for the initial MF step (3 step
292	process), with 17 and 8.5% SP removal reported for subsequent DF steps. When comparing
293	the cumulative SP removal in this study based on the SP content in the MF/DF2 permeate
294	(1.28 kg) relative to the skim feed (1.42 kg) this equates to a 90.1% SP removal efficiency on
295	a mass basis. It should be noted that although Zulewska and Barbano (2014) reported an
296	improved SP removal efficiency compared with this study, these authors used twice the
297	volume of diafiltrant, which would have significant implications relative to equipment sizing
298	and subsequent water recovery requirements in commercial installations.
299	
300	3.2. Determination of SP partition efficiency based on MF retentate composition
301	
302	In contrast to determining SP removal efficiency based on the permeate
303	mass/composition, when assessing cumulate SP removal based on the MF retentate
304	mass/composition (Eqn 7) a higher rejection of SP was observed with an overall removal

- 305 efficiency of 55.63% (Table 3) in line with the observations of Tremblay-Marchand et al.
- 306 (2016). The study of Hurt et al. (2010) calculated SP removal using both approaches, MF
- 307 permeate and MF retentate composition and reported it as 95.2 and 78.6% respectively. These

308	observations highlight the challenges in accurate determination of SP removal rates using the
309	retentate composition, as the precipitation step which is part of the NCN analysis (IDF 29-
310	1:2004) fails to precipitate all CN, leading to an overestimation of NCN (and hence SP) in
311	MF retentates. For the current study, when directly assessing the total cumulative SP in the
312	final MF retentate (0.63 kg) (0.51 + 0.12 kg) and MF permeate (1.28 kg) of 1.91 kg (1.28 + (1.28 kg))
313	0.63 kg) relative to the 1.42 kg of SP in the skim feed, this gives a recovery of 134.51% of
314	the SP in the skim feed considering analysis of NCN content by Kjeldahl. In corollary, while
315	the CN concentration in the final MF retentate was ~2.8 times that of the skim milk (Table 2),
316	the cumulative CN recovery was only 89.4% indicating underestimation of CN and
317	overestimation of SP according to the standard methodology for NCN determination (IDF 29-
318	1:2004). The standard methodology for NCN determination by Kjeldahl requires
319	precipitation of caseins at their isoelectric point and determination of the remaining proteins
320	soluble at pH 4.6. High protein/casein contents in MF retentates are a particular challenge if
321	they are not standardised to the protein content for which the test is designed, i.e., raw milk.
322	In this study the pH was monitored during NCN analysis of MF retentates to ensure
323	that pH 4.6 was achieved and that casein micelles were fully precipitated (Southward, 2002).
324	This pH monitoring step was also suggested by Zhang and Metzger (2011) to account for
325	variation in casein solubility relative to the altered ionic environment in MF retentates.
326	Further investigation of the protein profile contained within the NCN filtrates derived
327	from the MF/DF2 retentates by SDS-PAGE, identified the presence of serum caseins therein.
328	These serum caseins are reported as SP according to the NCN test when in fact they are
329	clearly CN (Fig. 3). Nelson and Barbano (2005) also detected intact casein in NCN filtrates
330	and suggested that the standard NCN test (IDF 29-1:2004) may underestimate CN
331	concentration in MF retentates. To address this challenge Di Marzo, Pranata, and Barbano
332	(2021) compared the results from quantitative approaches for CN determination and found

that CN/TP% was overestimated by NCN analysis (IDF 29-1:2004) compared with SDSPAGE, again highlighting the challenges of accurate determination of N fractions. In the
current study, serum CN in NCN filtrates post isoelectric precipitation of colloidal caseins
was not observed for the skim milk feed (Fig. 3), indicating significantly higher levels of
serum CN relative to total protein contents in the MF/DF2 retentates.

