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Dehydrated tofu whey as cryoprotectant in protein-stabilized oil-in-water emulsions

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ABSTRACT

The cryoprotective effect of dehydrated tofu whey (DTW) and sucrose on freeze–thaw stability of oil-in-water emulsions stabilized by soy protein isolate was comparatively evaluated at equivalent carbohydrate content (0.066, 0.33 and 1.5 g/100 g emulsion). The influence of storage time at -18 ± 2 °C for different periods (1–130 days) was evaluated. Emulsion stability was assessed by oiling off and particle size measurements. For unfrozen and freeze-thawed emulsions, flocculation index (FI) was determined from De Brouckere mean diameters ($D_{4,3}$) in the absence and presence of sodium dodecyl sulfate (SDS). Coalescence index (CI) was evaluated through variation of $D_{4,3}$ values before and after freeze-thawing. Control emulsion, without cryoprotectant, was unstable whatever the storage period. Both cryoprotectants improved emulsion stability by decreasing the amount of freezable water. However, the protective effect of DTW was better than that of sucrose. After a prolonged storage (130 days), a high stability to coalescence ($CI < 2$) was observed when DTW was added at the highest concentration. In contrast, sucrose only was effective as cryoprotectant at relatively short periods of storage (<30 days). This study has demonstrated that the addition of DTW is helpful to improve the stability of protein-stabilized o/w emulsions subjected to freeze-thawing.

1. Introduction

Proteins are important ingredients in food systems. Soy proteins play an important role in several food systems due to their high nutritional value and functional behavior (Molina, Papadopoulou, & Ledward, 2001). Soy protein isolates (SPI) are widely used as emulsifiers in food emulsions due to the surface active properties of their constitutive proteins, the storage globulins 7S (β-conglycinin, ~270 kDa) and 11S (glycinin, ~360 kDa). SPI are generally obtained in non-denatured state from isoelectric precipitation by acidifying (pH 4.5–4.8) an aqueous extract of defatted soy flour, and further solubilization and neutralization of precipitate (Sorgentini & Wagner, 1999; Yamauchi, Yamagishi, & Iwabuchi, 1991).

Tofu is generally made from a filtered water extract of whole soybean called soy milk. During the preparation of this product, a cooking process is necessary to eliminate off-flavor components and inactivate the antinutritional factors (Kunitz trypsin inhibitor, KTI and lectin, L). The curd is obtained by coagulation of 7S and 11S globulins, following by molding and pressing to remove whey. The tofu whey (TW) contains the majority of components that remains soluble after coagulation, mainly calcium, oligosaccharides and biologically active proteins (Sobral & Wagner, 2007). Espinosa-Martos and Ruperez (2006) analyzed the carbohydrate composition of TW, reporting the following contents (g/100 g): sucrose, 53, stachyose, 39, xylose, fructose and inulin, 1.0. At present, TW as residual liquid represent an important problem due to its negative environmental impact. A strategy to minimize this impact and allow the utilization of TW in formulated foods should include a concentration stage and the complete denaturation of KTI and L (Sobral & Wagner, 2007). Among the main components of TW, the soybean oligosaccharides have prebiotic effects and studies have shown that their consumption is related to several health benefits, such as lowering blood cholesterol, reducing blood pressure and

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preventing some types of cancer (Robertfroid, 2007). Moreover, thermal denatured KTI and L have a high biological value, similar to that of storage globulins 7S and 11S (Kishi & Inoue, 1987).

Freezing is one the most important preservation methods for maintenance of microbiological and chemical stability of food products (Xiong, 1997). Nevertheless, protein-stabilized oil-in-water (o/w) emulsions are highly destabilized when they are stored at temperatures where the water crystallizes (Ghosh & Coupland, 2008; McClements, 2004). In the absence of cryoprotectants, freeze–thaw stability of o/w emulsions prepared with native and thermally denatured soybean isolates depends on various factors such as protein concentration and sample aging (Palazolo, Sobral, & Wagner, 2011; Palazolo & Wagner, 2010). Indeed, cryoprotectants have been used to prevent cold protein denaturation and losses in functional properties in a wide variety of food systems (Xiong, 1997). In model o/w protein-stabilized emulsions, sugars and polyols improved the freeze–thaw stability (Ghosh, Cramp, & Coupland, 2006; Thanasukarn, Pongsawatmanit, & McClements, 2004). The addition of these low-molecular solutes increases the viscosity of continuous phase, depresses the freezing temperature and modifies both the amount of ice at given temperature and the glass transition. Sugars were reported to exert an inhibiting effect on ice crystal growth (Thiebaud, Dumay, & Cheftel, 2002). Moreover, Carpenter and Crowe (1988) proposed that the cryoprotection of sugars or polyols on isolated proteins can be accounted for by fact that these low molecular solutes are preferentially excluded from contact with the protein surface. Hence, any factor that alters protein stability in non frozen solution will tend to have the same qualitative effect during freeze-thawing (Arakawa, Prestrelski, Kenney, & Carpenter, 2001).

As a consequence of its high sucrose, oligosaccharides and protein contents, this article focuses on the cryoprotective effect of dehydrated tofu whey (DTW) on freeze–thaw stability of model o/w emulsions prepared with SPI and refined sunflower oil. A comparative analysis with sucrose at equivalent carbohydrate content was carried out. The effect of DTW or sucrose concentration in the continuous phase and frozen storage time was evaluated.

2. Materials and methods

2.1. Materials

Defatted soy flour was provided by Solae Latin America (Barueri, SP, Brazil). The soy flour contains 95 g dry solids per 100 g powder and the composition (in dry basis, as given by the producer) was: 56.0 g/100 g crude protein (N × 6.25), 7.0 g/100 g ash, 3.5 g/100 g total lipids and 14.0 g/100 g dietary fiber. Refined sunflower oil (Molinos Cañuelas, Argentina) was purchased in a local supermarket. Tofu whey (TW: 21.2 ± 0.3 g dry matter/L, pH 5.8 ± 0.1) was provided by Soyana S.H. (San Martín, Argentina). Sudan III (CI number 26100) was purchased from Chroma Gesselschaft (Schmidt GmbH & Co., Germany). The chemical reagents used in this work were all of analytical grade.

2.2. Preparation of soybean protein isolates

Soy protein isolates (SPI) was prepared according to experimental procedure reported by Sorgentini and Wagner (1999). The defatted soy flour was extracted for 2 h at 20 °C at pH 8.0 with deionized water (water: flour ratio: 10:1). The mixture was centrifuged at 10,400× *g* for 15 min at 20 °C (Beckman Coulter Avanti J25 centrifuge, Beckman Coulter, USA). The supernatant was adjusted to pH 4.5 with HCl, then kept for 2 h at 4 °C and subsequently centrifuged at 10,400× *g* for 20 min in the same conditions. The precipitate was washed with water, resolubilized in water by neutralization at pH 8.0 with NaOH at room temperature, freeze-dried and finally ground. SPI was stored as a freeze-dried powder at 4 °C and was rapidly utilized in further experiments for avoid the sample aging. The crude protein (N × 6.25), ash and moisture contents (g/100 g) of SPI sample were 90.25, 3.08 and 2.10, respectively.

