Chemical fractionation of caseins by differential precipitation: influence of pH, calcium addition, protein concentration, and temperature on the depletion in α - and β -caseins

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Chemical fractionation of caseins by differential precipitation: influence of pH, calcium addition, protein concentration, and temperature on the depletion in α - and β -caseins

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Abstract

The impact of pH, calcium/casein molar ratio, casein concentration and temperature on differential precipitation of caseins by calcium affinity was investigated. One of the most recent methods for extract caseins is the addition of calcium at alkaline pH. Increasing the pH to 11 disturbed the micellar structure by enhancing electrostatic repulsion of caseins, leading to a marked viscosity increase and a significant particle size decrease, indicating casein micelle disruption. The increase in negative charges enhanced the affinity of individual caseins for calcium, proportionally to the number of phosphate groups carried by each casein. The addition of calcium first led to a progressive increase in the proportion of precipitated caseins, before reaching a plateau. Hence, an optimal calcium/casein molar ratio of about 40 was evidenced to optimise casein precipitation (and fractionation), leading to significant depletion of α -casein (around 80 %) and, in a lesser extent β -casein (around 65 %) and κ -casein (around 55 %). This method led to a casein solution composed of 31 % of α -casein, 45 % of β -casein and 24 % of κ -casein, which differs significantly from usual casein proportions in milk products. This new dairy product enriched in κ -casein is expected offer new interesting functionalities.

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Key words

Casein / differential precipitation / calcium affinity / HPLC / rheology / dynamic light scattering

Introduction

From time immemorial, milk was used as raw material for many dairy products¹ such as cheese, yoghurt, cream, etc. Dairy products or milk derivatives (fractionated proteins, peptides, etc.) exhibit many bioactive properties and functional properties (emulsification, foaming abilities)⁴. These diverse and unique properties^{2,3} led to the development of new dairy products of high added-value, like protein isolates. Milk is a complex medium composed of water, proteins (caseins, whey proteins), carbohydrates (mainly lactose), lipids, and minerals (calcium, phosphates, etc.). Some milk minerals (sodium, potassium, etc.) are soluble and very available for interaction with other molecules. The rest of the minerals exist as colloidal species involved in casein micelles. The most important is calcium, which is mainly present within casein micelles (65 %) and is essential to their stability.

Proteins are found in milk at a concentration varying between 30 and 40 g/L depending on season, cow's nutrition, race, age, etc. There are two groups of protein in milk: casein micelles and (soluble) whey proteins. This study intended to apply fractionation methods to the casein fraction, which represents approximately 80 % of total milk proteins, in order to provide casein-derived dairy products with new functionalities. The typical composition of casein micelles is displayed in table 1. Dairy protein composition is variable in terms of total protein concentration, but also relative proportions of whey proteins and caseins, as well as proportions of each casein type. This implies that total protein concentration is standardised in food processing applications to guarantee the repeatability of dairy product features.

According to the more recent consensus⁵, caseins are structured in micelles of size ranging from 80 to 400 nm, constituted by submicelles of caseins bound by nanoclusters of calcium phosphate. The

internal structure of casein micelles is mainly composed of α - and β -caseins, because of their higher hydrophobicity. κ -casein, being a glycosylated protein, has an amphiphilic nature making it prone to be located at the water/protein interface constituted by the micelle surface, thus providing stability to the micelle structure. Nevertheless, the interaction between phosphorylated caseins (α - and β -caseins essentially) and calcium phosphate nanoclusters also contributes to strengthen casein binding in micelles.

The micellar organisation of caseins is strongly affected by the physical and chemical properties of the surrounding environment, especially temperature, pH, and chemical composition (especially, mineral composition). Temperature mostly influences the structuration of micelles by strongly affecting their size. For instance, heating milk at 50 °C results in a decrease in micelle size from 180 to 140 nm, as hydrophobic interactions are favoured at high temperatures, leading to micelle contraction⁶ and thus reduction of micelle porosity. However, in other milk derivatives (for example when caseins are reconstituted in simulated milk ultrafiltrate⁷ which corresponds to a solution of the mineral fraction of milk), heating leads to an increase in casein micelle diameter by a mechanism that is still not totally understood. This shows that interactions with other milk components markedly affect casein micelle features.

Several studies have showed that a pH decrease to 4.6 induces the progressive solubilisation of micellar calcium, thus disturbing micelle structure. A further pH decrease to the isoelectric point of caseins may induce their precipitation. pH increase has an inverse effect⁸. Indeed, casein alkalisation affects their electric charge: amino acids undergo an increase in negative charge, enhancing casein electrostatic repulsion, which in turns disturbs ionic equilibrium between micellar and soluble phases. The subsequent loss of interactions between caseins allows solubilisation of micelles, which lowers milk turbidity.

Currently, caseins are mainly used in dairy factories, mostly as protein supplements for improving milk gelation in cheese and yoghurt production. Because of the difficulty to fractionate caseins (coming

from the similarity of chemical structure of individual caseins), isolated caseins⁹ are rarely employed in the food industry, although several academic studies put in light various technofunctional^{10,11} and biological properties that could be used with benefits by dairy industrials.

β -casein is surely the most studied casein. First, it is rather abundant, accounting for about 33 % (w/w) of total caseins (table 1). It has the particularity to leave the micelle and partially solubilise at low temperature (around 4 °C), as in these conditions hydrophobic interactions are lowered. Thus, it can be isolated by cold filtration¹² followed by membrane filtration. β -casein has great amphiphilic properties (in spite of the absence of glycosylated group) owing to its amino acid composition, making it suitable for emulsification and foaming applications¹³. β -casein can be enzymatically hydrolysed to produce various peptides with a large range of biological activities: for example, antioxidant¹⁴, antibacterial¹⁵, and antihypertensive¹⁶ effects have been reported.