338 To further clarify the relationship between serum to colloidal proteins, the skim milk 339 feed and MF/DF2 retentates were ultracentrifuged to gravimetrically precipitate colloidal 340 from serum components, followed by analysis of CP and TP contents in the ultracentrifugates 341 (Table 4). Refrigerated samples were pre-heated to 50 °C prior to ultracentrifugation to reflect the temperature at which the MF process was performed and reverse any solubilisation 342 343 of caseins during storage at lower temperatures (Schiffer, Scheidler, Kiefer, & Kulozik, 344 2021). Direct comparison of TP in the skim milk with that of the ultracentrifugate thereof indicated that 31% of the TP in the skim milk remained in the serum phase post 345 346 ultracentrifugation. When performing the same calculation for the MF/DF2 retentates it was 347 found that 27% of the TP remained in the serum phase after ultracentrifugation. The high 348 SP/TP% in the MF/DF2 retentate indicates a relationship between casein concentration and 349 changes in the ionic environment with respect to ratios of serum to colloidal casein. 350 Identification of the protein profile in the ultracentrifugates of the skim milk and 351 MF/DF2 retentates by SDS PAGE indicated the presence of serum α_{S1} -, α_{S2} -, and β - and κ -352 caseins (Fig. 2). This was supported by subsequent HPLC analysis which identified serum 353 caseins in the ultracentrifugates of the skim milk and MF/DF2 retentates with a large increase 354 in serum κ -case in in particular observed in the latter (Fig. 4). Studies have shown that at 355 elevated pH and temperatures, κ-casein tends to dissociate from the casein micelle (Anema & 356 Klostermeyer, 1997; Anema & Li, 2000; Anema, Creamer, & Singh, 1993). The study by

Anema (2007) reported that in heated skim milk samples at pH values above 6.7, the level of

358	serum κ -case in increases, with ~70% solubilisation at pH 7.1. In the current study the MF
359	retentate had a pH of 7.06, which could explain the presence of elevated levels of serum κ -
360	caseins in the ultracentrifugates (Figs. 2 and 4). Additionally the loss of soluble/serum ionic
361	species to the permeate during MF/DF processes may increase surface charge of the micelles,
362	thus increasing the solubility of surface κ -casein in particular. Analysis of the MF/DF2
363	permeates (Figs. 2 and 4) indicate that serum caseins are retained while whey proteins
364	permeated through the membrane. According to Eigel et al. (1984) individual casein fractions
365	have a molecular mass in the range of 19 to 24 kDa while that of α -lactalbumin and β -
366	lactoglobulin is 14 and 18 kDa, respectively, thus serum caseins may have a higher rejection
367	coefficient relative to their size and shape. This observation is reinforced by the fact that high
368	molecular mass globular SP such as lactoferrin (~78 kDa) and bovine serum albumin (~69
369	kDa) permeated through the MF membrane while serum CN did not (Fig. 2).
370	Measurement of N fractions in skim milk and MF permeates (MF/DF1/DF2) also
371	highlights areas for potential underestimation of SP. The skim milk itself had a CN/TP%
372	value of 85.6%, which could be considered high given that expected average casein to whey
373	proteins ratios of 80:20 are widely reported in the literature (Fox & Brodkorb, 2008; Fox,
374	McSweeney, & Paul, 1998; Jenness & Patton, 1959). The high-temperature short-time
375	(HTST) pasteurisation of the skim milk at 73 °C for 15s, may result in higher levels of whey
376	protein denaturation compared with raw milk (Guinee et al., 1997) and unpasteurised skim
377	milk (Svanborg et al., 2014). However, the CN/TP% results in this study are similar to those
378	reported by Beckman et al. (2010), Mercier-Bouchard, Benoit, Doyen, Britten, and Pouliot
379	(2017) and Tremblay-Marchand et al. (2016), which were reported at 83.6, 83.86 and 83.2%,
380	respectively. The skim milk was subjected to pasteurisation and mechanical treatment (e.g.,
381	pumping) prior to and during MF, which could damage/partially unfold whey proteins
382	(Brodkorb et al., 2016), affecting their solubility at pH 4.6 during subsequent NCN testing. In

