Freeze-dried phosphatidylcholine liposomes encapsulating various antioxidant extracts from natural waste as functional ingredients in surimi gels

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Abstract

Three antioxidant extracts (collagen hydrolysate, pomegranate peel extract, shrimp lipid extract) were encapsulated in soy phosphatidylcholine liposomes with the addition of glycerol. The particle size of the fresh liposomes ranged from 75.7 to 81.0 nm and zeta potential from –64.6 to –88.2 mV. Freeze-drying increased particle size (199–283 nm), and slightly decreased zeta potential. The lyophilized liposomes were incorporated in squid surimi gels at 10.5 % concentration. An alternative functional formulation was also prepared by adding 2 % of non-encapsulated bioactive extract. The gels were characterized in terms of colour, texture and oxidative stability (TBARS) after processing and also after frozen storage. The incorporation of the freeze-dried liposomes caused a slight decrease in gel strength and contributed to maintaining the stability of the gels during long-term frozen storage. The antioxidant properties of the bioactive extracts, liposomes and *in vitro* digested surimi gels were determined.

Keywords: soy phosphatidylcholine, liposomes, freeze-drying, antioxidants, surimi gels, storage.

1. Introduction

Growing consumer demand for a healthier lifestyle means that more attention is paid to a balanced diet which guarantees a good nutritional status and prevention of chronic diseases. Some years ago the food industry began incorporating healthy ingredients in prepared foods, so-called “functional foods”, with the aim of providing important nutrients and other bioactive substances in the diet. Food products with antioxidant properties are indicated for the prevention of numerous diseases related to oxidative stress, such as cardiovascular and neurodegenerative diseases, diabetes and cancer (Pham-Huy, He, & Pham-Huy, 2008). Raw materials from animal and plant waste represent an important source of antioxidant compounds that could be included in the composition of functional foods. There has been growing interest in the recovery of fish waste by enzymatic hydrolysis of protein residues. The tunics of giant squid are an excellent source of antioxidant peptides that may protect living cells against free radical-mediated oxidative damage (Mendis et al., 2005; Giménez et al., 2009). The high levels of hydroxyproline, hydrophobic residues and glycosylated peptides in squid tunic gelatin hydrolysates are related to their noticeable antioxidant capacity (Alemán, Giménez, Pérez-Santín, Gómez-Guillén, & Montero, 2011; Alemán, Gómez-Guillén, & Montero, 2013). Large amounts of cephalothorax and cuticle waste produced by the crustacean processing industry may also provide a source of bioactive compounds with health-promoting effects. Shrimp (*L. vannamei*) lipid extract, with high proportions of -3 polyunsaturated fatty acids (DHA and EPA), astaxanthin and α-tocopherol, also presents antioxidant properties (Gómez-Estaca, Montero, Fernández-Martín, Calvo, & Gómez-Guillén, 2016; Gómez-Estaca, Calvo, Álvarez-Acero, Montero, & Gómez-Guillén, 2017). Among plant waste, pomegranate peel and pulp extracts, rich in polyphenols, mainly ellagitannins, ellagic acid and anthocyanins, are well recognized for their health-promoting effects based on their high antioxidant and

antiproliferative activities (Masci et al., 2016). Surimi gels offer an acceptable food matrix for the incorporation of functional ingredients leading to products with high versatility of presentation. They provide a source of muscle proteins which, after gastrointestinal digestion, could be capable of releasing peptides with biological activities (Ueki, Wan, & Watabe, 2014). The activities, however, may be decreased by thermal degradation during the gelling process or by excessive interactions with the protein matrix (Montero, Giménez, Pérez-Mateos, & Gómez-Guillén, 2005), which could affect their bioaccessibility. A way of enhancing the efficacy and stability of bioactive substances in food systems could be encapsulation in nanoliposomes (Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008). Liposomes are colloidal vesicular structures composed of one or more lipid bilayers that have the ability to act as target delivery carriers for both lipophilic and hydrophilic compounds. The hydrodynamic properties, encapsulation efficiency and stability of the loaded liposomes might be affected, depending on the nature and concentration of the loading material (Tan et al., 2013). In food applications, the use of non-synthetic lecithins for liposomal encapsulation, such as soy lecithin, has a reasonably low cost, does not raise any food legislation concerns and provides nutritional value due to the high essential polyunsaturated fatty acid profile (Laye, McClements, & Weiss, 2008). Moreover, natural soybean phospholipids are able to interact with cellular membranes, changing their fatty acid compositions; therefore, they could act as active ingredients for the treatment of various diseases, such as inflammatory processes, cardiovascular risks, neurological disorders, cancer, etc. (Küllenberg, Taylor, Schneider, & Massing, 2012). The use of polyunsaturated phospholipids for liposomal encapsulation and their addition in the surimi gels contributes greatly to reinforcing the overall functional design of the product. Owing to the chemical instability of liposomes for long-term storage, lyophilization is the main approach used to extend their shelf life. The incorporation of dry liposomes brings about the technological possibility of adjusting the moisture content in the resulting gel products, which is of extreme importance to modulate their textural properties. However, freeze-drying may