α_s -casein, the major type of casein (accounting for 44 % (w/w) of total caseins, cf. table 1), has been well less studied. The few available information deals with reinforcement of egg protein gels¹⁷ or production of peptides with health benefits, like anxiolytic effect, by enzymatic hydrolysis¹⁸.

The most interesting casein is probably the κ -casein. This glycoprotein is very effective in promoting emulsification and foaming of food products¹⁹. Furthermore, enzymatic hydrolysis of κ -casein may lead to various peptides with interesting biological activities, like kappacin presenting a strong antibacterial activity²⁰. The most studied peptide deriving from κ -casein is surely the glycomacropeptide which is issued from the C-terminus of κ -casein; it presents various functional properties like foaming and emulsification abilities²¹, but it also allows improving health by contributing to nutrition of phenylketonuria patients or intestinal health prevention, etc. Finally, as κ -casein greatly contributes to micellar stabilisation, modifying its concentration in milk and/or its relative proportion toward other caseins may allow controlling acid gelation or enzymatic hydrolysis of milk derivatives²². Thus, milk products enriched or depleted in κ -casein may lead to dairy gels with innovative physicochemical and textural properties.

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3 102 Several chemical and/or physical methods have been developed to separate milk caseins from other
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5 103 proteins. The chemical process is widely used in the dairy industry for caseinate powder production.
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7 104 Acid casein can be obtained by decreasing milk pH²³, thus making caseins precipitate. Coagulated
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10 105 caseins are isolated, then neutralised and resolubilised with an alkali, and finally dried to be stored
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12 106 under the form of a powder. Since the discovery of the process of acid precipitation of caseins, some
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14 107 novel methods have been described, like ethanolic²⁴ or phosphate precipitation²⁵.
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17 108 Physical processes are also available for casein separation. For example, centrifugation enables the
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19 109 physical separation of compounds on the basis of density difference by applying centrifugal force to
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21 110 liquid samples. However, caseins being soluble, it is necessary to precipitate caseins to be isolated by
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23 111 chemical process prior to centrifugation.
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26 112 Electrophoresis is another separation technique, whose principle is to apply an electric field to liquid
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28 113 samples to separate molecular compounds according to their electrophoretic mobility that depends
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30 114 on electric charge and molecular weight²⁶. Following this technique, caseins could be separated from
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32 115 other milk proteins, and even each casein type could be isolated, with a high level of purity.
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34 116 Unfortunately, this method involves the use of chemicals incompatible with food safety requirements
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36 117 (urea, acetonitrile, methanol, etc.) and the amounts of isolated caseins that can be produced are too
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38 118 low to envisage production at industrial scale.
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43 119 For better separation efficiency, chromatographic methods are mostly used²⁷. Caseins in liquid solution
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45 120 (such as milk) are injected in a column filled with an apolar stationary phase (often, C18-bonded silica),
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47 121 which retains molecular compounds. After that, sample molecules are gradually eluted by a polar
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49 122 mobile phase (generally acetonitrile or methanol). This method allows to separate the different caseins
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51 123 and obtain isolates of α -, β -, and κ -caseins. Preparative chromatographic columns do now permit to
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53 124 produce greater amounts of isolated caseins than electrophoretic methods, making chromatographic
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55 125 methods suitable for industrial applications. Another advantage lies in the fact that recovered proteins
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57 126 are obtained in native form. Elution solvents can be evaporated to increase casein concentration and
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3 127 eliminate chemicals that are not suitable for food applications. But this method has several drawbacks:
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5 128 it requires specific equipments (a large HPLC column and a recycling system for solvents), large
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7 129 amounts of solvents should be handled with care and correctly reprocessed, and produced quantities
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9 130 are low in comparison with traditional methods of casein isolate production.
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13 131 Last, the most common method for producing casein isolates still remains the membrane filtration
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15 132 process, either by using organic or inorganic membranes²⁸. Generally, tangential filtration is operated
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17 133 by pumping the solution to be concentrated and making it circulate under pressure. After the filtration
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19 134 step, two products are obtained: a retentate in which large molecules (caseins for instance in the case
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21 135 of milk microfiltration) are concentrated and a permeate composed of water and soluble molecules of
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23 136 size inferior to the diameter of membrane pores. Membrane cut-off is selected according to the
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25 137 molecules to be concentrated and defines the type of filtration process, for which pore size decreases
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27 138 in the following order: microfiltration > ultrafiltration > nanofiltration > reverse osmosis²⁹. Membrane
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29 139 filtration is used to produce a great variety of milk derivatives: milk protein concentrates, native
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31 140 phosphocaseinate (NPC), whey protein concentrate, etc. is the first step of casein purification is
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33 141 generally a microfiltration in order to remove the majority of whey proteins, minerals, and lactose. The
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35 142 second step often consists in an ultrafiltration of the microfiltration retentate to increase casein
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37 143 concentration.
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42 144 Our study aims at adapting the differential precipitation method of Post et al. (2012) for producing two
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44 145 casein concentrates, one being enriched and the other depleted in κ -casein. In this study of Post et al.,
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46 146 casein was first alkalised, leading to destabilisation of casein micelles by an increase in negative charges
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48 147 of caseins. At alkaline pH, soluble caseins have a high negative charge, allowing them to interact with
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50 148 divalent ionic calcium. Calcium addition at this point induces calcium-casein interactions resulting in
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52 149 casein solubility decrease. Solubility of calcium-casein complexes being minimal at acid and neutral
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54 150 pH, the alkalised casein solution containing calcium is then acidified up to pH 7. Precipitated calcium-
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56 151 casein complexes can then be recovered by centrifugation, decantation, or filtration. The resulting
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supernatant phase is expected to be enriched in κ -casein, and the precipitated phase in α - and β -caseins, owing to their difference in calcium affinity. The precipitate is then resuspended in water to obtain a liquid phase enriched in α - and β -caseins. α - and β -caseins have the highest affinity for calcium, hence the precipitate obtained by the method of Post et al. (2012) is mainly composed by these caseins. However, κ -casein is not insensible to calcium, so the precipitation of a fraction of κ -casein is also expected too, together with some whey proteins like β -lactoglobulin³⁰.

