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RHEOLOGY OF DOUBLE (W/O/W) EMULSIONS PREPARED WITH SOYBEAN MILK AND FORTIFIED WITH CALCIUM

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ABSTRACT

The objective of this work was to study the rheological behavior of water-in-oil-in-water (w/o/w) emulsions prepared with soybean milk and sunflower oil, with different calcium solutions as the internal aqueous phase, in order to evaluate them as a vegetable substitute of whipped dairy cream. The obtained systems exhibited a creamy texture, which was attributed to the swelling of w/o droplets because of the osmotic gradient generated by the inclusion of soluble salts in the internal aqueous phase. A secondary factor could be the flocculation of w/o droplets due to the interaction of released calcium with soybean proteins at the interface. Consequently, the increase of calcium chloride content produced emulsions with higher consistency. A pasteurization produced flocculation and coalescence of w/o droplets only at high calcium chloride content. These double emulsions could be a potential alternative to the whipped dairy cream, because of their texture, reduced fat content and calcium contribution.

PRACTICAL APPLICATIONS

This article deals with the formulation of novel calcium-fortified food emulsions prepared with soybean milk and sunflower oil. Because calcium needs to be isolated from soybean milk components (proteins and

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phospholipids), we proposed to include calcium salts in the internal aqueous phase of a water-in-oil-in-water (w/o/w) emulsion. The practical applications of this research could include the formulation of low lipid content emulsions and the isolation of a component which is incompatible with the continuous aqueous phase. Particularly, this work leads to the understanding of how the inclusion of calcium salts in the internal aqueous phase of a w/o/w emulsion prepared with soybean milk affects the rheology and microstructure of the system. The results led to the conclusion that these emulsions can work as a whipped dairy cream substitute with vegetal components, low lipid content and important calcium contribution.

KEYWORDS

Calcium, pasteurization, rheology, soybean milk, w/o/w emulsions

INTRODUCTION

Soybean milk is a very nutritive beverage, free of cholesterol and lactose, and with similar or higher protein content than cow's milk. However, soybean milk has much lower calcium content than cow's milk: 20–30 mg Ca/100 mL versus 120 mg Ca/100 mL (Chaiwanon *et al.* 2000). Since calcium is an essential mineral for bone growth and dental health, soybean milk should be fortified with this cation to overcome this deficiency. Nevertheless, calcium fortification is limited because this cation tends to bind by the soybean proteins and phospholipids, leading to the formation of aggregates and the destabilization of the system (Appu Rao and Narasinga Rao 1975; Molina and Wagner 1999; Whittinghill *et al.* 2000; Pathomrungsiyounggul *et al.* 2007; Wagner and Tomás 2007). A mild heat treatment like pasteurization also induces the coagulation of soybean milk proteins in the presence of calcium (Pathomrungsiyounggul *et al.* 2010). Previous works showed that a heat stable soybean milk fortified with calcium at relatively high concentrations can be obtained by the addition of citrate and phosphate as sequestering agents (Yazici *et al.* 1997; Pathomrungsiyounggul *et al.* 2010). The present work proposes an alternative solution that leads to the formulation of a novel calcium-fortified food emulsion.

Whipped dairy cream is an oil-in-water (o/w) emulsion that owes its creamy texture to a partial coalescence process, by which oil droplets form a rigid tridimensional network (Boode and Walstra 1993; Boode *et al.* 1993; Goff 1997). This phenomenon requires the presence of saturated fat in the dispersed lipid phase, as oil globules are joined because of hydrophobic interactions between fat crystals that penetrate the interfaces; this allows the characteristic consistency increase of dairy cream after whipping or shaking (Vanapalli and Coupland 2001). Formulation and characterization of cream-like emulsions prepared with soybean milk were successfully accomplished in previous works (Márquez *et al.* 2005a,b,c), but the addition of high melting fraction of dairy fat was needed to simulate the creamy texture of traditional dairy creams; this is opposed to the objective of obtaining a healthy, free of cholesterol vegetable system. Also, calcium fortification was a parallel challenge for these soybean milk-based emulsions.

Double or water-in-oil-in-water (w/o/w) emulsions are multiple emulsions composed of two aqueous phases and a lipid one. The first aqueous phase is dispersed in oil, which, in turn, is dispersed in a continuous second aqueous phase. The most common method to prepare this type of emulsion involves the following two stages (Matsumoto and Kang 1989): (1) preparation of a water-in-oil (w/o) emulsion with a lipophilic emulsifier by a homogenization method of high energy; and (2) dispersion of this w/o emulsion in a second aqueous phase with a hydrophilic emulsifier, with lower homogenization energy. Differences in homogenization energies are necessary to obtain large enough oil droplets to contain very small water droplets (Dickinson and McClements 1995). As multiple emulsions are able to entrap important substances in the internal droplets, they are used in applications where controlled (slow and prolonged) release of important ingredients is desired. Due to increasing knowledge of the stabilization of multiple emulsions, these kinds of emulsions represent a very promising encapsulation method for both hydrophilic and lipophilic compounds (Auweter 2001). It was previously demonstrated that the rheological properties of a w/o/w emulsion are similar to those of a simple o/w emulsion having the same volume fraction of dispersed phase, but lower oil content (de Cindio and Cacace 1995).