383	this scenario damaged SP is measured as CN according to the test (IDF 29-1:2004) leading to
384	an overestimation of CN/TP%. This hypothesis is supported by the high levels of CN/TP%
385	reported in the MF permeates at each filtration stage at 10.8, 10.09 and 9.37% for MF, DF1
386	and DF2 steps respectively. It is therefore possible that all TP transmitted through the
387	membrane is SP based on optical clarity of the permeate, supported by particle size
388	measurements (results not shown). In addition, neither SDS PAGE (Fig. 2) nor HPLC (Fig. 4)
389	identified the presence of serum casein in the MF/DF2 permeates. However, during NCN
390	determination a precipitate wass formed which consisted of SP insoluble at pH 4.6, which
391	almost certainly consists of whey proteins denatured during processing. Thus, the separation
392	efficiencies for MF, based on determination of N fractions, reported in the literature (Hurt et
393	al., 2010; Nelson & Barbano, 2005; Zulewska et al., 2009) are challenged as follows:
394	1. SP available for permeation in the starting pasteurised skim milk is underestimated
395	based on NCN analysis which overestimates CN/TP% contents therein, due to the
396	presence of pH 4.6 insoluble whey proteins.
397	2. Denatured whey proteins present in MF permeates are reported as CN without
398	performing confirmatory tests of the protein profile therein.
399	3. Protein profiles determined by HPLC and gel electrophoresis indicate that ceramic
400	MF permeates contain negligible amounts of casein.
401	4. SP present in MF retentates is overestimated as a proportion of serum CN is reported
402	as SP according to NCN analysis.
403	5. Reliability of true SP partition efficiency reported in the literature is impacted by
404	errors associated with determination of N fractions in processed dairy streams.
405	These observations clearly highlight challenges concerning accurate determination of
406	N fractions and in particular SP in MF retentates/permeates by NCN analysis (IDF 29-
407	1:2004). Hence, relying solely on N fractions analysis to determine SP partition efficiency

408	leads to compounding analytical errors, masking true SP separation behaviour during MF.
409	Complementary qualitative SDS-PAGE and HPLC analysis performed in tandem with N
410	fractions determination by Kjeldahl, may be a better approach to gauge SP removal efficiency
411	for MF processes.
412	

413 414 3.3.

Energy consumption

415 The energy required per litre of permeate was calculated for each discrete MF/DF step at 0.043 (MF), 0.042 (MF/DF1) and 0.041 (MF/DF2) kW h L^{-1} (Table 5). There was no 416 417 significant difference in energy usage per unit of permeate produced between the MF/DF1 418 and MF/DF2 filtration steps [based on energy consumption of feed and recirculation pumps 419 (Eqn 8)], which is not unexpected considering the flux consistency across the process cycle 420 (Fig. 1). Tremblay-Marchand et al. (2016) reported higher energy consumptions of 0.089, 0.083 and 0.077 kW h L⁻¹ of permeate removed for their 3 step ceramic GP MF/DF process. 421 422 They reported that the recirculation pump only accounted for 34% of the energy consumed 423 during MF of skim, stating that their plant design utilised two feed pumps, as the membrane 424 plant was a multipurpose system also capable of delivering the high pressures required for 425 RO. It is likely that this membrane plant was not equipped with frequency inverters to control the speed of each pump, potentially overestimating the required kW h L⁻¹ of permeate 426 427 separated. In contrast, the pumps in the current study were equipped with frequency inverters 428 to minimise the energy required to deliver a given cross flow velocity/TMP. Thus the recirculation pump, which provides the high cross flow velocities (~4.8 m s⁻¹), accounted for 429 430 93% of the energy used by the filtration plant.

Broadening the scope of the energy consumption calculation relative to the mass of SP
separated within each MF/DF stage (Eqn 9) provides insights into the impact of diafiltrant

