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Characterization and storage stability of spray dried soy-rapeseed lecithin/trehalose liposomes loaded with a tilapia viscera hydrolysate

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ABSTRACT

Drying-induced stabilization is a challenge that delivery systems still face. This study aims to investigate the effects of adding trehalose to spray dried soy-rapeseed lecithin liposomes, and the storage stability of the dried liposomes loaded with a tilapia viscera protein hydrolysate, during 42 days at 4 °C and 23 °C, and at different relative humidity (RH). Particle size increased from 215 to 250 nm in fresh liposomes to 258–314 nm after spray drying according to trehalose concentration, all preparations showing a strong electronegative ζ Potential (−48.5 to −59.9 mV). Dried liposomes stored at 4 °C maintained lower polydispersity and higher solubility than those stored at 23 °C. Changes in water activity (A_w), FTIR and DSC revealed structural changes in samples stored at 23 °C and high RH. Spray dried hydrolysate-containing liposomes could be considered as a functional food ingredient due to the substantial antioxidant activity and angiotensin-converting enzyme (ACE) inhibitory capacity after *in vitro* simulated gastrointestinal digestion.

Industrial relevance: The availability of a natural liposomal preparation in dry powder form, capable to maintain its bioactive properties, is a great advantage for the functional food industry, since it favours the stability, transport and storage of the ingredient prior to use, and allows great versatility to be incorporated in solid restructured products.

1. Introduction

Peptides from fish protein hydrolysates have shown a number of bioactive properties such as antihypertensive, antioxidant, and antimicrobial activities (Morales-Medina, Tamm, Guadix, Guadix, & Drusch, 2016). However, some difficulties regarding food applications may exist due to their strong or bitter taste, high hygroscopicity, and possible matrix incompatibility (Sarabandi et al., 2019). The use of nanocarriers such as liposomes can be a useful strategy to avoid these problems. Liposomes are one of the most widely used delivery systems to protect and release bioactive compounds, as they can be manufactured from food compounds using simple methods (McClements, 2015). Moreover, they are of great interest due to their biocompatibility and ability to transport both, hydrophilic and hydrophobic compounds (Tan et al., 2014). As well, and in addition to the properties of the encapsulated bioactive compounds, liposomes formulated from natural lecithin provide a nutritional contribution owing to their content in essential polyunsaturated fatty acids and antioxidant compounds (Li & Guo, 2016; Marín, Alemán, Montero, & Gómez-Guillén, 2018). In a previous study, an equimolar soy-rapeseed lecithin mixture was successfully used to prepare liposomes with antioxidant capacity, which was further increased by the loading with a protein hydrolysate from tilapia viscera (Sepúlveda et al., 2021).

Liposome destabilization during storage can produce changes such as membrane fusion, aggregation, and leakage of the encapsulated material (Tan et al., 2014). Also, phospholipids can be degraded by peroxidation of unsaturated acyl chains or hydrolysis of ester bonds (Abdelwahed, Degobert, Stainmesse, & Fessi, 2006). Liposomal powder formulations are more stable and more appropriate for long-term storage compared with liposomal dispersions, which may allow for a better adjustment to the properties required for certain specific applications (Ingvarsson, Yang, Nielsen, Rantanen, & Foged, 2011). Spray drying is less expensive, and less time and energy consuming compared with freeze drying, making it a suitable strategy to dry liposomal dispersions.

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However, one of the challenges of using liposome spray drying is to avoid membrane bilayer instability caused by heat-induced phase transitions (Van Den Hoven, Metselaar, Storm, Beijnen, & Nuijen, 2012). The removal of water molecules that are spatially separating phospholipid membrane polar heads promotes van der Waals interactions, leading to an increase in the melting temperature (Crowe, Carpenter, & Crowe, 1998). Furthermore, both heat and high shear forces involved in the process may also induce degradation of the liposomal bilayer structure (Ingvarsson et al., 2011). Therefore, compounds that protect the membrane during the drying process are necessary. Some disaccharides, such as sucrose, lactose, and trehalose, can be used as stabilizers during dehydration (Patist & Zoerb, 2005). In order to protect liposomal membranes, the stabilizer must be present in excess with respect to lipids (Viera, Alonso-Romanowski, Borovyagin, Feliz, & Disalvo, 1993). Trehalose is a non-reducing disaccharide of glucose, frequently found in high concentrations in organisms that are capable of surviving dehydration (Crowe, Crowe, & Rudolph, 1985). Due to the flexibility of its structure, trehalose improves the interaction between sugar and liposome phospholipid heads (Patist & Zoerb, 2005). Furthermore, trehalose has low hygroscopicity and high glass transition temperature, both useful properties to increase storage stability of dried liposomal powders (Abdelwahed et al., 2006).

This work aims to evaluate the effects of increasing concentrations of trehalose on the physicochemical properties of liposomes - formulated from a mixture of soy and rapeseed lecithin- before and after spray drying. After selecting the most convenient trehalose concentration, the stability of spray-dried liposomes loaded with a tilapia viscera protein hydrolysate was evaluated in the course of 42 days under different storage conditions in terms of particle properties, water activity, solubility, hygroscopicity, and antioxidant activity. Furthermore, the antioxidant activity and angiotensin-converting enzyme (ACE) inhibitory capacity of dried liposomes and free hydrolysate were investigated after *in vitro* simulated gastrointestinal digestion. Microscopic observation of liposomal morphology was carried out after 11 months of storage at 4 °C.

2. Materials and methods

2.1. Materials

The chemicals, including 2.2-azino-bis(3-ethylbenzothiazoline-6-sul- phonic acid) diammonium salt (ABTS), bovine serum albumin, and hippuril-L-histidyl-L-leucine (HHL), were purchased from Sigma-Aldrich. Potassium persulphate was obtained from Panreac. 2.4.6-tri (2-pyridyl)-*s*-triazine (TPTZ) was obtained from Fluka. All other reagents were of analytical grade.

2.2. Fish protein hydrolysate

The protein hydrolysate from red tilapia viscera used in the present work was produced and characterized (proximate and amino acid composition, molecular weight distribution, and antioxidant capacity) in a previous work (Sepúlveda et al., 2021). Briefly, endogenous enzymes were inactivated by heating viscera at 90 °C for 20 min and fat was mechanically separated after 24 h of frozen storage at −20 °C. Enzymatic hydrolysis was carried out with Alcalase 2.4 L (Novozymes, Denmark) (enzyme/substrate ratio of 1:10, *w*/w) for 3 h at 59 °C and pH 10, using a pH-stat. After enzyme inactivation at 90 °C for 10 min, the hydrolysate was centrifuged at 4500 ×*g* (30 min, 4 °C) and the supernatant was freeze-dried and stored at −20 °C until further experiments.

2.3. Liposome preparation

The procedure for liposome preparation was the same as described by Sepúlveda et al. (2021). Very briefly, a mixture (50:50, w:w) of soy lecithin and rapeseed lecithin was dispersed (5%, *w/v*) in 0.2 M phosphate buffer (pH 7) by gently stirring for 1 min, heated at 80 °C for 2 h in a water bath and sonicated by probe-tip in a pulsed mode for 5 min, with a 60 s stop every min. Increasing concentrations (5, 10, 15, and 20%, w/v) of trehalose with respect to total liposomal dispersion were added after sonication, namely LT-5, LT-10, LT-15, and LT-20, respectively. Based on particle properties, drying yield and solubility results, the liposomal formulation with 15% trehalose was selected for encapsulating the fish protein hydrolysate. These liposomes, which were designated as HL, were prepared following the same procedure mentioned above by previously mixing the soy-rapeseed lecithin blend with the freeze-dried hydrolysate in a ratio of 5:1 (lecithin:hydrolysate, *w*/w).