In the current work, it was tried to improve the differential precipitation method by using milk protein concentrates instead of milk as feeding material. The objective of the study was to focus on the interactions between caseins and calcium, hence casein concentrate presents many advantages: mainly, it contains a higher proportion of compounds of interest (caseins) than milk, while having reduced amounts of minerals, lactose, and whey proteins that may interfere with casein-calcium interactions. In milk, the casein/whey protein ratio is known to be close to 80/20, but in milk concentrate (by a combination of microfiltration and ultrafiltration) is around 92/8. The impact of calcium addition was thoroughly investigated in order to select the optimal calcium concentration dependently from the composition of milk derivative to be treated. Also, the influences of initial protein concentration and nature of added acid were studied with a view to meet economical and ecological concerns (treatment of samples having the highest possible protein concentration and reduction of added volumes of acid, calcium, and alkaline solutions in order to obtain products as much concentrated as possible while limiting environmental impact). Prior to these assays intending to optimise the differential precipitation process, DLS analyses were performed on the casein concentrate at different pH ranging from 7 to 11 to identify the critical pH at which alkaline micelle disruption occurs. Also, rheological characterisation of casein concentrate at native pH (6.85) and pH 11 were carried out at 4, 25, and 50 °C to find the temperature to be used in the differential precipitation process in order to avoid a too high viscosity of the casein concentrate, which may impair casein concentrate agitation and handling.

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Material and methods

Preparation of native phosphocaseinate solution

In general, NPC is industrially obtained from two-step tangential filtration (microfiltration and ultrafiltration) of pasteurised skim milk followed by stabilisation by spray-drying. In this work, the NPC used for differential precipitation of caseins was Promilk 852 B produced by Ingredia (Arras, France). Its composition was provided by Ingredia and checked by HPLC quantification: 81 % (w/w) total proteins, with caseins and whey proteins respectively representing 92 and 8 % (w/w) of total proteins, 8.5 % (w/w) minerals, 1.5 % (w/w) fat, and 2 % (w/w) lactose. NPC powder was resuspended in distilled water to reach the following concentrations: 22.5 and 55 g/kg. Appropriate amounts of powder were slowly poured into distilled water at 25 °C under magnetic stirring for 1 h. Then, casein concentrates were stirred at 4 °C overnight, in order to ensure complete powder solubilisation.

Prior to differential precipitation assays, temperature of casein concentrates was adjusted to 25 °C with a warming plate and their proteic composition was checked by HPLC following the method described later.

Chemical and reagents

During differential precipitation assays, alkalisation was performed with NaOH 1 M (VWR, Radnor, USA). Calcium addition was achieved with a 0.7 M CaCl₂ solution, prepared by dissolving CaCl₂·2H₂O (Carlo Erba reagents, Val de Reuil, France) in distilled water. Acidification was realised either by HCl (Sigma-Aldrich, Saint-Louis, USA) or citric acid (citric acid monohydrate powder, Honeywell Riedel-de-Haën, Sellze, Germany) either at 0.5 and 1 M.

For HPLC quantification of caseins, acetonitrile (Sigma-Aldrich) and trifluoroacetic acid (Sigma-Aldrich) were used for the preparation of the mobile phase. Calibration was performed using casein and whey protein standards (Sigma-Aldrich).

Chemical fractionation of caseins by differential precipitation

The differential precipitation process of the current study was developed on the basis of Post and al.'s work³¹. The main differences with the approach of Post et al. resided on one hand in the nature of feeding material, casein concentrate, which has a greater proportion of compounds of interest, and on the other hand in the proteic concentration of the feeding material: 55 g/kg in our study (with 92 % of casein) vs. 27 g/kg (with 89 % of casein) for Post et al.. The NPC solution was alkalised by the drop-by-drop addition of molar NaOH under vigorous stirring until reaching pH 11, as measured with a Five Easy Plus pH meter (Mettler-Toledo, Viroflay, France). During this step, the addition of NaOH induces the progressive destructuration of casein micelles, thus affects ionic equilibrium and leading to a small pH reincrease. Consequently, it was necessary to let solution at rest for 2 minutes minutes to reach pH equilibrium after addition of each NaOH drop.

Different amounts of 0.7 M CaCl_2 were added to the alkalised NPC solution at pH 11 and the mixtures were stirred for 30 min to allow calcium-casein interaction. Depending on the assay, calcium additions were carried out to reach different molar ratios between added calcium and total caseins: 10, 20, 30, 40, and 50. The information about the initial calcium concentration of the casein concentrate were not provided by the supplier.

Then, pH was decreased to 7 by addition of HCl or citric acid either at 0.5 or 1 M. Acid addition was performed drop-by-drop to limit acid hydrolysis of caseins, while avoiding exceeding the target pH of 7. The acidification step leads to calcium complexation by caseins followed by precipitation of complexes, thus inducing a slow pH reincrease during equilibration. Consequently, solution was let at rest for 20 min after each acid drop addition.

224 It is important to notice that added volumes of CaCl_2 and citric acid solutions were different according
225 to the calcium/casein molar ratio. So, the volume of all samples was adjusted with distilled water
226 before performing the last step of the differential precipitation process.

227 Finally, the precipitated was separated from the supernatant phase by centrifugation (Megafuge 8R,
228 Thermo Scientific, Massachusetts, USA) at 5 000 x g and 25 °C, then collected. Each differential
229 precipitation assay was replicated three times.

