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PII: S0023-6438(20)31193-2

DOI: https://doi.org/10.1016/j.lwt.2020.110204

Reference: YFSTL 110204

To appear in: *LWT - Food Science and Technology*

Received Date: 1 June 2020

Revised Date: 5 September 2020

Accepted Date: 9 September 2020

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Abstract

The objective of this work was to analyze the changes in the physicochemical and rheological characteristics of milk fortified with different calcium salts. Reconstituted milk samples using skim milk powder with different concentrations of calcium chloride and calcium lactate (0, 5 and 30 mmol kg^{-1}) were obtained. Several physicochemical and rheometric techniques were used to analyze the effect of milk fortification. According to the results, all the applied techniques indicated that some of the added calcium migrates into the casein micelle forming colloidal calcium phosphate, and that the calcium added as lactate enters the micelles to a greater extent. A part of whey proteins would also be integrated into the micellar structure. An addition of 5 mmol kg^{-1} of calcium chloride and calcium lactate would be practically feasible, due to the mineral balance and the thermal stability that were not significantly affected at that concentration level. In conclusion, the results obtained with physicochemical techniques commonly used in literature are in agreement with those obtained in this study by rheometry, demonstrating that this simple and rapid technique allows inferring about the changes in mineral balance and effects on thermal stability when different salts are used for milk fortification.

Keywords: milk, calcium fortification, rheology, physicochemical changes, heat stability.

1. Introduction

Currently, mineral-supplemented foods are both on the market and in development to prevent mineral deficiencies. Milk is a good option for mineral fortification, mainly due to its massive consumption, high nutritional value, buffering effect on digestion and absorption processes, and positive effects on growth (Lombardi et al., 2016).

Fortification of milk with calcium is a common practice to improve its nutritional properties. Several soluble and less-soluble calcium salts are used for calcium fortification of milk, e.g. calcium carbonate, calcium chloride, calcium phosphate, tribasic calcium phosphate, calcium citrate, calcium lactate, calcium gluconate, calcium lactate gluconate, and natural milk calcium (Deeth & Lewis, 2014; Ramasubramanian, D'Arcy, & Deeth, 2012; Singh et al., 2007). Calcium addition in milk can lead to changes in the physicochemical properties and cause irreversible coagulation during industrial high-temperature heat treatment and unacceptable off-flavors (Ramasubramanian et al., 2012; Singh et al., 2007). Therefore, the selection of the appropriate salt or a combination of them is generally based on avoiding undesirable effects and improving bioavailability.

Numerous studies have been carried out mostly to analyze the changes in physicochemical characteristics and distribution of ions between the different phases present in milk (Bijl, van Valenberg, Huppertz, & van Hooijdonk, 2013; Gaucher, Piot, Beaucher, & Gaucheron, 2007; Omoarukhe, On-Nom, Grandison, & Lewis, 2010; Philippe, Gaucheron, Le Graet, Michel, & Garem, 2003). In milk, calcium is in equilibrium between the micellar (or colloidal) and continuous (or serum) phases. In serum, it is mainly present in free form or associated with citrate and, to a lesser extent, with inorganic phosphate, chloride, and α-lactoalbumin (Gaucheron, 2005; Ramasubramanian, Webb, D'Arcy, & Deeth, 2013). In the colloidal phase, calcium is present as colloidal calcium phosphate (CCP) bound to casein micelles (CM). Most of the calcium (70%) is found in this phase (Bijl et al., 2013; Koutina, Knudsen, & Skibsted, 2015a; Omoarukhe et al., 2010). CCP is in dynamic equilibrium with calcium phosphate present in the serum. This balance depends on physicochemical conditions such as temperature, pH, presence of different minerals, and ionic strength (de la Fuente, 1998; Nogueira Silva, Bahri, Guyomarc´h, Beaucher, & Gaucheron, 2015).

The enrichment of milk with calcium salts influences the level of CCP, the proportion of caseins in the colloidal and serum phases, the activity of Ca^{2+}, and the ionic strength of milk. It also produces a decrease in the hydration of CM and the zeta potential (Famelart, Le Graet, & Raulot, 1999; Koutina et al., 2015a; Philippe, Le Graët, & Gaucheron, 2005). The addition of this mineral neutralizes negatively charged residues on the surface of CM, making them more susceptible to aggregation. Consequently, the stabilizing properties of the κ-casein layer surrounding CM are affected by calcium concentration (Ye & Harte, 2013). Moreover, milk stability during heat processing can be affected by calcium addition (Koutina, Christensen, Bakman, Andersen, & Skibsted, 2015b). Among the wide variety of techniques to evaluate the effect of calcium addition on milk stability, rheological studies are relatively easy methods that can provide useful complementary information (Meza, Zorrilla, & Olivares, 2019).