433	volume on the energy efficiency of SP removal. The initial MF step operating at VCF3
434	removed ~ 200 kg of permeate, with each sequential DF step adding 100 kg of diafiltrant and
435	hence generating an additional ~100 kg of a diluted permeate (Table 3). The initial MF (VCF
436	3) and subsequent diafiltration (DF1/DF2) steps had a corresponding energy usage of 13.1,
437	13.7 and 20.6 kW h kg ⁻¹ of SP removed respectively. In contrast, Tremblay-Marchand et al.
438	(2016) reported energy consumptions of 25.83, 63.99 and 124.85 kW h kg ⁻¹ of SP removed,
439	for their 3 stage MF/DF process, although these authors utilised a higher volume reduction
440	ratio (VRR) of 3 during DF compared with a volume reduction ratio of 2 applied in this
441	study. Operation of the plant at VCF3 while applying an overall VRR of 2 during the DF1
442	step had a clear benefit in terms of SP removal efficiency as there was no significant
443	difference observed in the energy consumption in kW h kg ⁻¹ SP removed between the initial
444	MF and DF1 steps (Table 5). Further diafiltrant addition during the DF2 step did not
445	significantly increase flux; however; energy consumption kW h kg-1 SP removed
446	significantly increased. Minimising DF volumes is beneficial in terms of energy usage per kg
447	of SP separated, as a point of diminishing returns is quickly reached due to dilution of soluble
448	phase components in the retentate when using large DF volumes. Further implications
449	concerning membrane area and associated capital/operational costs and water recovery/reuse
450	or discharge to effluent strategies, all require careful consideration in determining optimal DF
451	processes.

4. Conclusion

A comprehensive mass balance indicated overall recoveries on a dry matter basis for
DM (99.5%), CP (94.9%) and TP (95.3%). However, CN (89.4%) and SP (134.51%)
recoveries indicated analytical errors associated with determination of NCN and indirectly

458	CN according to standard methodologies for determination of N fractions. A cumulative SP
459	removal rate for a $3 \times MF/DF$ process of 90.14% on a mass basis was achieved, based on
460	comparative analysis of NCN contents in the feed and permeate streams. When the same
461	comparison was performed based on the SP content in the MF retentate the cumulative
462	removal rate was 55.63%. These observations highlight the challenges of determination of N
463	fractions for evaluation of the separation efficiency of SP by MF, based on analysis of the
464	retentate stream. Combining quantitative N fractions analysis of the feed/permeate streams by
465	Kjeldahl with qualitative analysis by HPLC/SDS PAGE provides better insights into the true
466	partition efficiency of SP during MF. In parallel, the relationship between energy
467	consumption in kW h kg ⁻¹ SP removed, diafiltrant volumes used and plant operational
468	conditions can enhance the energy efficiency of SP removal.
469	
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Reproved

Parameter	Stage 1 MF	Stage 2 DF	Stage 3 DF	RO
		-		
Recirculation flow rate (L h^{-1})	13687	13602	13474	12700
Feed flow rate (L h ⁻¹)	73.87	74.52	73.5	
Retentate flow rate (L h ⁻¹)	25.1	25.13	24.7	2055
Permeate flow rate (L h^{-1})	47.73	48.38	48.8	345.7
Permeate flux (L m ⁻² h ⁻¹)	45.45	46.08	46.48	24.7
Feed pressure (kPa)	306.6	307	306.7	1741
Recirculation pressure (kPa)	100.2	100.6	100.6	1395
Back pressure (kPa)	98	98	98	1574
TMP (kPa)	105.4	105.8	105.7	
Processing temperature (°C)	50.3	50.1	49.9	48.7

Operational parameters during 3× ceramic MF/DF process.