2.3. Preparation of dehydrated tofu whey (DTW)

Tofu whey (TW) was concentrated using a rotavapor (R-124, Büchi Labortechnik, Switzerland) at 50 °C. In this device, a final pressure of 8.10^3 Pa was reached. The obtained syrup was dried at 50 °C under vacuum (400 Pa) for 4 h. Then, the sample, obtained as sticky scales, was frozen in liquid N_2, ground and dried consecutively at room temperature in desiccators containing silica gel (5 days), CaO (7 days) and finally P_2O_5 (5 days). A fine yellow powder of dehydrated tofu whey (DTW) was finally obtained. The chemical composition of dehydrated tofu whey sample (DTW, g/100 g) was: total carbohydrates, 38.6 ± 3.2, crude protein (N × 6.25), 14.5 ± 0.1, ash, 17.2 ± 0.2 and total calcium, 1.9 ± 0.1. A DSC analysis of proteins isolated from DTW by cold acetone precipitation revealed that KTI and L are totally denatured (Sobral, Palazolo, & Wagner, 2010; Sobral & Wagner, 2009). For DTW, the antitryptic activity (AA) was negligible. AA was determined through the inhibitory effect of an acid extract of the sample (0.05 mol/L HCl, 24 h) on trypsin activity (from porcine pancreas, Sigma Co, USA) at pH 8.0 and 37 °C, using denatured hemoglobin as substrate (Sobral & Wagner, 2009).

2.4. Preparation of aqueous dispersions

SPI aqueous dispersions (2.0 g/100 g) were prepared in 0.1 mol/L sodium phosphate buffer pH 7.0 by magnetic stirring for at least 5 h at room temperature to ensure complete dispersion and hydration. Sodium azide (0.03 g/100 mL) was added in order to avoid the microbial spoilage.

DTW sample was dispersed in 0.1 mol/L sodium phosphate buffer pH 7.0 at 0.4, 2.0 and 9.0 g/100 g by using a magnetic stirrer. The free calcium content was negligible due to phosphate ions (mainly as HPO_4^{2-}) are in excess respect to divalent ion. In the same buffer, three solutions of sucrose were also prepared at an equivalent concentration of total carbohydrates (TCH) respect to those of DTW. These sucrose concentrations were 0.13, 0.66 and 3.0 g/100 g, respectively.

2.5. Preparation of o/w emulsions

A two-step process was used to prepare the stock o/w emulsion. First, SPI aqueous dispersion were mixed with refined sunflower oil (oil mass fraction, $\phi_m = 0.33$) in a high-speed blender (Ultraturrax T-25, IKA Labortechnik, Germany) at 20,000 rpm for 60 s. Coarse emulsion was then re-circulated three times through a twin-stage valve high pressure homogenizer (Panda 2K, GEA Niro Soavi, Italy) to finally obtain the stock o/w emulsion. The homogenization pressure was 40 and 4 MPa in the first and second valve, respectively. Then, diluted o/w emulsions without and with cryoprotectant were prepared using vertical containers with plastic caps. Aliquots (10 g) of stock emulsion were gently mixed with 10 g of 0.1 mol/L sodium phosphate buffer pH 7.0 (control emulsion) or 10 g of aqueous dispersions containing sucrose or DTW at equivalent TCH content (Section 2.4). For diluted emulsions ($\phi_m = 0.165$), the cryoprotectant concentration (g/100 g emulsion) were the following: DTW: 0.2, 1.0 and 4.5; sucrose: 0.066, 0.33 and 1.5, respectively. On these diluted emulsions, the freeze–thaw stability was evaluated.

2.6. Freeze–thaw protocol

Emulsion samples were isothermally stored in still air at −18 ± 2 °C for 1.5, 16, 30, 42, 60 and 130 days. The cooling curves were registered using a Lufft Opus C-10 datalogger (Lufft, Meβ und Regeltechnik, Germany). K thermocouples were located half way through emulsion at center of the sample. Temperature data were recorded every 1 min. After the undercooling of the aqueous phase, the further increase of temperature is attributed to latent heat release. Hence, the freezing temperature of the aqueous phase (T_f) was determined as the maximum temperature reached once the latent heat was removed from the sample (Reid, 1997). Frozen emulsions were thawed at room temperature for at least 4 h before characterization analyses. Photographs at bulk scale of thawed emulsions were made using a digital camera (Kodak Easy Share M853, Eastman Kodak Company, USA).

2.7. Modulated DSC measurements

Modulated DSC (MDSC) technology was employed to measure the amount of freezable water (FW) of o/w emulsions. Emulsions were frozen according to experimental procedure mentioned above (Section 2.6). Then, aliquots of frozen emulsions (15–18 mg) were placed in low mass aluminum pans and rapidly transferred to modulated calorimeter (Q200, TA Instruments, USA) which was previously cooled to −20 °C. Then, the cooling–heating sequence was: isotherm at −20 °C, 5 min, cooling to −60 °C. at 10 °C min^{-1}, isotherm at −60 °C, 5 min and finally, heating to 25 °C at 5 °C min^{-1}. The temperature modulation amplitude and period were set at ±1.0 °C and 60 s, respectively.

The same calorimetric measurements were performed for pure water. The melting enthalpy was measured for a known mass of emulsion and for a known mass of pure water. Freezable water was then calculated as:

$$\mathrm{FW(g/100g)} = (\Delta H_{em}/\Delta H_w) \times 100 \quad (1)$$

ΔH_{em} and ΔH_w were the enthalpy changes per unit mass of water for emulsions and pure water, respectively.

2.8. Particle size distribution

The particle size distribution (PSD) of unfrozen and freeze-thawed emulsions was determined as differential volume in the diameter range of 0.1–1000 μm by laser scattering using a Malvern Mastersizer 2000E (Malvern Instruments Ltd, UK). The optical parameters were the following: refractive indexes of sunflower oil and water 1.47 and 1.33, respectively; adsorption coefficient: 0.001 (Palazolo et al., 2011). Two aliquots of each emulsion were diluted separately with 0.1 mol/L sodium phosphate buffer pH 7.0 without and with 1.0 g/100 mL sodium dodecyl sulfate (SDS). The samples prepared with and without SDS, were dispersed in 600 mL of water at 2000 rpm in the dispersion unit (Hydro 2000MU). From PSD, the Sauter ($D_{3,2}$) and De Brouckere ($D_{4,3}$) moment mean diameters were obtained. In measuring without SDS, dilution and stirring were likely to disrupt any weakly flocculated droplets, but leave strongly flocculated droplets intact; therefore this method allows evaluating flocs stable in the measurement conditions, which are formed by a bridging mechanism (Thanasukarn et al., 2004). For unfrozen and freeze-thawed emulsions, flocculation index (FI) was calculated employing the size particle data as:

$$\mathrm{FI} = \left[(D_{4,3} - D_{4,3\ \mathrm{SDS}})/D_{4,3\ \mathrm{SDS}}\right] \quad (2)$$

$D_{4,3}$ and $D_{4,3\ \mathrm{SDS}}$ are the volume-weighted diameters, measured in the absence and presence of SDS, respectively (Palazolo et al., 2011).