In the present work, we analyze the changes in the physicochemical characteristics of milk fortified with calcium chloride and calcium lactate. Furthermore, we explore the efficiency of rheometry to analyze the effect of milk fortification with calcium salts.

2. Materials and methods

2.1. Preparation of milk samples

Low-heat commercial-grade skim milk powder (4% w/w moisture, 1.5% w/w fat, 35% w/w protein, 8.5% w/w ash, WPNI = 7 mg undenatured whey protein nitrogen per gram of milk powder, SanCor Cooperativas Unidas Ltda., Sunchales, Argentina) was used. Milk samples were reconstituted to 10% w/w, following the manufacturer's recommendation. The required amount of powder was gradually added to purified water at 25 °C while stirring at 1200 rpm. Samples were sealed and stirred for 4 h at 25 °C. To prevent microbial growth, sodium azide (0.02% w/v) was added to the reconstituted milk samples before being stored overnight at 25 °C to ensure complete hydration of casein micelles and equilibration of mineral content. The next day, milk samples with different added calcium chloride and calcium lactate concentrations (0, 5 and 30 mmol kg^{-1}) were prepared under stirring at moderate speed for 5 min. Again, the samples were stored overnight at 25 °C to ensure the equilibration of mineral content. pH was determined in all samples, with a pHmeter pH spear (Oakton Instruments, Vernon Hills, IL, USA). Each sample preparation was carried out in duplicate.

2.2. Milk ultracentrifugation

The separation of micellar and serum phases was obtained by ultracentrifugation (Biofuge 28RS centrifuge, Heraeus Sepatech, Osterode, Germany) of reconstituted milk samples at 50,000g for 2 h at 25 °C (Koutina et al., 2015a). Proteins and minerals of the supernatant were expressed as components of the serum phase.

2.3. Protein analysis

Total and serum protein contents of milk samples were determined using the Bradford method (Kruger, 2002). The protein composition (caseins, α-lactalbumin and β-lactoglobulin) of the serum phase was analyzed by SDS-PAGE (Walker, 2002); the resolving and stacking gels contained 12% w/v and 4% w/v acrylamide, respectively. The current for the electrophoretic runs was set at 70 mA. Gels were stained using 0.125% w/v Coomassie Brillant Blue R250 in a 1:1 mixture of 95% w/v ethanol and 10% w/v acetic acid and destained in a 2:3 mixture of 95% w/v ethanol and 5% w/v acetic acid.

2.4. Mineral analysis

Total and serum calcium contents of milk samples were determined using an atomic absorption spectrometric method (USEPA, 1991). Micellar calcium was determined as the difference between the total calcium and serum calcium.

Total and serum phosphorus contents of reconstituted milk samples were determined using the standard molecular absorption spectrometry method (IDF, 2006). The samples were digested by a wet digestion method using sulfuric acid and hydrogen peroxide. Molybdenum blue was formed by the addition of a molybdate/ascorbic acid solution. The absorbance was measured at 820 nm.

2.5. Osmolality

Osmolality of milk samples was measured using a vapor pressure osmometer VAPRO® model-5520 (Wescor Inc, Puteaux, France). Following the instructions of the manufacturer, 10 μL of the sample was inoculated into a solute-free paper disc in the sample holder, whereupon the sample holder was pushed into the instrument and the sample chamber was locked to carry out an automatic measurement. Previously, the osmometer was calibrated with NaCl standards of 100, 290 and 1000 mmol kg^{-1}.

2.6. Rheometry

Milk samples were evaluated using a speed-controlled rheometer Brookfield DV3TLVCP (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) with a cone-plate geometry consisting of a lower hermetic sample cup (plate) and an upper cone CPA-40Z (0.8° angle and 48 mm diameter). The sample volume (0.5 mL) was placed into the hermetic sample cup that was designed to prevent water vaporization during measurements. The viscosity of the milk samples was measured as a function of temperature in the range of 20-80 °C at a constant value of shear rate of 100 s^{-1}. The cell temperature increased linearly with a rate of heating of 2.4 °C min^{-1}. Under these conditions, the viscosity of milk samples changes as temperature increases to reach a critical temperature (T_c) when viscosity suddenly diverges (Meza et al., 2019). To obtain representative critical temperatures, the experimental data of viscosity versus temperature were analyzed following the procedure reported by Meza et al. (2019). Briefly, a linear regression of each linear segment (before and after the beginning of the aggregation process) was obtained. Then, the intersection between the two linear segments was used to determine T_c, which can be considered as an estimation of a critical aggregation temperature.