2.4. Spray-drying of liposomes

The aqueous dispersions were spray dried using a Büchi B-290 Mini Spray Dryer (Büchi Labortechnik, Flawil, Switzerland). Liposomal dispersion was feed into the dryer by a peristaltic volumetric pump at a feed flow rate of 630.5 mL/h. The pressure nozzle with an internal diameter of 0.7 mm was used to spray the liposomes into the chamber in current flow with a compressed air flow rate of 35 m^3/h. The inlet and outlet air temperatures were 130 and 62 °C, respectively. The collected powders were stored in airtight containers at 4 °C for further analysis. The spray-drying yield was calculated as the mass ratio of spray-dried powder to the total solids content in the feed solution.

2.5. Determination of size, polydispersity and ζ potential

The mean particle size (z-average, nm), polydispersity index (PDI) and ζ Potential (mV) of fresh and rehydrated liposomal dispersions were determined using a Zetasizer Nano series (Malvern Instruments. UK) at 25 °C as previously described (Taladrid et al., 2017).

2.6. Hygroscopicity of spray-dried liposomes

Hygroscopicity was evaluated according to the method described by Cai and Corke (2000) with some modifications. An amount of 0.2 g of spray-dried liposomes was weighed and placed in desiccators containing saturated solutions of NaBr and NaCl (≈60% and ≈75% RH, respectively) at 23 °C for 42 days. In parallel, samples were also stored at 4 °C. Hygroscopicity was determined in triplicate by measuring the mass of water absorbed per 100 g of sample.

2.7. Water activity and solubility of spray-dried liposomes

Water activity was determined in triplicate using a LabMaster-aw equipment (Novasina, Switzerland). Solubility was determined by a gravimetric method described by Gomez-Estaca, Comunian, Montero, Ferro-Furtado, and Favaro-Trindade (2016). Briefly, 0.5 g dry powder were diluted in 50 mL of distilled water and homogenized at 100 rpm using an AG-200 orbital shaker (COMECTA S.A., Barcelona, Spain) for 150 min. The dispersions were centrifuged at 5000 ×*g* for 5 min at 4 °C, and 25 mL of supernatant was heated to 105 °C until complete evaporation. The solubility was calculated according to Eq. 1:

$$Solubility\ (\%) = \frac{Final\ weight \times 2}{Initial\ powder\ weight} \times 100 \quad (1)$$

2.8. Entrapment efficiency (EE) of spray-dried liposomes

Entrapment efficiency in rehydrated liposomal dispersions was estimated as described previously in Sepúlveda et al. (2021). The amount of encapsulated hydrolysate was calculated as the difference between the total hydrolysate used for liposome preparation and the non-encapsulated hydrolysate.

The non-encapsulated hydrolysate was separated from the liposomes

by filtration using 10 kDa centrifugal filter device (Amicon® Ultra-15, Merck Millipore Ltd., Ireland) and centrifugation at 5000 ×*g* for 40 min at 4 °C. The non-encapsulated hydrolysate, which passed through the membrane, and the total hydrolysate were determined by measuring the protein content (determined by the Lowry method) and antioxidant activity (ABTS method). The EE was calculated according to Eq. 2:

$$\%EE = (\text{encapsulated hydrolysate}/\text{total hydrolysate}) \times 100 \quad (2)$$

2.9. Storage stability of spray-dried liposomes

The liposomal formulation added with 15% trehalose was selected for further analysis. Empty liposomes (EL) and hydrolysate-loaded liposomes (HL), both added with 15% of trehalose, were spray dried, and the resulting dry powders were placed in several containers at different relative humidity levels (≈75% and ≈60%, namely EL-75, HL-75, EL-60, HL-60). Samples were stored at both 23 °C and 4 °C for 42 days. Hygroscopicity, water activity (A_w), and water solubility of powders stored at the different conditions were determined as described in sections 2.6 and 2.7. All measurements were made in triplicate.

2.9.1. Fourier transformed infrared spectroscopy (ATR-FTIR)

Infrared spectra of spray-dried liposomes and the freeze-dried hydrolysate were obtained using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin Elmer Inc., Waltham, MA, USA) equipped with an ATR prism crystal accessory. Scans with spectral resolution of 4 cm^{-1} were recorded in the frequency between 4000 and 650 cm^{-1}.

2.9.2. Thermal properties

Thermal analysis of spray-dried liposomes was performed using a TA-Q1000 differential scanning calorimeter (DSC) (TA Instruments, New Castle, DE, USA). The samples (2–10 mg) were placed in a hermetically sealed aluminum pan, and an empty pan was used as the reference. The powder samples were scanned at a heating rate of 10 °C/min from −50 °C to 90 °C, under dry nitrogen purge (50 mL/min). Endothermic peak and glass transition temperatures (T_{peak} and T_g) were determined in duplicate.

2.9.3. Antioxidant activity

Antioxidant properties of spray-dried liposomes were determined after liposomes were dissolved in distilled water, following the ABTS radical scavenging capacity and ferric ion reducing power (FRAP) methods described by Alemán, Giménez, Pérez-Santin, Gómez-Guillén, and Montero (2011). Determinations were made in triplicate, and the results were calculated by extrapolation in a calibration curve with Trolox as standard. The values obtained were expressed as equivalent micromoles of Trolox per gram of dry sample (μmol TE/g).

2.10. In vitro simulated gastrointestinal digestion

The selected spray-dried liposomes and the freeze-dried hydrolysate were subjected to an *in vitro* gastrointestinal digestion (GID) assay, which was conducted according to the harmonized INFOGEST protocol (Minekus et al., 2014) with a slight modification. Briefly, gastric digestion was simulated, mixing 10 mL of liposomes or hydrolysate with 7.5 mL of simulated gastric fluid (SGF). 1.6 μL of porcine pepsin (2000 $U.mL^{-1}$, EC 3.4.23.1, Sigma-Aldrich, St. Louis, MO, USA) was added followed by 5 μL of $CaCl_2$ to achieve 0.075 mM in the final mixture. 2 N HCl was used to reduce the pH to 3.0; water was added to reach a final volume of 10 mL, and the samples were incubated for 2 h in a water bath at 37 °C under constant stirring. Afterwards, 11 mL of simulated intestinal fluid (SIF), 5 mL of pancreatin (100 $U.mL^{-1}$ in the final mixture), 2.5 mL of 160 mM bovine bile, and 40 μL of 0.3 M $CaCl_2$ were added. The pH was adjusted with 1 M NaOH to reach pH 7.0, and water was added to obtain a final volume of 20 mL. Intestinal digestion was simulated by incubation at 37 °C for 2 h under constant stirring. Samples were collected after 1, 2, 3, and 4 h, and the enzymes were inactivated by heating at 90 °C for 10 min.

2.11. Angiotensin-converting enzyme inhibitory capacity

Angiotensin-converting enzyme (ACE) inhibitory capacity of the selected spray-dried liposomes and the freeze-dried hydrolysate was determined as described by Alemán, Gómez-Guillén, and Montero (2013) using hippuril-L-histidyl-L-leucine (HHL) as substrate. The hippuric acid (HA) produced was quantified by RP-HPLC. Both HA and HHL were monitored at 228 nm, and the determinations were made in triplicate.

2.12. Transmission electron microscopy (TEM) of spray-dried liposomes

The hydrolysate-loaded liposomes were suspended in sterile water. An aliquot was placed on a complete membrane grid. Sample loaded grids were stained with 2% uranyl acetate and were left to stand for 20 min. The grid was washed with sterile water and allowed to dry at room temperature (25 °C). The sample was visualized using a Tecnai F20 Super Twin TMP transmission electron microscope (FEI Company, Hillsboro, USA) operating at 200 kV and recorded using a Gatan US 1000XP-P camera.