The effect of freeze-thawing on emulsion stability was evaluated through the coalescence destabilization. Coalescence index (CI) was calculated from:

$$\mathrm{CI} = \left[\left(D_{4,3\ \mathrm{SDS\ freeze-thawed}} - D_{4,3\ \mathrm{SDS\ unfrozen}}\right)\Big/ D_{4,3\ \mathrm{SDS\ unfrozen}}\right] \quad (3)$$

$D_{4,3\ \mathrm{SDS\ freeze\text{-}thawed}}$ and $D_{4,3\ \mathrm{SDS\ unfrozen}}$ are the volume-weighted mean diameters of freeze-thawed and unfrozen emulsions, respectively. Both parameters were obtained from PSD measurements with 1.0 g/100 mL of SDS (Palazolo et al., 2011).

$D_{4,3}$ parameter is more sensitive to particle aggregation processes than $D_{3,2}$ (Relkin & Sourdet, 2005). Thus, volume-weighted diameters were used to calculate FI and CI values.

2.9. Oiling off

The dye-dilution technique was utilized to determine the amount of free oil of o/w emulsions after freeze-thawing. The experimental procedure of Palanuwech, Poniteni, Roberts, and Coupland (2003) was followed with some modifications (Palazolo et al., 2011). Sudan III solution was gently mixed with emulsions, incubated for 60 min and then centrifuged at 700× g for 20 min (Rolco CM 4080 Millenium, Argentina). An aliquot of dyed free oil on top of emulsion was carefully sucked off with a Pasteur pipette and centrifuged at 10,000× g for 20 min (Beckman Coulter GS-15R centrifuge, Beckman Coulter Inc., USA). The absorbance of supernatant at 508 nm was then determined. The change in the absorbance due to dye dilution was related to the amount of free oil (FO), according to the expression:

$$\mathrm{FO(g/100\ g\ oil)} = [M_o \times (A-1)/(M_e \times \phi_e)] \times 100 \quad (4)$$

M_o is the mass (g) of added dye solution, M_e is the mass (g) of emulsion, ϕ_e is the mass fraction of oil in the emulsion and $A = A_b/A_a$ is the ratio of the measured absorbances of the dye before (A_b) and after (A_a) extraction process. The corresponding procedures of calibration were carried out according to previous works (Palanuwech et al., 2003).

2.10. Statistical analysis

Each of the measurements described above was conducted at least in duplicate and the results were reported as the mean and standard deviation. Data were analyzed by analysis of variance (ANOVA) and differences between mean values by the Fisher's Least Significant Differences (LSD). Statistical analysis was carried out using Statgraphics Centurion XV software (StatPoint Inc., USA). Significance was considered at $p < 0.05$.

3. Results and discussion

3.1. Characterization of unfrozen o/w emulsions

In this section, the characterization of unfrozen emulsions, without and with cryoprotectants, was carried out. For control emulsion, the particle size distribution (PSD) was monomodal and asymmetrical when measurements were performed without sodium dodecyl sulfate (SDS). A broad peak in the range of 2–300 μm was effectively observed (Fig. 1a). $D_{3,2}$ and $D_{4,3}$ values were 9.71 ± 0.09 and 20.02 ± 0.11 μm, respectively. SDS is able to displace proteins from oil/water interface and to induce electrostatic repulsions between droplets due to its negative charge (Smulders, Caessens, & Walstra, 1999). Therefore, flocs are eliminated if SDS is present during the diffraction analysis. In the

presence of SDS, PSD of control SPI emulsion was monomodal, showing a single particle population in the range 0.2–3.0 μm (Fig. 1a). $D_{3,2}$ and $D_{4,3}$ values were 0.84 ± 0.01 and 1.06 ± 0.01 μm, respectively. The results are in accord with the presence of flocs stable in the measurement conditions (Thanasukarn et al., 2004). Indeed, flocculation index (FI) was 17.91 ± 0.21. SPI sample is constituted by storage globulins 7S and 11S, which have both a high molecular size and a complex quaternary structure (Yamauchi et al., 1991). Moreover, glycinin retains its oligomeric structure at intermediate ionic strength (Wagner & Guéguen, 1995). Hence, during high-pressure homogenization, protein adsorption of oligomers on freshly created oil droplets may become the rate-determining process which favors the bridging flocculation. SPI emulsions prepared with higher protein concentration (3.0 g/100 g at the same ionic strength) were also flocculated (data not shown) so that the emulsifier concentration was not limiting during the homogenization process, i.e. there is enough sufficient protein to cover the interface. Unflocculated SPI emulsions were obtained when they were prepared at low ionic strength with the same protein concentration, pressure homogenization in the valves and similar oil mass fraction (Palazolo et al., 2011). This fact reflects the noticeable effect of ionic strength on the droplet aggregation ability of SPI emulsions.

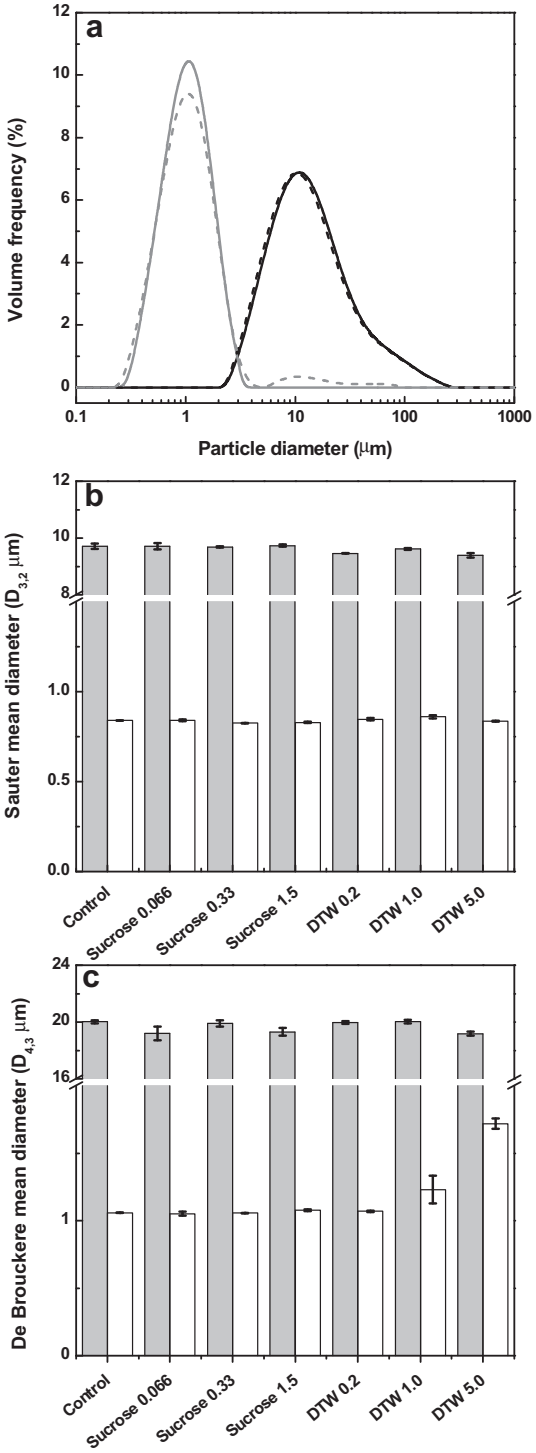

Fig. 1. a) Particle size distributions (PSD, expressed as volume frequency %) of SPI-stabilized oil-in-water emulsions in the absence (control) and presence of dehydrated tofu whey (DTW, 4.5 g/100 g emulsion). PSD measurements were carried out without (−) and with (+) 1.0 g/100 mL SDS: (—) Control − SDS, (—) Control + SDS, (– –) DTW − SDS, and (– – – –) DTW + SDS; b) Sauter ($D_{3,2}$) and (c) De Brouckere mean diameters ($D_{4,3}$) of SPI-stabilized oil-in-water emulsions obtained from PSD without (■) and with (□) 1.0 g/100 mL SDS. Error bars indicate ± standard deviation for $n = 2$ replicates.