Sample	DM	СР	TP	NPN	NCN	CN	SP	CN/TP
	(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	(%)
Stage 1: microfiltration								
Skim milk	8.65 ± 0.17	3.43 ± 0.07	3.24 ± 0.05	0.19 ± 0.02	0.66 ± 0.05	2.77 ± 0.01	0.47 ± 0.04	85.5
Permeate	$4.8^{a}\pm0.13$	$0.53^{a}\pm0.01$	$0.37^{a}\pm0.01$	$0.16^{a} \pm 0.01$	$0.49^{a} \pm 0.01$	$0.04^{ab} \pm 0.01$	$0.33^a\pm0.01$	10.8
Retentate	$12.42^{A} \pm 0.3$	$7.58^{\rm A}\pm0.2$	$7.4^{\rm A}\pm0.2$	$0.18^{\rm A}\pm0.01$	$0.94^{A}\pm0.07$	$6.64^{A}\pm0.13$	$0.76^{A}\pm0.06$	89.72
Stage 2: after 1 diafiltration Permeate	$\begin{array}{c} 4.14^{b}\pm 0.18\\ 13.71^{B}\pm 0.2\end{array}$	$\begin{array}{c} 0.49^{b} \pm 0.03 \\ 8.55^{B} \pm 0.3 \end{array}$	$\begin{array}{c} 0.34^{b} \pm 0.02 \\ 8.36^{B} \pm 0.3 \end{array}$	$\begin{array}{c} 0.15^{a} \pm 0.01 \\ 0.19^{A} \pm 0.01 \end{array}$	$\begin{array}{c} 0.46^{b} \pm 0.01 \\ 1.02^{B} \pm 0.02 \end{array}$	$\begin{array}{c} 0.03^{a} \pm 0.01 \\ 7.53^{B} \pm 0.2 \end{array}$	$\begin{array}{c} 0.32^{ab} \pm 0.02 \\ 0.81^{B} \pm 0.04 \end{array}$	10.09 90.1
Retentate								
Stage 3: after 2 diafiltrations								
Permeate	$3.64^{c}\pm0.14$	$0.41^{c} \pm 0.01$	$0.32^{b} \pm 0.1$	$0.10^{b} \pm 0.02$	$0.36^{c}\pm0.05$	$0.05^{b} \pm 0.02$	$0.3^{b}\pm0.02$	9.37
Retentate	$10.77^{C} \pm 0.7$	$8.61^{\text{B}} \pm 0.7$	$8.53^{\circ} \pm 0.6$	$0.11^{\text{B}} \pm 0.02$	$0.79^{\rm C} \pm 0.15$	$7.82^{\text{B}} \pm 0.6$	$0.68^{\circ} \pm 0.1$	91.7
Flush	2.52 ± 0.4	1.81 ± 0.3	1.75 ± 0.3	0.06 ± 0.01	0.2 ± 0.02	1.6 ± 0.21	0.14 ± 0.1	91.4

Compositional analysis of skim milk, MF/DF retentates and permeates.^a

^a Abbreviations are: DM, dry matter; TP, total protein; CP, crude protein; NPN, non-protein nitrogen; CN, casein; SP, serum protein. Values calculated as: TP = CP–NPN; CN = CP–NCN; SP= NCN–NPN; CN/TP% = CN as a percent of TP = (CN/TP) × 100. Results are the means \pm standard deviations of data from three independent trials. Permeate and retentate values in the same column without common superscript lowercase and uppercase letters, respectively, are significantly different (p < 0.05).

Mass balance of fractionated skim milk proteins relative to dry matter and protein when subjected to 3× ceramic MF/DF process at 50 °C.^a

Process stage	Mass collected	DM	СР	TP	CN	SP	
	(kg)	(kg)	(kg)	(kg)	(kg)	(kg)	
Stage 1: microfiltration							
Skim milk	301.67 ± 2.89	26.08 ± 0.75	10.35 ± 0.31	9.78 ± 0.25	8.37 ± 0.11	1.42 ± 0.15	
Permeate	209.17 ± 5.96	10.05 ± 0.44	1.11 ± 0.03	0.77 ± 0.02	0.08 ± 0.01	0.69 ± 0.04	
Retentate	88 ± 6.24	10.93 ± 0.62	6.67 ± 0.5	6.51 ± 0.5	5.85 ± 0.45	0.66 ± 0.08	
Stage 2: after 1 diafiltration							
Permeate*	319 ± 16.25	13.21 ± 0.59	1.58 ± 0.06	1.09 ± 0.04	0.11 ± 0.02	1.03 ± 0.05	
Retentate	79.4 ± 7.3	10.89 ± 0.5	6.79 ± 0.3	6.64 ± 0.5	5.98 ± 0.4	0.64 ± 0.06	
Stage 3:after 2 diafiltrations	443.67 ± 12.03	16.13 ± 1.44	1.8 ± 0.07	1.43 ± 0.05	0.24 ± 0.09	1.28 ± 0.3	
Permeate*	75.67 ± 6.44	8.04 ± 0.7	6.44 ± 0.12	6.36 ± 0.08	5.85 ± 0.05	0.51 ± 0.1	
Retentate	87 ± 7.2	2.19 ± 0.04	1.57 ± 0.02	1.52 ± 0.03	1.39 ± 0.02	0.12 ± 0.01	
Flush							
% Recovery		99.52	94.87	95.33	89.4	134.5	

^a Abbreviations are: DM, dry matter; TP, total protein; CP, crude protein; NPN, non-protein nitrogen; CN, casein; SP, serum protein. Values calculated as: TP = CP–NPN; CN = CP–NCN; SP= NCN–NPN; CN/TP% = CN as a percent of TP = (CN/TP) × 100. Results are the means \pm standard deviations of data from three independent trials. An asterisk indicates collected permeate masses are cumulative values representing 2 or more filtration stages.