2.13. Statistical analysis

Data were presented as means ± standard deviation ($n = 3$) and analyzed using the analysis of variance (ANOVA). Fisher's minimum significant difference (LSD) method was used to discriminate between the means. The statistically significant difference was established at $p < 0.05$.

3. Results and discussion

3.1. Effect of trehalose concentration

Mean particle size (z-average), polydispersity index (PDI), and ζ Potential of fresh and spray-dried liposomes with trehalose are shown in Table 1. The size of fresh liposomes tended to increase with increasing trehalose addition, although not significantly ($p > 0.05$) in LT-15. The mean size of fresh liposomes without trehalose (193.2 ± 3.0 nm) was reported in a previous work (Sepúlveda et al., 2021), being lower compared to any of the trehalose-stabilized liposomes in the present study. It should be noted that spray-dried liposomes in the absence of trehalose collapsed, making impossible the obtaining of a suitable dry powder. Despite trehalose addition, and regardless the concentration, all samples showed a significant increase ($p < 0.05$) in size after the spray-drying process, as compared with their respective fresh preparations. This effect could be attributed to partial fusion and aggregation of liposomes (Charnvanich, Vardhanabhuti, & Kulvanich, 2010; Ingvarsson et al., 2011). Van Den Hoven et al. (2012) also found an increase in spray-dried liposome size despite the addition of disaccharides. Both the damage to the membrane due to dehydration stress and the grouping of vesicles during reconstitution, could explain the increase in liposome size (Sarabandi et al., 2019). The polydispersity index (PDI) did not change with a higher trehalose concentration in fresh liposomes (Table 1), however, it became lower ($p < 0.05$) as the amount of trehalose increased in spray-dried liposomes, indicating that trehalose induced more homogeneity in particle size distribution. According to these results, which agree with those reported by Ingvarsson et al. (2013) in liposomes stabilized with mannitol, lactose, and trehalose, 15% trehalose was selected as an optimal concentration to prevent the increase in both particle size and PDI in spray-dried liposomes.

The ζ Potential in fresh liposomes remained the same regardless trehalose concentration. A slightly lower value of ζ Potential (−46.4 ± 1.1 mV) was previously reported for similar liposomes in the absence of

Table 1
Physico-chemical properties of fresh (F) and spray-dried (SD) liposomes with addition of trehalose at 5, 10, 15, and 20%.

Sample	Size (nm)		PDI		ζ Potential (mV)		Drying yield (%)	Solubility (%)	Moisture (%)
	F	SD	F	SD	F	SD	SD	SD	SD
LT-5	$215 \pm 4^{a/A}$	$258 \pm 4^{a/B}$	$0.49 \pm 0.04^{a/A}$	$0.42 \pm 0.02^{a/B}$	$-48.5 \pm 2.5^{a/A}$	$-53.6 \pm 0.2^{a/B}$	59.6 ± 6.3^{a}	65.2 ± 1.3^{a}	3.0 ± 0.0^{a}
LT-10	$229 \pm 7^{b/A}$	$314 \pm 5^{b/B}$	$0.47 \pm 0.03^{a/A}$	$0.37 \pm 0.03^{b/B}$	$-49.2 \pm 0.6^{a/A}$	$-59.9 \pm 0.9^{b/B}$	$67.1 \pm 0.1^{a, b}$	82.8 ± 6.0^{b}	3.5 ± 0.8^{b}
LT-15	$219 \pm 6^{a/A}$	$291 \pm 5^{c/B}$	$0.46 \pm 0.03^{a/A}$	$0.34 \pm 0.05^{b/B}$	$-48.7 \pm 2.7^{a/A}$	$-58.0 \pm 0.5^{b/B}$	68.6 ± 0.1^{b}	93.2 ± 1.3^{c}	3.8 ± 0.2^{b}
LT-20	$250 \pm 7^{c/A}$	$283 \pm 3^{d/B}$	$0.46 \pm 0.06^{a/A}$	$0.30 \pm 0.02^{c/B}$	$-49.2 \pm 0.1^{a/A}$	$-58.6 \pm 3.7^{b/B}$	$63.8 \pm 0.2^{a, b}$	95.3 ± 0.8^{c}	3.8 ± 0.0^{b}

Different letters (a, b, c, d, e, f) in the same column indicate significant differences ($p < 0.05$) as a function of trehalose concentration. Different letters (A, B) indicate significant differences between F and SD at the same concentration of trehalose.

trehalose (Sepúlveda et al., 2021). Higher ($p < 0.05$) ζ Potential values were observed after spray drying, with no significant changes from 10% trehalose upwards. According to Mohan, McClements, and Udenigwe (2016) absolute values of ζ Potential higher than ±30 mV indicate excellent stability since repulsion charges prevent aggregation due to collisions between adjacent particles.

Drying yield, moisture, and water solubility of spray-dried liposomes with increasing concentrations of trehalose are also shown in Table 1. The maximum value for powder recovery was 68.6% in LT-15. This yield was noticeably higher than the 52% reported by Sarabandi et al. (2019) for spray-dried liposomes using maltodextrin as wall material. The solubility was higher ($p < 0.05$) with increasing trehalose concentrations, being above 93% in LT-15 and LT-20. This is an important functional property that allows predicting the behavior of the powder in water reconstitution, and widens the application of the product in different foods and formulations (Sarabandi, Sadeghi Mahoonak, Hamishekar, Ghorbani, & Jafari, 2018). The moisture values for all dried samples were less than 3.8%, with no significant differences ($p > 0.05$) among LT-10, LT-15, and LT-20. Moisture content in powdered products is an indicator of stability, being 1–6% a desirable range to ensure their shelf life during storage (Zhang, Zhang, Chen, & Quek, 2020). Liposomes with 15% trehalose were then selected for further analysis due to their good particle properties, low moisture content, and high drying yield and solubility.

3.2. Encapsulation of fish protein hydrolysate

The entrapment efficiency (EE) of the tilapia hydrolysate in liposomes formulated with 15% trehalose was determined according to the total protein content and the ABTS radical scavenging capacity, being the results 81.3% and 80.7%, respectively. These results were noticeably higher than the ones reported for similar fresh liposomes without trehalose (49% and 64%, respectively) (Sepúlveda et al., 2021), suggesting that both, trehalose addition and spray drying could have favored the entrapment of the hydrolysate and/or prevented its leakage. Furthermore, irreversible changes in protein concentration induced by drying and subsequent rehydration could also have contributed to the increase in EE. In another work, da Rosa Zavareze et al. (2014) reported an EE of about 80% in soy phosphatidylcholine liposomes loaded with hydrolysates of croaker proteins, while Ramezanzade, Hosseini, and Nikkhah (2017) found values between 55.2 and 80.2% for rainbow trout (*Oncorhynchus mykiss*) gelatin hydrolysate loaded in chitosan-coated DPPC liposomes. Sarabandi et al. (2019) found an EE between 85 and 90% in lecithin liposomes loaded with different flaxseed protein hydrolysates and concluded that the high EE could be attributed to a better placement of smaller peptides within the liposome structure. Anyhow, proper comparisons among different works reported in the literature are quite difficult due to differences in methodology, raw material chemical properties and ratios between encapsulating materials and loaded compounds.

3.3. Storage stability of spray-dried liposomes

The stability of spray-dried EL and HL, both containing 15% trehalose, was evaluated along storage under different conditions of relative humidity (≈60% and ≈75%) and temperature (23 °C and 4 °C).