The presence of cryoprotectants did not alter the PSD of diluted SPI emulsions where measurements were made without SDS: $D_{3,2}$ and $D_{4,3}$ values were similar whatever the cryoprotectant type and concentration ($p < 0.05$) (Fig. 1b and c). Nevertheless, in the presence of SDS, PSD exhibited some little differences as a function of DTW concentration. Although $D_{3,2}$ values were similar, $D_{4,3}$ exhibited an increasing tendency as DTW concentration increased from 0.2 to 5.0 g/100 g ($p < 0.05$) (Fig. 1b and c). This behavior was not observed when sucrose was mixed at equivalent carbohydrate content. The DTW aqueous dispersions exhibited a turbid appearance probably as a consequence of the presence of insoluble calcium phosphate and possibly also proteins. Although these insoluble particles (range 4.0–100 μm) represented a very low proportion of total volume of the particles in PSD, their presence was sufficient to induce an increase the $D_{4,3}$ values (Fig. 1a and c). When PSD were made in the absence of SDS both populations (DTW insoluble particles and droplet flocs) were overlapped, which explains the similar values of $D_{4,3}$ obtained for SPI emulsions without and with DTW in this measurement condition ($p < 0.05$, Fig. 1). For emulsions mixed with DTW aqueous dispersions at 1.0 and 5.0 g/100 g, it is worth noting that FI values were lower respect to those of other emulsions (Table 1). As was mentioned above, FI is a parameter that directly depends on $D_{4,3}$ values measured without and with SDS. Therefore, an increase of $D_{4,3}$ in the presence of surfactant produces a decrease in the flocculation index (Eq. (1)). Indeed, the emulsions mixed with DTW at the lowest content (0.2 g/100 g) or sucrose exhibited similar FI values respect to that of control ($p < 0.05$).

3.2. Evaluation of freeze–thaw stability of o/w emulsions

In conditions where the water crystallizes and in the absence of cryoprotectants, the instability of most of protein-stabilized o/w emulsions is noticeably enhanced (Cornacchia & Roos, 2011; Ghosh et al., 2006; Magnusson, Rosén, & Nilsson, 2011; Palazolo et al., 2011; Thanasukarn et al., 2004). Several destabilizing effects can occur in o/w emulsions in frozen state. First, the crystallization of water molecules leads to the formation of an unfrozen aqueous phase, which is concentrated in all solutes. Hence, high salt concentration can screen out any repulsion between droplets. Second, the dispersed phase volume fraction increases as the amount of liquid water is reduced during the freezing process. The actual oil mass fraction will approach close packing for typical emulsions. The flocculation, coalescence and further free oil release is promoted since the droplets are forced into close proximity (Ghosh & Coupland, 2008; McClements, 2004; Thanasukarn et al., 2004).

Table 1
Flocculation index (FI) values of SPI-stabilized oil-in-water emulsions without freezing treatment and after different periods of frozen storage. Cryoprotectants (dehydrated tofu whey, DTW and sucrose) were added to emulsions after homogenization at equivalent total carbohydrate (TCH, g/100 g) content.

Emulsion samples	TCH (g/100 g)	Unfrozen	Frozen storage time (days)				
			1.5	16	30	42	60
DTW	0.066	$17.45 \pm 0.11^{a,A}$	$11.95 \pm 0.32^{b,A}$	$8.41 \pm 0.06^{c,A}$	$8.00 \pm 0.15^{d,A}$	$5.00 \pm 0.01^{e,A}$	$4.76 \pm 0.09^{e,A}$
	0.33	$15.42 \pm 0.21^{a,B}$	$16.86 \pm 0.35^{a,B}$	$30.56 \pm 2.17^{b,B}$	$38.78 \pm 4.25^{b,c,B}$	$42.73 \pm 6.30^{c,B}$	$61.86 \pm 4.00^{d,B}$
	1.50	$10.04 \pm 0.33^{a,C}$	$7.61 \pm 0.38^{b,C}$	$8.88 \pm 0.65^{c,A}$	$7.47 \pm 0.52^{b,A}$	$8.09 \pm 0.64^{b,c,C}$	$5.87 \pm 0.07^{d,A}$
Sucrose	0.066	$17.23 \pm 0.90^{a,A}$	$3.88 \pm 0.66^{b,D}$	$3.08 \pm 0.18^{b,C}$	$2.96 \pm 0.15^{b,C}$	$1.22 \pm 0.02^{c,D}$	$3.13 \pm 0.21^{b,A}$
	0.33	$17.67 \pm 0.23^{a,A}$	$10.01 \pm 0.22^{b,A}$	$11.32 \pm 0.68^{b,c,D}$	$12.51 \pm 0.71^{c,D}$	$4.56 \pm 0.16^{d,A}$	$5.18 \pm 1.27^{d,A}$
	1.50	$16.92 \pm 0.04^{a,A}$	$25.86 \pm 3.90^{b,E}$	$26.13 \pm 3.31^{b,E}$	$33.85 \pm 4.12^{c,B}$	$41.56 \pm 1.65^{d,B}$	$49.40 \pm 0.58^{e,C}$

Mean ± standard deviation for $n = 2$ replicates. Within the same row, numbers followed by the same lowercase letters are not significantly differences from each other (least significant differences, $p \leq 0.05$). Within the same column, numbers followed by the same uppercase letters are not significantly differences from each other (least significant differences, $p \leq 0.05$).

As an example, Fig. 2 shows the cooling kinetics of SPI emulsions in the absence and presence of cryoprotectant at the highest total carbohydrate content (TCH, 1.5 g/100 g). The cooling rate was approximately 1.5 °C/min from 22 to 0 °C. The presence of cryoprotectant decreased the freezing temperature (T_f) of the aqueous phase (−0.35 ± 0.05 °C) in a colligative fashion. When sucrose and DTW were added to emulsions (TCH = 1.5 g/100 g) T_f were −0.60 ± 0.10 and −0.90 ± 0.06 °C, respectively, i.e, DTW promoted a higher decrease of T_f respect to that of control emulsion ($p < 0.05$). Moreover, at the intermediate and low cryoprotectant concentration (TCH = 0.33 and 0.066 g/100 g, respectively), a higher, but non significant decrease of T_f values was observed for emulsions containing DTW ($p < 0.05$). For all samples, a undercooling was always observed whatever the absence or presence of cryoprotectant. After 350 min, the mean temperature (−18 °C) was reached both in the absence or presence of cryoprotectant. Then, during frozen storage, refrigerator temperature fluctuated between −16 °C and −20 °C (amplitude: 4 °C) for a period of 48 min (Fig. 2). As a consequence of thermal fluctuations, ice recrystallization was favored during the frozen storage (Hartel, 1996; Phimosiripol, Siripatrawan, Tulyathan, & Cleland, 2008).