Multiple emulsions are classified into three broad groups depending on the number of internal droplets present in the multiple emulsion droplets (Florence and Whitehill 1981; Garti 1997; Garti and Benichou 2001): (1) type-A multiple emulsion, where the multiple emulsion droplet consists of only one large internal droplet, that is, the multiple emulsion droplet is of "core-shell" type; (2) type-B multiple emulsion, where the multiple emulsion droplet consists of several small internal droplets; and (3) type-C multiple emulsion, where the multiple emulsion droplet consists of a large number of internal droplets. Applications of multiple emulsions in the food industry include: (1) encapsulation and controlled release of nutrients; (2) manufacture of low-calorie food products; and (3) manufacture of food products with improved sensorial characteristics (Dickinson *et al.* 1991; Owusu *et al.* 1992; Garti 1997; Van der Graff *et al.* 2005; Kim *et al.* 2006). The isolation of a substance in the internal aqueous phase of a w/o/w emulsion can also serve to avoid the destabilization of the system when there is an incompatibility of this substance with other ones present in the continuous aqueous phase, as it occurs with calcium ions and the soybean milk components.

The objective of this work was to obtain w/o/w emulsions prepared with soybean milk and sunflower oil, in order to study the effect of the addition of calcium in the internal aqueous phase on the rheology of these systems. Although the interaction of calcium with soybean proteins should have an effect on the rheology of the emulsions, other factors such as the osmotic gradient were also analyzed. Variations of added calcium amount, salt type, lipophilic emulsifier concentration, oil content and dispersed aqueous phase percentage, and the effect of pasteurization were studied. In this way, we studied the effectiveness of calcium isolation from the soybean milk components and evaluated these emulsions as a potential vegetable and reduced lipid content substitute of whipped dairy cream.

MATERIALS AND METHODS

Materials

Fluid soybean milk (provided by SOYANA SH, San Martín, Argentina); refined sunflower oil (Molinos Río de la Plata S.A., Avellaneda, Argentina); distilled water; polyglycerol polyricinoleate (PGPR 90; Grindsted-Danisco; provided by CALSA, Lanús, Argentina); xanthan gum (Parafarm, Buenos Aires, Argentina); calcium chloride, anhydrous (Anedra, San Fernando, Argentina); calcium lactate (Parafarm); calcium carbonate, precipitated (Parafarm); sodium chloride, crystal (J.T. Baker, Xalostoc, Mexico). All salts were of analytical grade.

The solubility in water of the salts was determined at defined concentration (0.25 M) and temperature (25C). Calcium and sodium chloride, which are totally dissociable, were 100% soluble; calcium lactate, organic salt less dissociable than calcium chloride, was 73% soluble; and calcium carbonate, the least dissociable salt, was 0.025% soluble.

Preparation of Emulsions

W/o/w emulsions were obtained by the two-stage emulsification method. The first stage consisted on the preparation of a w/o emulsion by homogenization of the aqueous phase (distilled water without or with added salts) and sunflower oil (containing PGPR as lipophilic emulsifier) using an Ultraturrax T-25 (IKA-Works, Wilmington, NC) with a S25-NK-19G rotor (IKA-Labortechnik, Staufen, Germany; rotor/stator distance, 0.3 mm; rotor diameter, 12.7 mm) at 24,000 rpm during 2 min (sample weight, 70 g). In the

TABLE 1.
VARIATIONS OF DIFFERENT PARAMETERS IN THE STUDIED W/O/W EMULSIONS: ADDED SALT TYPE, CALCIUM AMOUNT, DISPERSED LIPID PHASE (DLP) CONTENT, PGPR CONCENTRATION, AND DISPERSED AQUEOUS PHASE (DAP) PERCENTAGE

Salt	Calcium concentration (mg/100 g emulsion)	DLP* (% w/w)	PGPR† (% w/w)	DAP† (% w/w)
Calcium chloride	120	40	1.0	20
Calcium lactate	120	40	1.0	20
Calcium carbonate	120	40	1.0	20
Sodium chloride‡	–	40	1.0	20
Calcium chloride	**30**	40	1.0	20
Calcium chloride	**60**	40	1.0	20
Calcium chloride	**120**	40	1.0	20
Calcium chloride	60	**30**	1.0	20
Calcium chloride	60	**40**	1.0	20
Calcium chloride	60	**50**	1.0	20
Calcium chloride	60	40	**0.5**	20
Calcium chloride	60	40	**1.0**	20
Calcium chloride	60	40	**2.0**	20
Calcium chloride	60	40	1.0	**10**
Calcium chloride	60	40	1.0	**20**
Calcium chloride	60	40	1.0	**30**
Calcium chloride	60	40	1.0	**40**

* DLP includes the whole primary w/o emulsion.
† PGPR and DAP percentages are expressed in relation with DLP.
‡ The system with sodium chloride in the DAP was prepared with 263 mg NaCl/100 g emulsion, corresponding to the same osmolarity (or ions molarity) than the emulsion with calcium chloride at a level of 120 mg Ca/100 g.
Bold indicates which factor is varied.

second stage, the primary w/o emulsion was mixed with soybean milk (containing 0.2% w/w xanthan gum as stabilizer) and the mixture was homogenized by Ultraturrax with a S25N-10G rotor (IKA-Labortechnik; rotor/stator distance, 0.35 mm; rotor diameter, 7.5 mm) at 24,000 rpm for 1 min (sample weight, 50 g).

The effect of added calcium amount, salt type, PGPR concentration, dispersed lipid phase (DLP) content and dispersed aqueous phase (DAP) percentage on the characteristics of the w/o/w emulsions was studied under the conditions described in Table 1. Comparative o/w emulsions with soybean milk (without or with calcium chloride at 60 mg Ca/100 g of emulsion) and 40% w/w oil were prepared at the conditions of the second stage of homogenization. The emulsions were stored at 4C for 1 day before the corresponding characterizations. In order to evaluate the effect of storage on particle size, the emulsions were stored at 4C until 15 days.