3.3.1. Particle properties

Mean particle size (z-average), PDI, and ζ Potential were measured after 0, 7, 14, 28, and 42 days (Fig. 1). The particle size varied between 247 and 379 nm throughout the entire analysis. From day 14, liposomes EL-60 and HL-60 stored at 23 °C (Fig. 1b) showed a pronounced increase ($p < 0.05$) in particle size. A small increase ($p < 0.05$) was only observed in HL-75 stored at 4 °C from day 28, while HL-60 at 4 °C showed the lowest fluctuations during the 42 days of analysis (Fig. 1a). In general, samples stored at 23 °C showed a noticeable increase ($p < 0.05$) in PDI after two weeks of storage, which did not occur at 4 °C (Fig. 1c and d). The size increase might indicate particle destabilization due to aggregation phenomena and possible swelling and phase transition of the liposomal membrane during rehydration (Chen, Han, Cai, & Tang, 2010; Stark, Pabst, & Prassl, 2010). Similar results were reported in nanoliposomes loaded with flaxseed protein hydrolysates (Sarabandi et al., 2019) or with thyme essential oil using β-cyclodextrin as a cryoprotectant (Lin, Zhu, Thangaraj, Abdel-Samie, & Cui, 2018). The ζ Potential varied between −31.4 mV and − 59.4 mV (Fig. 1 e and f), indicating that optimal liposomal stability after rehydration was not lost during the entire storage. In general, samples stored at 60% RH presented lower fluctuations in ζ Potential values, especially those stored at 4 °C ($p > 0.05$).

3.3.2. Solubility, water activity, and hygroscopicity

The solubility of spray-dried liposomes during storage at 4 °C and 23 °C is shown in Fig. 2 a and b, respectively. At the beginning of storage, hydrolysate-loaded liposomes had higher ($p < 0.05$) solubility compared with empty liposomes. This effect could be attributed to structural changes in the liposome membrane due to peptide interactions, and also to the non-encapsulated fraction of the hydrolysate, which could prevent liposome associations during rehydration. In most samples water solubility decreased significantly ($p < 0.05$) over time, which is attributed to a noticeable particle aggregation. In general, liposomes stored at 23 °C showed a faster decrease in solubility than those stored at 4 °C. EL-75 at 4 °C also showed a pronounced decrease ($p < 0.05$) after two weeks of storage; in contrast, HL-60 stored at 4 °C showed the highest solubility ($p < 0.05$) after 42 days. This sample was the most stable over time in terms of particle size, polydispersity and ζ Potential.

Water activity (A_w) could be used as an indicator of liposomal membrane stability, considering it determines variations in free water associated to structural changes affecting surface properties, permeability, or swelling behavior of liposomes (Disalvo, & Frias, M. de los A., 2019). Initially, HL presented a markedly lower ($p < 0.05$) A_w than EL (Fig. 2 c and d), suggesting that the presence of peptides modified the hydration properties of liposomes, probably by altering the hydration shell of phospholipids. It should be noted that the initial A_w value of HL (0.14) was lower than that reported for liposomes loaded with flaxseed protein hydrolysates and spray-dried in the presence of maltodextrin (2.27–0.29) (Sarabandi et al., 2019). The water activity of all samples increased ($p < 0.05$) with storage time, suggesting changes in the

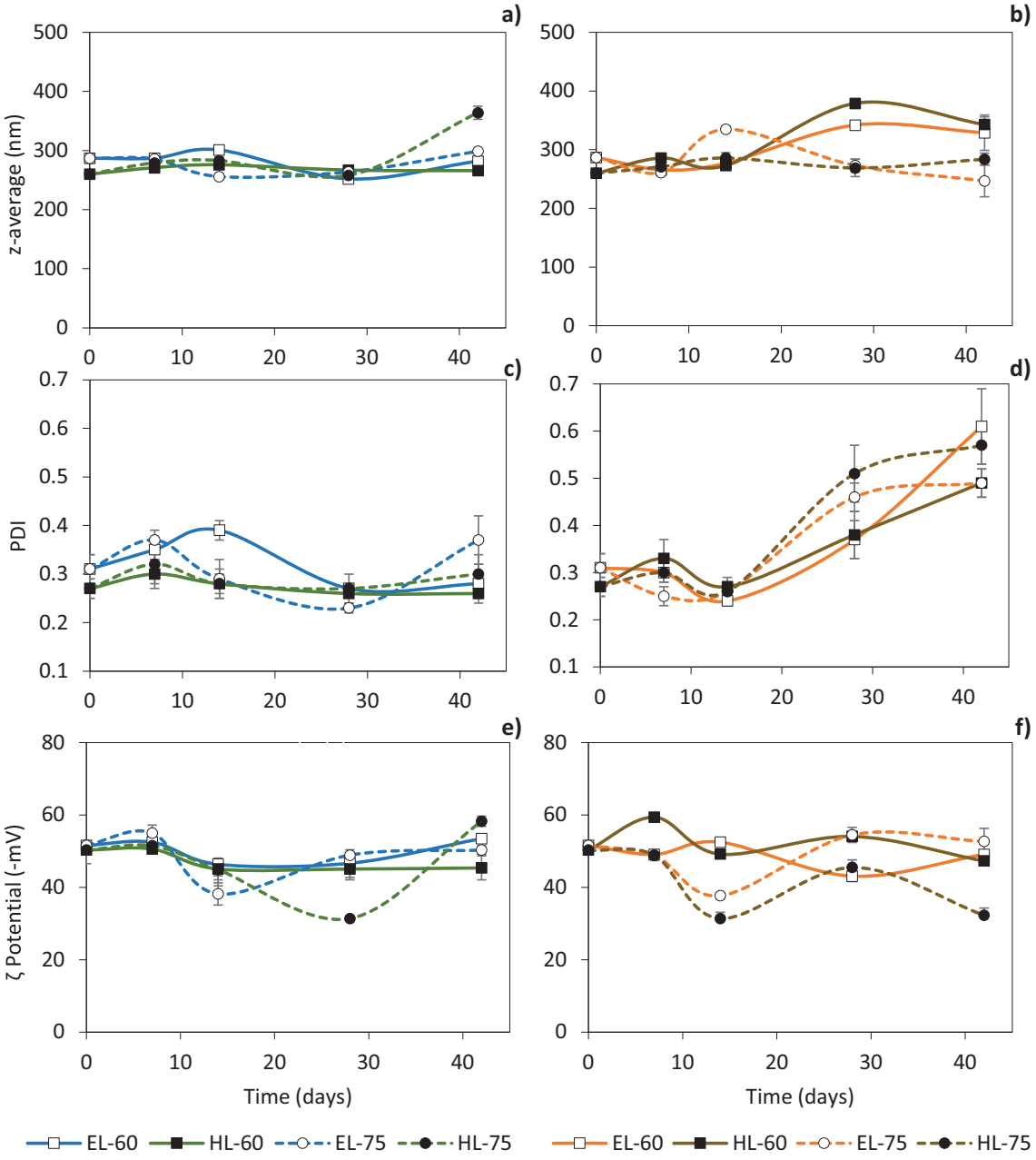

Fig. 1. Mean particle size, polydispersity index (PDI) and ζ Potential of spray-dried liposomes stored for 42 days at 4 °C (a,c,e) and at 23 °C (b,d,f). EL-60: empty liposomes at 60% RH; HL-60: hydrolysate-loaded liposomes at 60% RH; EL-75: empty liposomes at 75% RH; HL-75: hydrolysate-loaded liposomes at 75% RH. RH = relative humidity.

membrane by water adsorption; this effect was more noticeable in samples stored at 23 °C and high RH (Fig. 2d). The sample with the lowest increment ($p < 0.05$) of A_w at the end of storage was HL-60 stored at 4 °C (Fig. 2c). This finding suggests a remarkable stabilizing effect of the hydrolysate on the liposomal membrane, which prevented water from permeating the lipid bilayer. This effect, however, was not observable at 23 °C, possibly due to some structural change in the bilayer at higher storage temperatures.