The freeze–thaw stability of SPI emulsions after a short period of frozen storage (40 h) was firstly evaluated. An important degree of destabilization was observed after freeze-thawing without cryoprotectant. PSD of SPI-emulsions after freeze-thawing in the presence of DTW or sucrose at different concentrations are shown in Fig. 3. In the absence of SDS, a main peak at larger particle sizes (100–900 μm) was effectively observed for control emulsion. This peak was split in two particle populations when the measurement was made with SDS (Fig. 3a–d). The formation of a coagulated creamy layer formed by flocs, coalesced droplets and protein aggregates, previously observed by Palazolo et al. (2011) and Palazolo and Wagner (2010) was consistent with this result. For control freeze-thawed emulsion $D_{4,3}$ values were fairly higher than >200 μm. Nevertheless, the particle size data on highly destabilized control emulsions should be treated with some caution, due to stirring may have promoted additional destabilization during the particle size measurements. Emulsion stability was progressively improved with increasing DTW or sucrose concentration. However, DTW exhibited a better protecting behavior than that of sucrose. At intermediate and high DTW concentration (TCH = 0.33 and 1.5 g/100 g, respectively) the cryoprotectant addition avoided the formation of larger particles (>100 μm) both in the absence and presence of SDS (Fig. 3a and b). However, for this purpose, sucrose was only effective at the highest content (1.5 g/100 g) (Fig. 3c and d). After 40 h of frozen storage, the amount of free oil (FO) of all emulsions was negligible when DTW or sucrose were added regardless their concentration. In the absence of cryoprotectant a value of FO <10 g/100 g oil was obtained.

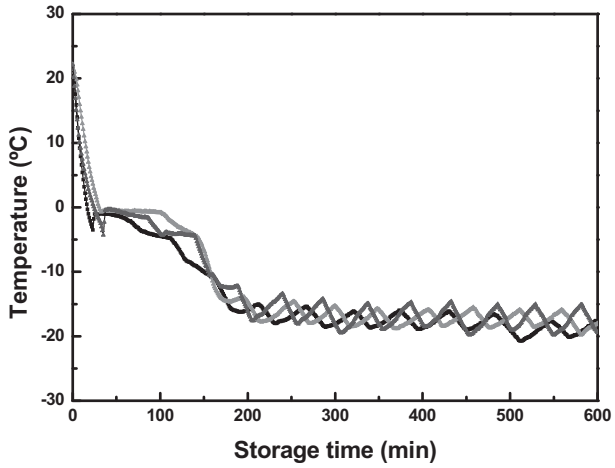

Fig. 2. Cooling kinetics of oil-in-water emulsions prepared with soy protein isolate (SPI) and sunflower oil in the absence and presence of cryoprotectans (sucrose and dehydrated tofu whey, DTW) at the highest total carbohydrate (TCH) content (1.5 g/100 g): (▲) Without cryoprotectant, (■) DTW, and (▲) sucrose.

After 40 h of frozen storage, FI and CI values were also determined (Tables 1 and 2). At the lowest cryoprotectant concentration (TCH = 0.066 g/100 g), the unfrozen emulsions showed similar FI values ($p < 0.05$, Table 1). However, after freeze-thawing, higher CI values and lower FI values were simultaneously observed when sucrose was used as cryoprotectant ($p < 0.05$, Tables 1 and 2). It is worth noting that, unlike CI parameter, a low FI value is not necessary related to a high stability of emulsion after freeze-thawing. When ice crystals form in the aqueous phase, the oil droplets are forced close together (Thanasukarn et al., 2004). Hence, for emulsions prepared with soy protein isolate as the sole emulsifier, the freezing of aqueous phase promotes the flocculation. If the interfacial film is further disrupted during the frozen storage, these flocs will be destabilized by coalescence (Palazolo et al., 2011). In this situation, a decrease of FI value is associated with an increase of CI parameter. In the presence of DTW, the flocs present in unfrozen emulsion are more resistant to the stress exerted by the presence of ice, which was evidenced both as lower decrease of initial FI value and a lower CI value (Tables 1 and 2).

As was observed in Fig. 3, after 40 h of frozen storage, the increase in the cryoprotectant concentration enhanced the emulsion stability. This observation may be supported through a comparative analysis of CI values for each cryoprotectant at different TCH contents (Table 2). Notwithstanding this, some important differences were observed between freeze-thawed emulsions with both cryoprotectants. At intermediate cryoprotectant concentration (TCH = 0.33 g/100 g), DTW was clearly more effective to protect emulsions against coalescence (CI ~ 0) and the