Light Microscopy

Micrographs were obtained with a Leica DMLB optical microscope (Leica Microsystems, GmbH, Wetzlar, Germany) with an adapted digital camera (Leica DC100) operating at 100× or 200× magnification.

Particle Size Distribution

Particle size distributions of the emulsions were obtained with a particle analyzer (Malvern Mastersizer 2000E, Malvern Instruments Ltd., Worcestershire, U.K.). Mean particle diameters (d_{43}) were obtained from the volume particle size distributions. Samples were diluted in water in the dispersion system (Hydro 2000MU) at different speeds (600, 2,000 and 3,000 rpm). All measurements were performed at least in duplicate.

Rheology

Rheological analysis of the emulsions was performed with a Haake RS600 rheometer (Haake, Karlsruhe, Germany) with a serrated PP35-S rotor using a 1-mm gap parallel-plate sensor. Experimental data were obtained by recording the storage or elastic modulus (G'), loss or viscous modulus (G''), tan δ (G''/G'), and complex viscosity (η^*) as a function of oscillation frequency (0.01–10 Hz range) within the linear viscoelasticity range (stress = 1 Pa). Tests were conducted at least in duplicate and temperature was maintained at 15C during the experiment.

Pasteurization Assays

Assays simulating the pasteurization process of emulsions were performed in a thermostatic bath at 75C during different times (15, 30 and 60 s), with later cooling in water. Two grams of sample located in the bottom of a short assay tube was pasteurized. The additional time to reach pasteurization temperature was 15 s. Changes produced by heating were evaluated by optical microscopy on diluted emulsions and by particle size determination.

Statistical Analysis

The statistical analysis was performed by analysis of variance and test of least significant difference ($P < 0.05$) using the statistical program Statgraphics Plus 5.1.

RESULTS AND DISCUSSION

Increasing of Consistency by Osmotic Gradient

The w/o/w emulsions with soluble salts (calcium chloride or lactate, and sodium chloride) in the internal aqueous phase showed a creamy texture, similar to a whipped dairy cream. It was observed an increase of the consistency of these systems during and rapidly after the second stage of homogenization. The absence of salts or the inclusion of little soluble calcium carbonate produced liquid systems, either as an o/w or w/o/w emulsion. According to Matsumoto and Sherman (1981), this rheological behavior of w/o/w emulsions with soluble salts in the internal aqueous phase can be explained by the swelling of w/o droplets during and immediately after homogenization, as a consequence of the osmotic gradient generated by the inclusion of soluble salts in the internal aqueous phase. A much lower concentration of diluted components in the external aqueous phase would lead to the passage of water from this phase toward the internal aqueous phase. The transportation of water through the oil layers is thought to involve solubilization into reverse micelles of lipophilic emulsifier (Matsumoto *et al.* 1980), which is PGPR in our case. This phenomenon seems to produce an important increase of volume fraction of dispersed w/o phase, with respect to the initial percentage of primary w/o emulsion, explaining the consequent increase of consistency because of a greater contact between droplets. It should be taken into account that an increase of only 10% in the w/o droplets diameter produces an increase of 33% in the volume of dispersed w/o phase. This increase of consistency can be obtained in a simple o/w emulsion as a result of higher oil concentration (Campanella *et al.* 1995). In our systems, water acted as an oil substitute, with the double benefit of reducing the required lipid content and increasing the dispersed w/o phase.

By optical microscopy, a w/o/w emulsion prepared with calcium chloride was observed as compacted dark droplets (Fig. 1a). The darker appearance of the dispersed w/o phase in this emulsion (as well as in w/o/w emulsions with calcium lactate and sodium chloride) would be attributed to a higher amount of water inside the w/o droplets. As a contrast, clearer droplets were observed in both liquid emulsions, o/w without added salt (Fig. 1b) and w/o/w with calcium carbonate (Fig. 1c). On the other hand, when the osmolarity in the internal and external aqueous phases was balanced in a w/o/w emulsion (with $CaCl_2$ in the internal aqueous phase) by adding an amount of NaCl in the soybean milk (to achieve an equivalent ions molarity), liquid w/o/w emulsions were obtained (Fig. 1d). This result confirms that the increment of consistency of w/o/w emulsions was mainly a result of the osmotic gradient, since the addition of salt in soybean milk counteracted that effect. In this way, the partial coalescence that produces the characteristic texture of whipped dairy creams (Boode and Walstra 1993) was substituted by the increase of dispersed phase, despite the relatively low lipid content (32% of oil in w/o/w emulsions with 40% DLP and 20% DAP) and giving away the need of addition of saturated fats.