The hygroscopicity of spray-dried liposomes increased dramatically within the first week of storage and remained with slight variations thereafter (Fig. 2 e and f). In samples stored at 4 °C, values were higher ($p < 0.05$) for HL than for EL, being logically higher ($p < 0.05$) at greater relative humidity. The small non-encapsulated hydrolysate fraction, as well as the peptides associated to the membrane surface, could probably be the main contributors of the highest hygroscopicity values, since low molecular peptides from enzymatic hydrolysis are highly hygroscopic (Kurozawa, Park, & Hubinger, 2009). However, at 23 °C, only HL-75 showed a noticeably high value ($p < 0.05$) for hygroscopicity, which was lower than for its HL-75 counterpart stored at 4 °C. This finding also indicated thermally-induced changes in the disposition of phospholipid polar head groups and peptides in the external face of the bilayer, which would alter their interactions with water molecules.

3.3.3. *Fourier transform infrared spectroscopy*

Fourier transform infrared (FTIR) spectroscopy was used to obtain information on the conformational changes of dried liposomes with and without the hydrolysate at different storage conditions (Fig. 3). First of all, Fig. 3a shows the IR absorbance of the hydrolysate (H), which presented a broad band at 3270 cm^{-1} due to O—H and N—H stretching, followed by two bands at 2923 and 2853 cm^{-1} related to CH_2 stretching

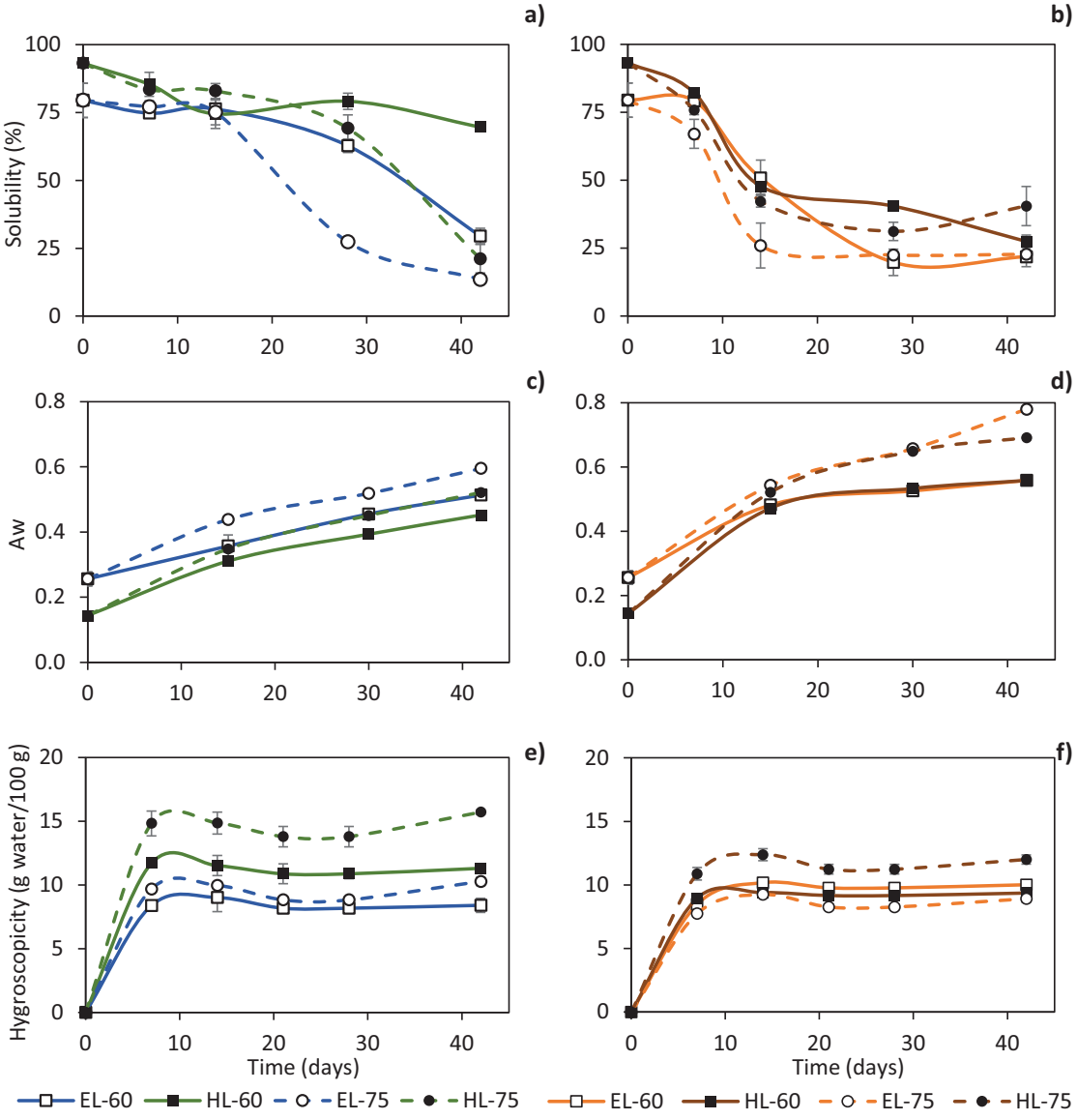

Fig. 2. Solubility, water activity (A_w) and hygroscopicity of spray-dried liposomes stored for 42 days at 4 °C (a,c,e) and at 23 °C (b,d,f).
EL-60: empty liposomes at 60% RH; HL-60: hydrolysate-loaded liposomes at 60% RH; EL-75: empty liposomes at 75% RH; HL-75: hydrolysate-loaded liposomes at 75% RH.
RH = relative humidity.

vibrations of aliphatic chains. The small band at 1744 cm^{-1}, associated to C═O stretching, could be related to residual fat, considering it is a typical signal of carbonyl groups of triglycerides and phospholipids. The shoulder at 1645 cm^{-1} corresponding to protein amide I was barely visible, probably due to the overlapping caused by the absorption of water molecules; in contrast, the amide II, which can be highly influenced by hydration, appeared as a prominent band at 1563 cm^{-1} (Gómez-Guillén et al., 2010). These events would agree with the highly hygroscopic nature of the hydrolysate. The small band at 1234 cm^{-1} was related to the protein amide III. Residual carbohydrates, among others, could explain the intensity of the band at 1044 cm^{-1} associated with C—O vibrations (Gómez-Guillén et al., 2010). In this connection, the viscera hydrolysate presented a high content of dietary fiber (≈15.3%) (Sepúlveda et al., 2021).

Fig. 3b shows the IR spectra of EL and HL, which presented great similarity. In this case, the bands at 1740 and 1645 cm^{-1} could be mostly assigned to the C═O stretching of the polar head ester groups of phospholipids (Hosseini, Ramezanzade, & Nikkhah, 2017). The slight up-shift reached for HL to 1742 and 1646 cm^{-1} was attributed to the interaction of the hydrolysate with the carbonyl ester groups at the interfacial part of the membrane (Marín et al., 2018). The prominent band at 1563 cm^{-1} in the hydrolysate was not visible in the HL sample, in agreement with the high liposomal entrapment efficiency (>80%).

The results of the different liposomes stored for 42 days at 4 °C (Fig. 3c) and 23 °C (Fig. 3d) showed a down-shift from 2925 cm^{-1} at day zero (Fig. 3b) to 2920 cm^{-1} after storage, indicating an increase in hydrophobic interactions between acyl chains in the deep interior of the bilayer (Marín et al., 2018). Noticeable modifications observed in the region between 1456 and 1337 cm^{-1} (CH_2 bending) and in the region of 1146 to 1029 cm^{-1} (symmetrical PO^{2-} stretch vibrations) in liposomes stored at 23 °C with respect to liposomes on day zero indicated changes in the polar regions of phospholipids and the formation of hydrogen bonds (Sarabandi et al., 2019). A similar effect was also observed in EL-75 stored at 4 °C. These results indicated intrinsic structural changes in the membrane strongly related to storage temperature, and also to the high relative humidity in the case of empty liposomes stored at 4 °C.