damage lipid bilayers by ice crystal formation during freezing, vesicle fusion/aggregation following dehydration and changes in phase transition during rehydration (Chen et al., 2010). To prevent these drawbacks, the use of cryoprotectants, such as glycerol or oligosaccharides, has been proposed (Mozafari, 2005; Stark et al., 2010).

The aim of this work was to encapsulate three antioxidant extracts (collagen hydrolysate, pomegranate peel extract and shrimp lipid extract) in soy phosphatidylcholine liposomes, which were freeze-dried and incorporated in surimi gels. The particle and antioxidant properties of liposomes and the colour, texture and oxidative stability (TBARS) of the resulting gels (newly prepared and after 3 and 7 months of frozen storage) were analysed. The surimi gels incorporating the freeze-dried liposomes were subjected to *in vitro* simulated gastrointestinal digestion (sGID) in order to determine the antioxidant potential of the corresponding digests. An alternative functional formulation was also tested by including the non-encapsulated bioactive extracts in the gels.

2. Materials and methods

2.1. Materials

Frozen cleaned tunics and surimi blocks of giant squid (*Dosidicus gigas)* were provided by PSK Océanos S.A. (Madrid, Spain). Peel and albedo of pomegranate (*Punica granatum*) were collected from a local market and transported in ice to the laboratory. Shrimp waste was obtained from frozen shrimp (*L. vannamei*) kindly provided by Angulas Aguinaga Burgos (Burgos, Spain). Soybean lecithin was purchased from Manuel Riesgo S.A. (Madrid, Spain). Alcalase® 2.4L, pepsin and pancreatin enzymes were obtained from Sigma Aldrich, Inc. (St. Louis, MO, USA). Epigallocatechin, epicatechin-3-gallate, epigallocatechin-gallate, catechin, rutin, hyperoside and kaempferol-3-O-glucoside were purchased from Extrasynthese (Genay,

Cedex, France). Quercetin, gallic acid, ellagic acid, caffeic acid, punicalagin, chlorogenic acid, coumaric acid, ferulic acid, syringic acid, vitamin C, vitamin B12, aprotinin, hippuryl histidyl leucine (HHL), dimethyl sulfoxide and glycine were from Sigma Aldrich. All other reagents were of analytical grade.

2.2. Extraction and characterization of active compounds

2.2.1. Squid collagen hydrolysate (HC)

Giant squid tunics were thawed, profusely washed in water and slowly stirred with 0.5 M acetic acid (1:20 w/v) at 3 °C for 96 hours. The mixture was filtered with Whatman No. 4 filter paper and the filtrate, which constitutes the acid-soluble collagen, was then freeze-dried. Dried collagen was suspended with distilled water (30 % w/v) and subjected to enzymatic hydrolysis in a pH-stat (TIM 856, Radiometer analytical, Villeurbanne, France) using Alcalase® 2.4L, as described previously (Alemán et al., 2011), with an enzyme substrate ratio of 1:60 (w/w). The collagen hydrolysate (HC) was freeze-dried and stored at −20 °C until use. The molecular weight distribution and the amino acid composition of HC were determined by using, respectively, size-exclusion chromatography and ion exchange chromatography, as described by Aleman et al. (2011).

2.2.2. Pomegranate extract (PG)

Peel and albedo of pomegranate (*Punica granatum*) were dried in an oven (FD 240, Binder, Tuttlingen, Germany) at 50 °C and ground in an Osterizer (Sunbeam Par, 4153-50, Mexico). The resulting dry powder was suspended in ethanol/water (70/30) at a ratio of 1:20 (w/v), gently stirred at 40 °C for 4 h and left to stand at 21 °C for 24 h. The mixture was centrifuged at 12000 g for 15 min at 4 °C and the supernatant was filtered through Whatman No. 1 paper, rota-evaporated at 40 °C and stored at –20 °C until use. Total phenolic compound content was

123 determined by the Folin–Ciocalteu method, using gallic acid as standard. To identify the main
124 phenolic compounds, PG was dissolved in dimethyl sulfoxide and analysed by reverse phase
125 high performance liquid chromatography (RP-HPLC), following the procedure described in a
126 previous work (Giménez, Moreno, López-Caballero, Montero, & Gómez-Guillén, 2013).
127

