

## Physicochemical characterization of calcium-supplemented skim milk

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**Abstract** – Addition of  $\text{CaCl}_2$  to skim milk, from 0 to  $13.5 \text{ mmol}\cdot\text{kg}^{-1}$ , leads to significant physicochemical changes in milk salts equilibrium. Indeed, acidification, insolubilizations of calcium, inorganic phosphate and citrate are observed. Calculation of ion equilibria in the resulting milk ultrafiltrates indicates that the observed ion migrations, controlled by solubilities of calcium-phosphate and calcium-citrate, do not cause change in ion activity products of dicalcium-phosphate (calculated as  $\text{CaHPO}_4\cdot 2\text{H}_2\text{O}$  and  $\text{Ca}(\text{HPO}_4)_{0.7}(\text{PO}_4)_{0.2}$ ). In the same way, the acidification of calcium-supplemented milks in the range of pH 6.7–3.5 shows a solubilization of calcium-phosphate similar to non-enriched milk. So whatever the added calcium concentration, the behaviour of colloidal calcium-phosphate seems to be unchanged. However, at room temperature, addition of calcium to milk originates a decrease in soluble casein concentration and tightly bound water of ultracentrifuged pellets. The consequences are (i) an increase in milk protein hydrophobicity; (ii) a decrease in zeta potential of casein micelles, without modification of their hydrodynamic diameter; and (iii) an increase in milk lightness and turbidity. Furthermore, calcium supplementation of milk seems to lead to a proteinic reorganization of micellar casein, responsible for an increase in micellar density.

**Milk / calcium / physicochemical characterization / calcium phosphate / acidification / supplementation**

**Résumé** – **Caractérisation physicochimique d'un lait écrémé enrichi en calcium.** L'addition de  $\text{CaCl}_2$  à du lait écrémé, pour des concentrations allant de 0 à  $13,5 \text{ mmol}\cdot\text{kg}^{-1}$ , induit d'importants changements physicochimiques du lait. En effet, une acidification, une insolubilisation du calcium, du phosphate inorganique et du citrate sont observées. Le calcul des équilibres ioniques dans les ultrafiltrats des différents laits montre que les flux ioniques observés, contrôlés principalement par les solubilités du phosphate et du citrate de calcium, ne modifient pas les produits d'activité ionique du phosphate dicalcique (calculés pour  $\text{CaHPO}_4\cdot 2\text{H}_2\text{O}$  et  $\text{Ca}(\text{HPO}_4)_{0.7}(\text{PO}_4)_{0.2}$ ). De la même manière, l'acidification de ces laits supplémentés en calcium entre pH 6,7 et 3,5 montre une solubilisation du phosphate de calcium similaire à celle d'un lait non enrichi. Quelle que soit la concentration en calcium ajouté, le comportement du phosphate de calcium colloïdal semble donc inchangé. Par ailleurs, l'addition de calcium à du lait écrémé est responsable, à température ambiante, d'une diminution de la concentration en caséines ultracentrifugeables et de l'eau

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fortement liée aux culots d'ultracentrifugation. Les conséquences sont (i) une augmentation de l'hydrophobicité des protéines laitières; (ii) une diminution du potentiel zéta des micelles de caséines, sans modification de leur diamètre hydrodynamique; et (iii) une augmentation de la blancheur et de la turbidité du lait. Une supplémentation en calcium du lait conduirait également à un réarrangement protéique au sein des micelles de caséines, responsable d'une augmentation de la densité micellaire.

## **Lait / calcium / caractérisation physicochimique / phosphate de calcium / acidification / supplémentation**

### **1. INTRODUCTION**

In milk, calcium is present in various forms [18]. For a total concentration of  $32 \text{ mmol}\cdot\text{L}^{-1}$ ,  $22 \text{ mmol}\cdot\text{L}^{-1}$  are in the colloidal state and  $10 \text{ mmol}\cdot\text{L}^{-1}$  are diffusible. Only  $2 \text{ mmol}\cdot\text{L}^{-1}$  of this diffusible calcium are free ionic  $\text{Ca}^{2+}$ . The remainder is essentially complexed with citrate, phosphate, caseins or whey proteins. In the colloidal state, calcium can be complexed with phosphoester, carboxyl groups of micellar caseins or with colloidal phosphate and citrate associated with casein micelles.

In the dairy industry, enrichment of milk with calcium is a common practice in order to improve the functional, technological and sometimes nutritional properties of milk [25, 30, 38]. To describe the effects of calcium addition to milk, numerous physicochemical studies have been carried out [3, 4, 7, 8, 10, 11, 14, 15, 21, 23, 24, 26, 27, 31, 34, 35, 37, 39]. These studies principally showed that addition of calcium to milk resulted in a new distribution of ions between milk serum and casein micelles, leading to physicochemical changes in casein micelles.

In the present study,  $\text{CaCl}_2$  was added, at room temperature, to raw skim milk in a concentration range from 0 to  $13.5 \text{ mmol}\cdot\text{kg}^{-1}$ . A computer program [19] was used to (i) quantify free ionic forms (ionic calcium, free inorganic phosphate and free citrate) and free salts (ultrafiltrable calcium-phosphate and calcium-citrate); and (ii) calculate log (ion activity product) for colloidal calcium phosphate regarded as dicalcium phosphate ( $\text{CaHPO}_4\cdot 2\text{H}_2\text{O}$  and  $\text{Ca}(\text{HPO}_4)_{0.7}(\text{PO}_4)_{0.2}$ ) and tricalcium phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ ).