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231 **Dynamic light scattering measurement**

232 Dynamic light scattering (DLS) was used to determine the average micelle diameter in the casein
233 concentrate at different pH (7, 8, 9, 10, 11 and 12) with a Zetasizer Nano (Malvern). Prior to analysis,
234 the casein concentrate was diluted at 2 g/kg of total proteins and adjusted to the target pH with the
235 Five Easy Plus pH meter by using molar NaOH under agitation with a magnetic bar. Then, temperature
236 was equilibrated to 25 °C during 5 min and DLS analysis was repeated three times for each sample.
237 Recorded size estimators were mean diameter in number and mean diameter in volume. Casein
238 standards ($\kappa/\alpha/\beta$) were also analysed with the same method.

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240 **Rheological characterisation**

241 Rheological behaviour of NPC at protein concentrations of 22.5 and 55 g/kg was evaluated with a
242 Kinexus rotational rheometer (Malvern Instruments, Malvern, UK). A cone-plate geometry of 50 mm
243 diameter and 2° angle was used. Shear stress measurements were realised between 0.0001 and 1 000
244 s^{-1} shear rate (which corresponded to 71 analytical points) during 10 min.

245 First, 100 g sample was adjusted to the target pH (from 7 to 12) by adding molar NaOH and the dilution
246 induced by alkalisation was compensated between samples by adding an appropriate amount of
247 distilled water to reach a final sample mass of 106 g. Three temperatures were investigated: 4, 25, and

248 50 °C. Prior to analysis, the sample was equilibrated at the target temperature for 10 h and
249 temperature equilibration of the viscometer was achieved in 20 min.

250 All analysed concentrates presented newtonian rheological behaviour and this was modelled with
251 Microsoft Excel 2013 (Washington, United State) using linear regressions of the shear stress vs. applied
252 shear rate curve. In our case, the coefficient of determination (R^2) was high enough (between 98 and
253 100 %) to apply a linear regression.

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255 **Reverse-phase high performance liquid chromatography**

256 Chromatographic analysis was performed with a Prominence HPLC apparatus (Shimadzu Tokyo,
257 Japan). The chromatographic system was composed of a 3-way degasser (Degasys Populaire), a SCL-
258 10 A system controller (Shimadzu), FCU-10AL proportioning valves (Shimadzu), an LC-10AT pump
259 (Shimadzu), a SPD-M10 autosampler (Shimadzu), and a light diffusion detector EUROSEP Chromachem
260 (VWR, Radnor, USA). Chromatograms were acquired with the LC Solution software (Shimadzu).
261 Separation was achieved with an Agilent (Santa Clara, USA) 300 Extend column (250 mm length x
262 4.6 mm diameter, C18-bonded silica, 5 μ m bead size, 30 nm pore size). Mobile phases were: 89.9 %
263 (v/v) ultra-pure water, 10 % (v/v) acetonitrile (Sigma-Aldrich), and 0.1 % (v/v) trifluoroacetic acid
264 (Sigma-Aldrich) (eluant A); 10 % (v/v) ultra-pure water, 89.9 % (v/v) acetonitrile, and 0.1 % (v/v)
265 trifluoroacetic acid (eluant B). Gradient elution was carried out as follows: the initial composition of
266 the mobile phase was 80 % eluant A and 20 % eluant B. After injection, the proportion of eluant B was
267 linearly increased to 46 % in 35 min. After that step, 100 % eluant B was passed during 10 min for
268 cleaning purposes. Finally, during the last 20 min, column was re-equilibrated with 80 % eluant A and
269 20 % eluant B. Total method duration (analysis, column cleaning, equilibration) was 65 min. Flow rate
270 was set at 1 mL/min and room temperature was regulated at 25 °C.

271 The supernatant obtained after application of differential precipitation process was analysed by RP-
272 HPLC. Before analysis, it was filtered at 0.45 μ m.

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3 273 The three replicates of differential precipitation supernatants were each analysed three times by HPLC.
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9 275 **Results and discussion**
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12 276 **Size of casein micelles upon pH change**
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15 277 Table 2 presents results of DLS analysis of NPC at pH ranging from 7 to 11.
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18 278 In the investigated pH range, casein micelles are expected to be negatively-charged, as the isoelectric
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20 279 point of caseins has been reported around 4.8³². Thus, pH increase may enhance the negative charge
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22 280 of caseins and lead to their disruption. It is apparent from DLS measurements that no significant size
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24 281 change of casein micelles occurred between pH 7 and 9, showing that electrostatic repulsion between
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26 282 caseins was not sufficient to disrupt micelles in this pH range. At pH 10, the mean diameter in number
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28 283 of caseins was markedly decreased to about 30 - 40 nm, indicating the presence of a major population
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30 284 of isolated caseins. Also, the mean diameter in volume increased to 282 nm, showing that some casein
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32 285 micelles were still present at pH 10, but their swelling confirms that electrostatic repulsion between
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34 286 caseins began to induce their destabilisation. When further increasing the pH at 11, two populations
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36 287 appeared from measurements of size in volume: one constituted of partially-disrupted micelles of high
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38 288 size (366 nm) and the other of low mean size (55 nm), likely corresponding to individual caseins. These
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40 289 results show that casein micelles disruption, triggered between pH 9 and 10, significantly occurred
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42 290 between pH 10 and 11, which justifies the alkalisation at pH 11 in the differential precipitation process.
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44 291 But when we increase the pH to 12, we could see that the decrease is more significant, and the
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46 292 population of particles is more homogenous than pH 11.
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52 293 To confirm these interpretations, the size of individual caseins was measured by analysing casein
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54 294 standards (table 3).
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57 295 Table 3 shows that the size in number of isolated caseins is found 10 - 13 nm. This confirms that the
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59 296 differential precipitation process applied to casein concentrates allows disrupting casein micelles, as
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the mean particle size of casein concentrate alkalisated at pH 11 tended to the size of individual caseins. The higher size of caseins in NPC alkalisated at pH 11 may result from the difference in chemical composition and manufacturing process.