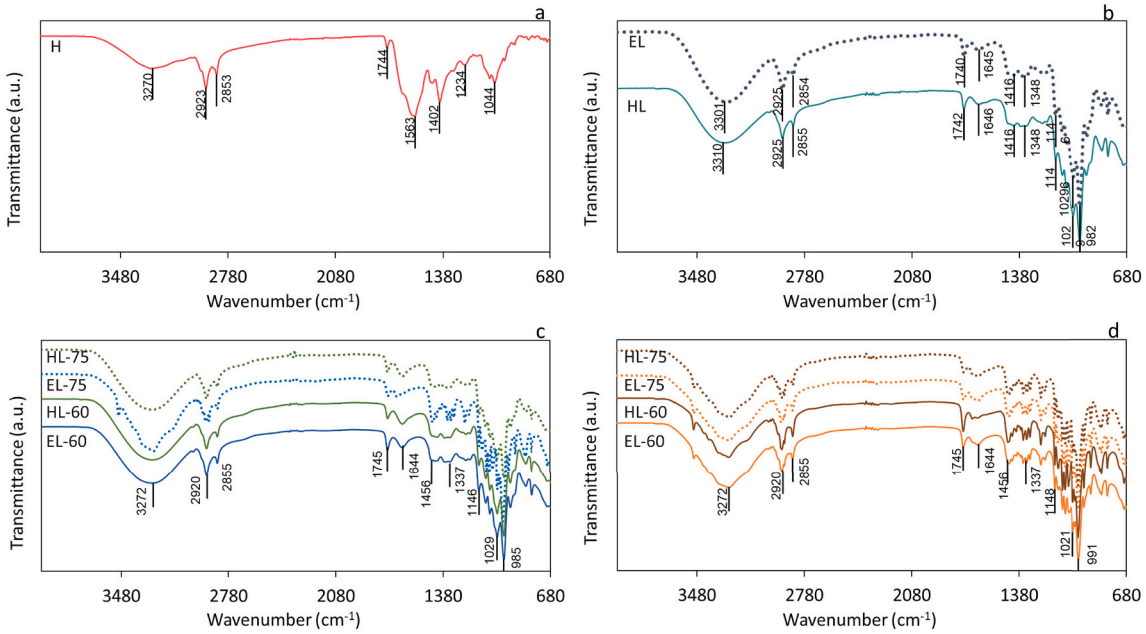

Fig. 3. Infrared spectra of: (a) fish hydrolysate; (b) spray-dried liposomes at the zero day; spray-dried liposomes stored for 42 days at 4 °C (c) and at 23 °C (d). EL-60: empty liposomes at 60% RH; HL-60: hydrolysate-loaded liposomes at 60% RH; EL-75: empty liposomes at 75% RH; HL-75: hydrolysate-loaded liposomes at 75% RH.

3.3.4. *Differential scanning calorimetry (DSC)*

The thermal profiles of EL and HL stored for 42 days are shown in Fig. 4. All thermograms were characterized by a first glass transition (T_g) around −23 °C which tended to be more pronounced at high storage temperatures and relative humidity, but was barely affected by the hydrolysate loading. Subzero gel to liquid-crystalline transitions are typical of unsaturated phospholipids from natural sources, due to an increase on free volume and molecular rotational degree of freedom by the presence of *cis* double bonds (Biltonen & Lichtenberg, 1993). Liposomes stored at 4 °C (Fig. 4a) showed a well-defined second endothermic transition at T_{peak} 29 °C, 40 °C, 13 °C, and 24 °C for EL-60, HL-60, EL-75, and HL-75, respectively. This second thermal event denoted the complex lipid composition of the soy-rapeseed lecithin blend stabilized with trehalose and revealed the increase in thermal stability of liposomes caused by a reduction of the relative humidity and the loading with the hydrolysate. An up-shift in T_{peak} has been previously reported in partially purified soy phosphatidylcholine liposomes loaded with collagen hydrolysate (Marín et al., 2018) or with sea fennel phenolic extracts (Alemán, Marín, Taladrid, Montero, & Gómez-Guillén, 2019). The second thermal transition in liposomes stored at 23 °C were observed at 7 °C and 9 °C for EL-60 and HL-60, respectively (Fig. 4b), denoting the pronounced loss in thermal stability by increasing storage temperature, and again evidencing a certain stabilizing effect caused by the hydrolysate. This thermal event was not found in the corresponding liposomes stored at the higher RH, indicating a considerable change in liposomal structure by the combined effect of high storage temperature and humidity. According to Sun, Leopold, Crowe, and Crowe (1996), both temperature and relative humidity have a strong influence on glass

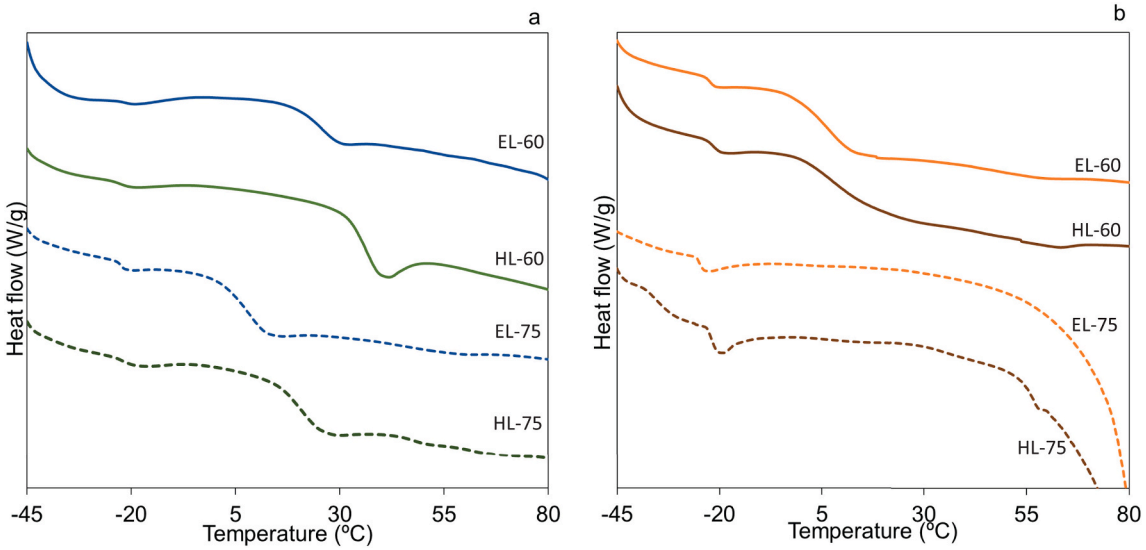

Fig. 4. DSC of spray-dried liposomes stored for 42 days at 4 °C (a), and 23 °C (b).
EL-60: empty liposomes at 60% RH; HL-60: hydrolysate-loaded liposomes at 60% RH; EL-75: empty liposomes at 75% RH; HL-75: hydrolysate-loaded liposomes at 75% RH.

transition temperatures (T_g), being a low storage temperature and low relative humidity required to increase the stability of liposomes. The increase in phase transition temperature in the hydrolysate-loaded liposomes could be the result of a more rigid and stable membrane caused by the interaction of peptides with phospholipid polar head groups, as well as by their possible orientation, parallel to the membrane surface (Orädd, Schmidtchen, & Malmsten, 2011; Yokota, Moraes, & Pinho, 2012).