Information on the behaviour of colloidal calcium phosphate of calcium-supplemented milks was also obtained by calcium and phosphate solubilizations during acidification. After calcium addition, distributions of caseins and water between milk serum and casein micelles were determined as well as changes in (i) the hydrophobicity of milk proteins; (ii) the zeta potential and average diameter of casein micelles; and (iii) the lightness and turbidity of milk, in order to relate changes in ion, protein and water distributions with physicochemical properties of milk and casein micelles. Special attention was paid to the acidification effect occurring during calcium addition.

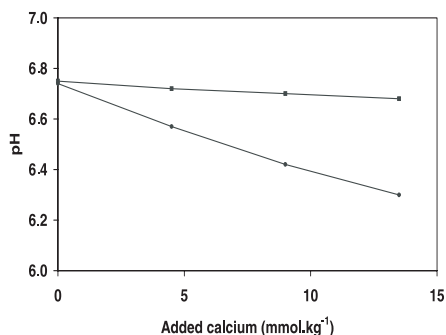
### **2. MATERIALS AND METHODS**

#### **2.1. Skim milk and calcium**

Raw whole milk was obtained from Triballat (Noyal-sur-Vilaine, France). It was skimmed at  $40^\circ\text{C}$  on a centrifugal separator (Elecrem 3, Vanves, France), and 0.03% thiomersal (w/w) (Sigma, Saint Louis, USA) was added to prevent bacterial growth.  $\text{CaCl}_2$  was purchased from Merck (Darmstadt, Germany), and a stock solution of calcium ( $1 \text{ mol}\cdot\text{L}^{-1}$ , pH 4.61) was prepared just before its addition to the skim milk.

#### **2.2. Preparation of calcium-supplemented skim milks and their ultra-centrifugates and ultrafiltrates**

Skim milk was supplemented, at room temperature, with  $\text{CaCl}_2$  to reach final



**Figure 1.** Influence of added calcium concentration on milk pH. (●) pH not readjusted and (■) pH readjusted to about 6.75 after calcium addition.

added calcium concentration ranging from 0 to 13.5 mmol.kg<sup>-1</sup>. The dilution caused by this CaCl<sub>2</sub> addition was kept constant for each sample by adding an appropriate amount of Milli Q-water. These mixtures were vigorously stirred to ensure rapid and complete mixing. One hour after calcium addition, the pH of an aliquot of the samples was adjusted to about 6.75 with 1 mol.L<sup>-1</sup> NaOH. The other part of samples was not adjusted in pH (Fig. 1). In parallel, a sample that was not enriched with calcium was acidified to pH 6.35 with 1 mol.L<sup>-1</sup> HCl. Milli Q-water was added to this acidified milk to have a dilution equal to that obtained in the calcium-supplemented milks. Both types of calcium-supplemented skim milk (with or without pH adjustment) and the acidified milk were analysed in parallel. Before analysis or acidification (section 2.4), the different calcium-supplemented milks (with or without pH adjustment) were left standing overnight at room temperature. Then, pH values were readjusted if necessary just before analysis. Preparations of the different calcium-supplemented milks and subsequent analyses were carried out in duplicate.

To characterize the serum of the different calcium-supplemented skim milks, ultracentrifugation of these samples was

carried out at 100 000 g (Beckman L-8-55 ultracentrifuge, Gagny, France) for 1 h at 20 °C. Each supernatant was recovered and filtered on a 0.42 µm filter (Sartorius AG, Göttingen, Germany) before analysis, or ultrafiltered on an Ultra free 15 membrane (molecular mass cut-off: 10 000 g.mol<sup>-1</sup>) (Millipore, Saint-Quentin-en-Yvelines, France) after centrifugation at 1 800 g for 30 min at 20 °C.

### 2.3. Determination of cation and anion contents

Cation concentrations (calcium, magnesium, sodium and potassium) in the ultrafiltrates were determined by atomic absorption spectrometry (Varian AA300 spectrometer, Les Ulis, France) [5], and chloride, inorganic phosphate and citrate concentrations by ion chromatography [12]. The experimental error for cation and anion determinations was ± 5%. Concentrations in colloidal ions were deduced from the differences between total ion concentrations and diffusible ion concentrations measured in the ultrafiltrates (ion concentrations in ultrafiltrates were converted into diffusible ion concentrations by multiplying by a 0.96 correcting factor, in order to take into account the excluded volume, as described by Pierre and Brulé [29]).

### 2.4. Study of the behaviour of colloidal calcium phosphate

The behaviour of colloidal calcium phosphate was studied by calcium and phosphate solubilizations during acidification by 1 mol.L<sup>-1</sup> HCl (from pH 6.7 to 3.5) of skim milks supplemented with 0, 4.5, 9 and 13.5 mmol.kg<sup>-1</sup> CaCl<sub>2</sub>. After acidification, samples were left standing overnight at room temperature. Then, calcium and phosphate concentrations were determined on the milk serum obtained by ultrafiltration, as described previously. Relations between colloidal calcium concentration and colloidal inorganic phosphate concentration in the pH range 6.7–5.4 were also

determined. Concentrations in colloidal ions were deduced from the difference between total ion concentrations and ion concentrations in the ultrafiltrates. Contrary to Section 2.3, no correcting factor was used because only relative values were necessary.

### **2.5. Calculation of salt partitions and ion activity products for dicalcium phosphate ( $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ and $\text{Ca}(\text{HPO}_4)_{0.7}(\text{PO}_4)_{0.2}$ ) and tricalcium phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ )**

From ion concentrations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{Pi}$  and citrate) measured in the ultrafiltrates of the different calcium-supplemented milks, a computer program was used to calculate the ion partitions and activities of each ion [19]. From the activities, and in the pH range 6.7–5.4, the ion activity products for dicalcium phosphate ( $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  and  $\text{Ca}(\text{HPO}_4)_{0.7}(\text{PO}_4)_{0.2}$ ) and tricalcium phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ ) were calculated [6, 19].