2.7. Statistical analysis

For statistical analysis, type of salt and salt concentration were selected as main factors for ANOVA with test for interaction, performed using Minitab (Minitab Inc., State

College, PA, USA). When differences between treatment effects were significant (P<0.05), a multiple comparison of means was performed by Least Significant Differences (LSD) test using Statgraphics (Statgraphics Inc., Rockville, MD, USA).

3. Results and discussion

3.1. pH

Table 1 shows the average pH values for all samples studied. Values for samples without calcium salt addition were in agreement with those reported for milk (Anema, 2009; Gaucheron, 2005; On-Nom, Grandison, & Lewis, 2012; Philippe et al., 2003; Walstra, Wouters, & Geurts, 2006; Williams, D'Ath, & Augustin, 2005).

The addition of calcium salts reduced the pH values in milk. A significant interaction (P<0.05) between type of salt and calcium concentration was found (Table 1). At the same concentration level, the pH values of samples without salt addition and those with 5 mmol kg^{-1} of added salt showed no significant differences, while the pH values of calcium lactate-added samples were significantly higher than those of calcium chloride-added samples at 30 mmol kg^{-1}.

The decrease in the pH of milk after the addition of calcium salts has been previously reported (Gaucheron, 2005; Lewis, 2010; Philippe et al., 2003; Ramasubramanian et al., 2013). It is related to (i) the formation of calcium phosphate and calcium citrate, (ii) changes between the added calcium and protons present in the micellar phase, and (iii) the acidity of the added calcium salt solution (Philippe et al., 2003).

3.2. Protein analysis of milk and serum phase samples

The total protein content of the reconstituted skim milk was 32.9 g L^{-1}, which is in agreement with values reported in the literature (Bijl et al., 2013; Koutina et al., 2015a; Walstra et al., 2006).

Table 1 shows the average protein concentrations in the serum phase. The values in milk samples without salt addition are lower than those expected in fresh milk due to the thermal treatment that takes place during the production of the skim milk powder, which causes the whey protein to denature and attach to CM surface (Dalgleish & Corredig, 2012; Koutina et al., 2015a; Singh & Fox 1987).

Serum protein concentrations of all calcium-added samples were significantly lower than that of unfortified milk sample. These results are in agreement with those reported by other authors. Philippe et al. (2003) studied the physicochemical characterization of skim milk supplemented with calcium chloride and suggested that the concentration of caseins in serum phase decreases after the addition of the calcium salt and that caseins from the serum phase either become part of existing micelles or form new casein micelle structures. Also, Williams et al. (2005) concluded the same through experiments in which calcium chloride in combination with tri-potassium orthophosphate was added to skim milk. More recently, Koutina et al. (2015a) carried out studies of characterization of skim milk enriched with calcium D-lactobionate. They observed a decrease of phosphorous and caseins in serum phase and suggested that the additional calcium could be bound to serum phosphorus and serum caseins or remain as free ions, which can enter the micellar structure giving a different conformation of casein micelles.

A significant interaction ($P<0.05$) between type of salt and calcium concentration was observed (Table 1). At 5 mmol kg^{-1} of added calcium, the serum protein concentration of the calcium lactate-added samples was lower than the value for calcium chloride-added samples, suggesting that the protein migration to the interior of CM is higher when calcium lactate is used.

The protein nature of the serum was also analyzed by SDS-PAGE. Figure 1 shows the gel images. It is observed that α_{s1}-, β- and κ-caseins and β-lactoglobulin decrease their band intensity as the calcium concentration increases for the two salts used. In addition, these results correspond to those obtained by quantification using the Bradford method. In the case of β-lactoglobulin, the decrease is not present. These results are in agreement with those reported by Koutina et al. (2015a) and Williams et al. (2005). Koutina et al. (2015a) used calcium D-lactobionate at different pH conditions and quantified the protein fractions by SDS-PAGE, while Williams et al. (2005) used 20 mM of added calcium chloride and quantified the protein fractions by capillary zone electrophoresis.

3.3. Mineral composition of milk and serum phase samples

3.3.1. Calcium content

Table 1 shows the average values of calcium content in milk and the serum phase. The calcium content in milk samples without salt addition agrees with that reported in the literature (Walstra et al., 2006). Also, calcium content increased as the amount of added salt increased. At 30 mmol kg^{-1}, calcium concentration (1.95 mg/g) was significantly higher than at 5 mmol kg^{-1} (1.13 mg/g); this was independent from the type of salt used.