3.3.5. Antioxidant activity

Fig. 5 shows the antioxidant activity of spray-dried liposomes, determined by ABTS and FRAP, after rehydration in water. The ABTS values increased markedly ($p < 0.05$) in hydrolysate-loaded liposomes, being attributed to the radical scavenging capacity of the latter (Fig. 5 a, b). Within the first week of storage, all samples showed a decrease ($p < 0.05$) in ABTS values, which continued to descend ($p < 0.05$) very slowly in HL, regardless the storage temperature or RH. Thus, changes in the physical properties of dry powders, as well as structural changes in the liposomal structure during storage, reduced the scavenging capacity of the encapsulated hydrolysate to a certain extent. In contrast, Fig. 5c and d showed a continuous and more pronounced decrease ($p < 0.05$) in antioxidant activity determined by the FRAP method. Initially, HL had slightly more activity ($p < 0.05$) than EL. Contrary to ABTS values, the antioxidant activity measured by FRAP showed that liposomes notably contributed to this activity and hence, the decreased values during storage could be associated to physicochemical changes. Even so, the decrease in FRAP values proceeded slower in HL, which showed minimal differences regarding storage temperature and RH. After the first week, the reducing capacity of EL at 23 °C was not significantly affected by the RH ($p > 0.05$), while at 4 °C, EL-60 evolved as in the HL samples up to day 28 ($p > 0.05$), decaying later. All these findings denoted that the spray-dried liposomes loaded with the hydrolysate maintained a relatively high radical scavenging capacity for 42 days, despite the physicochemical or structural changes associated to storage temperature and relative humidity, which contributed to a decrease the ferric ion reducing power. Conversely, Guldiken et al. (2019) reported that storing spray-dried liposomes loaded with black carrot extract at room temperature caused the degradation of bioactive compounds.

3.4. In vitro simulated gastrointestinal digestion

The radical scavenging (ABTS), iron reducing (FRAP) and ACE-inhibitory activities of HL were evaluated during simulated GID (Fig. 6). For comparison purposes, the results for the plain hydrolysate (H) were also determined. The digestion pattern was quite similar in both samples. After the first hour of digestion (G-1 h, gastric phase), the ABTS activity decreased significantly ($p < 0.05$), in both H and HL (Fig. 6a). This effect was attributed to structural changes in peptides caused by pepsin-induced degradation (You, Zhao, Regenstein, & Ren, 2010). Furthermore, the low pH may also induce liposome coalescence leading to peptide release (Beltrán, Sandoval-Cuellar, Bauer, & Quintanilla-Carvajal, 2019). During the subsequent intestinal digestion (GI-1 h and GI-2 h), the activity increased ($p < 0.05$) in both samples. The rise in the activity of the hydrolysate was attributed to the release of new oligopeptides after pancreatin digestion (Alemán et al., 2013). This effect was similar in HL, since the bile salts in the intestinal phase promoted vesicle swelling or alteration, causing the leakage of the bioactive peptides (Tan et al., 2014).

The FRAP values for H remained unchanged during the gastric phase, while for HL they decreased markedly ($p < 0.05$) (Fig. 6b). Liposome

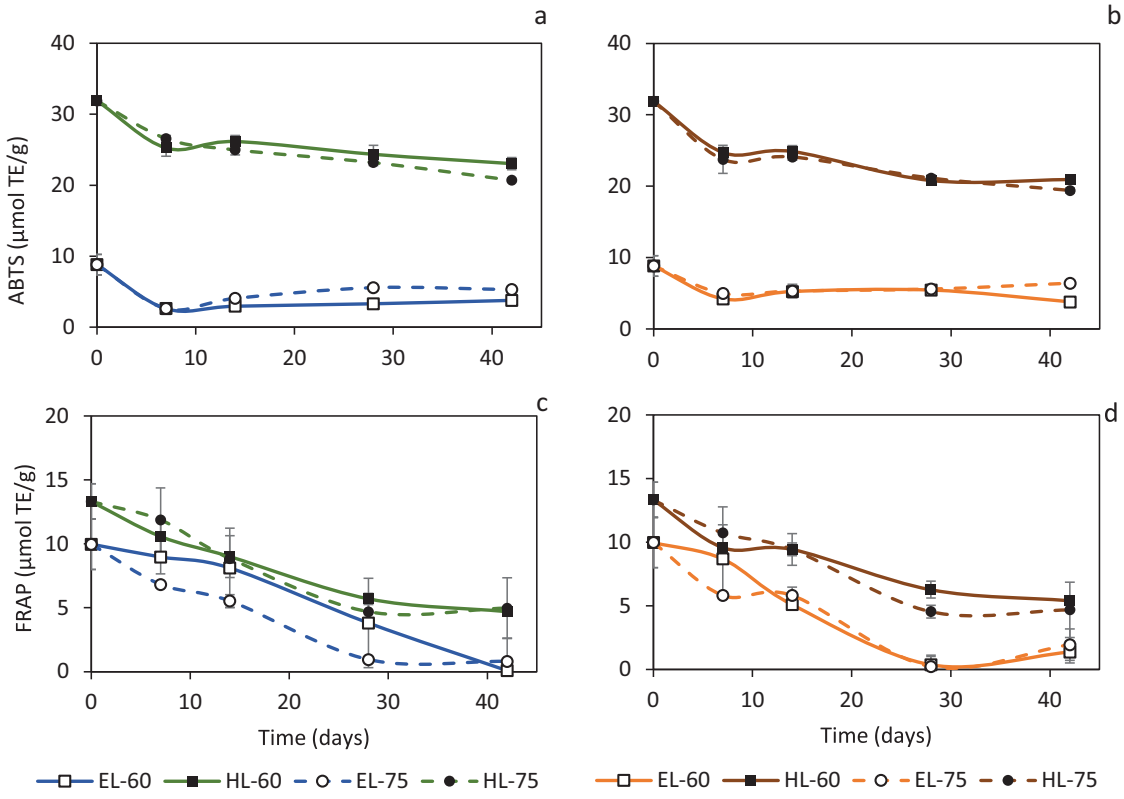

Fig. 5. Antioxidant activity determined by ABTS and FRAP of spray-dried liposomes stored for 42 days at 4 °C (a,c) and at 23 °C (b,d).
EL-60: empty liposomes at 60% RH; HL-60: hydrolysate-loaded liposomes at 60% RH; EL-75: empty liposomes at 75% RH; HL-75: hydrolysate-loaded liposomes at 75% RH.
RH = relative humidity.

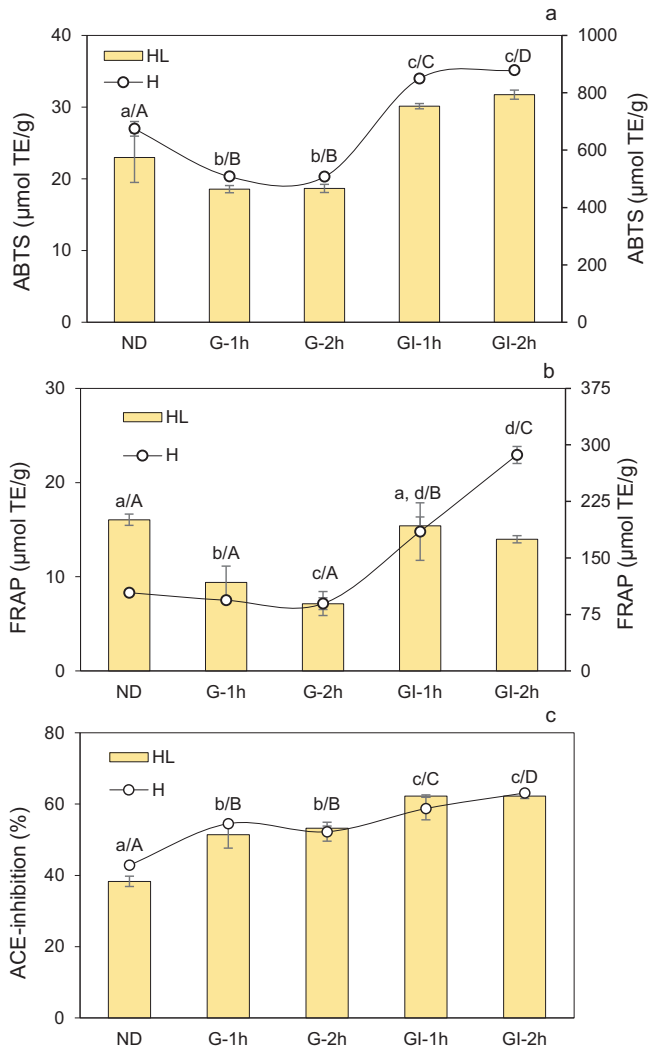

Fig. 6. Antioxidant activity by ABTS method (a) and FRAP method (b), and ACE inhibition (c) of hydrolysate-loaded liposomes (HL) and fish protein hydrolysate (H) during *in vitro* gastrointestinal digestion. Different lowercase or uppercase letters indicate, respectively, significant differences ($p > 0.05$) for HL or H samples.