Statistical tests (linear model with 2 variables: added calcium (qualitative variable with 4 levels) and pH (covariable)) were applied.

### **2.6. Analysis of phosphorylated compounds**

$^{31}\text{P}$  nuclear magnetic resonance ( $^{31}\text{P}$ -NMR) spectra of non-enriched milk and milk supplemented with  $13.5 \text{ mmol} \cdot \text{kg}^{-1}$  calcium (with and without pH regulation) were conducted. Spectra were obtained on a Bruker AM 300 WB spectrometer (Wissembourg, France) operating at 121.5 MHz, with a delay time of 3 s, acquisition time of 0.254 s and 29 °C with broad-band proton decoupling. 4000 transients were accumulated, leading to an analysis time of about 3.5 h. Chemical shifts were measured relative to 85%  $\text{H}_3\text{PO}_4$  external reference;  $\text{D}_2\text{O}$  was used for the reference lock. The assignment of

the different phosphorylated compounds (peaks 1, 2 and 3) was made according to Belton et al. [1]. The area of peak 3 was assumed to be constant and corresponds to an arbitrary value of 100.

### **2.7. Determination of the nitrogen content in ultracentrifugal supernatants**

Nitrogen content in the ultracentrifugal supernatants was determined by the Kjeldahl method [20].

### **2.8. Determination of caseins ( $\alpha_{s1}$ - and $\beta$ -caseins) and whey proteins ( $\alpha$ -lactalbumin and $\beta$ -lactoglobulin) in ultracentrifugal supernatants**

Reverse-phase high-performance liquid chromatography (RP-HPLC) analysis was carried out as described by Gaucheron et al. [13]. Each chromatographic peak was integrated and expressed in %. The chromatographic peak areas corresponding to each individual casein ( $\alpha_{s1}$ - and  $\beta$ -caseins) and to  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin present in the ultracentrifugal supernatant of non-enriched milk corresponded to an arbitrary value of 100.

### **2.9. Determination of tightly bound water of ultracentrifuged pellets**

From the wet pellet obtained by ultracentrifugation, the tightly bound water of ultracentrifuged pellet was determined as follows: the drained casein pellet was weighed and then dried at 103 °C for 7 h. The difference between the weight before and after drying, expressed as g of water per g of dry pellet was taken as the amount of tightly bound water of ultracentrifuged pellets. The experimental error was  $\pm 0.02 \text{ g}$ .

### **2.10. Determination of milk lightness**

A microcolor tristimulus colorimeter (Minolta chromameter CR-300, Carrières-

sur-Seine, France) was used for color testing. Calibration was performed using the Minolta calibration plate (standard tristimulus values:  $Y = 92.4$ ;  $x = 0.3161$ ;  $y = 0.3325$ ). Results were expressed using the  $L^*$  value which defines the position of the sample on the light-dark axis. The experimental error for the milk lightness determination was  $\pm 0.4$ .

### 2.11. Determination of milk turbidity

The turbidity of the different milks was measured at 500 nm (Uvikon spectrophotometer UV 922, Saint-Quentin-en-Yvelines, France) immediately after diluting the sample sixty-three-fold with its corresponding ultrafiltrate. The experimental error for the milk turbidity determination was  $\pm 0.003$ .

### 2.12. Determination of protein hydrophobicity

The spectrofluorimetric measurements were made on a LS 50B Perkin Elmer spectrofluorimeter (Saint-Quentin-en-Yvelines, France). The exposure of hydrophobic groups was measured by the fluorescent probe binding method [2] using fluorophor 8-aniline naphthalene 1-sulphonate (ANS) (Sigma, St Louis, USA). ANS is known to bind hydrophobic areas accessible to the aqueous solvent. Upon binding, its fluorescence is dramatically enhanced so that exposed hydrophobic surface areas may be quantitatively determined. Experimentally, 3 mg of the different calcium-supplemented milks, supplemented with an aqueous solution of ANS, were diluted into their ultrafiltrates to a final weight of 3 g. The final concentration of ANS was  $200 \mu\text{mol}\cdot\text{kg}^{-1}$ . Samples were immediately analyzed at excitation and emission wavelengths of 390 nm and 480 nm, respectively. The emission and excitation slits were both set at 2.5 nm bandwidth. The experimental error for the relative fluorescence intensity was  $\pm 10$  units.

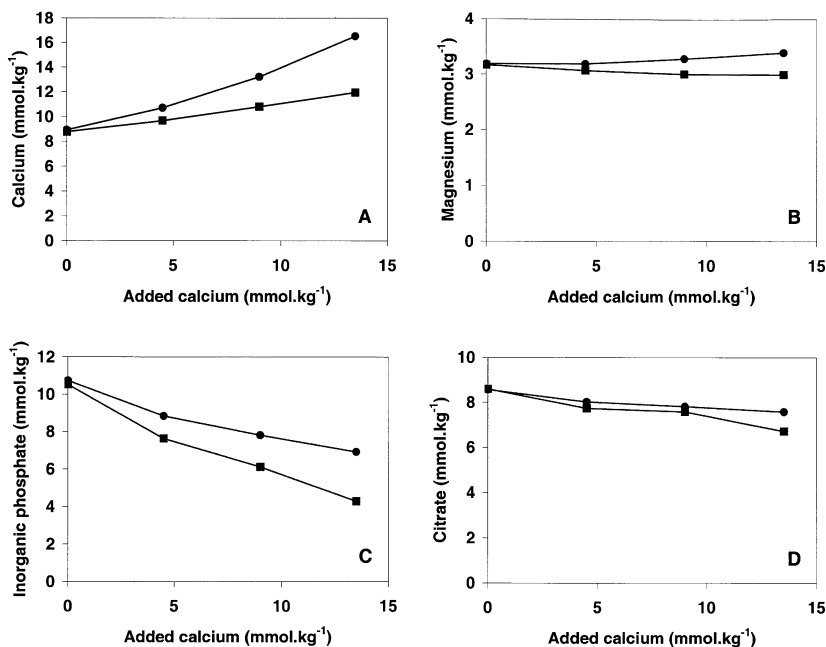
### 2.13. Determinations of zeta potential and average diameter of casein micelles