The calcium content in the serum phase increases as the concentration of added calcium increases for both salts studied. A significant interaction ($P<0.05$) between type of salt and calcium concentration was found (Table 1). At 5 mmol kg^{-1} of added salt, no significant differences were observed depending on the type of salt. However, at 30 mmol kg^{-1} of added salt, calcium chloride-added samples showed higher calcium values in the serum phase than those of calcium lactate-added samples. These results are in agreement with those reported by Williams et al. (2005) and Zuraw, Smietana, Szpendowski, & Chojnowski, (1986). Also, Koutina et al. (2015a) obtained similar results when studying the milk fortification with calcium D-lactobionate at concentrations ranging from 0 to 50 mM.

In our case, it can be inferred that some of the added calcium is incorporated into the micellar structure as it was postulated in previous studies (Philippe et al., 2003; Sievanen, Huppertz, Kelly, & Fox, 2008; Williams et al., 2005, Zuraw et al., 1986). Also, it can be concluded that the calcium from calcium lactate is incorporated to a greater extent into the micellar structure, which is in agreement with the results reported by Singh et al., (2007). These results are in agreement with the behavior observed for pH values, at 30 mmol kg^{-1} calcium lactate-added samples showed higher values than calcium chloride-added samples, probably due to the lower amount of calcium outside of CM available to affect the equilibrium of ionic species in milk, particularly H^+ ions.

3.3.2. Phosphorus content

The total phosphorus content was 31.65 ± 0.54 mmol kg^{-1}, which is in agreement with the phosphorus concentration reported by Koutina et al. (2015a).

Phosphorus content in the serum phase is also shown in Table 1. ANOVA indicated that only the added salt concentration had a significant effect on the phosphorus in serum content. Concomitantly with serum calcium values, it was observed that the phosphorus content decreased with the concentration of the added calcium salt. At 30 mmol kg^{-1}, phosphorus concentration (10.21 mmol/kg) was significantly lower than at 5 mmol kg^{-1} (12.00 mmol/kg); this was independent from the type of salt used. These results agree with those previously reported (Gaucheron, 2005; Koutina et al., 2015a; Philippe et al. 2003; Udabage, McKinnon, & Augustin, 2000).

3.4. Osmolality

Osmolality is defined as the concentration, expressed on a molar base, of the osmotically active particles in a true solution. The dissolved substances in milk result in osmotic pressure of approximately 700 kPa (7 bar) and a freezing point decrease close to 0.53 K (Walstra et al., 2006). Using the van't Hoff equation for dilute solutions, which relates osmotic pressure to the concentration of the solute, this osmotic pressure value corresponds to a theoretical concentration of dissolved solutes in milk of 282 mmol kg^{-1}. The osmolality of milk only depends on the concentration of each solute in the aqueous phase. The suspended fat particles and CM do not contribute to this colligative property (Bachmann, Schmidt, Rauwolf, Wenge, & Coenen, 2012; Novo, Reija, & Al-Soufi, 2007). Therefore, through osmolality measurements, it is possible to analyze the variation of the concentration of osmotically active species dissolved in the serum phase.

Table 1 shows the average values of osmolality obtained for milk fortified with the different salts. The values obtained for milk without the addition of calcium salt agree with those reported for this food (Novo et al., 2007) and the value predicted using the van't Hoff equation. For the two salts studied, it was observed that osmolality increased as the concentration of added calcium salt increased. A significant interaction ($P<0.05$) between type of salt and calcium concentration was found (Table 1).

It was observed that at 5 and 30 mmol kg^{-1} of added salt, the osmolality values of calcium lactate-added samples were higher than those for calcium chloride-added samples. Although beforehand these results seem opposed to those obtained for calcium content in serum (if calcium ions enter the micelles in greater extent when calcium lactate is added, osmolality should decrease further in these samples), it should be taken into account that calcium lactate (CaL_2) in solution undergoes into a two-step equilibrium process (Kubantseva & Hartel, 2002; Vavrusova, Munk, & Skibsted, 2013; Vavrusova & Skibsted, 2014):

$$CaL_2(s) \leftrightarrow CaL^+ + L^- \qquad (1)$$

$$CaL^+ \leftrightarrow Ca^{2+} + L^- \qquad (2)$$

The second step does not go completely to the end point and an equilibrium state is established (Kubantseva & Hartel, 2002). Side reactions of lactate ion may occur (lactic acid, a weak acid may form by association with hydrogen ions) and the presence of common and uncommon ions may modify the equilibrium state (2). In addition, the anions distribution between micellar and serum phases should also be taken into account. Hence, it is difficult to estimate the number of osmotically active species in these samples.

3.5. Rheometry

Figure 2 shows the results obtained through temperature sweeps. From 20 to 60 °C, the viscosity slowly decreases as temperature increases. Several changes in milk equilibria with temperature can explain the changes in viscosity at this temperature range. Between 4 and 40 °C, the amount of calcium in the milk serum phase is reduced with increasing temperature due to the reduction in calcium phosphate solubility (Koutina et al., 2015b; Walstra et al., 2006; Wang & Ma, 2020). In our case, the viscosity value increased as calcium salts were added, when a constant temperature is considered (Figure 2, magnified insert). It appears that the sample with the addition of 5 mmol kg^{-1} of calcium lactate increased less its viscosity. This behavior indicates that the addition of calcium may modify the viscosity of the aqueous phase (serum) or the disperse phase (CM) structure, even at relatively low temperatures, generating physicochemical changes in milk samples.

An Arrhenius-type equation can be used to represent the decrease of viscosity with temperature in the range of 20-60 °C as proposed by Meza et al. (2019) (Eq. 1),

$$\eta = A_0\, exp\left(\frac{E_A}{RT}\right). \qquad (1)$$

Here A_0 is the pre-exponential factor, E_A is the activation energy, R is the universal gas constant, and T is the absolute temperature. The quantity E_A is the barrier of energy that must be overcome before the elementary flow process can occur (or viscous flow). E_A values for the different conditions studied are listed in Table 2. Values of E_A are in the same order of magnitude than those obtained for reconstituted skim milk in a previous study in the range of 25 to 60 °C (Meza et al., 2019). ANOVA indicated that the factor salt concentration had a significant effect, while the type of salt added and interaction did not have a significant effect. It was observed that E_A increased with the addition of salt but no differences were detected between samples with 5 and 30 mmol kg^{-1} of added calcium, indicating that calcium addition affects the change of milk viscosity during heating.

Above 60 °C, the viscosity sharply increases at a salt concentration of 30 mmol kg^{-1} but at different temperatures depending on the type of salt (Figure 2). The samples with 5 mmol kg^{-1} of both calcium salt studied did not show a divergence of the viscosity in the temperature range evaluated.

In the case of samples with calcium lactate, a destabilization in viscosity can be observed between 65 °C and 70 °C until finally the divergence occurs. This feature was exhibited in all the replicates. As it was discussed above, it is well known that calcium lactate in solution undergoes into a two-step equilibrium process (Kubantseva & Hartel, 2002; Vavrusova et al., 2013). The presence of other ions in milk and the changes induced by the temperature probably alter both equilibria, as suggested by Vavrusova et al., (2013). As temperature increases, the concentrations of calcium in serum, inorganic phosphate and citrate decrease, suggesting the formation of calcium phosphate structures (Singh, 2004; Wang & Ma, 2020). These changes possibly affect lactate equilibrium and the dissociation during heating, causing this instability in viscosity previous to divergence at 71.2 °C (Table 2).

The viscosity of samples with calcium chloride diverged at a significantly lower temperature of 64.6 °C (Table 2). Other changes besides alteration in mineral balance are expected with milk heating above 60 °C. Denaturation of whey proteins takes place at temperatures higher than 65 °C. Besides, at pH ≤ 6.5, denatured serum proteins form aggregates and also partially cover CM via–S-S- linkages (Koutina et al., 2015b; Singh, 2004; Walstra et al., 2006).

The pH of milk decreases during heating, the lower the initial pH, the lower the temperature at which coagulation occurs (Walstra et al., 2006). Lowering pH weakens electrostatic and steric repulsions of CM. Also, the addition of salts increases the ionic strength, effect that contributes to the weakening of interactions. The excess of calcium ions enhances the possibilities of -Ca- bridge formation between negatively charged groups of the overlapped hairy layers of two casein micelles. Additionally, at high temperatures covalent bonds between amino acid residues can be formed, strengthening the junction (Considine, Flanagan, & Loveday, 2014; Walstra et al., 2006).

As it was discussed before, the decrease in pH was more pronounced in the calcium chloride-added samples than the case of calcium lactate-added samples at similar concentrations. Furthermore, calcium and phosphorus contents revealed that the calcium added in the form of lactate enters the micelles to a greater extent. Thus, the amount of calcium and phosphate ions outside the CM is higher in calcium chloride-added samples. The combined effect of calcium addition (and the consequent increase in ionic strength) and pH reduction affect the coagulation phenomenon and the temperature at which it starts. It is relevant to note that this study shows how a macroscopic parameter that can be easily determined as viscosity allows detecting the microstructural differences of milk fortified with different salts and may help to analyze the colloidal stability.

4. Conclusions

In this work, milk fortified with calcium chloride and calcium lactate was characterized by the physicochemical and rheometric point of view. The results obtained allowed to relate how the physicochemical changes modify the micellar structure and the thermal stability of milk. All the techniques applied indicate that some of the added calcium migrate into the CM forming CCP and that the calcium added in the form of lactate enters the micelles to a greater extent. A part of whey proteins would also be integrated into the micellar structure.

From the information obtained, it is concluded that an addition of 5 mmol kg^{-1} of calcium chloride and calcium lactate would be feasible, due to the mineral balance and the thermal stability were not significantly affected at this concentration level. Though, calcium lactate would be more appropriate for formulations with higher calcium concentrations (e.g. intermediate concentrations in the range 5-30 mmol kg^{-1}).

Finally, as the results obtained with physicochemical techniques commonly used are in agreement with those obtained by rheometry, we demonstrate that this simple and rapid technique allows inferring about the changes in mineral balance and effects on thermal stability when different salts are used for milk fortification.

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6. Acknowledgments

This study was conducted with the financial support of Universidad Nacional del Litoral (project CAI+D: 504 201501 00051 LI) (Santa Fe, Argentina), Consejo Nacional de Investigaciones Científicas y Técnicas (project CONICET: 11220150100606) (Argentina), Agencia Nacional de Promoción Científica y Tecnológica (project ANPCyT PICT 2016-249) (Argentina) and Instituto Nacional de Tecnología Agropecuaria (INTA) (project 2019-PD-E7-I152-001).

Conflict of interest

Nadia Belén Acosta, Guillermo Adrián Sihufe, Bárbara Érica Meza, Fernanda Marino, Luciana María Costabel, Susana Elizabeth Zorrilla, and María Laura Olivares declare that they have no conflict of interest.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects performed by any of the authors.

Table 1: Average values and standard deviations corresponding to the physicochemical parameters of milk and serum samples studied.

Type of salt	Concentration of added salt (mmol kg^{-1})	pH	Calcium in milk (mg g^{-1})	Calcium in serum (mg g^{-1})	Micellar calcium (mg g^{-1})	Phosphorus in serum (mmol kg^{-1})	Protein in serum (g L^{-1})	Osmolality (mmol kg^{-1})
Calcium lactate	0	6.65 ± 0.00^{a}	1.01 ± 0.02	0.31 ± 0.00^{d}	0.70 ± 0.02^{c}	12.51 ± 0.01	3.05 ± 0.11^{a}	285.2 ± 10.7^{d}
	5	6.54 ± 0.01^{b}	1.09 ± 0.01	0.38 ± 0.01^{c}	0.71 ± 0.02^{c}	11.90 ± 0.35	2.42 ± 0.13^{c}	300.5 ± 3.8^{c}
	30	6.18 ± 0.00^{c}	1.97 ± 0.07	0.94 ± 0.02^{b}	1.04 ± 0.05^{a}	10.04 ± 0.42	2.03 ± 0.07^{d}	349.3 ± 2.2^{a}
Calcium chloride	0	6.71 ± 0.07^{a}	1.01 ± 0.02	0.31 ± 0.00^{d}	0.70 ± 0.02^{c}	12.51 ± 0.01	3.05 ± 0.11^{a}	285.2 ± 10.7^{d}
	5	6.53 ± 0.01^{b}	1.17 ± 0.01	0.42 ± 0.01^{c}	0.75 ± 0.01^{c}	12.11 ± 0.02	2.75 ± 0.08^{b}	280.7 ± 3.0^{d}
	30	6.09 ± 0.01^{d}	1.94 ± 0.01	1.05 ± 0.04^{a}	0.89 ± 0.03^{b}	10.38 ± 0.06	2.07 ± 0.06^{d}	337.5 ± 5.2^{b}
Type of salt		NS	NS	*	NS	NS	*	*
Salt concentration		*	*	*	*	*	*	*
Interaction		*	NS	*	*	NS	*	*

NS: no significant effect (*P*>0.05); *: significant effect (*P*<0.05).

$^{a\text{-}d}$: Average values in the same column with different superscript letters are significantly different (*P*<0.05).

Table 2. Values of activation energy and critical temperature in the calcium fortified milk samples analyzed.

Type of salt	**Concentration of added salt (mmol kg^{-1})**	E_A **(kcal mol^{-1})**	T_c **(°C)**
Calcium lactate	0	4.48 ± 0.44	-
	5	5.00 ± 0.19	-
	30	5.46 ± 0.23	71.20 ± 0.54^a
Calcium chloride	0	4.48 ± 0.44	-
	5	5.28 ± 0.36	-
	30	5.12 ± 0.36	64.60 ± 0.07^b
Type of salt		NS	*
Salt concentration		*	
Interaction		NS	

NS: no significant effect (*P*>0.05); *: significant effect (*P*<0.05).