Beyond their rheology, all these emulsions were stable against creaming for at least 1 month, which was attributed to the presence of 0.2% xanthan

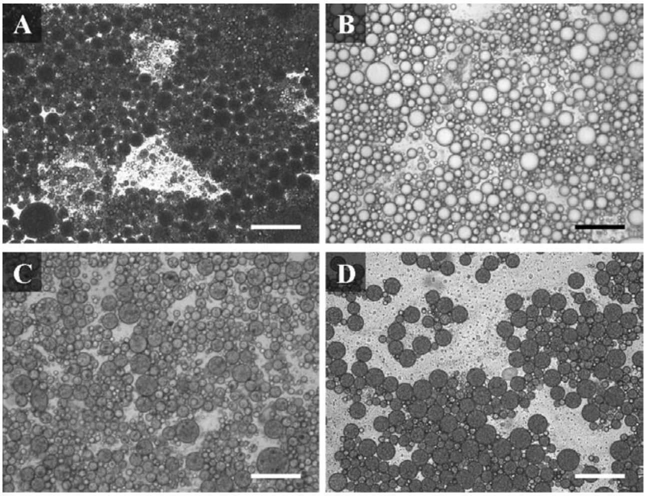

FIG. 1. OPTICAL MICROGRAPHS OF EMULSIONS PREPARED WITH SOYBEAN MILK
Image A: w/o/w emulsion with 120 mg Ca/100 g (40% DLP with 1% PGPR and 20% DAP containing calcium chloride). Image B: o/w emulsion with 40% oil and without added salt. Image C: w/o/w emulsion with 120 mg Ca/100 g (40% DLP with 1% PGPR and 20% DAP containing calcium carbonate). Image D: w/o/w emulsion with 120 mg Ca/100 g (40% DLP with 1% PGPR and 20% DAP containing calcium chloride) and sodium chloride in the continuous aqueous phase (balanced osmolarity in both aqueous phases). Bar = 100 μm.

gum in the continuous aqueous phase before homogenization, as it was previously demonstrated in stability studies with o/w emulsions prepared with soybean milk (Márquez *et al*. 2005a). The creamy texture of w/o/w emulsions with soluble salts in the internal aqueous phase was also observed in the absence of xanthan gum. Moreover, it was previously demonstrated that calcium reduces the viscosity of aqueous solutions containing xanthan gum (Bergmann *et al*. 2008); thus, the increase of consistency when calcium salts were included would not be a consequence of an interaction of the cation with the hydrocolloid.

Effect of Including Calcium In and Out the Dispersed Phase

In the studied emulsions containing soybean milk as continuous aqueous phase, the interface surrounding the w/o droplets is composed mainly of proteins and phospholipids. It is known that calcium affects the functionality of both soybean milk components, then the addition of calcium in emulsions prepared with soybean milk is expected to affect their stability (Appu Rao and Narasinga Rao 1975; Molina and Wagner 1999; Whittinghill *et al*. 2000; Pathomrungsiyounggul *et al*. 2007; Wagner and Tomás 2007). It is, therefore,

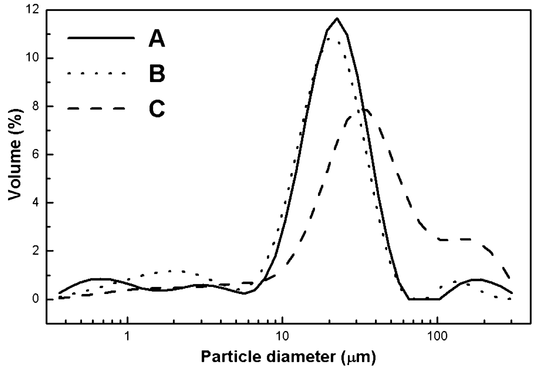

FIG. 2. VOLUME PARTICLE SIZE DISTRIBUTIONS FOR EMULSIONS PREPARED WITH SOYBEAN MILK

A: w/o/w emulsion with 60 mg Ca/100 g (40% DLP with 1% PGPR and 20% DAP containing calcium chloride). B: o/w emulsion with 40% oil and without added salt. C: o/w emulsion with 60 mg Ca/100 g (40% oil and calcium chloride added in the aqueous phase). Dispersion speed: 2,000 rpm.

required to prevent or reduce the interaction of calcium with soybean milk or its components. In order to evaluate the functionality of the w/o/w emulsions about the cation isolation in the internal aqueous phase, a comparative o/w emulsion with equivalent calcium content was prepared. Figure 2 shows the particle size distributions of a w/o/w emulsion fortified with calcium and two o/w emulsions, with and without added calcium. It was observed that the inclusion of calcium chloride in the dispersed aqueous phase of the w/o/w emulsion (Fig. 2a) produced similar particle sizes than the o/w system with same (initial) dispersed phase (40%) and without added cation (Fig. 2b). The increase of volume fraction of dispersed w/o phase does not necessarily imply an important increase of particle size, since swelling and rupture of w/o droplets would simultaneously occur during homogenization. Thus, the addition of calcium in the internal aqueous phase produced w/o/w emulsions with a higher number of oil droplets whose size was not considerably different than the droplets of an o/w emulsion without calcium. When the same calcium amount (60 mg Ca/100 g) was added in the soybean milk immediately before homogenization, the particle size of the o/w emulsion was considerably increased (Fig. 2c), which corresponds to the presence of flocs and/or free big oil droplets; this was also evidenced by a yellowish, less homogeneous appearance of the emulsion. This last emulsion also showed a slightly viscous texture attributed to the interaction of soybean proteins with calcium, which promotes both coagulation and droplet flocculation. The larger oil droplet sizes could be explained by a lower solubility of soybean proteins in presence of calcium and their incapability of being adsorbed in the interface during homogenization. Thus, the addition of calcium in the dispersed aqueous phase of w/o/w emulsions seems to allow the isolation of the cation from the soybean milk proteins, bringing the benefit of production of systems with smaller particle sizes and a whipped cream-like appearance.