Interpretations of DLS results meet visual observations of NPC solutions realised during the alkalisation process. Indeed, at pH 7, the NPC was white and opaque, owing to light diffraction by casein micelles, whereas upon alkalisation above pH 10, it became translucent, consistently with literature. This can be explained by the effect of pH increase on caseins: the higher the pH, the greater the negative charge of caseins, the more repulsion between caseins. Around pH 10, electrostatic repulsion between caseins causes the destabilisation of micelle structure and the solubilisation of individual caseins, making the NPC translucent.

So, the significant decrease of mean particle size at pH 11 suggests that casein micelles are disturbed and individual caseins are present as monomers or very small polymers. The pH 12 seems to be more efficient for the casein micelle destructure, but it didn't mean the differential precipitation will be more efficient.

Impact of protein concentration, temperature, and pH on rheological behaviour of casein concentrates

Preliminary differential precipitation assays (data not shown) showed that a tremendous increase in NPC viscosity occurred around pH 10 at ambient temperature. Also, protein concentration in the NPC had a strong impact on viscosity: over 55 g/kg total proteins, stirring of the NPC was impaired, which could lead to a lower degree of homogenization of the NPC and reduce calcium-caseins interactions. Besides, caseins, like other proteins, are sensitive to acid hydrolysis; hence, during the acidification step of the differential precipitation process, the poor agitation of the NPC could locally induce a marked pH decrease upon acid addition, leading to undesired precipitation of caseins (especially κ -casein) and lowering the efficiency of the differential precipitation process. To validate that

observation, viscosity of NPC at 22.5 and 55 g/kg total proteins at various pH was evaluated. This was achieved at 4, 25, and 50 °C to investigate temperature influence, as viscosity is known to decrease at increased temperature. Table 4 displays viscosity of NPC at 22.5 g/kg total proteins.

At 22.5 g/kg total proteins, no great change in NPC viscosity was denoted upon alkalisation, especially at 50 °C. At 25 °C, viscosity seemed to slightly decrease upon alkalisation, while at 4 °C it slightly increased up to pH 10 and slightly decreased above. These results show that pH had a small impact on NPC viscosity at 22.5 g/kg total proteins whatever the temperature, which could be expected at this low total protein concentration (inferior to the one of cow's milk that is around 32 g/kg). Moreover, table 4 shows that viscosity of NPC decreases with temperature, which is the common behaviour of proteic solutions. It seems that increase the pH to pH 12 lead to a small decrease of viscosity compare to the pH 11

The impact of pH on viscosity of NPC at 55 g/kg total proteins was also investigated at 4, 25, and 50 °C (table 5).

The decrease in viscosity at higher temperature was also found for the NPC at 55 g/kg total proteins, whatever the pH. NPC viscosity was also increased at higher total protein concentration, which is logical.

Regarding the impact of pH on NPC viscosity, it was more important at 55 g/kg total proteins than at 22.5 g/kg total proteins. Whatever the temperature, viscosity increased up to pH 10: first slightly from pH 7 to 9, then markedly between pH 9 and 10 (in accordance with the beginning of micelle destabilisation, previously evidenced with DLS analyses). Between pH 10 and 11, a slight increase in viscosity was denoted at 4 °C, whereas at 25 and 50 °C viscosity markedly decreased. This difference may indicate a better stability of casein micelles at 4 °C: it can be presumed that pH 11 is not enough at 4 °C to completely disrupt swollen casein micelles. When we observed the result at pH 12, we could see that at 4°C there is a significant decrease of the viscosity, but the decrease were less significant for the other temperatures. Maybe the stability of the isolated casein is better at an higher pH at 4 °C. On

one hand, these results confirm the mechanism of casein micelle destabilisation already evidenced with DLS analyses. On the other hand, they show that the differential precipitation process should be carried out at low protein concentration or at high temperature to avoid homogenisation issues due to elevated viscosity, which may lower the efficiency of the differential precipitation process, as previously mentioned.

HPLC quantification of caseins

The developed HPLC method intended to separate caseins between them, but also from whey proteins, in order to allow accurate quantification of each casein in untreated casein concentrates as well as supernatants resulting from the differential precipitation process. Standards of α -, β -, κ -caseins, α -lactalbumin, β -lactoglobulin, and bovine serum albumin were analysed by the HPLC method in order to identify their retention times (figure 1).

The elution order is apparent in the caption of figure 1. As expected with a C18 HPLC column, retention time increased for hydrophobic compounds: α -casein was slightly more retained than β -casein, and both were markedly more retained than κ -casein. This is in agreement with the hydrophobicity of caseins, decreasing in the following order: β -casein > α -casein > κ -casein. Whey proteins are known to be more hydrophilic than caseins, thus lower retention times could be expected from this point of view, but their smaller molecular size make them more prone to interact with the stationary phase, leading to retention times close to those of caseins. Nevertheless, a correct separation of all peaks of major caseins and whey proteins was achieved with applied elution conditions.

The knowledge of retention times of the major compounds found in NPC (α -, β -, κ -caseins, α -lactalbumin, β -lactoglobulin, bovine serum albumin) made possible their quantification from the area of related peaks on the chromatogram of NPC (figure 2).

Indeed, figure 2 shows that the peaks corresponding to three major caseins are relatively well separated on the chromatogram of NPC at a total protein concentration of 22.5 g/kg. Retention times of α - and β -caseins were rather close but the resolution was sufficient to allow a correct quantification of their respective concentrations. Modifications of the elution gradient were attempted to improve the separation of α and β -caseins, unfortunately it led to κ -casein peak spreading, decreasing its signal-to-noise ratio and strongly impairing quantification accuracy. Whey proteins were correctly separated from caseins. The NPC has a low whey concentration (only 8 % of total proteins), so peaks corresponding to whey proteins were small on the chromatogram of NPC. Figure 3 illustrates the effect of calcium in the differential precipitation process, as it displays the chromatogram obtained for the resulting supernatant at a calcium/casein molar ratio of 40.