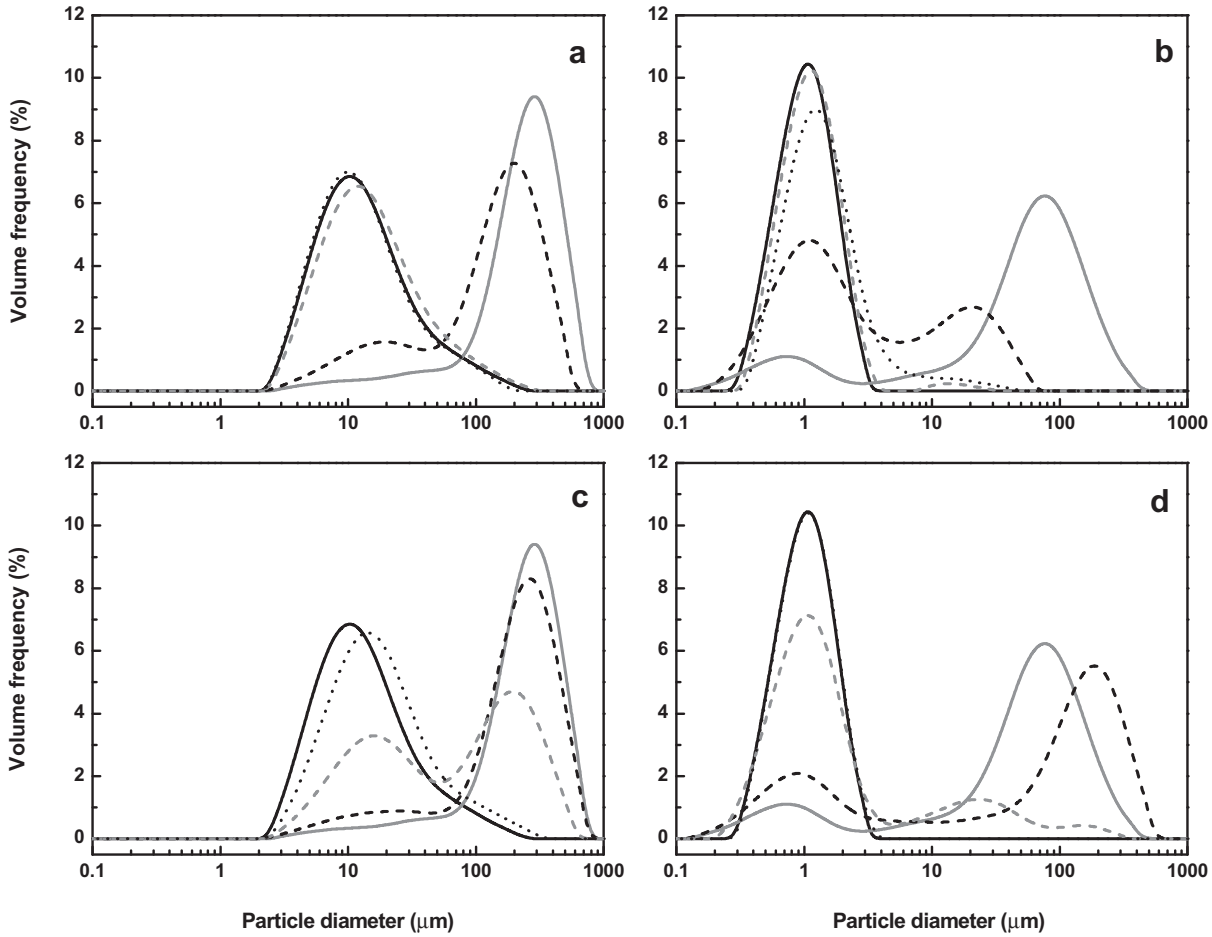

Fig. 3. Particle size distributions (PSD, expressed as volume frequency %) of freeze-thawed SPI-stabilized oil-in-water emulsions (storage time: 40 h) in the presence of DTW (a, b) or sucrose (c, d) at equivalent total carbohydrate content, TCH). Measurements were carried out without (a, c) and with 1.0 g/100 mL SDS (b, d). PSD of unfrozen and freeze-thawed emulsion without cryoprotectant (control) were also included. (—) unfrozen, (—) control, (——) cryoprotectant 0.066 g TCH/100 g, (– – – –) cryoprotectant 0.33 g TCH/100 g, and (••••) cryoprotectant 1.5 g TCH/100 g.

flocs present in unfrozen emulsions remain stable after 40 h of frozen storage. Conversely, in the presence of sucrose, a lower FI value respect to that of unfrozen emulsion was effectively observed, which was consistent with a relatively high CI value (Tables 1 and 2). At the highest TCH content (1.5 g/100 g), emulsions were stable to coalescence. Nevertheless, the formation of new flocs during the frozen storage cannot be avoided when sucrose was used as cryoprotectant, which was evidenced as a significant increase of FI value respect to its initial value ($p < 0.05$, Tables 1 and 2).

In this paper, the effect of long storage time at subzero temperatures was also studied. In this condition, two important factors would play a decisive role on freeze–thaw stability of SPI emulsions. First, the ice recrystallization becomes important since thermal fluctuations enhance this process. Second, the negative effect of oil crystallization must also be considered. According to Ghosh et al. (2006), under the presence of the ice, the liquid oil droplets are presumably able to deform to some extent while the crystalline oil droplets focuses the stress at the point of contact between droplets. This favors the disruption of interfacial film by a partial coalescence mechanism and further full coalescence after thawing. In this work, refined sunflower oil with a high content of polyunsaturated fatty acids (~60 g/100 g linoleic acid) was used as

Table 2
Coalescence index (CI) values of SPI-stabilized oil-in-water emulsions after different periods of frozen storage. Cryoprotectants (dehydrated tofu whey, DTW and sucrose) were added to emulsions after homogenization at equivalent total carbohydrate (TCH, g/100 g) content.

Emulsion samples	TCH (g/100 g)	Frozen storage time (days)				
		1.5	16	30	42	60
DTW	0.066	$10.26 \pm 2.02^{a,A}$	$18.80 \pm 2.05^{a,A}$	$21.12 \pm 0.01^{b,A}$	$27.38 \pm 0.09^{c,A}$	$26.74 \pm 2.60^{c,A}$
	0.33	0	0	$0.15 \pm 0.06^{a,B}$	$0.17 \pm 0.03^{a,B}$	$0.22 \pm 0.09^{a,B}$
	1.50	$0.25 \pm 0.10^{a,B}$	$0.25 \pm 0.04^{a,B}$	$0.54 \pm 0.12^{b,c,B}$	$0.45 \pm 0.05^{a,b,B}$	$0.76 \pm 0.15^{c,B}$
Sucrose	0.066	$40.30 \pm 2.12^{a,C}$	$41.37 \pm 4.37^{a,C}$	$64.41 \pm 1.96^{b,C}$	$83.55 \pm 3.32^{c,C}$	$72.67 \pm 5.60^{b,C}$
	0.33	$8.23 \pm 0.01^{a,D}$	$7.54 \pm 1.17^{a,D}$	$7.78 \pm 0.73^{a,D}$	$26.01 \pm 7.60^{b,A}$	$24.00 \pm 5.60^{b,A}$
	1.50	0	0	$0.01 \pm (<0.01)^{a,B}$	$0.02 \pm 0.01^{a,B}$	$0.06 \pm 0.02^{a,B}$

Mean ± standard deviation for $n = 2$ replicates. Within the same row, numbers followed by the same lowercase letters are not significantly differences from each other (least significant differences, $p \leq 0.05$). Within the same column, numbers followed by the same uppercase letters are not significantly differences from each other (least significant differences, $p \leq 0.05$).

dispersed phase. According to theoretical calculations carried out by Magnusson et al. (2011), at −18 °C the solid fat content of sunflower oil is quite important (~30 g/100 g). However, the calculation assumes isothermal and equilibrium conditions, which is far from the case of freezing in still air at −18 °C. Negligible oil crystallization was observed by Palazolo et al. (2011) when frozen o/w emulsions (24 h) prepared with native and denatured soybean isolates and sunflower oil were analyzed by DSC. A mayonnaise-type o/w emulsion stable to freeze-thawing was obtained by Magnusson et al. (2011) after 24 h of frozen storage when sunflower oil was used as dispersed phase. However, after 15 days of storage the emulsion was fully destabilized. These results are in accord with a low crystallization rate of sunflower oil triglycerides. Equilibrium conditions will be less distant when the emulsion is stored for a larger period of time. The higher amount of solid fat, indicated to be formed according to calculations, may then be reached for sunflower oil, even though oil is emulsified within tiny droplets.

FI and CI values of emulsions at long periods of frozen storage (16–60 days) are shown in Tables 1 and 2. At the lowest cryoprotectant concentration (TCH = 0.066 g/100 g), the stability of emulsions to coalescence was always higher when DTW was used as cryoprotectant. For both cryoprotectants a progressive increase of CI values as a function of storage time was effectively observed (Table 2). In the presence of sucrose, a sharp decrease of FI value was observed after freeze-thawing at 40 h, which remained relatively constant throughout the frozen storage period. In contrast, when DTW was added to emulsions, a progressive decrease of FI values was evidenced, due to flocs were destabilized by coalescence (Table 1).