Effect of Added Calcium Amount

Figure 3 shows the effect of the variation of calcium content on the rheology of the studied emulsions. It was observed a conclusive increase of G′ and G″ values as calcium content was increased (Fig. 3a), evidencing the formation of more thick systems as a consequence of an osmotic gradient generated by higher differences of salt concentration between the continuous and the internal aqueous phases. Another probable simultaneous factor is the

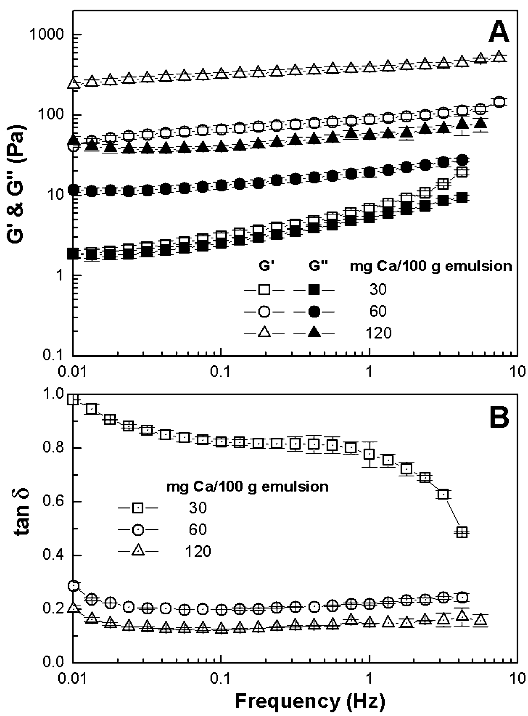

FIG. 3. EFFECT OF CALCIUM CONTENT ON (A) ELASTIC MODULUS (G′) AND LOSS MODULUS (G″), AND (B) TAN δ (G″/G′) OF W/O/W EMULSIONS
Systems with 40% DLP (with 1% PGPR and 20% DAP containing calcium chloride). Values are means of two replicates and error bars indicate SD.

TABLE 2.
EFFECT OF DISPERSION SPEED (RPM) ON MEAN PARTICLE DIAMETER (D_{43}) OF W/O/W EMULSIONS STORED AT 4C FOR 1 DAY

Salt	Osmolarity in internal aqueous phase (Osm)	Calcium concentration (mg/100 g emulsion)	d_{43} (μm)		
			At 600 rpm	At 2,000 rpm	At 3,000 rpm
$CaCl_2$	0.281	30	57.4 ± 2.0 b	44.2 ± 2.1 d, e	40.4 ± 2.5 e, f
$CaCl_2$	0.563	60	57.7 ± 3.6 b	43.4 ± 0.5 d, e	40.0 ± 1.7 e, f
$CaCl_2$	1.125	120	72.4 ± 3.3 a	49.5 ± 2.3 c	40.5 ± 1.8 e, f
NaCl	1.125	–	46.8 ± 2.1 c, d	40.6 ± 2.0 e, f	36.6 ± 0.8 f

Assayed emulsions contain 40% DLP (with 1% PGPR and 20% DAP) and calcium chloride or sodium chloride in internal aqueous phase.
Values are means of two replicates ± SD.
Mean values with different letters are significantly different ($P < 0.05$).

higher flocculation degree of w/o droplets at high calcium concentrations, which was evidenced by the higher d_{43} value at low dispersion speed (600 rpm) in the emulsion with 120 mg Ca/100 g (Table 2). At higher dispersion speeds (2,000 and 3,000 rpm) the unstable flocs were dissociated, as the d_{43} values decreased in all cases; it was observed that the higher the calcium content the higher the difference between the d_{43} values at 600 and 3,000 rpm (Table 2), indicating a higher floculation degree. This result could be attributed to the release of a proportion of calcium to the continuous aqueous phase and its interaction with the soybean proteins at the interface, favoring the formation of flocs; this effect would be more important at higher calcium amounts in the formulation. Flocculation increases the viscosity of an emulsion because the effective volume fraction of a floc is greater than the sum of the volume fractions of the individual droplets due to the presence of the continuous phase trapped within it (McClements 1999). At high dispersion speed (3,000 rpm) the d_{43} values were similar at every calcium concentration, indicating that the individual w/o droplets size did not vary (Table 2). O/w and w/o/w emulsions are known to have higher viscoelastic parameters with decreasing particle size at same volume fraction of dispersed phase (Clark and Pilehvari 1993; Márquez *et al*. 2007). Since in our w/o/w emulsions (with same initial volume fraction of dispersed w/o phase), the w/o droplets size did not decrease from 30 to 120 mg Ca/100 g, the increase of G' and G'' values would be a consequence of a higher final volume fraction of dispersed w/o phase and/or a higher flocculation degree of w/o droplets. In Fig. 3b, it can be observed that tan δ was lower than 1 ($G' > G''$) in these w/o/w emulsions; a higher calcium content increased the viscoelastic parameters favoring the elastic character over the viscous one. The w/o/w emulsion with 120 mg Ca/100 g reached a lower value

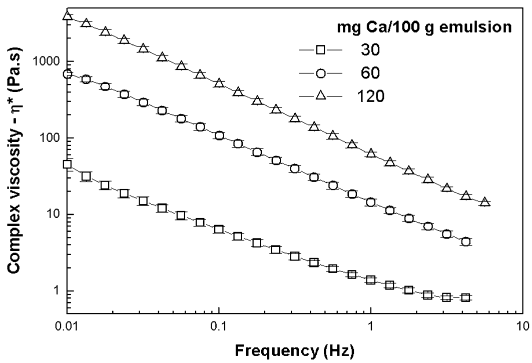

FIG. 4. EFFECT OF CALCIUM CONTENT ON THE COMPLEX VISCOSITY (η*) OF W/O/W EMULSIONS

Systems with 40% DLP (with 1% PGPR and 20% DAP containing calcium chloride). Values are means of two replicates and error bars indicate SD.

of tan δ (<0.15) in all frequency ranges, which corresponds to a rheological behavior of a highly hydrated gel.