Mean (% by weight) CP and TP values of skim milk and MF retentate pre and post ultracentrifugation.^a

Sample	% CP		% TP			
	Before	After	Before	After		
	ultracentrifugation	ultracentrifugation	ultracentrifugation	ultracentrifugation		
Skim milk	3.43 ± 0.07	1.3 ± 0.09	3.24 ± 0.05	1.01 ± 0.06		
MF Retentate	8.61 ± 0.2	2.85 ± 0.2	8.53 ± 0.6	2.31 ± 0.4		

^a After ultracentrifugation, serum phase layer collected was sampled and analysed. Results are the means \pm standard deviations of data from three independent trials.

Total energy consumption during the MF/DF process. ^a

Filtration stage	Feed pump	Recirculation pump	Permeate flow rate	Energy consumption per unit of	Total permeate	Total energy consumption	Total SP removed	Energy consumption
	(kW h)	(kW h)	(L h ⁻¹)	permeate (kW h L ⁻¹)	(L)	(kW h)	(kg)	(kW h kg ⁻¹ SP removed)
MF	0.14 ± 0.02	1.93 ± 0.03	47.7 ± 0.4	$0.043^a\pm0.04$	209.2 ± 6	$9.07^{a} \pm 0.03$	0.69 ± 0.03	$13.14^{a} \pm 0.06$
DF1	0.14 ± 0.01	1.91 ± 0.01	48.8 ± 0.3	$0.042^{ab}\pm0.03$	109.8 ± 16	$4.66^b\pm0.03$	0.34 ± 0.02	$13.7^{a} \pm 0.04$
DF2	0.14 ± 0.01	1.87 ± 0.02	48.8 ± 0.3	$0.041^{b} \pm 0.01$	124.7 ± 12	$5.15^{c}\pm0.01$	0.25 ± 0.02	$20.6^{b} \pm 0.05$

^a Results are the means \pm standard deviations of data from three independent trials; values in the same column without a common superscript letterare significantly different (p < 0.05).

Figure legends

Fig. 1. Permeate flux as a function of time during step 1 microfiltration (MF), step 2 diafiltration (DF1) and step 3 diafiltration (DF2).

Fig. 2. SDS-PAGE protein profiles of (1) serum phase of skim milk, (2) skim milk, (3) RO retentate representing the MF permeate, (4) serum phase of MF retentate, (5) final MF retentate and (6) molecular mass standards. Samples of serum phase of skim milk and MF retentate were obtained by collecting supernatant post ultracentrifuge. Bands are identified on the gel as: α_{S1} -CN, α_{S1} -casein; α_{S2} -CN, α_{S2} -casein; κ-CN, κ-casein; β-CN, β-casein; β-LG, β-lactoglobulin; α-LA, α-lactalbumin; LF, lactoferrin; BSA, bovine serum albumin.

Fig. 3. SDS-PAGE profile of NCN filtrates of (1) skim milk, (2) MF retentate, (3) RO retentate obtained from the three stage $3 \times$ ceramic microfiltration process and (4) molecular mass standards. Bands are identified on the gel as: β -LG, β -lactoglobulin; α -LA, α -lactalbumin.

Fig. 4. HPLC profiles of (A) skim milk, (B) RO retentate, (C) Skim milk ultracentrifugate and (D) MF Retentate ultracentrifugate. Peaks identified as: 1, κ-casein; 2, α_{s2} -casein; 3, α_{s1} -casein; 4, β-casein; 5, α-lactalbumin; 6, β-lactoglobulin b; 7, β-lactoglobulin a.



Figure 1



Figure 2



Figure 3



Figure 4

Declaration of interests

 \boxtimes The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

□The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: