Effect of Salt Addition on the Micellar Composition of Milk Subjected to pH Reversible CO₂ Acidification

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ABSTRACT

Response surface methodology was used to investigate the effect of salt supplementation on the micellar composition of reconstituted skim milk subjected to acidification by CO₂ pressure to pH 5.8, followed by depressurization under vacuum. Using a Doehlert design, calcium and phosphate were added to skim milk in the range of 0 to 25 mmol/kg and 0 to 16 mmol/kg of milk, respectively, and the pH was adjusted to 6.65 ± 0.02. After carbonation, the milk sample was depressurized, and the pH returned to its initial value without modification of the ionic strength. Micellar composition was assessed by the concentration of micellar Ca, P, Mg, and protein, and the buffering properties of milk. The second order polynomial models satisfactorily predicted the effect of salt supplementation on the micellar composition ($R^2_{\text{adj}} > 0.75$). Added calcium was the most determinant factor, and favored the removal of Ca, P, Mg, and proteins from the soluble phase to the micellar phase when this addition was less than 17.5 mmol/kg of milk. Above this concentration, only the concentration of micellar Ca increased. The buffering response surface showed that the amount of micellar calcium phosphate increased to a maximum upon addition of 17.5 mmol of Ca/kg. By comparison with a control sample (supplemented but untreated skim milk), changes were essentially due to salt supplementation and not to the CO₂ treatment. We suggest that Ca formed micellar calcium phosphate when added at a concentration less than 17.5 mmol/kg; whereas above this concentration, Ca bound directly to micellar proteins.

(Key words: salt supplementation, CO₂ acidification milk, micellar mineral)

Abbreviation key: BCA = bicinehonic acid, MSD = mean standard deviation, RMSE = root mean square error, RSM = response surface methodology.

INTRODUCTION

Casein micelles represent roughly colloidal spherical particles composed of caseins and minerals in equilibrium with a soluble phase (Walstra and Jenness, 1984). Micellar calcium exists partly as a calcium phosphate salt, also containing citrate, Mg, and Zn (McGann et al., 1983), and partly as Ca bound to protein (Holt et al., 1986). This mineral fraction is essential for maintaining the micelle structure and stability as demonstrated by the dissociation effects of milk dialysis (Holt et al., 1986) or Ca-chelating agents (Ward et al., 1997). Salt and casein balance is influenced by parameters such as temperature (Pierre and Brulé, 1981; Law, 1996), pH (Visser et al., 1986; Dalglish and Law, 1989), and ionic strength (Le Graet and Brulé, 1993; Famelart et al., 1996). Many reports have been published on the effect of calcium and/or phosphate supplementation on acid or rennet milk gelation properties (Dalglish, 1983; Gastaldi et al., 1994; Lagoueyte et al., 1995), but only few reports have described the mineral partitioning between the serum and colloidal phases of milk.

Milk acidification is generally obtained by a biological process or by direct addition of acid, but some authors have used CO₂ under pressure (Jordan et al., 1987; Tomasula et al., 1999). During classic milk acidification, Gastaldi et al. (1996) observed four stages of coagulation as a function of pH. The beginning of micelle demineralization and a decrease in micelle solvation mainly mark the first stage, between pH 6.7 and pH 6.0 to 5.8. The second, from pH 5.8 to pH 5.2 is a micellar fusion state with all the colloidal calcium phosphate in a soluble form. The third stage, from pH 5.2 to pH 4.8 to 4.7, is the formation of new casein particles and the solubilization of colloidal calcium. The last one is the onset of milk gelation. Lucey et al. (1996) noted that changes in the buffer capacity of milk acidified to pH 5.5 and reneutralized to pH 6.6 were reversible in contrast to the same milk acidified to pH 5.0 to 4.6. After acidification to pH 4.9 by CO₂ treatment followed by depressurization under vacuum, the pH returns to its initial value, and the amounts of soluble Ca, Mg, and P_i remain unchanged, whereas the buffering curves are modified (Gevaudan et al., 1996). Acidification to a pH

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below 5.5 seems to involve the inability to completely reform colloidal calcium phosphate or to changes in its salt form.

In this study, CO₂ acidification to pH 5.8 was performed at 4°C on salt-supplemented and pH-adjusted milk samples in a laboratory pilot plant. We chose to acidify the milk by the carbon dioxide method because of the pH reversibility of this treatment, which, in addition, does not modify the ionic strength. The pH of acidification chosen represents the limit of the first step of acidification described by Gastaldi et al. (1996), corresponding to partial demineralization. The aim of the work described here was to study, by response surface methodology (RSM) (Doelhart, 1970), the effect of salt supplementation on the micellar composition of reconstituted skim milk subjected to pH-reversible acidification.

MATERIALS AND METHODS

Milk Sample Preparation

Milk was reconstituted at room temperature from a low, heat-spray dried skim milk powder (Isigny Ste Mère, Cazouls les Béziers, France) to obtain a suspension of 120 g of milk solids/kg. Sodium azide at 0.02% (wt/wt) was added to prevent bacterial growth. The mineral stock solutions used were 250 mM Ca (from anhydrous CaCl₂) and 200 mM P_i, pH 8 (composed of a mixture of K₂HPO₄ and KH₂PO₄). The salt solutions were added to the milk during reconstitution, at a concentration ranging from 0 to 25 mmol/kg of milk for calcium and 0 to 16 mmol/kg of milk for phosphate. Then, the pH was adjusted to 6.65 ± 0.02 for all samples by the addition of 1 N NaOH or HCl. After standing for 1 h with stirring, the samples were stored overnight at 4°C to allow the components to reach equilibrium.

Acidification of Reconstituted Milk

Cooled milk was poured into a stainless steel refrigerated reactor in the laboratory carbonation pilot plant. Samples were pressurized with stirring (Micropump Corporation, Vancouver, BC, Canada) by injection of CO₂ at 4 ± 1°C until the pH dropped to 5.8. The pH was measured with a high-pressure combination probe (Dynaprobe II, Broadley-James Corp., Santa Ana, CA). After a contact time of 15 min, the vat was depressurized and the milk was racked. At this stage, the pH of the milk was about 5.95 ± 0.05 in accordance with the measurements of Jordan et al. (1987) and Tomasula et al. (1995). Depressurization of the milk samples was achieved under vacuum using a diaphragm pump (Vacubrand, GMBH-CO, Wertheim, Germany) at room temperature for approximately 1 h; at which time, the

pH of the CO₂-treated milk samples had returned to their initial value (i.e., pH 6.65 ± 0.02) (Gevaudan et al., 1996).

Mineral Analysis

The milk was centrifuged at 160,000 × *g* and 20°C for 55 min using a Beckman L7-65 ultracentrifuge (Beckman Instruments France, Gagny, France) to separate the supernatant (aqueous phase) from the pellet (colloidal phase). We verified that during this high-speed centrifugation, there was no shift in the balance between micellar and serum minerals compared with the centrifugation at 70,000 × *g* for 120 min (Dalglish and Law, 1989). The Ca, P, and Mg contents in the milk sample and the supernatant were determined in duplicate by inductively coupled plasma spectrometry (Jobin Yvon 24, Jobin Yvon Instruments S.A., Longjumeau, France) after mineralization of the samples. In all the analyses, the composition of the micellar phase was determined by subtracting the composition of the supernatant from that of the milk sample.

Protein Analysis

Protein was assayed by the bicinchoninic acid (BCA) method (Smith et al., 1985). The BCA stock solution and BSA were purchased from Sigma Chemical Co (St Louis, MO). Samples were diluted in water 1:200 and 1:400 for milk and 1:20 and 1:40 for supernatant. Next, 0.05 ml of each dilution was combined with 0.02 ml of the BCA working reagent in the wells of a microtiter plate and incubated at 40°C for 30 min before reading at 540 nm in an MRX microplate reader (Dynex Technologies, Grafton, OH). All assays were performed in triplicate. The micellar concentration of proteins was calculated as described above for the mineral micellar concentration.

Buffering Determination

Titration were performed in duplicate on 30-ml samples at 25°C on a Titrino 702 SM Autotitrator (Metrohm, Herissau, Switzerland), using 0.5 N HCl or NaOH, added in 0.1-ml increments at 30-s intervals as described by Lucey et al. (1993). The samples were titrated from the initial pH of 6.65 ± 0.02 to pH 2.0 with HCl and back titrated to pH 11.0 with NaOH. Buffering values (dB/dpH) were calculated according to the formula of Van Slyke (1922) and plotted as a function of pH:

$$\frac{dB}{dpH} = \frac{(\text{volume of acid or alkali}) \times (\text{normality factor})}{(\text{volume of milk}) \times (\Delta pH)}$$

Table 1. Variation of the total and micellar concentrations of minerals and protein in salt-supplemented CO₂-treated milk.

Variables ¹		Ca ²		P ²		Mg ²	Protein ³
X ₁	X ₂	Total	Micellar	Total	Micellar	Micellar ⁴	Micellar ⁵
12.50	8.00	49.90	38.79	48.14	32.38	2.98	29.70
25.00	8.00	63.37	52.19	48.43	38.58	2.92	28.25
18.75	14.93	56.64	48.29	54.89	39.10	3.11	29.39
6.25	14.93	44.41	35.69	55.21	32.08	2.70	29.45
0.00	8.00	37.43	27.43	47.97	24.43	2.36	23.05
6.25	1.07	43.91	32.14	41.01	27.34	2.45	27.13
18.75	1.07	56.89	43.42	40.68	31.97	2.73	29.10
12.50	8.00	50.21	38.91	48.05	32.24	2.85	29.50

¹X₁ = concentration of added calcium, X₂ = concentration of added phosphate; both in terms of mmol/kg of milk.

²Mean of two replicate assays expressed in mmol/kg of milk.

³Mean of three replicate assays expressed in g/kg of milk.

⁴Mean value of total magnesium = 5.76 mmol/kg of milk.

⁵Mean value of total protein = 39.7 g/kg of milk.

Experimental Design

RSM was used to determine the influence of Ca and P supplementation on the micellar composition of CO₂-treated milk. The experimental design adopted was a Doehlert design (Doehlert, 1970) for two coded variables (X₁ and X₂) at five levels and three levels, respectively, with two replicates at the central point. The two independent variables were the concentration of added calcium (X₁) and the concentration of added phosphate (X₂) with a variation domain from 0 to 25 mmol/kg of milk for X₁ and 0 to 16 mmol/kg milk for X₂. The responses observed were the micellar concentrations of Ca (Y₁), P (Y₂), Mg (Y₃), and protein (Y₄), and the maximal buffering capacity in a pH range of 4.5 to 5.5 (Y₅). The noncoded values of the two independent variables and the data obtained for each experiment are given in Table 1.

The results were analyzed to fit the following second order equation to all the chosen responses:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2$$

where b₀...b₂₂ are regression coefficients and X₁, X₂ are the coded independent variables. Statistical analyses were performed using the multiple regression of Statview Student (1991 version, Abacus Concepts Inc., Berkeley, CA), and response surfaces were drawn using Excel software (1998 version, Microsoft France, Les Ulis, France).

RESULTS AND DISCUSSION

The change in the micellar constituents concentration of CO₂-treated milk samples as a function of Ca and P supplementation was fitted to the experimental

data given in Table 1. Five equations were obtained and tested for adequacy and fitness by analysis of variance (Table 2). The adjustment quality of the model was evaluated by the adjusted coefficient of determination (R²_{adj}). R²_{adj} values greater than 0.75 (Henika, 1982) demonstrated that the model was accurate enough for prediction of responses. Models developed for micellar Ca concentration (Y₁), micellar P concentration (Y₂), micellar Mg concentration (Y₃), micellar protein concentration (Y₄), and maximal buffering capacity in a pH range of 4.5 to 5.5 (Y₅) appeared to be adequate, since the R²_{adj} values were high and the P values significant. Root mean square error (RMSE) for the models and mean standard deviation (MSD) for the experiments were used to compare computed and observed values for each response (Y). For all models, the RMSE and MSD values were in the same range, suggesting that the models were well adjusted.

The statistical analysis with coefficient estimates and the significance for each response model is presented in Table 2. The concentration of added calcium appeared to be the most important variable and had a highly significant effect on all chosen responses. The concentration of added P_i seemed to be less determinant but was highly significant for the micellar mineral concentrations (Y₁, Y₂, and Y₃). No significant effect was observed on any model when added Ca was in association with added P_i. The relationship between the independent variables and the chosen responses is represented by three-dimensional response surfaces (Figures 1, 2, 3, 4, 5, and 6). The suitability of the model was tested by performing the two independent experiments reported in Table 3. The results showed close agreement between experimental and predicted values.

Table 2. Regression coefficients and analysis of variance of the second order equation¹ for the five chosen responses.²

Coefficient	Y ₁	Y ₂	Y ₃	Y ₄	Y ₅
b ₀	37.895	33.035	3.040	29.703	0.056
b ₁	12.053***	6.529***	0.293***	2.047**	0.008***
b ₂	2.331***	3.930***	0.191***	0.761	4.619E-4
b ₁₂	0.880	1.354	0.064	-1.152	0.001
b ₁₁	1.953**	-1.795*	-0.420***	-4.057**	-0.005***
b ₂₂	1.628*	-0.160	-0.230***	0.095	0.002**
R ² adjusted	0.995	0.992	0.974	0.768	0.945
MSD ³	0.488	0.592	0.038	0.871	0.002
RMSE ⁴	0.120	0.482	0.003	0.484	0.002
P	0.0001	0.0001	0.0001	0.0031	0.0001

¹Equation: $Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2$; where X_1 = added calcium and X_2 = added phosphate.

²Y₁ = micellar calcium concentration, Y₂ = micellar phosphorus concentration, Y₃ = micellar magnesium concentration, Y₄ = micellar protein concentration, Y₅ = maximum value buffering in the pH range of 4.5–5.5.

³Mean standard deviation.

⁴Root mean standard error.

*P = 0.05.

**P = 0.01.

***P = 0.001.

Micellar Calcium Concentration

The micellar Ca concentration (Y₁) of CO₂-treated milk strongly increased from 28 to 50 mmol/kg of milk with Ca supplementation between 0 to 25 mmol/kg (Figure 1). As expected by the significant regression coefficient (Table 2), the concentration of micellar Ca was strongly influenced by the addition of Ca as seen in the non-CO₂-treated milk sample (Holt et al., 1986; Zoon et al., 1988). Figure 1 also shows that P_i supplementation from 0 to 8 mmol/kg did induce no real change in the

colloidal calcium concentration as observed by Le Ray et al. (1998) on reconstituted casein micelles. On the other hand, for P_i addition from 8 to 16 mmol/kg, a slight increase of the micellar Ca concentration from 28 to 31 mmol/kg of milk was observed. At least 8 mmol of added P_i/kg was necessary to displace Ca from the soluble to the colloidal phase. Udabage et al. (2000) noted a small decrease in soluble Ca of reconstituted skim milk upon addition of P_i from 10 to 30 mmol/kg, corresponding to a small change in the Ca balance in favor of micellar Ca. Added simultaneously, Ca and P_i

Figure 1. Response surface of the micellar calcium concentration (Y₁) in salt supplemented, CO₂-treated milk samples.

Figure 2. Response surface of the micellar phosphorus concentration (Y₂) in salt supplemented, CO₂-treated milk samples.

Figure 3. Response surface of the micellar magnesium concentration (Y_3) in salt supplemented, CO_2 -treated milk samples.

caused a slight increase in the colloidal Ca concentration values relative to the corresponding individual supplementation of Ca and P_i .

Micellar Phosphorus Concentration

Micellar P concentration (Y_2) of CO_2 -treated milk slightly increased from 22 to 32 mmol/kg of milk for Ca addition from 0 to 25 mmol/kg with an asymptotic effect at 17.5 mmol of added Ca/kg (Figure 2). Famelart et al. (1999) and Tessier and Rose (1958) also observed

Figure 4. Response surface of the micellar protein concentration (Y_4) in salt supplemented, CO_2 -treated milk samples.

Figure 5. Buffering curves of CO_2 -treated milk supplemented with 8 mM of P_i only ($\times$), with 6.25 mM of Ca ($\circ$), with more than 12.5 mM of Ca ($\blacklozenge$). (a) Titrated from the initial pH (6.65 ± 0.02) to pH 2.0 with 0.5 N HCl and (b) back titrated from pH 2.0 to 11.0 with 0.5 N NaOH.

an increase of the micellar P concentration upon addition of Ca to reconstituted casein micelles or milk. On

Figure 6. Response surface of the maximum buffering value (Y_5) in the pH range of 4.5-5.5 in salt supplemented, CO_2 -treated milk samples.

Table 3. Comparison between micellar composition of CO₂-treated milk, non-CO₂-treated milk and predicted model milk.

Milk	Variables		Micellar composition				Maximum value of dB/dpH in the pH range of 4.5–5.5 ⁴
	X ₁ ¹	X ₂ ¹	Ca ²	P ²	Mg ²	Proteins ³	
CO ₂ -treated	0	0	27.5	23.5	2.31	22.6	0.040
	25	16	56.1	43.3	3.02	28.6	0.057
Non-treated	0	0	27.2	23.8	2.28	23.2	0.042
	25	16	55.8	44.3	2.97	29.8	0.056
Predicted model	0	0	28	22	2	21.8	0.041
	25	16	56.7	43	3	27.4	0.058

¹X₁ = concentration of added calcium, X₂ = concentration of added phosphate; both in terms of mmol/kg of milk.

²Expressed in terms of mmol/kg of milk.

³Expressed in terms of g/kg of milk.

⁴Calculated according to the formula of Van Slyke (1922).

adding 0 to 16 mmol of P_i/kg of milk, colloidal P values rose to 27 mmol/kg of milk, but most of the added P_i remained in the serum phase as Udabage et al. (2000) observed. When Ca and P_i were added simultaneously, the micellar P concentrations increased to a maximum value of 43 mmol/kg of milk at the maximum enrichment level. This tendency was also observed by Udabage et al. (2000) for pH-adjusted milk supplemented with P_i and Ca. Added simultaneously, Ca and P_i increased micellar P concentration values relative to the corresponding individual supplementation.

Micellar Magnesium Concentration

The response surface of micellar magnesium concentration (Y₃) showed an optimum of 3.14 mmol of micellar Mg/kg of milk for addition of 17.5 mmol of Ca/kg and 12 mmol of P_i/kg (Figure 3). This trend was conserved with individual supplementation. Indeed, colloidal Mg increased from 1.97 to 2.65 mmol/kg of milk with Ca enrichment from 0 to 17.5 mmol/kg and then decreased to 2.43 on adding 25 mmol/kg. Concerning non-CO₂-treated milk, Van Hooydonk et al. (1986) observed a slight increase of colloidal Mg when 3mM CaCl₂ was added, whereas Udabage et al. (2000) noted a slight decrease on adding 10 to 30 mmol CaCl₂/kg. Our observations could explain their results since there was a critical value of Ca supplementation at which micellar Mg seemed to be exchanged with Ca. On adding P_i from 0 to 12 mmol/kg milk, the micellar Mg concentration increased from 1.97 to 2.34 mmol/kg of milk and decreased to 2.22 with a P_i supplementation of 16 mmol/kg.

Micellar Protein Concentration

A strong increase in micellar protein concentration (Y₄) was observed from 21.8 to 29.7 g of micellar protein/

kg of milk on adding Ca from 0 to 17.5 mmol/kg (Figure 4). From 17.5 to 25 mmol of added Ca/kg milk, the micellar protein concentration (Y₄) decreased to 28.2. Other authors also demonstrated that added Ca causes an increase in micellar protein in non-CO₂-treated milk compared with nonsupplemented milk (Le Ray et al., 1998; Udabage et al., 2000). This could be due to aggregation of calcium-sensitive caseins. We found that the optimal concentration for increasing the micellar protein concentration of CO₂-treated milk was 17.5 mmol of Ca/kg of milk.

On adding P_i from 0 to 16 mmol/kg, the micellar protein concentration slightly increased to 25.6 g/kg of milk. Le Ray et al. (1998) found that phosphate addition from 0 to 8 mmol/kg to reconstituted casein micelles increased the amount of supernatant protein but without modification of the percentage of solubilized α_{s1} - and β -caseins. In contrast, Udabage et al. (2000) reported that the amount of soluble caseins in P_i supplemented milk was lower than the control level but increased with P_i addition from 10 to 30 mmol/kg. A critical value of added P_i seemed to be necessary to insert the soluble caseins into the colloidal phase. These observations concerning P_i addition (X₂) must be taken with caution because the effect of the regression coefficient b₂ on Y₄ was not significant (Table 2). The maximum value of 30.6 g/kg of milk of micellar proteins was obtained with 17.5 mmol of Ca/kg and 16 mmol of P_i/kg.

Buffering Curves of Salt-Supplemented, CO₂-Treated Milk

Buffering curves for salt supplemented CO₂-treated milk samples upon titration with acid (0.5 N HCl) are shown in Figure 5a. The titration curves had a similar shape and a buffering peak in the pH range of 4.5 to 5.5 for all experiments. According to Lucey et al. (1993), the maximum buffering value occurs at pH 5.1, but in

our experiments this maximum appeared at pH 4.85. This buffering peak is the result of the combination of H^+ with the phosphate ions liberated after complete solubilization of colloidal calcium phosphate. Three zones of maximum buffering capacity could be distinguished as a function of the Ca concentration. The highest values of dB/dpH, i.e., between 0.055 and 0.060, were obtained on CO_2 -treated milk samples supplemented with more than 12.5 mmol of Ca/kg milk. Values of approximately 0.050 were noted for samples supplemented with 6.25 mmol of Ca/kg milk, and the lowest values of about 0.042 were obtained on samples without Ca addition. Added P_i from 0 to 16 mmol/kg seemed to have no effect on the maximum values of dB/dpH.

Buffering curves for CO_2 -treated milks upon back titration with base (0.5 N NaOH) are shown in Figure 5b. The titration curves can be practically superimposed, and dB/dpH values are not significantly different. The peak of maximum dB/dpH occurred in the pH range of 5.5 to 6.5: at approximately pH 6.3 according to Lucey et al. (1993) and pH 6.0 in our experiments. The peak of maximum buffering value can be explained by the formation of $Ca_3(PO_4)_2$ with the release of H^+ which combines with OH^- .

Significant variation in buffering properties between samples occurred during acid titration in the pH range of 4.5 to 5.5. For this reason, the effect of mineral supplementation on the maximum buffering capacity values of CO_2 -treated milk samples (Y_5) is only presented in this pH range in Figure 5. Judging by the significant regression coefficients, the most important variable influencing Y_5 was the concentration of added Ca (Table 2). The response surface clearly showed that P_i had no real effect on this buffering value (Figure 5). The maximum value of dB/dpH strongly increased from 0.040 to 0.060 on adding Ca from 0 to 17.5 mmol/kg of milk. Above this level of supplementation, there was an asymptotic effect and Y_5 remained constant at the value of 0.060.

Effect of CO_2 Treatment

Upon acidification of skim milk below pH 5.1, modification of casein micelles corresponds to the formation of new casein particles and the solubilization of the micellar calcium phosphate (Dalgleish and Law, 1989; Gastaldi et al., 1996). Gevaudan et al. (1996) observed that acidification of milk below pH 5.1 using CO_2 under pressure followed by depressurization under vacuum causes the pH to return to its initial value, while the mineral partition remains unchanged. These authors also observed that the buffering properties changed and suggested that these changes arose from the formation of different forms of calcium phosphate during the CO_2

treatment. In our experiments, CO_2 acidification to pH 5.8 corresponded to the dissolution of moderate amounts of micellar calcium phosphate and a decrease in the micelle solvation (Gastaldi et al., 1996). After depressurization under vacuum, the pH of milk acidified by CO_2 to pH 5.8 returned to its initial value. Trends demonstrated by RSM on mineral-supplemented CO_2 -treated milk correlates with the results of supplemented non- CO_2 -treated milk reported in the literature, suggesting that our CO_2 treatment was entirely reversible. The micellar constituent concentration and the maximum buffering value in the pH range of 4.5 to 5.5 were similar for CO_2 -treated milk and untreated samples, both pH adjusted (Table 3). This suggests that the response surfaces obtained in this study can be applied to supplemented, non- CO_2 -treated milk. This reversibility is in accordance with the work of Lucey et al. (1996), who acidified milk with HCl to pH 5.5 followed by neutralization with NaOH without significant changes in the micellar calcium phosphate. Our results indicate that the CO_2 acidification to pH 5.8 followed by depressurization under vacuum was entirely reversible without changes in micellar composition nor in the structure of micellar calcium phosphate.

CONCLUSIONS

The mineral equilibria in milk are strongly influenced by salt supplementation (Udabage et al., 2000). The results presented in this study demonstrate that Ca supplementation in the range of 0 to 25 mmol/kg of milk is the most important factor affecting the micellar composition, as seen by the regression coefficients (Table 2) and the response surfaces. On the other hand, the effect of P_i supplementation from 0 to 16 mmol/kg of milk was only slight or not significant for modeling purposes. By using RSM, we were able to conclude that a calcium supplementation to about 17.5 mmol/kg of milk favored the increase in the concentration of micellar constituents: Ca (Y_1), P (Y_2), Mg (Y_3), and protein (Y_4). Above this level of Ca supplementation, only micellar Ca increased. Furthermore, an increase in the maximum buffering value, in the pH range of 4.5 to 5.5 (Y_5), was observed with a Ca supplementation up to 17.5 mmol/kg. Above 17.5 mmol of Ca/kg, added Ca did not cause further changes in this maximum buffering value. In milk, micellar calcium exists partly as Ca directly bound to protein and partly as calcium phosphate salts (Holt et al., 1986). It is probable that at a Ca supplementation less than 17.5 mmol/kg of milk, Ca integrates the micelle as calcium phosphate, but above this concentration, Ca integrates the micelle and binds directly to protein.

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