The complex viscosity (η*) of w/o/w emulsions with different calcium contents can be observed in Fig. 4. According to Ross-Murphy (1995), these emulsions would be classified as strong gels. The diminution of η* with the frequency indicates that these systems are pseudoplastic; this behavior can be explained by the gradual rupture of flocs as the oscillation frequency is increased. It should be taken into account that the system with 30 mg Ca/100 g, with lower flocculation degree and swelling of w/o droplets, showed a lower diminution slope of η* with the applied frequency and a considerably higher tan δ value (Fig. 3b), indicating a lower gel behavior.

With regard to the effect of storage at 4C on particle size, the only system that showed changes after 15 days was the w/o/w emulsion with 120 mg Ca/100 g (as calcium chloride), where the d_{43} value (measured at 3,000 rpm) was doubled in comparison to its value after 1 day. This result was attributed to the increased flocculation and coalescence of w/o droplets due to the release of calcium toward the interface. In w/o/w emulsions with lower calcium chloride contents (30 or 60 mg Ca/100 g) or with sodium chloride, no changes were observed in the d_{43} value after storage during 15 days (data not shown).

Effect of Added Salt Type

The addition of different salts in the inner aqueous phase also had a direct influence on the rheology of these emulsions. The w/o/w emulsion fortified

TABLE 3.
EFFECT OF ADDED SALT ON ELASTIC MODULUS (G′), LOSS MODULUS (G″), AND TAN δ (G″/G′) OF W/O/W EMULSIONS

Salt	G′ (Pa)	G″ (Pa)	tan δ
Calcium chloride	382.5 ± 27.6 a	55.9 ± 3.7 a	0.147 ± 0.001 a
Calcium lactate	105.5 ± 4.9 b	22.7 ± 1.4 b	0.216 ± 0.004 c
Sodium chloride	93.2 ± 6.6 b	19.2 ± 1.2 b	0.206 ± 0.001 b

Systems with 40% DLP (with 1% PGPR and 20% DAP). Emulsions with calcium salts contain 120 mg Ca/100 g. The emulsion with NaCl was prepared with the same osmolarity in the internal aqueous phase than the emulsion with $CaCl_2$.
Frequency: 1 Hz.
Values are means of two replicates ± SD.
Mean values with different letters are significantly different ($P < 0.05$).

with calcium lactate (120 mg Ca/100 g) had lower G′ and G″ values than the system with calcium chloride at same concentration of divalent cation (Table 3). A lower dissociation degree of the calcium salt produced less thick emulsions, since calcium needs to be free to produce the osmotic gradient. With calcium carbonate (the least dissociable salt), the system exhibited a liquid behavior (the applied stress was out of its linear viscoelasticity range). With sodium chloride, the emulsion had lower G′ and G″ values than the system with calcium chloride at the same osmolarity in the internal aqueous phase (Table 3); this result could be attributed to the higher flocculation degree in the emulsion with $CaCl_2$ and 120 mg Ca/100 g, according to the higher d_{43} value measured at 600 rpm (Table 2). This reinforces the previously formulated hypothesis – that a proportion of calcium would be released, promoting the formation of flocs because of its interaction with soybean proteins at the interface. Moreover, this interaction of calcium with the proteins would produce a higher rigidity of the interfacial film, affecting the viscoelasticity of the emulsion. Furthermore, part of the released calcium could increase the viscosity of the continuous aqueous phase because of the coagulation of soybean proteins.

Effect of Lipophilic Emulsifier Concentration

The rheology of these emulsions was also affected by the amount of lipophilic emulsifier used to stabilize the inner aqueous phase that contains the calcium salt and is dispersed inside the w/o droplets. Higher PGPR concentrations produced lower G′ and G″ values and a less elastic behavior (Table 4). Increasing the PGPR concentration reduces the water droplets size of the primary w/o emulsion (Márquez *et al.* 2010); this would lead to better isolation of the calcium salt, reducing the swelling of w/o droplets during the

TABLE 4.
EFFECT OF PGPR, DISPERSED LIPID PHASE (DLP), AND DISPERSED AQUEOUS PHASE (DAP) CONTENTS ON ELASTIC MODULUS (G′), LOSS MODULUS (G″), AND TAN δ (G″/G′) OF W/O/W EMULSIONS

Factors			G′ (Pa)	G″ (Pa)	tan δ
PGPR (%)	DLP (%)	DAP (%)			
0.5	40	20	152.0 ± 7.1 a	26.4 ± 0.9a	0.174 ± 0.003a
1.0	40	20	89.1 ± 8.9 b	19.5 ± 2.3b	0.218 ± 0.004a
2.0	40	20	49.8 ± 10.5c	16.4 ± 1.2b	0.335 ± 0.047b
1.0	**30**	20	10.9 ± 0.2 a	6.9 ± 0.0a	0.636 ± 0.011a
1.0	**40**	20	89.1 ± 8.9 b	19.5 ± 2.3b	0.218 ± 0.004b
1.0	**50**	20	212.0 ± 25.5c	38.3 ± 2.7c	0.182 ± 0.009c
1.0	40	**10**	135.0 ± 21.2a	27.0 ± 3.0a	0.201 ± 0.009a
1.0	40	**20**	89.1 ± 8.9 b	19.5 ± 2.3b	0.218 ± 0.004a
1.0	40	**30**	47.2 ± 9.2 c	15.0 ± 1.2b	0.322 ± 0.037b
1.0	40	**40**	10.2 ± 1.1 d	7.8 ± 0.6c	0.761 ± 0.021c