It is worth noting that the chromatogram obtained by performing the differential precipitation process without adding calcium was similar to native NPC, the only difference being that peaks were slightly broader but peak resolution remained sufficient for a correct quantification of caseins (resolution of α - and β -casein peaks was equal to 1.13). Calcium addition during the differential precipitation process had a marked impact on casein peaks: a significant decrease in α - and β -casein peak areas was observed (α -casein seemed a little more affected by calcium precipitation), whereas κ -casein was less affected by the presence of calcium (this will be confirmed in the next section through κ -casein concentration calculation). This demonstrates the efficiency of the differential precipitation process using calcium to fractionate casein concentrates: the relative proportion of κ -casein seemed increased in the supernatant phase, while α - and β -caseins should be enriched in the centrifugation pellet. The next section will present the main assays of differential precipitation process and the HPLC method improved in this study was employed to quantify caseins in resulting supernatant but also in the pellet resolubilized by acid addition.

Application of the differential precipitation process to NPC

The differential precipitation process of Post et al. (2012), using molar NaOH for alkalisation, 0.7 M calcium chloride for calcium addition, and molar HCl for acidification, was applied to NPC at a total protein concentration close to the one of milk (22.5 g/kg of total proteins, among which 20.7 g/kg of caseins). The evolution of the proportion of precipitated caseins, deduced from HPLC analysis of the supernatant obtained after the centrifugation step, was plotted against the molar calcium/casein molar ratio in Figure 4.

α -casein was the most impacted by calcium-induced precipitation, followed by β - and κ -caseins, as expected from calcium affinity. Indeed, at a calcium/casein molar ratio of 30, α -casein precipitation reached a plateau at 80 %, while the proportion of precipitated β - and κ -caseins was about 60 %. A further increase in calcium addition over 30 – 40 calcium/casein molar ratio led to the same proportions of precipitated caseins, indicating that this critical molar ratio range corresponded to maximal interaction level of caseins with calcium.

κ -casein precipitation was higher than expected from literature, lowering the efficiency of the separation process. Two hypotheses can be formulated to explain this pitfall. On one hand, the low κ -casein may have been sensitive to precipitation induced by acid hydrolysis: indeed, molar HCl, a strong acid at relatively high concentration, was added drop-by-drop in the NPC solution and could locally cause a marked pH decrease susceptible to hydrolyse κ -casein and make it precipitate. On the other hand, the NPC concentrate was prepared by reconstitution of a powder obtained by spray-drying, hence thermal treatments applied in the course of the manufacturing process (during pasteurisation, evaporation, and spray-drying steps) may have affected casein micelle structure and favoured the association of κ -casein with whey proteins, especially β -lactoglobulin. This chemical modification may have increased κ -casein sensitivity to calcium-induced precipitation, as β -lactoglobulin is known to strongly interact with calcium.

In order to limit the probable acid hydrolysis of κ -casein, another series of differential precipitation assays was performed with molar citric acid (Figure 5). Indeed, citric acid is a weak acid, thus local pH

increase should be reduced compared to HCl. Also, citric acid is already commonly used in the food industry, for instance in cheese factories, and benefits from a better image in food consumers' mind than chlorhydric acid. The last advantage of citric acid resides in its high affinity for calcium, which may compete with caseins for calcium binding and thus decrease the interaction of calcium with κ -casein, limiting its precipitation.

As expected, calcium-induced precipitation of caseins was reduced in the presence of citrates, which are able to complex calcium, thus lowering the proportion of free calcium able to interact with caseins. The difference between the action of HCl and citric acid is obvious when comparing figures 4 and 5: as affinity of chloride ions for calcium is very low compared to citrates, interaction of caseins with calcium is favoured upon HCl addition and thus the maximal precipitation of caseins was achieved at a lower casein/calcium molar ratio: 30 when using HCl against 40 when using citric acid. Interestingly, the proportions of precipitated caseins were increased when using citric acid for the acidification step: 88 % of α -casein, 68 % of β -casein, and 60 % of κ -casein. This was unexpected, as the weakness of citric acid should reduce acid hydrolysis of caseins and the complexation of calcium by citrates may have increased casein solubility. Actually, there are very few articles dealing with the interaction between citrates and casein but Mekmene and Gaucheron (2011) found that citrates were stronger complexants for calcium than caseins. This is consistent with the need to increase calcium addition to obtain the same level of casein precipitation when citrates were present in solution: indeed, as some calcium ions were complexed by calcium, the concentration of free calcium ions able to interact with caseins was lowered. Whatever it may be, even if more calcium needed to be employed to reach maximal casein precipitation, the use of citric acid instead of HCl permitted to precipitate more α - and β -caseins, while offering a better fractionation of β - and κ -caseins.

In order to improve the selectivity of the separation method, as local acid hydrolysis was still thought to be responsible for the excess of κ -casein precipitation, it was decided to try applying the differential precipitation process to a NPC concentrate at higher total protein concentration: 55 g/kg (figure 6).

This was expected to lower acid hydrolysis owing to the buffering capacity of caseins but also to improve HPLC quantification accuracy.

HPLC results showed that maximal α -casein precipitation equalled 81 %, while 74 % and 68 % of β - and κ -caseins, respectively, were insolubilized at the highest calcium/casein molar ratios. The precipitation of α -casein was comparable to results obtained in previously investigated separation conditions (cf. figures 4-6), but for β - and κ -caseins, an increase in the precipitation level was denoted. This may be caused by the higher protein concentration, favouring interactions between caseins themselves and thus their precipitation. Also, soluble caseins could have been entrapped in agglomerates of insoluble caseins and this phenomenon could have been intensified by the high viscosity of alkalisied casein concentrate at 55 g/kg (Table 5).