changes during gastric digestion could account for the noticeable decrease in FRAP values, evidencing that a significant part of the activity was due to the composition and structure of the encapsulating material, which could be susceptible to degradation induced by the low pH. Soy and rapeseed lecithins are known to contain high levels of tocopherols and phenolic compounds with high antioxidant activity (Naczk, Amarowicz, Sullivan, & Shahidi, 1998; Taladrid et al., 2017). After the first hour of the intestinal phase (GI-1 h) the activity increased significantly ($p < 0.05$) in both H and HL with respect to gastric digestion, due to further peptide breakdown, as explained above. However, at the end of the entire GID only the H sample showed values significantly higher ($p < 0.05$) than the ones obtained before digestion.

The percent ACE inhibition showed a remarkable and similar increase after the first hour of gastric digestion in both H and HL ($p < 0.05$) (Fig. 6c). From 1 to 2 h, the activity remained stable, and increased slightly ($p < 0.05$) during the intestinal phase in both samples. This finding evidenced that the gastrointestinal digestion enhanced the inhibitory activity of the hydrolysate. In a previous work the increase in ACE-inhibitory activity of a squid skin collagen hydrolysate after *in vitro* gastrointestinal digestion was related to the release of more bioactive peptides (Alemán et al., 2013). Different results were found in two fish hydrolysates, where ACE-inhibitory activity during pepsin and pancreatin digestion remained stable for one hydrolysate, while it decreased dramatically for the other one (Elavarasan, Shamasundar, Badii, & Howell, 2016). A remarkable increase in activity during the first hour of gastric digestion was also found in soy phosphatidylcholine liposomes loaded with a collagen hydrolysate (Marín-Peñalver, Alemán, Gómez-Guillén, & Montero, 2019).

3.5. Transmission electron microscopy (TEM)

Fig. 7 shows the morphology of spray-dried liposomes loaded with the fish protein hydrolysate and stabilized with 15% trehalose after 11 months of storage at 4 °C and ≈60% RH. Previously, under these temperature and humidity conditions dried hydrolysate-loaded liposomes

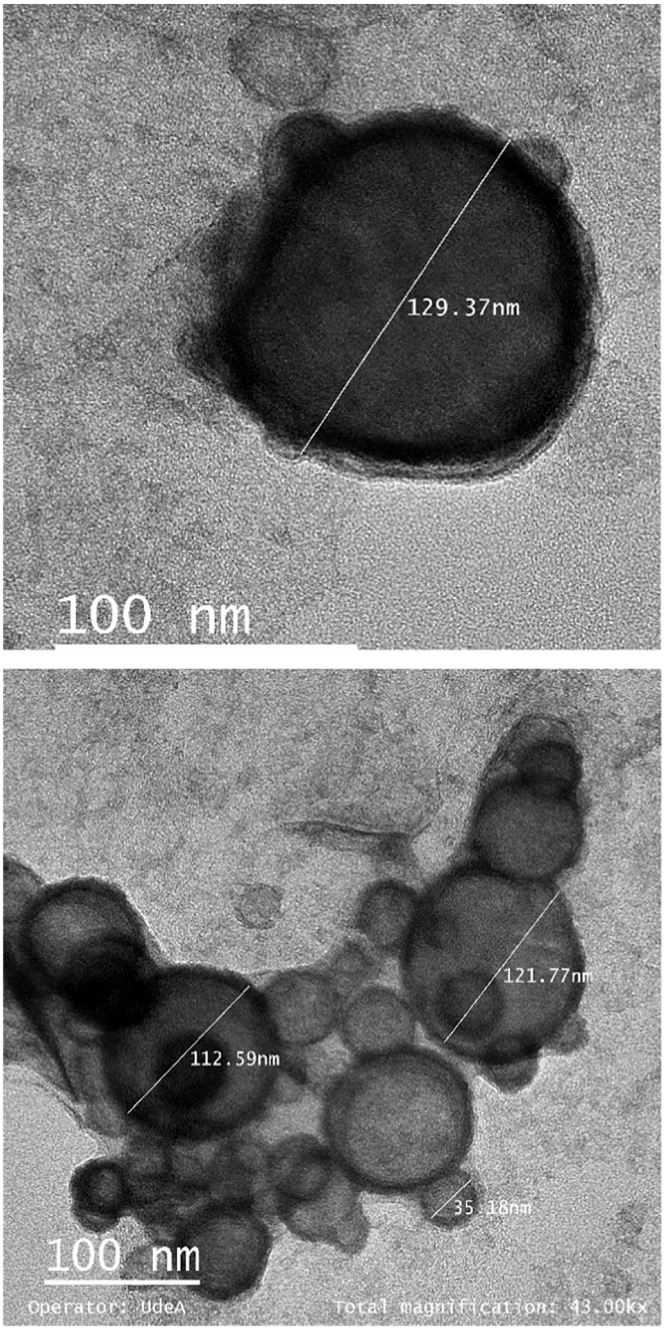

Fig. 7. Transmission electron micrograph of hydrolysate-loaded liposomes stabilized with 15% trehalose after 11 months of storage at 4 °C.

were found to change minimally in size and ζ potential during 42 days of storage. The micrographs confirmed the presence of individual spherical unilamellar vesicles and clusters, which could probably be indicative of vesicle agglomeration after the long-term storage. It should be noted that the particle size of liposomes visualized by TEM were noticeably smaller (≈ 100 nm) than the mean values obtained by dynamic light scattering (DLS) immediately after spray drying (260 nm) or 42 days after (266 nm). This discrepancy could be explained by the differences in the measurement principles between the two techniques. DLS measurements are pondered in terms of light intensity and considers the hydrodynamic diameter of the particle, while by TEM it is possible to measure directly the size.

4. Conclusions

Trehalose at 15% behaved as a good stabilizing agent for spray-dried liposomes, inducing favorable physical properties. The loading with the hydrolysate induced structural changes and higher thermal stability in the liposomal membrane, and also increased the antioxidant capacity of the liposomes by providing a noticeable radical scavenging capacity. Major physicochemical and structural changes in dried liposomes loaded with the protein hydrolysate were found during the 42-day storage at 23 °C instead of 4 °C and at ≈75% instead of ≈60% relative humidity. The radical scavenging capacity decreased very slightly during storage, and did not show substantial changes concerning the different storage conditions. On the contrary, a decrease of ferric iron reducing power was found, which was most likely associated to physicochemical changes in liposomes. The spray-dried liposomes loaded with the hydrolysate and stabilized with trehalose retained their membrane and spherical shape after 11 months of storage at 4 °C. This preparation could be considered as a functional food ingredient due to its substantial antioxidant activity and angiotensin-converting enzyme (ACE) inhibitory capacity after *in vitro* simulated gastrointestinal digestion.

Declaration of Competing Interest

None.

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