At intermediate TCH content (0.33 g/100 g), DTW promoted the coalescence stability but did not prevent the formation of new flocs during the frozen storage: a sustained increase of FI was observed as a function of storage time ($p < 0.05$, Tables 1 and 2). In contrast, the addition of sucrose was not effective to protect the emulsion against coalescence due to CI values were relatively high and a sharp increase of this parameter was especially observed after 42 days of storage. Simultaneously, FI values showed an important decrease after 42 days ($p < 0.05$, Tables 1 and 2). Thus, the flocs cannot resist the stress exerted by the presence of the ice at long storage periods.

When both cryoprotectants were added at the highest concentration, the corresponding emulsions were fairly stable (CI ~ 0, Table 2). In the presence of sucrose, FI values significantly increased as a function of storage time while for emulsions mixed with DTW, a slight and progressive decrease was observed ($p < 0.05$, Tables 1 and 2). It is worth noting that after 130 days of frozen storage all emulsions, except those stored with DTW at 5.0 g/100 g, were breakdown similarly to control. Hence, the calculation of CI and FI values was not possible for these highly destabilized emulsions. FI and CI values of emulsions with addition of DTW (TCH = 1.5 g/100 g) were 5.54 ± 0.33 and 1.80 ± 0.46, respectively. Although a significant increase of CI value was evidenced when this emulsions was stored for 130 days ($p < 0.05$), it is interesting to remark that this CI value is fairly low and almost no free oil was observed at the top of container. Moreover, no significant differences were observed between FI values at 60 and 130 days of frozen storage ($p < 0.05$). Evidently, at the highest TCH content, DTW exhibited a high ability to protect the emulsion integrity after a prolonged storage at subzero temperatures in conditions highly favorable to ice recrystallization.

In the present work, the amount of free oil was also determined as a function of frozen storage time (Fig. 4). In the absence of cryoprotectant, FO evidenced a rapid increase within the 30 days, reaching a maximum value (~40 g/100 g oil) at longer periods. A comparative analysis between DTW and sucrose at the lowest TCH content (0.066 g/100 g) revealed a similar behavior: after 130 days of freeze-thawing, FO values were similar to that of control ($p < 0.05$, Fig. 4a). In contrast, at higher concentrations the differences between both cryoprotectants were evident especially after 60 days of frozen storage (Fig. 4b and c). At 5.0 g/100 g DTW, the integrity of droplets was practically maintained: at 130 days, FO was <4 g/100 g (Fig. 4c). To better complement the results mentioned above, the visual appearances of emulsions were also analyzed through photographs at bulk scale. Images of thawed emulsions in the absence and presence of DTW after 40 h and 130 days of frozen storage are shown in Fig. 5a and b. Although in all

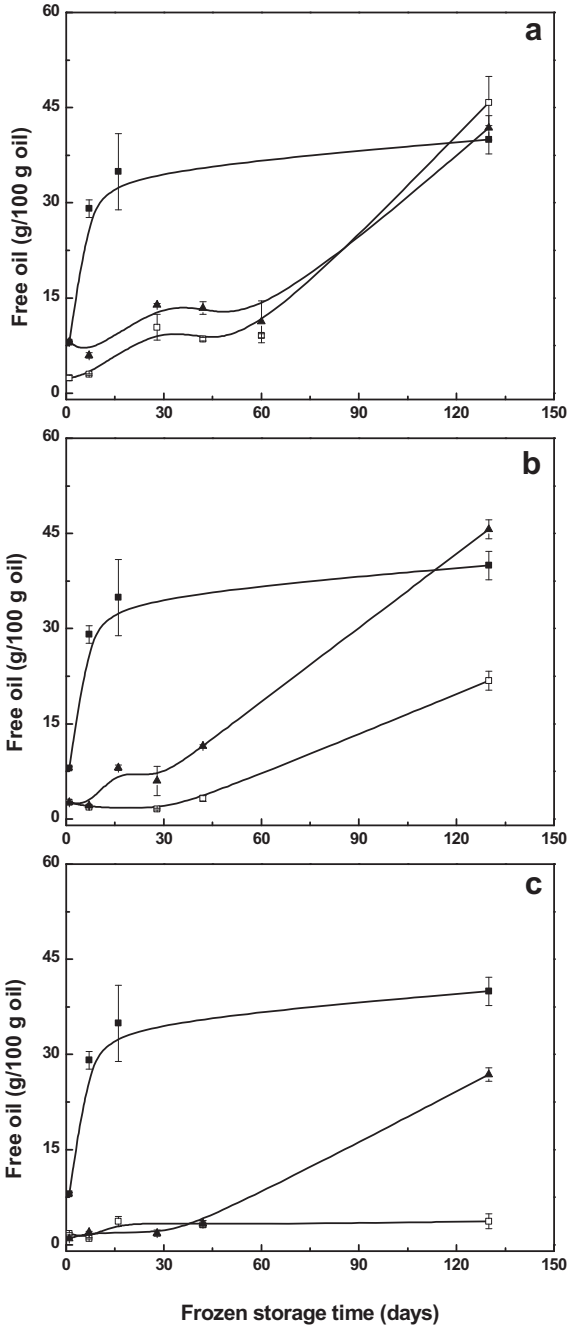

Fig. 4. Effect of cryoprotectant concentration (dehydrated tofu whey, DTW or sucrose) and storage time on the amount of free oil (FO) of SPI-stabilized oil-in-water emulsions. Total carbohydrate content (TCH, g/100 g): a) 0.066; b) 0.33; c) 1.5. (-■-) Without cryoprotectant, (-□-) DTW, and (-▲-) sucrose. Error bars indicate ± standard deviation for $n = 2$ replicates.

cases gravitational separation was observed, at low and intermediate DTW content (TCH = 0.066 and 0.33 g/100 g) the visual appearance of emulsions subjected to freeze-thawing for different times was quite different. After a prolonged frozen storage, besides free oil, a self-supporting cryo-gel was observed after thawing similarly to control emulsion. The presence of cryo-gel, which is formed by aggregated soy proteins and occluded oil molecules, is consistent with a high destabilization degree. Oil molecules remain, at least partially, within the cryo-gel matrix and hence, there is a density difference to that of the surrounding aqueous phase (Fig. 5b). Conversely, at the highest DTW content (TCH = 1.5 g/100 g) most of oil remained in an emulsified state at both storage periods (Fig. 5b). This observation was in agreement with the low values of FO throughout the frozen storage period (Fig. 4c). After 130 days of frozen storage, the presence of sucrose at equivalent TCH content was not effective to avoid neither the cryo-gel formation nor the extensive oiling off (FO = 26.9 ± 1.1, Figs. 4c and 5c). It is worth noting that the cryo-gel may be easily separated from the container (Fig. 5d).