The zeta potential and average diameter of casein micelles were determined using a Zetasizer 3000 HS (Malvern instrument, Malvern, UK) equipped with palladium electrodes and an avalanche photodiode detector, enhancing sensitivity. Before measurements, samples were prepared by suspending typically 5  $\mu\text{L}$  of the different milks in about 10 mL of their corresponding ultrafiltrates. Each sample was filtered on 0.80  $\mu\text{m}$  pore size filter (Pall, Cortland, USA) and measured 5 times at 25 °C. For zeta potential determination, the results were expressed in absolute values and the experimental error was  $\pm 0.5$  mV. The experimental error for diameter determination was  $\pm 7$  nm.

## 3. RESULTS

### 3.1. Ionic equilibria

Addition of calcium chloride resulted in milk pH decrease. For example, a supplementation of  $13.5 \text{ mmol}\cdot\text{kg}^{-1}$   $\text{CaCl}_2$  decreased milk pH from 6.75 to 6.30 (Fig. 1). When the pH was readjusted to 6.75, addition of calcium to milk in the range of  $4.5\text{--}13.5 \text{ mmol}\cdot\text{kg}^{-1}$  led to a binding of 80% of the added calcium to casein micelles. Thus, whatever the added calcium concentration, 20% stayed in the ultrafiltrate, resulting in a linear relationship between ultrafiltrable calcium and added calcium ( $+0.24 \text{ mmol}\cdot\text{kg}^{-1}$  calcium/ $\text{mmol}\cdot\text{kg}^{-1}$  added calcium, Fig. 2A). Concomitant transfers of magnesium (Fig. 2B), inorganic phosphate (Fig. 2C) and citrate (Fig. 2D) were, respectively,  $-0.01$ ,  $-0.45$  and  $-0.13 \text{ mmol}\cdot\text{kg}^{-1}/\text{mmol}\cdot\text{kg}^{-1}$  added calcium. Displacement of inorganic phosphate was confirmed by  $^{31}\text{P}$ -NMR measurements: a decrease of 67% of free inorganic phosphate was observed after addition of  $13.5 \text{ mmol}\cdot\text{kg}^{-1}$   $\text{CaCl}_2$  and pH regulation

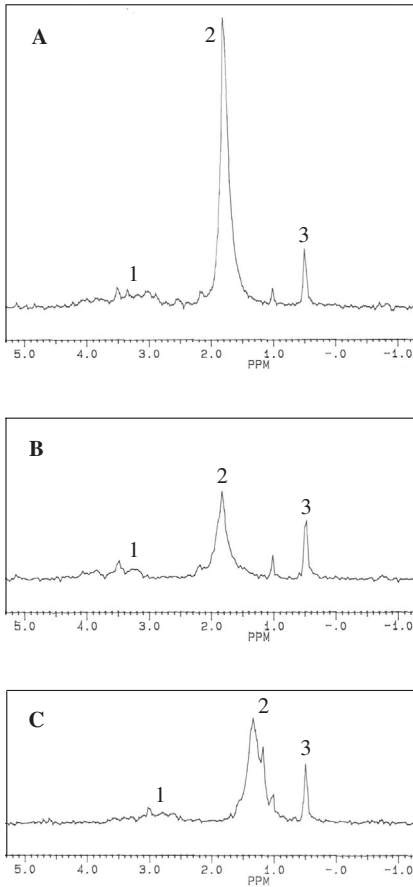


**Figure 2.** Influence of added calcium concentration on calcium (A), magnesium (B), inorganic phosphate (C) and citrate (D) concentrations in the milk ultrafiltrates. (●) pH not readjusted and (■) pH readjusted to about 6.75 after calcium addition.

(Fig. 3B). From the ion concentrations of the different ultrafiltrates, theoretical concentrations of free and complexed forms of calcium, magnesium, inorganic phosphate and citrate were calculated (Tab. I). Simultaneous increases in ionic calcium ( $\text{Ca}^{2+}$ ) and magnesium ( $\text{Mg}^{2+}$ ) and decreases in free inorganic phosphate ( $\text{H}_2\text{PO}_4^-$ ,  $\text{HPO}_4^{2-}$ ) and free citrate ( $\text{Cit}^{3-}$ ,  $\text{HCit}^{2-}$ ) were determined. A decrease in associated forms of inorganic phosphate and citrate with calcium and magnesium was also observed.

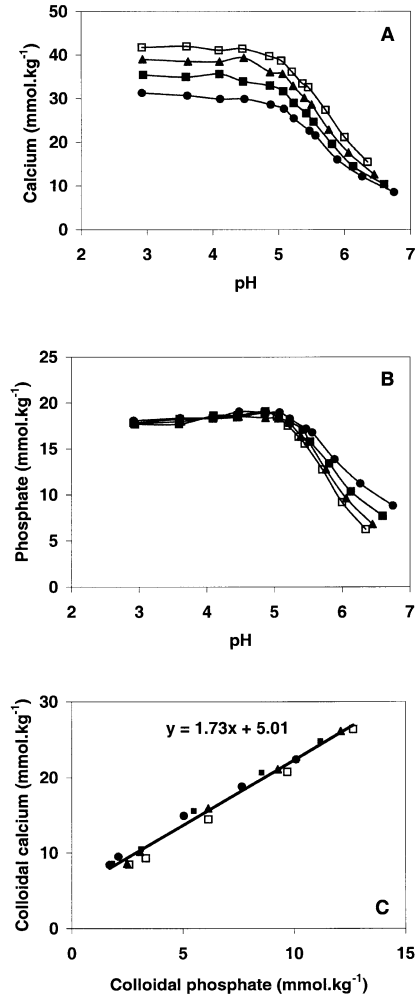
When the pH was not regulated to about 6.75, respective calcium bindings of 63%, 54% and 46% were determined after  $\text{CaCl}_2$  additions of 4.5, 9 and 13.5  $\text{mmol}\cdot\text{kg}^{-1}$ . These bindings, dependent on added calcium concentration, resulted in a linear relationship between ultrafiltrable calcium and added calcium with a slope of

+0.56  $\text{mmol}\cdot\text{kg}^{-1}$  calcium/ $\text{mmol}\cdot\text{kg}^{-1}$  added calcium (Fig. 2A). In parallel, solubilization of 0.01  $\text{mmol}\cdot\text{kg}^{-1}$  of magnesium/ $\text{mmol}\cdot\text{kg}^{-1}$  added calcium was calculated (Fig. 2B), and displacements of inorganic phosphate (Fig. 2C) and citrate (Fig. 2D) from the milk serum to the casein micelles were observed: the calculated slopes were  $-0.30$   $\text{mmol}\cdot\text{kg}^{-1}$  inorganic phosphate/ $\text{mmol}\cdot\text{kg}^{-1}$  added calcium and  $-0.07$   $\text{mmol}\cdot\text{kg}^{-1}$  citrate/ $\text{mmol}\cdot\text{kg}^{-1}$  added calcium. Displacement of inorganic phosphate towards the casein micelles was also confirmed by  $^{31}\text{P}$ -NMR measurements: decrease of 52% of free inorganic phosphate was observed after addition of 13.5  $\text{mmol}\cdot\text{kg}^{-1}$   $\text{CaCl}_2$  (Fig. 3C). As shown for pH-regulated milks, increases in free ionic forms were observed. On the other hand, increases in associated forms of inorganic phosphate and citrate with calcium and magnesium were determined (Tab. I).



**Figure 3.** <sup>31</sup>P-NMR spectra of non-enriched milk (A), milk enriched with 13.5 mmol·kg<sup>-1</sup> and readjusted in pH (to about 6.75) (B), milk enriched with 13.5 mmol·kg<sup>-1</sup> and not regulated in pH (C). According to Belton et al. [1], peaks 1, 2 and 3 correspond to phosphoserine residues, inorganic phosphate and to a phosphate diester, respectively. Area of peak 3 was assumed to be constant and corresponded to 100.

For each type of milk, ionic equilibria were also studied versus pH: whatever the added calcium concentration, the solubilization curves of calcium and inorganic phosphate versus pH were similar (Figs. 4A and 4B). Calcium and inorganic phosphate were totally solubilized at pH values of



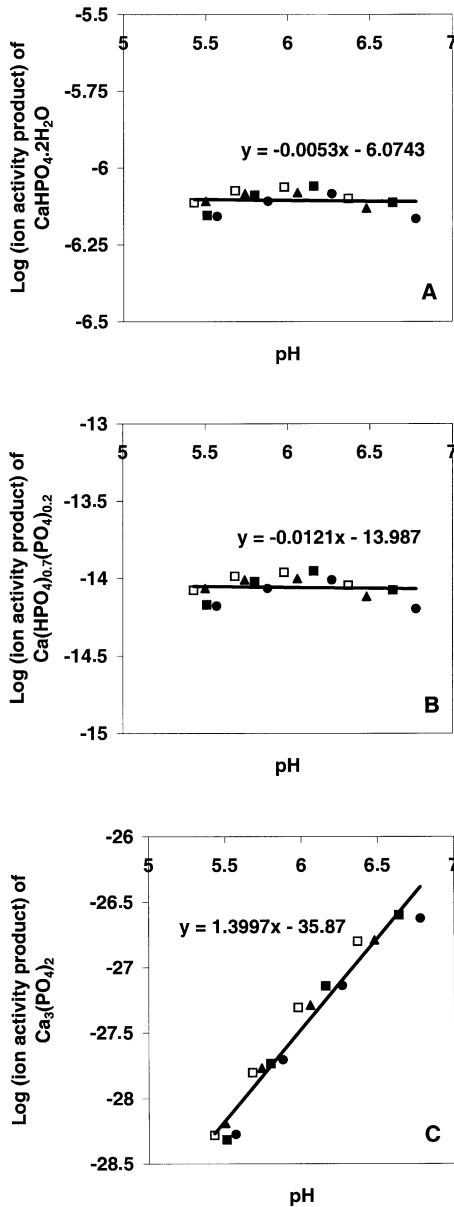
**Figure 4.** Effect of acidification of calcium-supplemented milks on the calcium (A) and inorganic phosphate (B) concentrations in the ultrafiltrates. Relation between colloidal calcium concentration and colloidal inorganic phosphate concentration in a pH range of 6.7–5.4 (C). Acidification was carried out on non-enriched milk (●), milk enriched with 4.5 mmol·kg<sup>-1</sup> (■), 9 mmol·kg<sup>-1</sup> (▲), and 13.5 mmol·kg<sup>-1</sup> (□) of calcium.

about 4.0 and 5.0, respectively. According to the added calcium concentration, relations between colloidal calcium and colloidal

**Table 1.** Theoretical concentrations ( $\text{mmol}\cdot\text{kg}^{-1}$ ) of the different associations of calcium, inorganic phosphate and citrate in the ultrafiltrate as a function of added calcium concentration. These values were determined as described by Holt et al. [19], from the ion concentrations measured in ultrafiltrates. Indice\* corresponds to milks whose pH was adjusted to about 6.75 after calcium addition. Symbol + is indicated when the concentration calculated was less than  $0.005 \text{ mmol}\cdot\text{kg}^{-1}$ .

Ca added	$\text{H}_2\text{Cit}^-$		$\text{HCit}^{2-}$		$\text{Cit}^{3-}$		$\text{H}_2\text{PO}_4^-$		$\text{HPO}_4^{2-}$		$\text{PO}_4^{3-}$		Free cation						
	Free anion	Ca complex	Free anion	Ca complex	Free anion	Ca complex	Free anion	Ca complex	Free anion	Ca complex	Free anion	Ca complex	Free anion	Ca complex					
0	+	+	0.05	0.02	+	0.27	6.01	1.98	6.34	0.05	0.03	2.39	0.45	0.30	+	0.01	+	1.56	0.74
4.5	+	+	0.04	0.02	+	0.15	6.03	1.70	5.70	0.08	0.04	1.47	0.49	0.28	+	+	+	2.86	1.08
9.0	+	+	0.04	0.02	+	0.11	6.19	1.37	5.30	0.12	0.04	0.99	0.50	0.24	+	+	+	4.56	1.47
13.5	+	+	0.03	0.03	+	0.08	6.25	1.16	5.00	0.16	0.05	0.73	0.52	0.21	+	+	+	6.94	1.74
4.5*	+	+	0.02	0.01	+	0.14	5.27	1.76	4.08	0.05	0.03	1.48	0.47	0.28	+	+	+	2.67	0.93
9.0*	+	+	0.03	0.01	+	0.14	5.75	1.52	3.45	0.05	0.02	1.23	0.42	0.24	+	+	+	3.11	1.12
13.5*	+	+	0.01	0.01	+	0.08	4.81	1.29	2.13	0.05	0.02	0.73	0.38	0.19	+	+	+	4.74	1.33





**Figure 5.** Influence of pH on log (ion activity products) calculated as dicalcium phosphate: (A) CaHPO<sub>4</sub>·2H<sub>2</sub>O, (B) Ca(HPO<sub>4</sub>)<sub>0.7</sub>(PO<sub>4</sub>)<sub>0.2</sub>; and tricalcium phosphate (C) Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> in the different calcium-supplemented milks. (●) non-enriched milk, (■) milk enriched with 4.5 mmol·kg<sup>-1</sup>, (▲) 9 mmol·kg<sup>-1</sup>, and (□) 13.5 mmol·kg<sup>-1</sup> of calcium.

inorganic phosphate concentrations in pH-adjusted (range 6.7–5.4) milks were also presented. As a function of added calcium concentration (0, 4.5, 9 and 13.5 mmol·kg<sup>-1</sup>), the same linear relationship between the two components was found: equations were not significantly different with a slope of 1.73 mmol·kg<sup>-1</sup> colloidal calcium/mmol·kg<sup>-1</sup> colloidal inorganic phosphate (Fig. 4C). In the same pH range, ion activity products for colloidal calcium phosphate were calculated for stoichiometry of dicalcium phosphate (CaHPO<sub>4</sub>·2H<sub>2</sub>O and Ca(HPO<sub>4</sub>)<sub>0.7</sub>(PO<sub>4</sub>)<sub>0.2</sub>) and tricalcium phosphate (Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>). The ion activity products of dicalcium phosphate were not statistically different as a function of added calcium concentration and pH at the 5% level of significance (Figs. 5A and 5B). Furthermore, the slopes (–0.0053 and –0.0121) were not different from 0. However, although the ion activity products of tricalcium phosphate were not statistically different on calcium concentration at the same level of significance, a dependence on pH was found (slope of 1.3997; Fig. 5C).

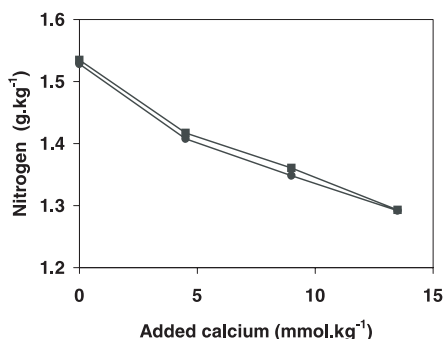
### 3.2. Protein distribution

A decrease in nitrogen content in ultra-centrifugal supernatants was observed after calcium addition to milk (Fig. 6). RP-HPLC analyses of supernatants showed no changes in α-lactalbumin and β-lactoglobulin contents, but a decrease of 70% in caseins (α<sub>s1</sub>- and β-caseins) after addition of 13.5 mmol·kg<sup>-1</sup> CaCl<sub>2</sub>. This decrease was not pH-dependent (Fig. 7).

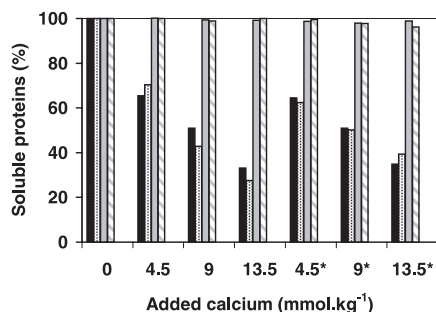
In the same way, analysis of phosphorylated compounds by <sup>31</sup>P-NMR of milks after calcium addition showed a decrease of about 45% of free seryl phosphate residues of caseins (Fig. 3).

### 3.3. Tightly bound water of the ultracentrifuged pellet

The tightly bound water of the ultracentrifuged pellet of skim milk decreased

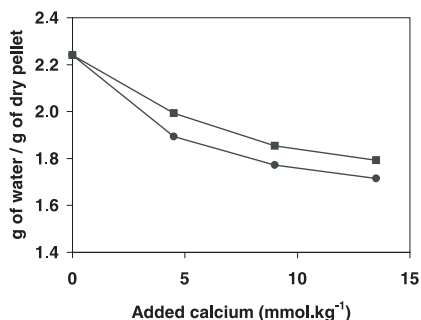


**Figure 6.** Influence of added calcium concentration on nitrogen content in the ultracentrifugal supernatants of the different calcium-supplemented milks. (●) pH not readjusted and (■) pH readjusted to about 6.75 after calcium addition.