Systems with calcium chloride in internal aqueous phase (60 mg Ca/100 g emulsion).
Frequency: 1 Hz.
Values are means of two replicates ± SD.
Mean values with different letters for each varied factor are significantly different ($P < 0.05$).
Bold indicates which factor is varied.

second stage of homogenization. Moreover, a higher PGPR content would diminish the flocculation degree due to a lesser amount of calcium released to the continuous aqueous phase. Previous studies have shown that the transport of water in multiple emulsions occurs by diffusion across the oil layer from one water phase to the other (Pays *et al.* 2002), and that this diffusion is influenced by the formation of micelles composed of the surfactant and the oil (Hino *et al.* 2001). Then, a relatively high PGPR concentration would induce the formation of micelles or an increase of its adsorption to the water/oil interface, which would lead to an effective stabilization of the system, reducing both the water diffusion and the calcium release.

Effect of Dispersed Lipid Phase Content

The effect produced by the content of DLP is shown in Table 4. It can be observed that a higher proportion of DLP led to the formation of emulsions with higher G′ and G″ values. A higher amount of oil would allow to produce more w/o droplets before and after their swelling, leading to the formation of more packed and thick systems. It is well known that the viscosity of an emulsion increases with the volume fraction of dispersed phase, ϕ (McClements 1999). Typically, the value of ϕ_c (critical volume fraction of dispersed phase), in which the spherical droplets become closely packed and the consistency increases, is between 0.6 and 0.7 if there are not via long-range colloidal interactions between droplets (McClements 1999). In our case, apart from the swelling of w/o droplets, there are colloidal interactions between w/o droplets since they are surrounded by a film composed of soybean proteins and phospholipids, indicating that the volume fraction of dispersed w/o phase of the systems reached its critical value.

Effect of Dispersed Aqueous Phase Percentage

It was also analyzed the variation of DAP that contains the calcium, with fixed DLP content (Table 4). A higher percentage of DAP produced less thick emulsions; when the DAP reached 40%, the decrease of G′ was much more pronounced than the decrease of G″, resulting in a value of tan $\delta \geq 0.7$ (less gel behavior). According to the classification of multiple emulsions made by Florence and Whitehill (1981), the studied w/o/w emulsions would be type C because of the large number of internal water droplets. In a recent paper, Pal (2008) proposed viscosity models in which the relative viscosity of multiple emulsions of types B and C depends on four variables: volume fraction of internal droplets within a multiple emulsion droplet (ϕ^{PE}), volume fraction of total dispersed phase in the whole multiple emulsion (ϕ^{ME}), ratio of primary-emulsion matrix viscosity to multiple-emulsion matrix viscosity (K_{21}), and ratio of internal droplet viscosity to primary-emulsion matrix viscosity (K_{32}). The viscosity of a multiple emulsion generally increases with the increase in ϕ^{PE}, ϕ^{ME}, K_{21}, or K_{32}. In our case, it should be expected an increase of the G′ and G″ values with increasing DAP and DLP (ϕ^{PE} and ϕ^{ME}, respectively). However, while the G′ and G″ values increased with the increase of DLP, they decreased with increasing DAP (Table 4). This last result can be explained by two parallel reasons: (1) the lower calcium concentration in the inner aqueous phase when DAP is increased, reducing the osmotic gradient; and (2) the lower oil content when DAP is increased, reducing the potential number of w/o droplets after their swelling.

General Overview of Rheology Results

By comparing the overall values of G′, G″ and tan δ of the different w/o/w emulsions, a high versatility of these systems can be observed. The variation of different parameters produced emulsions with behaviors that range from an almost creamy liquid (G′ or G″ lower than 20 Pa; tan $\delta > 0.7$) to a gel-like system (tan $\delta < 0.25$). Although these w/o/w emulsions prepared with soybean milk and sunflower oil have a consistency of cream, they are different from those obtained with soybean milk and a mixture of sunflower oil and milk fat (Márquez *et al.* 2005a,b,c). The latter emulsions, initially liquid after their preparation, reached a consistency of cream after subjection to agitation or thermal cycle, resulting in values of G″ > 100 Pa and tan δ > 1, due to the phenomenon of partial coalescence by the crystallization of fat. On the contrary, the w/o/w emulsions achieved in this work have a consistency of cream immediately after their preparation. Some of them reached a rheological behavior like strong gels (G′ > 100 Pa; tan $\delta < 0.25$), which was attributed to the degree of hydration and interaction of w/o droplets.

Pasteurization Assays

Finally, pasteurization assays were performed on some emulsions in order to analyze the heat stability and the effectiveness of calcium isolation. In w/o/w emulsions with 30 or 60 mg Ca/100 g (as calcium chloride), an increase of the d_{43} values (measured at 600 rpm) after heating the samples at 75C for 60 s (data not shown) was not observed; this result indicates that at those calcium concentrations, there was no increase in the flocculation degree in the emulsions after pasteurization, as can be observed in the optical micrographs (Fig. 5a–d). On the other hand, the w/o/w emulsion with 120 mg Ca/100 g (with visible initial flocculation, Fig. 5e) showed an increase in the flocculation degree (Fig. 5f), evidenced by an increment of the d_{43} value with the increase of heating time (Fig. 6). A maximum flocculation degree was detected after a short heating time (30 s), according to results obtained at low dispersion speed (600 rpm). The d_{43} increase was also observed at high dispersion speed (3,000 rpm), indicating that there was also an increase of the individual droplets size by coalescence; this change can be appreciated by optical microscopy (Fig 5e,f). Ryan *et al.* (2008) reported that the addition of calcium leads to decreased heat coagulation times in emulsions stabilized with soybean proteins. Thus, the pasteurization would induce the interaction of the calcium released toward the continuous aqueous phase with the soybean proteins at the interface, leading to the flocculation and coalescence of w/o droplets. In emulsions with 30 and 60 mg Ca/100 g the released calcium amount would not be enough to increase the size or number of flocs. With regard to the effect of the pasteurization on the rheology, a slight diminution of the viscoelastic parameters was observed after the heat treatment in the emulsion with 120 mg Ca/100 g (data not shown) despite the increased flocculation; this result could be explained by the coalescence of w/o droplets.