It should also be highlighted that maximal precipitation was reached from a calcium/casein molar ratio of 20, confirming that casein precipitation was markedly favoured when the concentration of the casein concentrate was increased. This seems logical, as casein precipitation in the cheese making process is known to be enhanced at higher casein concentration. The lower required volume of added calcium to reach maximal casein precipitation is particularly interesting for economical and ecological reasons.

Last, it was tried to lower the proportion of precipitated κ -casein by using 0.5 M citric acid instead of 1 M (Figure 7).

As shown by figure 7, the acid concentration had a major impact on the efficiency of the differential precipitation process. When using 0.5 M citric acid, the maximal proportion of precipitated α -casein was around 83 %. The precipitation of β - and κ -casein reached circa 65 % and 56 %, respectively. Compared to the differential precipitation process assay using molar citric acid, the proportion of precipitated α -casein was similar, but β - and κ -caseins were less precipitated when using 0.5 M citric

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3 469 acid in the acidification step. In these conditions, maximal casein precipitation was reached from a
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5 470 calcium/casein molar ratio of 30, consistently with previous results.
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8 471 In brief, differential precipitation assays showed that using a casein concentrate at a total protein
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10 472 concentration of 55 g/kg and 0.5 M citric acid in the acidification step allows fractionating caseins while
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12 473 meeting economical and ecological concerns. The relative proportion of caseins in supernatants
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14 474 resulting from these different assays was determined by HPLC quantification (table 6).
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18 475 First, it should be noted that the proportions of caseins after the differential precipitation process were
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20 476 very close to those obtained in the literature, confirming the suitability of the HPLC quantification
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22 477 method improved in the current study to characterise the casein concentrations in supernatants
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24 478 resulting from the differential precipitation process.
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27 479 Whatever the differential precipitation process conditions, the supernatants were strongly depleted
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29 480 in α -caseins, as their relative proportion was decreased from 50 % to 30 % approximately. A significant
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31 481 proportion of other caseins was precipitated too, but in a lesser extent than α -casein, leading to an
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33 482 apparent increase in their relative proportions (in general, β -casein proportion evolved from 35 % to
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35 483 45 %, whereas κ -casein proportion was increased from 15 to 22 %, approximately).
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39 484 The best results (higher amount of precipitated α -casein) were obtained at a total protein
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41 485 concentration of 22.5 g/kg when using molar citric acid, but a total protein concentration of 55 g/kg
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43 486 and 0.5 M citric acid gave very close results (the latter case even led to the highest κ -casein relative
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45 487 proportion).
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52 489 **Conclusion**
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55 490 The present study permitted to better understand the calcium-induced precipitation of caseins using
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57 491 alkalisation and acidification steps. It was confirmed by DLS and visual observations that the
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59 492 alkalinisation of the milk led to a destabilisation of casein micelles. This destabilisation is presumably
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caused by an increase in the negative charge of caseins at alkaline pH, enhancing repulsion between caseins and favouring their interaction with calcium, resulting in the formation of calcium-casein precipitates.

It was also evidenced an increase in NPC viscosity upon alkalisation, whatever the temperature and the protein concentration. This permitted to determine the best conditions for improving solution homogenisation and limiting the hydrolysis of κ -casein upon acidification occurring in the course of the differential precipitation process.

HPLC analysis of supernatants resulting from the differential precipitation process allowed showing that calcium addition preferentially precipitates α - and β -caseins, but the κ -casein was not insensitive to calcium. In optimal conditions (NPC at 55 g/kg total proteins and use of 0.5 M citric acid for the acidification step) were precipitated 83 % of α -casein, 65 % of β -casein, and 56 % of κ -casein. This permitted to obtain two casein fractions with relative proportion of caseins differing from the one of NPC. In particular, the supernatant was composed of 31 % α -casein, 45 % β -casein, and 24 % κ -casein, which is significantly different from NPC composition (49 % α -casein, 36 % β -casein, and 15 % κ -casein). This supernatant, after purification and concentration, may exhibit interesting properties, notably owing to the highest proportion of κ -casein.

Perspectives of this work concern the optimisation of alkalisation and acidification steps by investigating different kinds of acids and bases, in order to investigate the influence of acid and base strengths and concentrations, as well as their affinity for calcium. Moreover, there is a need to better understand why the precipitation of κ -casein during the differential precipitation process was so high for NPC reconstituted from powder. Thus, the influence of prior heat treatments on the propensity of κ -casein to interact with whey proteins, making it more sensitive to calcium-induced precipitation, should be investigated in future works.

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644 **Legends to Figures**

645 Figure 1: A chromatogram of all major milk proteins obtain by our HPLC method.

646 Figure 2: Separation of milk protein by using our method on a NPC solution at a protein concentration
647 of 22,5 g/kg.

648 Figure 3: A chromatographic representation of the impact of calcium on the three major caseins
649 concentration.

650 Figure 4: Evolution of the proportion of individual casein depending to the calcium ratio with NPC 22,5
651 g/kg and molar HCl.

652 Figure 5: Evolution of the proportion of individual casein depending to the calcium ratio with NPC 22,5
653 g/kg and molar citric acid.

654 Figure 6: Evolution of the proportion of individual casein depending to the calcium ratio with NPC 55
655 g/kg and molar citric acid.

656 Figure 7: Evolution of the proportion of individual casein depending to the calcium ratio with NPC 55
657 g/kg and 0,5 M citric acid.

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Table 1: Typical composition of micellar casein expressed on dry matter basis.

Table 2: Influence of pH on casein micelle size (mean diameters in number and volume) in diluted NPC (2 g/kg total proteins).

Table 3 : Casein standards size measurements (2 g/kg total proteins).