**Figure 7.** Influence of added calcium concentration on % of  $\alpha_{s1}$ - and  $\beta$ -caseins,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin in the ultracentrifugal supernatants of the different calcium-supplemented milks. The chromatographic peak areas of caseins,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin present in the ultracentrifugal supernatant of non-enriched milk corresponded to 100%.  $\alpha_{s1}$ -caseins (■);  $\beta$ -caseins (▤);  $\alpha$ -lactalbumin (▨) and  $\beta$ -lactoglobulin (▩). Indices\* corresponds to milks whose pH was readjusted to about 6.75 after calcium addition.

when added calcium concentration increased and pH decreased. For example, 13.5 mmol.kg<sup>-1</sup> CaCl<sub>2</sub> addition allowed a decrease of 0.53 g of water/g of dry pellet for pH-unadjusted milk against 0.45 for pH-adjusted milk (Fig. 8).



**Figure 8.** Influence of added calcium concentration on tightly bound water of ultracentrifuged pellet. (●) pH not readjusted and (■) pH readjusted to about 6.75 after calcium addition.

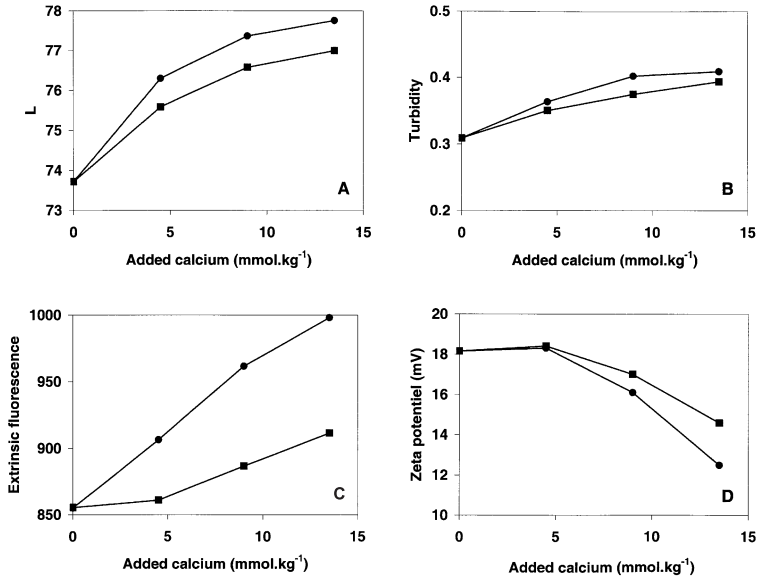
### 3.4. Physicochemical characteristics of milk and casein micelles

Addition of calcium led to an increase in milk lightness, turbidity and extrinsic fluorescence as well as a decrease in the zeta potential of casein micelles (Figs. 9A, 9B, 9C and 9D). These physicochemical characteristics of milk were also pH-dependent: addition of 13.5 mmol.kg<sup>-1</sup> CaCl<sub>2</sub> with pH regulation showed increases of 4.4%, 28.6% and 6.7% for, respectively, milk lightness, turbidity and extrinsic fluorescence and a decrease of 19.6% for the zeta potential of casein micelles. Without pH regulation, the respective increases were 5.5%, 32.4% and 16.8% and the decrease in zeta potential was 31.3%. These modifications did not lead to a change in the measured average diameter of casein micelles (178 ± 7 nm).

## 4. DISCUSSION

### 4.1. Influence of calcium addition on the behaviour of colloidal calcium phosphate

At room temperature, whatever the added calcium concentration and pH, the molar



**Figure 9.** Influence of added calcium concentration on milk lightness (A), milk turbidity (B), exposure of protein hydrophobic regions (C), and zeta potential of casein micelles (D). (●) pH not readjusted and (■) pH readjusted to about 6.75 after calcium addition.

ratio of transferred calcium/transferred inorganic phosphate from milk serum to casein micelles was  $1.80 \pm 0.07$ . These transfers led to a molar ratio of colloidal calcium/colloidal inorganic phosphate of  $1.90 \pm 0.04$ . However, such molar ratios are approximate because they do not take into account concomitant magnesium, citrate and organic phosphate transfers. Ion activity products for colloidal calcium phosphate calculated as dicalcium phosphate ( $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  and  $\text{Ca}(\text{HPO}_4)_{0.7}(\text{PO}_4)_{0.2}$ ) [19] were constant as a function of added calcium concentration and pH (Figs. 5A and 5B). These results suggest that, whatever the added calcium concentration, the colloidal calcium phosphate of calcium-enriched milks contains some dicalcium phosphate-like materials in near-equilibrium with the serum, like non-enriched milk. Such a hypothesis is in good accordance with Holt [16–18] and Chaplin [6] who found that colloidal calcium phosphate of skim milk closely resembled

brushite. In the same way, acidification of calcium-supplemented milks showed the same solubilization of colloidal calcium phosphate as that observed for reference milk: the same linear relationship between colloidal calcium and colloidal inorganic phosphate was obtained ( $1.73x \pm 5.01$ , Fig. 4C). According to these results, the behavior of colloidal calcium phosphate seemed to be unchanged by the calcium supplementation of milk. However, it is extremely unlikely that the calcium phosphate formed after addition of calcium to milk is the same as the natural micellar calcium phosphate, since about 90% of the casein phosphate groups are already incorporated in the nanoclusters (Holt, personal communication). Indeed, as described by Holt [18], a cluster of natural micellar calcium phosphate contains about 3 calcium ions for 1 phosphorylated amino acid residue. For each calcium supplementation, assuming that the 10% remaining of casein phosphate groups are mobilized in the

micellar calcium phosphate, it would be insufficient to be in the same proportion as initially. Further work is required to have some indication of the nature of the micellar calcium phosphate and consequently, to distinguish the different forms of micellar calcium phosphate (native and newly formed by calcium addition and pH adjustment). However, to our knowledge, techniques for such a study have not yet been developed.