For control emulsion MDSC studies showed that almost all water was in frozen state. As expected, in the absence of cryoprotectant, freezable water (FW) was higher than 99 g/100 g. Moreover, the higher cryoprotectant concentration, the lower amount of FW. Although DTW was slightly more effective to decrease the T_f of the aqueous phase, similar FW values were observed when cryoprotectans were added at equivalent TCH content ($p < 0.05$). At the highest TCH content, these values were 77.08 ± 3.07 and 79.02 ± 1.05 g/100 g for DTW and sucrose, respectively. This result is in agreement with a partial inhibition of ice crystallization. The observed differences in protecting effect between both cryoprotectants at longer periods of storage would be explained on the basis of other different mechanisms. First, the sucrose is the main soluble carbohydrate of TW (>50 g/100 g, Espinosa-Martos & Ruperez, 2006). However, others oligosaccharides, monosaccharides (as stachyose and glucose) and soluble peptides, jointly exert their cryoprotective role at long periods of frozen storage. Second, the presence of proteins in the aqueous phase could have and additional stabilizing effect on SPI emulsions. Ghosh et al. (2006) reported a positive effect of unadsorbed protein on freeze-thaw stability of model emulsions prepared with sodium caseinate and *n*-hexadecane. It is reasonable that unadsorbed protein between two protein-covered surfaces would produce a steric stabilization against coalescence. This argument is similar to those exposed by Carpenter and Crowe (1988) for the increased stability that is noted during freeze-thawing of enzymes as its concentration is increased or by addition of other proteins, as bovine serum albumin. According to these authors, for proteins that do not undergo self-association or aggregation, individual protein molecules cannot penetrate the hydration shell of neighboring proteins molecules. In this context, Palazolo et al. (2011) found that unadsorbed 11S globulin was not effective to exert a protective effect during the frozen storage due to its ability to aggregate and insolubilize at subzero temperatures. However, denatured whey soy proteins may be a different behavior respect to that of 11S globulin and could contribute to emulsion stability. Moreover, the preparation procedure of DTW induces an incipient glycosylation of whey soy proteins due to concentration stages and the presence of soluble sugars (Sobral & Wagner, 2009). The presence of glycosylated proteins in the aqueous phase could retard recrystallization and the associated growth of large ice crystals during frozen storage and thawing. As a consequence of extraordinary complexity of DTW sample, further experiments are needed to ascertain the

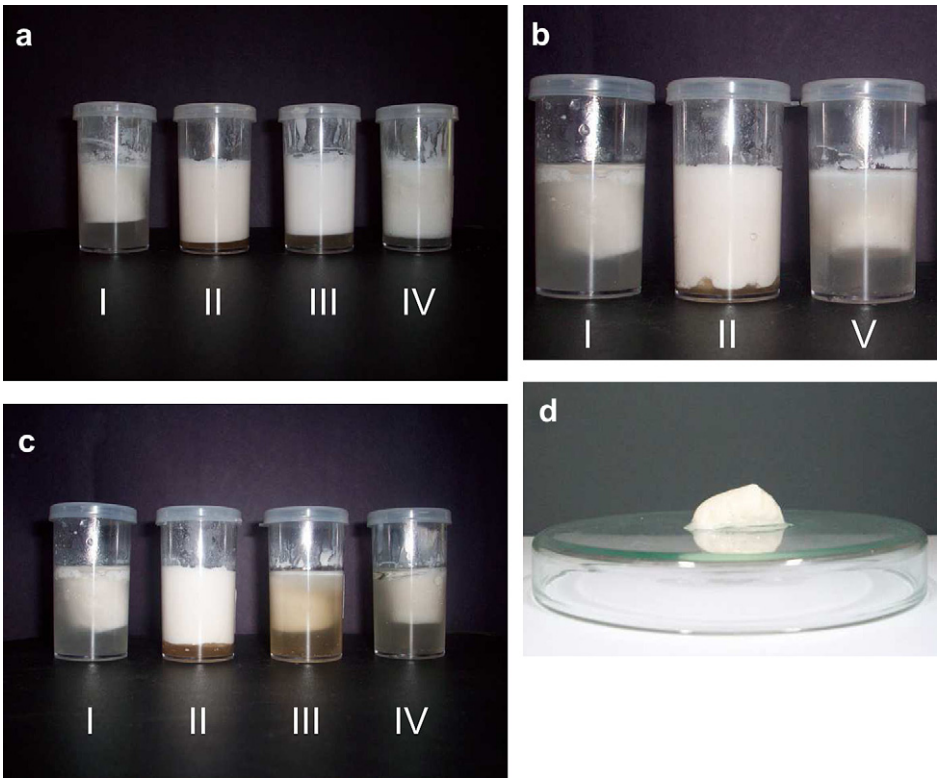

Fig. 5. Photographs at bulk scale of thawed SPI oil-in-water emulsions in the presence of dehydrated tofu whey (DTW) at different concentrations (0.2, 1.0 and 4.5 g/100 g): a) after 40 h of freeze-thawing, b) after 130 days of freeze-thawing, c) Comparative visual appearances between emulsions with DTW or sucrose at equivalent carbohydrate content after 130 days of freeze-thawing; d) Self-supporting cryo-gel separated from emulsion with sucrose addition. I) without cryoprotectant, II) DTW 4.5 g/100 g, III) DTW 1.0 g/100 g, IV) DTW 0.2 g/100 g, and V) sucrose 1.5 g/100 g.

relative importance of mentioned factors on freeze–thaw stability of o/w emulsions prepared with soy proteins and other protein samples. Nevertheless, in this article we have expose the high stabilizing effect of dehydrated tofu whey on model o/w emulsions at prolonged periods of frozen storage, in conditions highly favorable to ice recrystallization.

4. Conclusions

Dehydrated tofu whey clearly improved the freeze–thaw stability of model o/w emulsions prepared with soy protein isolate and sunflower oil. The cryoprotective effect was higher respect to that of sucrose at equivalent total carbohydrate content. At the highest cryoprotectant concentration, after 130 days of frozen storage, dehydrated tofu whey promoted the emulsion stability. Most of dispersed phase remained in an emulsified state and a high stability to coalescence was observed. However, sucrose only was effective as cryoprotectant at relatively short periods of frozen storage (<30 days). At longer periods, the oiling off and the formation of a self supporting cryo-gel formed by aggregated proteins and occluded oil was consistent with a high destabilization degree. Although both cryoprotectans partially inhibited the ice crystallization, the better protective effect of dehydrated tofu whey could be attributed to the presence of other components as glycosylated proteins.

Tofu whey is an effluent and hence, is discarded with a highly negative environmental impact. Notwithstanding this, it contains important components as denatured whey proteins (and hence, negligible antitryptic activity), oligosaccharides, calcium (and/or magnesium) and isoflavones. Drying and concentration reduces the transport cost and represents an interesting alternative to convert a contaminant effluent in a product of potential interest for the food industry. Freezing, freeze-concentration and freeze-drying processes are used in the production of many food products in order to prevent or delay deterioration. The addition of dehydrated tofu whey is helpful to improve the stability of protein-stabilized oil-in-water emulsions subjected to freeze-thawing. In future studies, we intend to examine the behavior of some individual components of tofu whey, as whey soy proteins on freeze–thaw stability of protein-stabilized oil-in-water emulsions, fundamentally the effect of glycosylation grade induced by concentration stages.

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