Table 4 : Newtonian viscosity of NPC at 22.5 g/kg total proteins at different temperatures (4, 25, and 50 °C) and pH (from 7 to 12).

Table 5: Newtonian viscosity of NPC at 55 g/kg total proteins at different temperatures (4, 25, and 50 °C) and pH (from 7 to 12).

Table 6: Evolution of the proportion of the different caseins in investigated selective precipitation process conditions.

Figure 1 : Chromatogram of milk protein standards. (1) κ -casein (19.6 min) / (2) BSA (21.7 min) / (3) α -lactalbumin (24.5 min) / (4) α -casein (26.2 min) / (5) β -casein (28.6 min) / (6) β -lactoglobulin (31.2 and 31.8 min).

Figure 2: Chromatogram of NPC at 22.5 g total proteins/kg. (1) κ -casein (20.2 min) / (2) BSA (21.4 min) / (3) α -casein (26.2 min) / (4) β -casein (28.6 min) / (5) β -lactoglobulin (31.2 and 31.8 min).

Figure 3: Chromatogram of the supernatant obtained from selective precipitation of NPC at a total protein concentration of 22.5 g/kg without calcium addition (straight line) and upon calcium addition at calcium/casein molar ratio of 40 (dashed line).

Figure 4: Evolution of casein precipitation upon calcium addition during the selective precipitation process for NPC at 22.5 g/kg of total proteins, using molar HCl for acidification. Circles, continuous line: α -casein; diamonds, dashed line: β -casein; triangles.

Figure 5: Evolution of casein precipitation upon calcium addition during the selective precipitation process for NPC at 22.5 g/kg using molar citric acid for acidification. Circles, continuous line: α -casein; diamonds, dashed line: β -casein; triangles, dotted li: κ -casein.

Figure 6: Evolution of casein precipitation upon calcium addition during the selective precipitation process for NPC at a total protein concentration of 55 g/kg using molar citric acid for acidification. Circles, continuous line: α -casein; diamonds, dashed line: β -casein; triangles, dotted line: κ -casein.

Figure 7: Evolution of casein precipitation upon calcium addition during the selective precipitation process for NPC at a total protein concentration of 55 g/kg, using 0.5 M citric acid for acidification. Circles, continuous line: α -casein; diamonds, dashed line: κ -casein.

Table 1

Total caseins	92
α_{S1} -casein (% w/w)	33.0
α_{S2} -casein (% w/w)	11.0
β -casein (% w/w)	33.0
κ -casein (% w/w)	11
γ -casein (% w/w)	4.0
Total minerals	8.0
Calcium (% w/w)	3.0
Magnesium (% w/w)	0.2
Phosphates (% w/w)	4.3
Citrates (% w/w)	0.5

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Table 2

pH	7	8	9	10	11	12
Mean diameter in number (nm)	173 ± 18	168 ± 13	171 ± 10	31 ± 10	38 ± 17	16 ± 4
Mean diameter in volume (nm)	215 ± 7	223 ± 8	237 ± 7	282 ± 24	366 ± 77 (55 %)	6.5 ± 1.9
					55 ± 22 (45 %)	

Table 3

Sample	α -casein	β -casein	κ -casein
Mean diameter in number (nm)	12 ± 3	10 ± 3	13 ± 3
Mean diameter in volume (nm)	14 ± 4 (96 %)	12 ± 4 (96 %)	16 ± 5 (75 %)
	88 ± 25 (1 %)	116 ± 90 (4 %)	81 ± 31 (13 %)
	424 ± 100 (3 %)	/	266 ± 106 (11 %)

Table 4

pH	Dynamic viscosity (mPa.s at 4 °C)	Dynamic viscosity (mPa.s at 25 °C)	Dynamic viscosity (mPa.s at 50 °C)
7	3.1 ± 0.0	1.9 ± 0.0	0.9 ± 0.0
8	4.3 ± 0.0	1.7 ± 0.0	0.9 ± 0.0
9	4.6 ± 0.0	1.9 ± 0.0	1.1 ± 0.0
10	15.1 ± 0.0	6.6 ± 0.0	2.2 ± 0.0
11	9.5 ± 0.0	5.4 ± 0.0	2.6 ± 0.0
12	6.2 ± 0.0	3.9 ± 0.0	2.1 ± 0.0

Table 5

pH	Dynamic viscosity (mPa.s at 4 °C)	Dynamic viscosity (mPa.s at 25 °C)	Dynamic viscosity (mPa.s at 50 °C)
7	5.1 ± 0.2	2.6 ± 0.0	1.6 ± 0.1
8	10.4 ± 0.2	6.3 ± 0.1	2.1 ± 0.2
9	16.1 ± 1.8	13.5 ± 0.7	4.5 ± 1.4
10	50.7 ± 2.9	37.0 ± 2.5	13.3 ± 0.4
11	59.9 ± 10.0	12.1 ± 1.9	4.5 ± 0.2
12	28.3 ± 1.1	9.4 ± 0.4	3.3 ± 0.4

Table 6

Differential precipitation process conditions	Calcium/casein molar ratio (-)	Proportion of α-casein (%)	Proportion of β-casein (%)	Proportion of κ-casein (%)
NPC 22.5 g/kg HCl 1 M	0	50 ± 2	36 ± 1	14 ± 2
	40	31 ± 2	49 ± 3	20 ± 1
NPC 22.5 g/kg Citric acid 1 M	0	51 ± 3	38 ± 2	11 ± 2
	40	27 ± 3	51 ± 4	22 ± 2
NPC 55 g/kg Citric acid 1 M	0	47 ± 2	37 ± 2	16 ± 2
	40	37 ± 3	41 ± 4	22 ± 2
NPC 55 g/kg Citric acid 0.5 M	0	49 ± 2	36 ± 3	15 ± 2
	40	31 ± 3	45 ± 4	24 ± 3

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7