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Impact of freeze-thaw treatment on the stability of calcium-fortified soy beverages

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ABSTRACT

The objective was to study the freeze-thaw stability of soy beverages (SB) obtained from active defatted soybean flakes and fortified with calcium at the level of bovine milk. Calcium chloride or calcium lactate was used to fortify the SB, and a third sample without calcium was prepared as control. Sunflower oil was added as lipid phase and high pressure homogenization was performed to obtain the systems. SB were prepared without or with sucrose (5.0 and 10.0 g/100 g) as cryoprotectant. In the absence of sucrose, freeze-thawing produced important aggregation of particles, observing a higher particle size increase (PSI) after 30 days of frozen storage in both SB with calcium (~700) than without addition of the cation (~500). Only the SB with 10.0 g/100 g sucrose presented an acceptable stability to freeze-thawing, and at that concentration of cryoprotectant the systems with calcium showed a lower PSI (~30) than in the absence of the cation (~100). In this case, because initial protein aggregation was favored in presence of calcium, new aggregation might have been limited during frozen storage at the conditions given by the cryoprotectant. The apparent viscosity of the samples decreased after freeze-thawing, probably because of the dehydration and compaction of aggregates.

1. Introduction

Soymilk is a beverage traditionally obtained by soaking soybeans in water (1:10 overnight), followed by grinding, boiling and filtration to eliminate the undispersible fiber residue, okara (Chen, 2006). A typical soymilk made from a ratio of 1:10 beans-to-water has much lower calcium content than bovine milk: 20–30 mg Ca^{2+}/100 g versus 120 mg Ca^{2+}/100 g (Chaiwanon, Puwastien, Nitithamyong, & Sirichakwal, 2000). The difficulty of adding calcium to soymilk lies on the fact that soy protein and phospholipids are highly sensitive to divalent ions (Appu Rao & Narasinga Rao, 1975; Pathomrungsiyounggul, Grandison, & Lewis, 2007; Wagner & Tomás, 2007; Whittinghill, Norton, & Proctor, 2000). Indeed, the soy protein aggregation by heating is noticeably enhanced by salt inclusion (Ryan, McEvoy, McSweeney, O'Callaghan, & FitzGerald, 2008). The formulation of heat-stable soymilk and soy beverages include the addition of insoluble calcium salts, the decrease of calcium ion activity by addition of organic calcium salts, the addition of calcium sequestering agents and the encapsulation of divalent ion with membrane systems (Pathomrungsiyounggul, Lewis, & Grandison, 2010; Yazici, Alvarez, Mangino, & Hansen, 1997). In the present work, the glycinin (11S) and β-conglycinin (7S) aggregation is promoted by heating soybean flour aqueous dispersions in the presence of soluble calcium salts and further treatment of protein aggregates by high pressure homogenization to increase the colloidal stability of soy beverages. This strategy was fairly similar to that previously reported by Nelson, Steinberg, and Wei (1976) but calcium salts were not used by these authors in the soymilk formulation.

Although freezing is recognized as an excellent method of preserving food, many undesirable changes in properties may take place during freezing, storage and thawing (Xiong, 1997). As a consequence of water crystallization, freeze-thawing promotes the colloidal destabilization of protein aqueous dispersions. This fact

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was exploited by other authors in order to obtain soymilk freeze-gels (Shimoyamada, Tômatsu, & Watanabe, 1999a,1999b; Shimoyamada, Tômatsu, Oku, & Watanabe, 2000). For oil in water (o/w) emulsions, the freeze-thaw stability can be affected by both the oil crystallization and expanding ice in the aqueous phase. The interfacial film must be able to resist the environmental stresses induced by these phase transitions (McClements, 2004). When an o/w emulsion is frozen, all components in the aqueous phase are cryo-concentrated and the oil droplets are forced together to very high volume or mass fractions; hence, the interactions between droplets are enhanced. Although the interfacial membranes stabilized by proteins are relatively resistant to environmental stresses, these emulsifiers usually exert a limited protection during frozen storage. Therefore, the addition of cryoprotectants is often necessary (Ghosh & Coupland, 2008; McClements, 2004; Thanasukarn, Pongsawatmanit & McClements, 2004). For model o/w emulsions prepared with soybean protein isolates as the sole emulsifier, the addition of dehydrated tofu whey as cryoprotectant enhanced their stability after frozen storage during short and long periods (Palazolo, Sobral, & Wagner, 2013). For Illinois soy beverages, sucrose and glucose was added to prevent visible freeze damage (Yeh, Wei, Nelson, & Steinberg, 1981).

Although the freeze-thaw stability of Illinois soy beverages obtained from dehulled cotyledons by high pressure homogenization was previously evaluated by Yeh et al. (1981), the influence of frozen storage on calcium-fortified soy beverages has been poorly addressed. The purpose of this article was to study the freeze-thaw stability of a soy beverage containing similar calcium content as in bovine milk. Active defatted soyflakes flour as main ingredient and refined sunflower oil as lipid phase were employed. Calcium chloride or lactate was used for calcium fortification and the influence of sucrose as cryoprotectant at different concentrations was also studied.