$^{a-b}$: Average values in the same column with different superscript letters are significantly different (*P*<0.05).

Figure captions

Figure 1: SDS–PAGE of serums. (a) Samples added with calcium chloride, (b) Samples added with calcium lactate. Salt content: 0 mmol kg^{-1} (lanes 1 and 2), 5 mmol kg^{-1} (lanes 3 and 4), 30 mmol kg^{-1} (lanes 5 and 6).

Figure 2: Temperature sweeps for milk samples fortified with different calcium salts. (○) milk without salt addition: Calcium chloride concentrations: (○) 5 mmol kg^{-1}; (○) 30 mmol kg^{-1}. Calcium lactate concentrations: (○) 5 mmol kg^{-1}; (○) 30 mmol kg^{-1}.

Figure 3: Examples of the procedure used to obtain the critical temperature T_c for milk samples fortified with different calcium salts with a concentration of 30 mmol kg^{-1}: (a) milk with calcium chloride, (b) milk with calcium lactate.

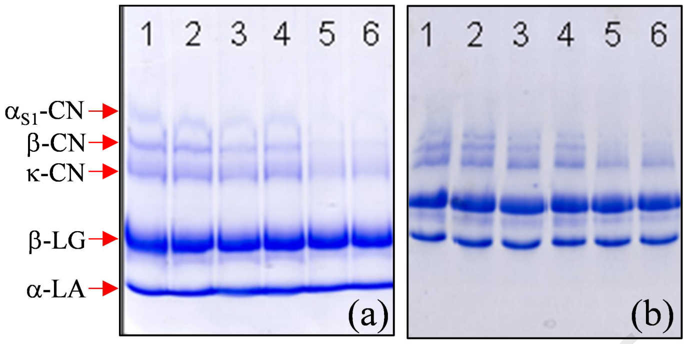

Figure 1

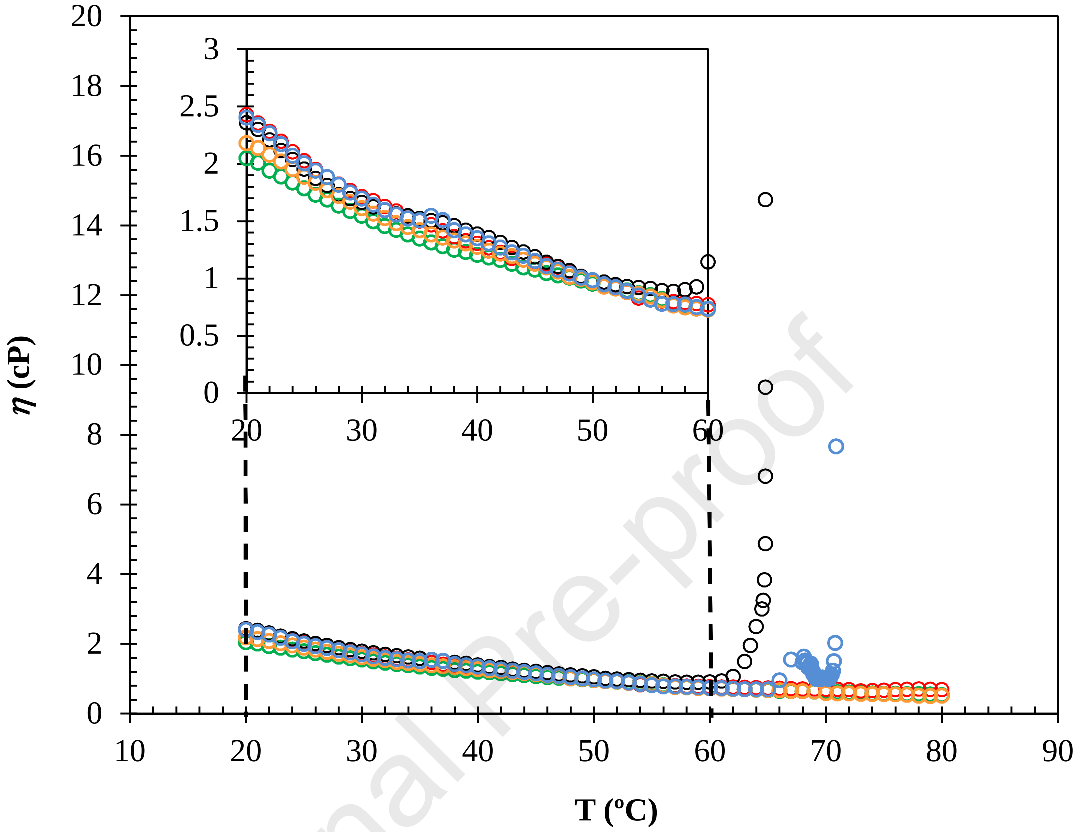

Figure 2

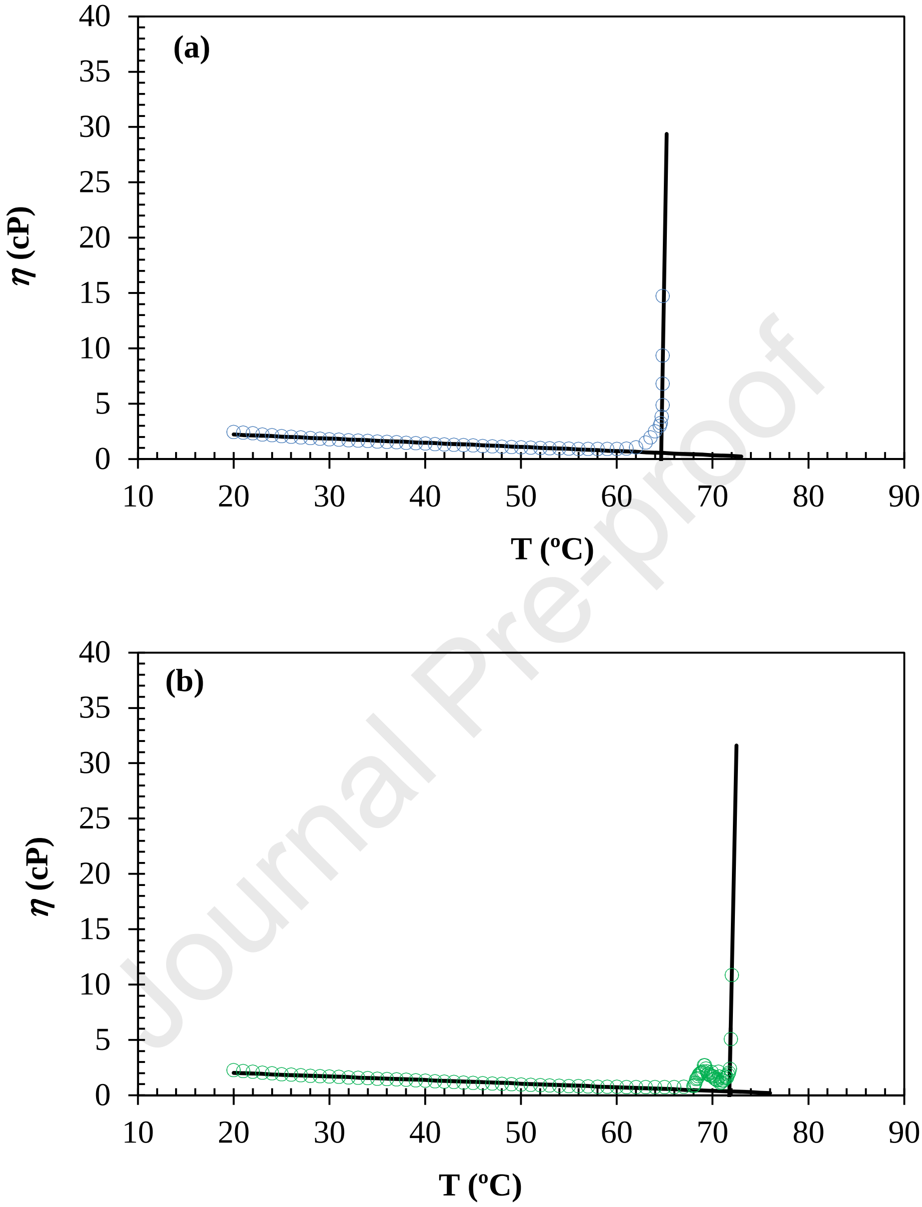

Figure 3

Milk fortified with calcium: changes in the physicochemical and rheological characteristics that affect the stability

By Acosta, N.B., Sihufe, G.A., Meza, B.E., Marino, F., Costabel, L.M., Zorrilla, S.E., Olivares, M.L.

Highlights:

- Physicochemical characteristics and rheometry of calcium fortified milk
- Different calcium salts and concentrations were analyzed
- Rheometric method helps to evaluate the thermal stability
- Information that may help to improve calcium-fortified dairy formulations

Conflict of interest

Nadia Belén Acosta, Guillermo Adrián Sihufe, Bárbara Érica Meza, Fernanda Marino, Luciana María Costabel, Susana Elizabeth Zorrilla, and María Laura Olivares declare that they have no conflict of interest.