#### **4.2. Influence of calcium addition on ion, protein and water distributions between milk serum and casein micelles**

Addition of calcium to milk resulted in a decrease in anion concentration in the ultrafiltrate (Figs. 2 and 3 and Tab. I). Similar displacements of calcium-inorganic phosphate and calcium-citrate complexes were observed after calcium or magnesium addition to milk [34, 37]. These transfers were quantitatively highly dependent on pH. Studies of complexed forms of ultrafiltrable ions give information about this pH-dependence (Tab. I). When the pH was not regulated, association of inorganic phosphate and citrate with calcium increased. This was related to a higher solubility of calcium salts at low pH. On the contrary, due to a lesser solubility of these salts after pH-regulation, association of anions with calcium decreased when the pH was regulated. These results suggest that low solubilities of calcium-phosphate and calcium-citrate resulted in a higher ion association with casein micelles. Whatever the pH and calcium concentration, this micellar casein supplementation was made possible because of calcium-salts saturation in the ultrafiltrate after calcium addition. In parallel with these incorporations of salts, a non-specific incorporation of caseins ( $\alpha_{s1}$ - and  $\beta$ -caseins) was observed (Figs. 3 and 7). Decreases in casein concentration, at room temperature, in ultracentrifugal supernatants detected by RP-HPLC or indirectly by  $^{31}\text{P}$ -NMR suggest that some casein molecules

would either become part of the existing micelles or constitute new casein structures. Release of water from casein micelles was also observed after calcium addition to milk. All these exchanges are in good accordance with the literature [14, 24, 37].

A decrease in pH value after calcium addition to milk has been described by many authors [37, 39]. It was related (i) to the formation of calcium-phosphate and calcium-citrate; (ii) to exchanges between added calcium and micellar  $\text{H}^+$ ; and (iii) to the acidity of the added calcium solution. According to the literature, the consequences of lowering milk pH with an acidifiant are solubilizations of micellar calcium, phosphate, citrate [9, 22] and magnesium [22], release of water [32, 33, 38] and caseins [9] from casein micelles to the milk serum. In the same way, comparison of non-pH-regulated milks with pH-regulated milks after calcium addition showed (i) a higher increase in ultrafiltrable calcium, an increase in ultrafiltrable magnesium and a lesser decrease in ultrafiltrable anions and (ii) a higher decrease in water of the ultracentrifuged pellet. The observed divergences of distribution between casein micelles and milk serum resulted from displacements due to either calcium alone or both calcium and pH. However, no pH effect was found on casein solubilization after calcium addition. This could be related to the fact that, considering the pKa values of phosphoserine, aspartic acid and glutamic acid residues (2.0/6.0, 4.1 and 4.6, respectively), charges were practically unmodified in the pH range studied (6.74–6.35). Thus, their affinity for calcium was unchanged.

#### **4.3. Influence of calcium addition on physicochemical characteristics of milk and casein micelles**

The hydrophobicity of milk proteins increased after calcium addition (Fig. 9C). These results indicate structural modifications of casein micelles, because when a

protein is partly disorganized, hydrophobic segments can be differently exposed. In the same way, the release of water described before may indicate an expulsion of water from cavities located in the hydrophobic core of casein micelles, caused by a solvent exposure change in casein amino acid chains. This reorganization is in good accordance with the literature. Indeed, several authors showed that rennet coagulation of heated or cooled milk was accelerated by calcium addition [3, 7, 10, 14, 15, 25, 26, 37]. This decrease in clotting time and aggregation time would be due to an acceleration of the secondary and the tertiary phases of rennet coagulation (aggregation of paracasein micelles and gel reticulation, respectively), caused by modifications of molecular interactions between paracasein micelles in the presence of calcium. Besides hydrophobic interactions, electrostatic interactions between casein micelles are modified by calcium supplementation [8, 14, 28]. Association of ionic material with casein micelles may shield the negative charge, resulting in a reduction of the micellar zeta potential. However, in the theory of zeta potential, changes in the electrophoretic mobility must be related to changes on the outer layers of the particles. Binding of calcium salt to macropeptide of the  $\kappa$ -casein cannot be exclusively envisaged because this casein is not a primary binding site for calcium. The reduction in zeta potential may likely arise from conformational changes in the micelle surface layer as a consequence of calcium binding to other parts of the surface and/or to the micellar core.

As a consequence of changes in the organization of proteins and ions in the micellar structure after calcium addition, increases in milk lightness and turbidity were observed (Figs. 9A and 9B). However, the average diameters of casein micelles were constant. In agreement with the study of Udabage et al. [35], these results suggested that (i) there was no bridge between casein micelles after calcium addition, and (ii) casein micelles were able to incorporate caseins and ions

and release water without detectable changes in their hydrodynamic diameters. Thus, from the increases in milk lightness and turbidity and in spite of the absence of variation in the average hydrodynamic diameter of casein micelles, we can suggest that the incorporation of ions and proteins into casein micelles and release of water from the casein micelles after calcium supplementation of milk increased the micellar density. These results are in good accordance with Jeurnink et al. [21] and Van Boekel et al. [36] who suggested that the decrease in voluminosity of casein micelles after calcium addition would result in a small shrinkage of these particles and thus in a more compact micelle core.

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