2. Material and methods

2.1. Materials

Active defatted soybean flakes were supplied by Bunge Argentina S.A. (Puerto General San Martín, Santa Fe, Argentina). Refined sunflower oil (Molinos Cañuelas S.A.C.I.F.I; Cañuelas, Argentina) was purchased in a local supermarket. Bovine serum albumin (>99 g/100 g, fatty acid free) was purchased from Sigma Chemical Co (St Louis, MO, USA). Folin-Ciocalteau reagent, sucrose (>99.5 g/100 g) and calcium chloride (>95 g/100 g) were provided by Anedra (San Fernando, Argentina). Calcium lactate (>98 g/100 g) was purchased from Parafarm (Buenos Aires, Argentina). Distilled water was used in all assays, and all other reagents were analytical grade reagents.

Soybean flakes were ground in all-purpose high speed smashing machine (Chincan, FW Model; China). The resultant defatted soyflakes flour (DSFF) was sieved in a two-step process: *i)* 500 μm (ASTM E-11-81, Zonytest; Buenos Aires, Argentina) and *ii)* 125 μm (ASTM E-11-70, Zonytest; Buenos Aires, Argentina) respectively. The DSFF contains 92.0 ± 0.1 dry solids per 100 g powder and its proximate composition (g/100 g, as dry basis) was: crude protein (N × 6.25, Nkonge & Murray Ballance, 1982), 55.1 ± 0.4; ash (dry ashing at 550 ± 10 °C), 7.7 ± 0.1; insoluble dietary fiber, 20.6 ± 0.6; soluble dietary fiber, 3.3 ± 0.2; galacto-oligosaccharides, 10.0 ± 0.2 (AOAC method 2009.01, AOAC, 2012) and lipids 1.9 ± 0.1 (AOCS Official Procedure Am 5-04, AOCS, 2005). All analytical assays were carried out in triplicate.

2.2. Preparation of soybean beverages

DSFF aqueous dispersions (7.5 g total solids/100 g) were prepared by dispersing the solid sample in distilled water under magnetic stirring for at least 3 h to ensure the complete dispersion and hydration. Three different SF aqueous dispersions were prepared: *i)* without added calcium, *ii)* with calcium chloride and *iii)* with calcium lactate. Calcium salts were added at equivalent total Ca^{2+} content (0.12 g/100 g), without considering the proper calcium content of SF. Dispersions were heated in boiling water bath for 40 min and subsequently cooled to room temperature (~25 °C). Refined sunflower oil (2.5 g/100 g in the total emulsion, $\phi_m = 0.025$) was then added. Pre-emulsions were obtained by mixing the aqueous and oil in a high-speed blender (Ultraturrax T-25, rotor/stator device, S25-20NK-19G dispersing tool, IKA Labortechnik, Staufen, Germany) at 24,000 rpm for 1 min. Pre-emulsions were recirculated three times through a twin-stage valve high pressure homogenizer (Panda 2K, GEA Niro Soavi; Parma, Italy) to finally obtain the soy beverages (SB). The homogenization pressure was 120 MPa and 12 MPa in the first and second valves, respectively. For all obtained SB, without and with calcium salts addition, sucrose was added (5.0 or 10.0 g/100 g in the total emulsion) as cryoprotectant. Emulsions without sucrose were also assayed for comparative purposes. Finally, SB were pasteurized at 75 °C for 1 min and rapidly cooled to room temperature (~25 °C) in a water-ice bath. Sodium azide (0.02 g/100 ml) was added to SB to prevent microbial growth.

2.3. Freeze-thaw protocol

SB were stored at −16.0 ± 2.0 °C for 1, 9 and 30 days. During frozen storage, the freezer temperature periodically varied from −14 to −18 °C and the variation period was 50 min. Thawing was made at room temperature (~25 °C).

Photographs at bulk scale of SB before and after frozen storage were made using a digital camera (Cannon Power Shot A570 IS, Cannon Inc., Malaysia).

2.4. Particle size distribution

The particle size distribution (PSD) of initial and stored SB was determined in the diameter range of 0.1–1000 μm by laser diffraction using a Malvern Mastersizer 2000E analyzer (Malvern Instruments Ltd; Worcestershire, UK). PSD were expressed as volume frequency (×100). Optical parameters applied were: relative refractive index, 1.14 and adsorption coefficient: 0.01. Before particle size measurements, samples were carefully mixed by turning the containers upside down to get a particle size for the whole sample. These aliquots were then dispersed in 600 ml of water at 2000 rpm in the dispersion unit (Hydro 2000 MU, Malvern Instruments Ltd; Worcestershire, UK). From PSD, the De Brouckere (volume-weighted, $D_{4,3}$) moment mean diameter was obtained.

From the obtained data, the particle size increase (PSI) was calculated as follows:

$$PSI = \left[\left(D_f - D_0\right)/D_0\right] \times 100 \quad (1)$$

where D_0 is the initial $D_{4,3}$ value and D_f is the $D_{4,3}$ value after frozen storage for 30 days.

2.5. Optical microscopy

Micrographs were obtained with an optical microscope operating at 400× magnification and using an adapted digital camera (Canon A570 IS; Malaysia) at 4× optical zoom.

2.6. Aqueous protein determination

To determine protein in the aqueous phase, SB samples were firstly centrifuged at 10,000× *g* for 30 min. Insoluble protein debris

were separated and the supernatant was again centrifuged at 10,000× *g* for 60 min to assure the total separation of tiny oil droplets and protein aggregates. After second step of centrifugation, appropriate dilutions of supernatants were made and protein concentration was determined using the modified Lowry method reported by Markwell, Haas, Bieber, and Tolbert (1978). Bovine serum albumin was used as standard. Assays were carried out in triplicate.

2.7. Apparent viscosity

A controlled-shear rate rheometer (AR-G2, TA instruments; Newcastle, USA) with a cone-and-plate geometry (gap = 55 μm; cone diameter, 40 mm; cone angle, 2°) was used to determine the apparent viscosity (η_{app}) of SB. The apparent viscosity was registered after increasing the shear rate from 0.1 to 30 s^{-1} for 100 s. Temperature was controlled at 21.0 °C with a water bath (Julabo ACW100, Julabo Labortechnik; Seelbach, Germany) associated to rheometer. For all freeze-thawed, highly-destabilized soy beverages, this assay was discarded to avoid sampling errors.

2.8. Statistical analysis

Two independent replicates were measured at least two times in each experiment and the results were reported as the mean and standard deviation. The statistical analysis was performed by analysis of variance (ANOVA) and test of least significant difference (LSD) using the statistical program Statgraphics Plus v5.1 (Statgraphics Corporation; USA, 2000). Significance was considered at $p < 0.05$.

3. Results and discussion

All the obtained SB were initially homogenous and fluid. After freeze-thawing, each SB with 10.0 g/100 g sucrose kept a homogeneous appearance until 30 days of frozen storage, because of the cryoprotective effect of the disaccharide. On the other side, the systems with 5.0 g/100 g sucrose or without the disaccharide were eventually destabilized, exhibiting a heterogenous appearance due to protein coagulation and gravitational separation. After freeze-thaw treatment, these samples were separated into a turbid serum layer at the top and an opaque layer at the bottom of the container. The visual appearances at different sucrose concentration can be appreciated, as an example, in the photographs at bulk scale of the SB with addition of calcium chloride before and after different times of frozen storage (Fig. 1). In the presence of 10.0 g/100 g sucrose, it is evident that SB samples remain stable whatever the frozen storage time.

Fig. 2 shows the PSD of the SB before and after freeze-thawing at different periods. In general, the treatment at subzero temperatures produced a displacement of the main population of particles toward higher diameters as the frozen storage time was increased. This effect was most evident in the absence of sucrose (Fig. 2a–c), showing a considerable increase of the $D_{4,3}$ values during frozen storage (Table 1). This result was attributed to the progressive aggregation of particles (proteins, insoluble fiber and oil droplets), because freezing causes an important stress in the microstructure of the systems as a consequence of water crystallization (Ghosh & Coupland, 2008; McClements, 2004). The increased aggregation after freeze-thawing, including protein insolubilization, can be appreciated by optical microscopy analyzing the SB without calcium and without sucrose before and after the treatment (Fig. 3a). This fact is supported by the progressive decrease of aqueous protein concentration in the absence of cryoprotectant (Fig. 4). The effect of sunflower oil crystallization would become important after frozen storage at long periods (Palazolo et al., 2013). Nevertheless, we consider that the SB destabilization upon freeze-thawing was mainly attributed to expanding ice due to low oil mass fraction

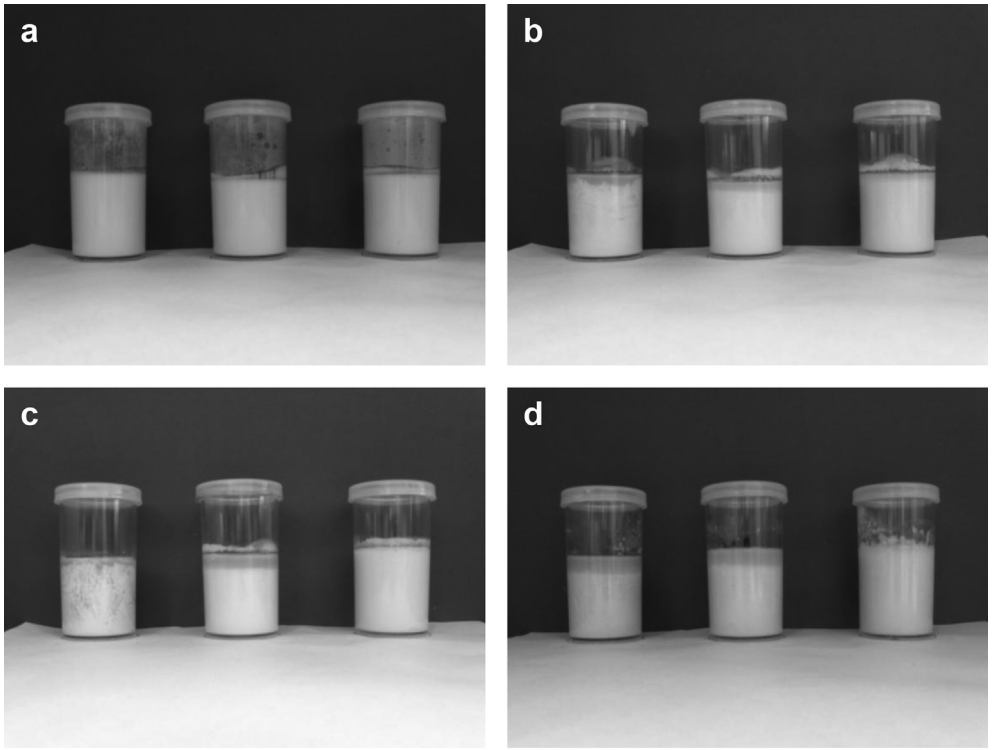

Fig. 1. Photographs of soy beverages with calcium chloride before a) and after frozen storage: b) 1 day; c) 9 days; d) 30 days. In each photograph, from left to right: 0, 5.0 and 10.0 g/100 g sucrose.

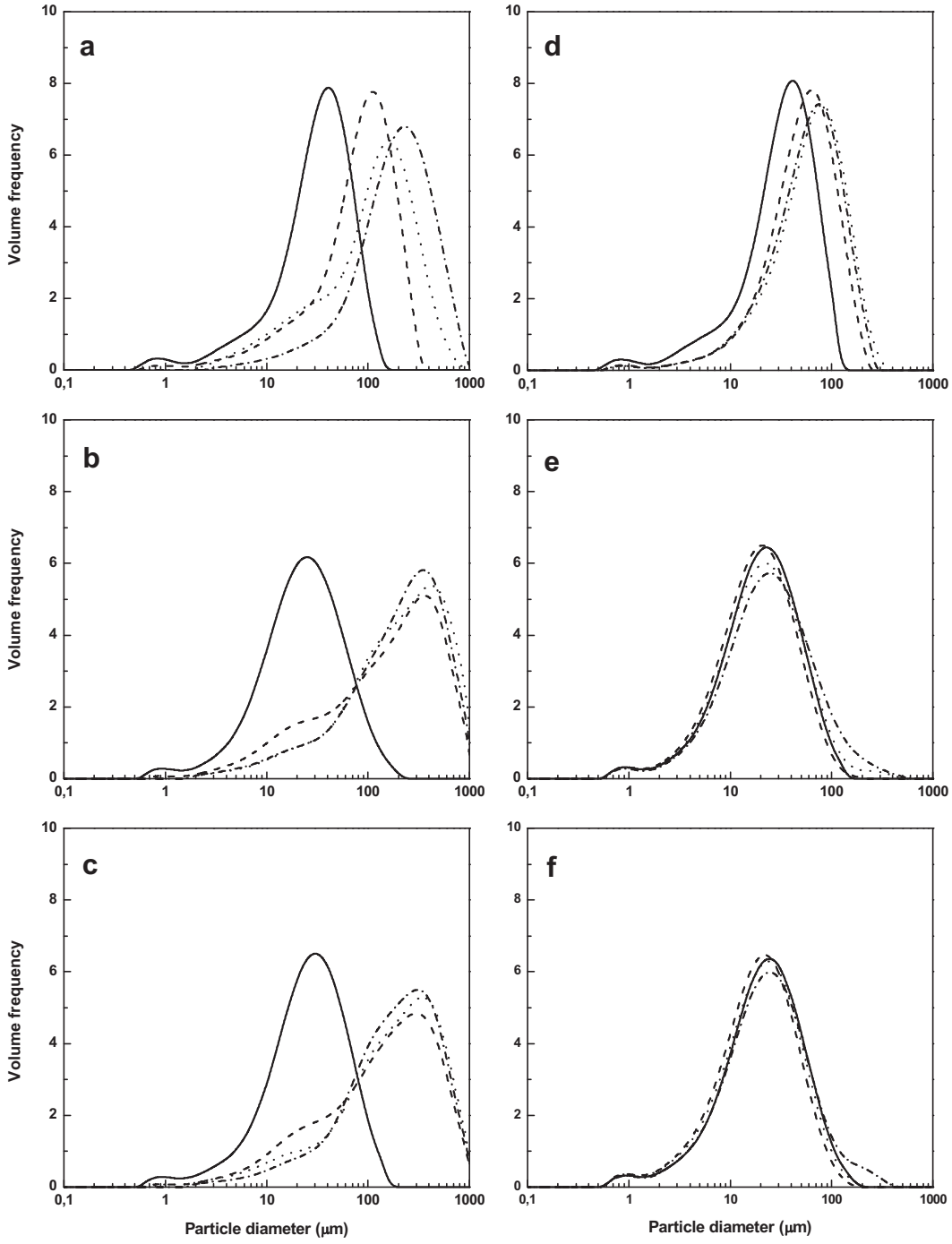

Fig. 2. Particle size distributions expressed as differential volume of soy beverages before (—) and after frozen storage for 1 (– – –), 9 (• • • •) and 30 days (—•—•). Samples without sucrose: a) without added calcium; b) with calcium chloride; c) with calcium lactate. Samples with sucrose (10.0 g/100 g): d) without added calcium; e) with calcium chloride; f) with calcium lactate.

($\phi_m = 0.025$) of these beverages. Because water crystallizes in pure form, two simultaneous phenomena occur: the cryo-concentration of soluble components and the confinement of particles in a smaller volume of unfrozen aqueous phase due to the expansion of ice (Thanasukarn et al., 2004). Thus, the unfrozen aqueous phase of the frozen SB has a noticeable higher viscosity than the aqueous phase of the initial, unfrozen SB; in this way, mass transference processes like protein aggregation or flocculation/coalescence of oil droplets would be unfavored. However, this effect is clearly masked by the screening of the electrostatic repulsion between particles as a consequence of the cryo-concentration of salts and the confinement of the particles in a small volume of unfrozen aqueous phase, thus, the aggregation processes prevail. Due to the low oil mass fraction ($\phi_m = 0.025$), protein aggregation play a preponderant role in the destabilization of SB after freeze-thawing. An observation made by Yeh et al. (1981) provides some support. In that work, Illinois SB prepared by high pressure homogenization without calcium salts addition were centrifuged at low speed and a fiber-rich debris was separated. Then, freeze-thaw stability of non-centrifuged and centrifuged SB was comparatively evaluated.

Table 1
Effect of frozen storage on the mean particle diameter $D_{4,3}$ (μm) of soy beverages.

Sample	Sucrose (g/100 g)	Initial	Frozen storage time (days)		
			1	9	30
No added calcium	0	35.364 ± 0.646[a]	88.986 ± 5.780[b]	136.078 ± 6.326[c]	223.429 ± 18.062[d]
	5.0	35.808 ± 1.235[a]	68.213 ± 5.305[b]	83.307 ± 7.811[b,c]	105.940 ± 13.951[c]
	10.0	34.076 ± 2.413[a]	58.817 ± 1.452[b]	72.798 ± 3.438[c]	68.324 ± 4.002[c]
Calcium chloride	0	29.387 ± 0.867[a]	222.029 ± 1.831[b]	268.246 ± 3.094[c]	250.664 ± 18,487[c]
	5.0	28.534 ± 0.659[a]	40.928 ± 1.105[b]	82.128 ± 2.086[c]	116.480 ± 3.443[d]
	10.0	26.793 ± 0.292[a]	25.305 ± 3.166[a]	29.088 ± 0.547[a,b]	35.798 ± 3.690[b]
Calcium lactate	0	31.989 ± 1.430[a]	206.815 ± 9.956[b]	234.669 ± 6.079[b,c]	250.028 ± 25.803[c]
	5.0	28.650 ± 1.294[a]	46.565 ± 2.662[a,b]	85.494 ± 19.540[b,c]	111.621 ± 24.659[c]
	10.0	26.434 ± 1.242[a]	23.811 ± 1.654[a]	26.185 ± 1.890[a]	32.850 ± 2.132[b]

Values are means of two replicates ($n = 2$) ± standard deviation.
Mean values with different lowercase letters indicate significant differences between samples with different storage time ($p < 0.05$).

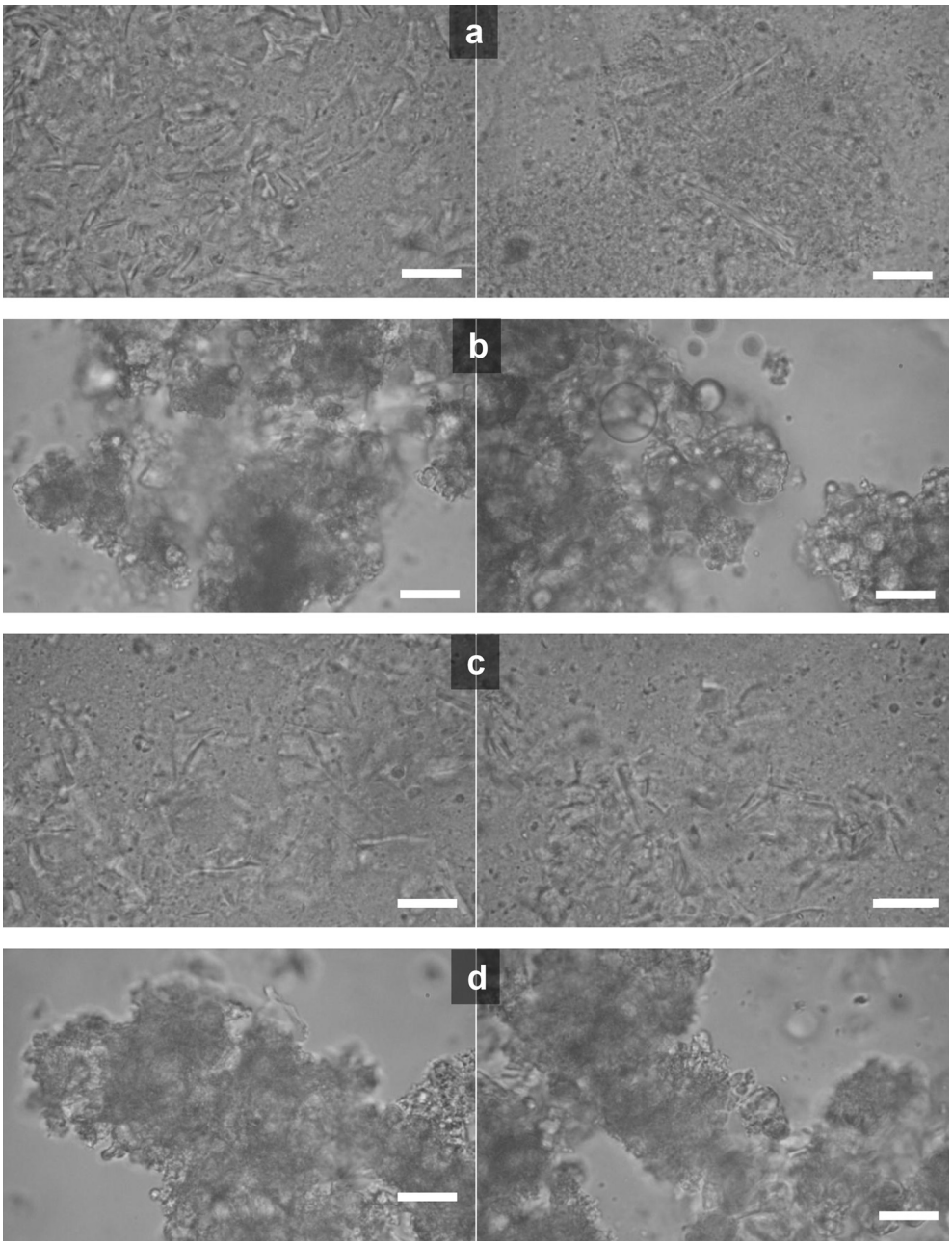

Fig. 3. Microstructure of soy beverages before (left micrographs) and after (right micrographs) frozen storage for 1 day: a) without calcium and without sucrose; b) with calcium chloride and without sucrose; c) without calcium and with sucrose (10.0 g/100 g); d) with calcium chloride and with sucrose (10.0 g/100 g). Bar = 20 μm.

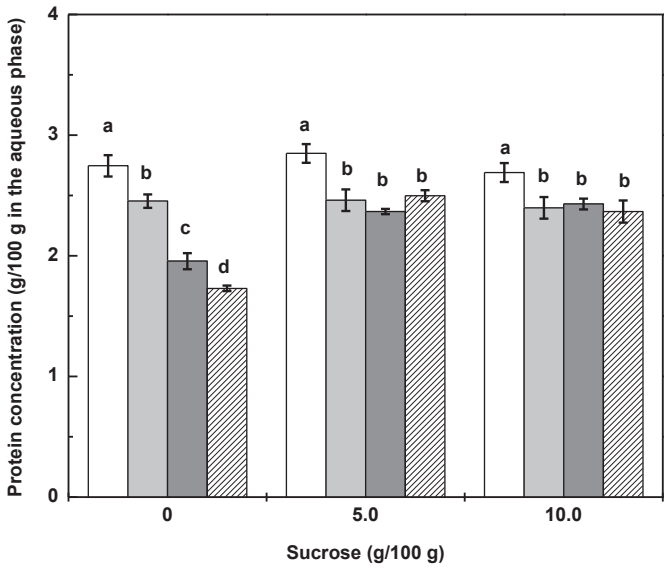

Fig. 4. Effect of freeze-thawing (F–T) on the protein concentration (g/100 g in the aqueous phase) of soy beverages without added calcium at different sucrose concentrations: unfrozen (white bars), after 1 (light gray bars), 9 (gray bars) and 30 (hatched bars) days of F-T. Values are means of two replicates ($n = 2$) and error bars indicate standard deviation. Mean values with different lowercase letters indicate significant differences between samples with different storage time ($p < 0.05$).

After freeze-thaw treatment, similar destabilization degree was observed so that the fiber would be entrapped within the protein aggregates, as it can be observed in Fig 3a.

The differences of stability to freeze-thawing between the SB with and without calcium were notorious in the absence of sucrose (Fig. 2a–c). In both SB fortified with calcium and without the disaccharide, a rapid destabilization was detected, observing high increases of the $D_{4,3}$ values after 1 day of frozen storage (Table 1).

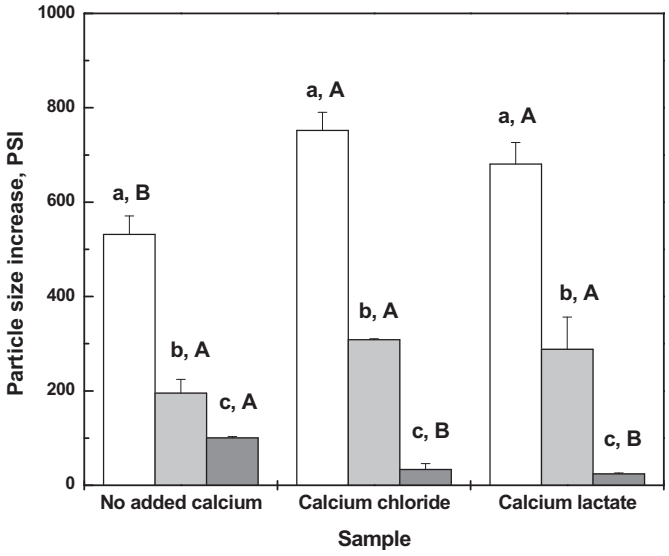

Fig. 5. Particle size increase (PSI) of soy beverages after frozen storage for 30 days without (white bars) and with sucrose at 5 g/100 g (light gray bars) and 10 g/100 g (gray bars). Values are means of two replicates ($n = 2$) and error bars indicate standard deviation. Mean values with different lowercase letters indicate significant differences between samples with different sucrose concentration and same calcium salt ($p < 0.05$). Mean values with different uppercase letters indicate significant differences between samples with different calcium salt and same sucrose concentration ($p < 0.05$).

While the presence of calcium produced aggregation of particles at initial conditions, this microstructure was substantially affected by freeze-thawing in the absence of sucrose, including coalescence of oil droplets (Fig. 3b). The SB without calcium and without sucrose eventually reached a relatively high $D_{4,3}$ value, but this increase was more gradual than in presence of calcium (Table 1). Divalent and multivalent counter-ions are noticeable more efficient than monovalent ones at promoting colloidal destabilization (McClements, 2004). The abrupt destabilization observed in the systems with calcium salts can be attributed to the effective screening of the electrostatic repulsion by Ca^{2+}, favoring the formation of new particle aggregates, especially when the divalent cation was cryo-concentrated in the unfrozen aqueous phase.

Calcium chloride ($CaCl_2$) is highly dissociated while calcium lactate (CaL_2) is only partially dissociated as a consequence of the following two step dissociation equilibrium (Vavrusova, Munk, & Skibsted, 2013):

$$CaL_{2\,(aq)} \leftrightarrow CaL^{+}{}_{(aq)} + L^{-}{}_{(aq)} \quad (2)$$

$$CaL^{+}{}_{(aq)} \leftrightarrow Ca^{2+}{}_{(aq)} + L^{-}{}_{(aq)} \quad (3)$$

The first dissociation step (Eq. (2)) is almost complete whereas the second (Eq. (3)) is reversible, so that in CaL_2 aqueous solutions, at the same total calcium concentration (0.12 g/100 g), free Ca^{2+} concentration and activity are lower than those of $CaCl_2$. Although both calcium salts exhibited different free Ca^{2+} concentration and activity, their influence on freeze-thaw stability of SB seems to be similar (Fig. 2b,c; Table 1). It is known that Ca^{2+} is highly effective to induce aggregation of storage soy proteins (Appu Rao & Narasinga Rao, 1975; Pathomrungsiyounggul et al., 2007, 2010; Ryan et al., 2008; Yacizi et al., 1997). Hence, the similar effect of both calcium salts could be explained by a variety of different mechanisms. First, the differences of free Ca^{2+} activity can be masked by the cryo-concentration during the expansion of ice so that in both calcium-fortified SB, Ca^{2+} activity is sufficient to completely aggregate the storage soy proteins. Second, the binary hydroxycarboxylate complex (CaL^{+}) would also exert a considerable screening of electrostatic repulsion favoring the aggregation after freeze-thawing. Third, due to reversibility of second dissociation step (Eq. (3)) the interaction of Ca^{2+} with soy proteins displaces the equilibrium towards CaL^{+} dissociation, producing new free Ca^{2+} able to interact with protein molecules.

The SB with 5.0 g/100 g sucrose showed a clearly more gradual destabilization than the systems without the disaccharide, but only the SB with 10.0 g/100 g sucrose presented an acceptable stability until 30 days of frozen storage (Table 1). The optical micrographs show that the higher concentration of sucrose prevented the aggregation of particles in the SB without added calcium (Fig. 3c) and the coalescence of oil droplets in the system with calcium chloride (Fig. 3d) after frozen storage. Sucrose has a cryoprotective effect because it partially inhibits the crystallization of water and, thus, the system is subjected to a lower stress degree during the freezing and frozen storage (Ghosh & Coupland, 2008; Thanasukarn et al., 2004;). Sucrose is also cryo-concentrated in the continuous phase and it increases the viscosity of the unfrozen aqueous phase, decelerating the mass transference processes like protein aggregation (Thiebaud, Dumay, & Cheftel, 2002). This was confirmed by the results observed in Fig. 4, corresponding to the SB without calcium. The systems with cryoprotectant only showed a diminution of soluble protein after 1 day of frozen storage and then this parameter was kept constant until 30 days at subzero temperatures, in contrast to the progressive aggregation observed in the absence of sucrose. Moreover, the PSI of every sample, obtained

from the $D_{4,3}$ values before and after freeze-thawing (Eq. (1)), decreased at higher sucrose concentration (Fig. 5), which was clearly consistent with a lower aggregation of particles in SB samples.

After freeze-thawing, in the presence of sucrose at the highest concentration (10.0 g/100 g), both SB with calcium salts showed slighter changes in the PSD and lower $D_{4,3}$ increases than the beverages without addition of the cation (Fig. 2d–f; Table 1). This was opposed to the previously observed result in the absence of sucrose, where the SB with calcium salts exhibited higher changes in the microstructure during frozen storage (Fig. 2a–c). These different results can be appreciated in Fig. 5: in the absence of sucrose, the SB without calcium showed a lower PSI than both systems with the divalent cation; in the presence of sucrose at 5.0 g/100 g, all systems exhibited a similar PSI; and in the presence of sucrose at 10.0 g/100 g, the SB without calcium showed a higher PSI than the other two systems ($p < 0.05$). Thus, calcium salts seemed to give a higher stability against aggregation only when an adequate quantity of cryoprotectant was added. Because aggregation was favored during the heat treatment in the presence of Ca^{2+}, and then the big aggregates were reduced by high pressure homogenization, new aggregation processes might have been limited during frozen storage. Both SB with $CaCl_2$ or CaL_2 showed a considerably lower protein concentration in aqueous phase at initial condition (~0.4 g/100 g) than the system without the divalent cation (~2.7 g/100 g), because the presence of Ca^{2+} produced high protein insolubilization; this led to different microstructures when comparing the SB with and without calcium, as it can be appreciated by optical microscopy (Fig. 3). Moreover, the protein concentration in aqueous phase did not decrease during frozen storage in both SB with $CaCl_2$ or CaL_2 whatever the presence of sucrose (data not shown), as it did in the absence of added calcium (Fig. 4). The absence of new protein insolubilization in the SB with calcium could explain their higher stability against aggregation. This effect was only manifested in the presence of sucrose at 10 g/100 g, because the formation of ice was more limited and the particles were not forced to occupy a small space in the unfrozen aqueous phase. Furthermore, the lower cryo-concentration of Ca^{2+} as a consequence of the sucrose addition would reduce the protein aggregation. Although Arakawa, Pertrelski, Kenney, and Carpenter (2001) reported that lactate ion is itself an effective cryoprotectant for proteins, no additional crioprotective effect was observed for SB with CaL_2 addition with respect to those with $CaCl_2$. Nevertheless, the crioprotective effect was reported for highly dissociated sodium lactate. According to Vavrusova et al. (2013), an important proportion of lactate ion is associated to binary CaL^+ complex (Eqs. (2) and (3)), so that the CaL_2 salt would not act as an effective cryoprotectant. It is highly probable that the crioprotective action of lactate ion is masked by the presence of Ca^{2+} and CaL^+ species.

On the other hand, for those SB that exhibited a good stability to freeze-thawing (10.0 g/100 g sucrose, Fig. 1), the η_{app} was also evaluated. The initial η_{app} values clearly decreased in the order $CaL_2 > CaCl_2 >$ without calcium ($p < 0.05$; Table 2). Undoubtedly, the presence of calcium salts induces the protein aggregation, which is consistent with the higher initial η_{app} values. Moreover, the SB with CaL_2 presented a higher initial η_{app} value than the system with $CaCl_2$, which can be attributed to a different initial structure of protein aggregates. Studies of microstructure of firm tofu by Kao, Su, and Lee (2003) indicated that an excess of calcium produced a too compact and less uniform structure of the protein network, as a result of too many cross-linkages. Because $CaCl_2$ provides a higher quantity of free Ca^{2+} than calcium lactate, it might produce more compact protein aggregates in the SB than the organic salt. In addition, the possible interaction of the proteins with the binary hydroxycarboxylate complex (CaL^+), produced as a consequence of the partial dissociation of CaL_2 (Eq. (2)), could reduce the number of cross-linkages and increase the hydration of protein aggregates. These differences in structure and hydration of protein aggregates are a plausible explanation of the higher initial η_{app} value observed in the SB with CaL_2.

Although no gravitational separation was observed after different periods of frozen storage, the SB samples showed a decrease of the η_{app} values (Table 2). For the SB without added calcium, the η_{app} values decreased only after 1 day of frozen storage, not observing significant changes in the rheological parameter during the subsequent measurements. As was mentioned above, new particle aggregation was observed during frozen storage in the absence of added calcium according to the $D_{4,3}$ values (Table 1). Nevertheless, the frozen storage would favor the dehydration and close packing of these particles in the unfrozen, cryo-concentrated aqueous phase, so that the degree of interaction between aggregates, and thus the η_{app} values, would be effectively decreased. In the presence of calcium salts (CaL_2 or $CaCl_2$), although the $D_{4,3}$ values were almost maintained (Table 1), the η_{app} values exhibited a progressive decrease as a function of the frozen storage time ($p < 0.05$; Table 2). In this case, the dehydration and close packing would be enhanced due to the presence of Ca^{2+} and CaL^+ (in the SB with added CaL_2) species in the unfrozen aqueous phase. Although the SB with CaL_2 showed a higher initial η_{app} value than the system with $CaCl_2$, both samples reached a similar η_{app} value after 30 days of frozen storage. This result suggests that both SB with added calcium salts tended to present similar dehydration and compaction of aggregates as a consequence of the freeze-thaw treatment.

4. Conclusions

In the absence of sucrose as cryoprotectant the freeze-thaw treatment induced a rapid destabilization of calcium-fortified soy beverages, whatever the added calcium salt (chloride or lactate). Conversely, the control, without added calcium, showed a progressive destabilization as a function of frozen storage time. These differences can be explained by the high ability of Ca^{2+} (and also probably, the monovalent binary complex between Ca^{2+} and lactate) to screen the electrostatic repulsion between particles due to their cryo-concentration in the unfrozen aqueous phase. The freeze-thaw stability of was highly enhanced with sucrose addition,

Table 2
Effect of frozen storage on the apparent viscosity (η_{app}, Pa·s·10^2) of soy beverages with sucrose addition (10.0 g/100 g).

Sample	Initial	Frozen storage time (days)		
		1	9	30
No added calcium	$8.501 \pm 0.117^{a,A}$	5.785 ± 0.465^{b}	5.568 ± 0.551^{b}	5.765 ± 0.300^{b}
Calcium chloride	$22.185 \pm 2.765^{a,B}$	15.643 ± 0.774^{b}	$11.067 \pm 2.331^{b,c}$	9901 ± 1.250^{c}
Calcium lactate	$32.200 \pm 2.998^{a,C}$	20.623 ± 0.074^{b}	15.555 ± 0.240^{c}	9.478 ± 0.140^{d}

Values are means of two replicates ($n = 2$) ± standard deviation.
Mean values with different lowercase letters indicate significant differences between samples with different storage time and same calcium salt ($p < 0.05$).
Mean values with different uppercase letters indicate significant differences between initial samples with different calcium salt ($p < 0.05$).

but cryoprotectant (10 g/100 g) was required to inhibit the gravitational separation and the extensive aggregation during frozen storage. Interestingly, calcium-fortified soy beverages showed a better behavior than the control because the mean De Brouckere particle diameter, which is especially sensitive to the presence of large particles or aggregates, was almost maintained even after 30 days of storage at subzero temperatures. In the presence of sucrose at the highest concentration (10.0 g/100 g), the apparent viscosity of all soy beverages decreased as a function of frozen storage time, presumably due to progressive dehydration and close packing in the unfrozen aqueous phase.

Undoubtedly, due to the high ionic strength of calcium-fortified soy beverages, a cryoprotectant is required at relatively high concentration. Moreover, as a consequence of different free Ca^{2+} activity and solubility associated to calcium salts, further studies will be necessary if other calcium salts (e.g. gluconate) are employed for fortification. In the same way, further investigation will be required if other sugars or non sweet cryoprotectans are used. The results of this study could facilitate the development of a calcium-fortified soy beverage with good stability to freeze-thawing.

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