CONCLUSIONS

The addition of calcium in the inner aqueous phase of w/o/w emulsions prepared with soybean milk, sunflower oil and PGPR as emulsifier, produced clear rheological changes. This effect was evidenced by the increase of the

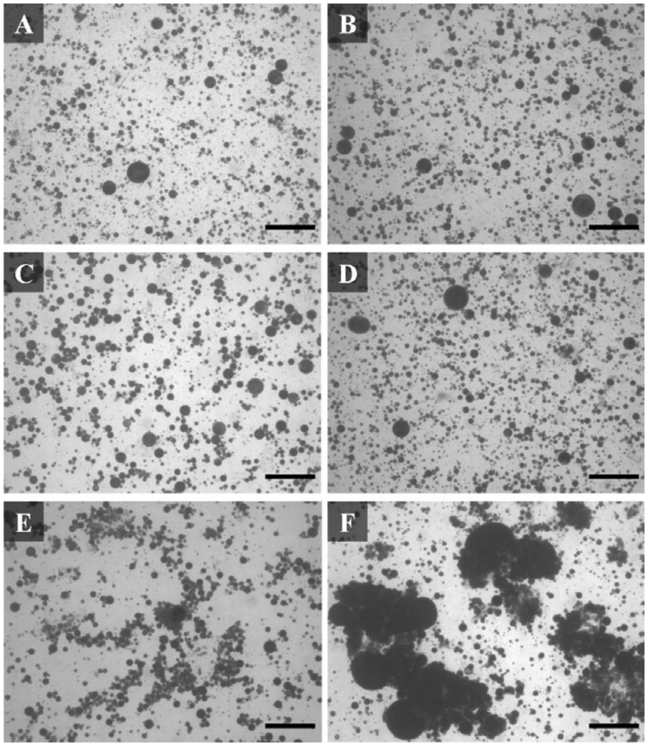

FIG. 5. OPTICAL MICROGRAPHS OF DILUTED W/O/W EMULSIONS
Systems with 40% DLP (with 1% PGPR and 20% DAP containing calcium chloride). 1:10 dilution.
Image A: 30 mg Ca/100 g, not pasteurized. Image B: 30 mg Ca/100 g, pasteurized (75C, 60 s).
Image C: 60 mg Ca/100 g, not pasteurized. Image D: 60 mg Ca/100 g, pasteurized (75C, 60 s).
Image E: 120 mg Ca/100 g, not pasteurized. Image F: 120 mg Ca/100 g, pasteurized (75C, 60 s).
Bar = 200 μm.

viscoelastic parameters of these systems, which can be explained by two main factors: the swelling of w/o droplets as a consequence of the osmotic gradient produced by the inclusion of soluble salts in the internal aqueous phase; and the flocculation of w/o droplets because of the interaction of released calcium with soybean proteins at the interface. It is possible to obtain w/o/w emulsions with different consistency by varying the added calcium amount, the salt type, the lipophilic emulsifier concentration, the oil content and the dispersed aqueous phase percentage. In this way, calcium not only contributes its nutritional property, but it also acts functionally by allowing the obtaining of systems with creamy texture without the need of addition of saturated fats. Only the w/o/w emulsion with high calcium chloride content showed increased flocculation and coalescence of w/o droplets after storage for 15

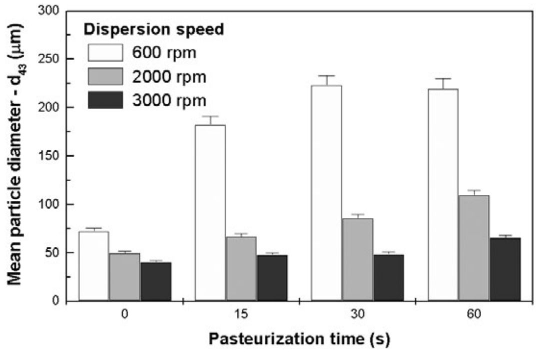

FIG. 6. EFFECT OF PASTEURISATION TIME (75C) ON MEAN PARTICLE DIAMETER (D_{43}) OF A W/O/W EMULSION
System with 120 mg Ca/100 g (40% DLP with 1% PGPR and 20% DAP containing calcium chloride). Values are means of two replicates and error bars indicate SD.

days or pasteurization treatment. In order to obtain stable w/o/w emulsions fortified with calcium at the level of cow's milk, future studies could include replacing part of calcium chloride with calcium carbonate and/or adding sequestering agents in the soybean milk; these strategies point to reduce the free calcium content and so its interaction with the soybean milk components. The obtained results lead to the conclusion that the studied emulsions can work as a reduced fat substitute of whipped dairy cream with important calcium contribution, and with the benefit of being free of cholesterol and rich in proteins and unsaturated fatty acids.

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