128 2.2.3. Shrimp lipid extract (SL)

129 The preparation and chemical characterization of the ethyl acetate-soluble lipid extract from
130 shrimp waste (SL) used in the present study was described in a recent work (Gómez-Estaca et
131 al., 2017). The SL was dried by rotary evaporation at 60 °C and nitrogen aspiration, and stored
132 at –20 °C until use. Fatty acid profile was analysed by gas chromatography with FID detector,
133 carotenoids by RP-HPLC with diode array detector, and α-tocopherol and cholesterol by HPLC
134 coupled to a quadrupole mass spectrometer.
135

136 2.3. Encapsulation in liposomes

137 2.3.1. Purification of phosphatidylcholine

138 Partially purified phosphatidylcholine (PC) from commercial soybean lecithin (Manuel Riesgo,
139 S.A., Madrid, Spain) was obtained by dissolving lecithin in ethyl acetate (1:5, w/v) and
140 subsequently performing five washes with acetone (1:2, w/v). The preparation and chemical
141 characterization of the PC powder used in the present study were described in a previous work
142 (Taladrid et al., 2017). The PC powder was stored at –20 °C until use.

143 2.3.2. Preparation of liposomes

144 The bioactive extracts (HC, PG and SL) (2.5 g) were first suspended in 250 mL of 0.1 M
145 phosphate buffer (pH 7). PC (62.5 g) was mixed with each bioactive solution and it was kept in

146 a water bath at 80 °C for 1 h with gentle stirring. Then 212.5 mL of the phosphate buffer and
147 37.5 mL of glycerol were added, and the mixture was kept in a water bath at 80 °C for 1 h with
148 gentle stirring. The volume of the suspension was completed with 750 mL of the phosphate
149 buffer, and then vortexed at 60 °C to produce multilamellar vesicles. The preparations were
150 subsequently sonicated in an ultrasonic cell disrupter (Model Q700, Qsonica sonicators,
151 Newton, CT, USA) at 90 % amplitude (power rating of 700 W) for 5 min, with a 60 s stop every
152 min to allow sample cooling. The composition of the liposomes, expressed in dry weight (dw),
153 was 2 % bioactive extract, 56 % PC and 42 % glycerol). Part of the newly prepared liposomal
154 dispersions (L-HC, L-PG and L-SL) was used for particle size, zeta potential and entrapment
155 efficiency determinations. The rest was freeze-dried and stored at –20 °C. Rehydration of
156 freeze-dried liposomes was performed by suspending 14 g of the liposomal pastes in 100 mL of
157 distilled water at 20 °C for 30 min under magnetic stirring.

158 2.4. Characterization of liposomes

159 2.4.1. Particle size and zeta potential

160 Particle size measurement (expressed as z-average in intensity), polydispersity (PDI) and zeta
161 potential of liposomes (fresh and rehydrated) were performed using a Zetasizer Nano ZS
162 (Malvern Instruments Ltd, Worcestershire, UK), as described previously (Mosquera et al.,
163 2016).

164 2.4.2. Water content

165 The moisture of freeze-dried liposomes was determined according to method 950.46
166 (A.O.A.C., 2005).

167 2.4.3. Water solubility

168 Freeze-dried liposomes were mixed with distilled water (1 % w/v) under agitation (100 rpm)
169 for 150 min at 20 °C and centrifuged at 3500 rpm (Multifuge 3 L-R, Heraeus, Madrid, Spain) for

5 min. The supernatant was dried at 105 °C for 24 h and the water solubility, expressed as a percentage, was calculated by weight differences.

2.4.4. Entrapment efficiency

The entrapment efficiency (EE) of the various encapsulated compounds was determined by measuring: i) protein content with a LECO FP-2000 nitrogen/protein analyser (LECO Corp., St. Joseph, MI, USA) for L-HC; ii) phenolic content by the Folin–Ciocalteu method (Slinkard and Singleton, 1977) for L-PG, and iii) astaxanthin concentration as reported in Gómez-Estaca et al. (2016) for L-SL. Encapsulated and non-encapsulated bioactive compounds were separated by mixing the liposomes with acetone (120 mg/mL) for L-HC, ethanol:methanol (1:1 v/v; 120 mg/mL) for L-PG, and hexane (80 mg/mL) for L-SL. Then all the mixtures were centrifuged at 5000 g for 30 min at 4 °C and filtered through a 0.45 µm filter device. EE was calculated using the following equation:

% EE = encapsulated bioactive / total bioactive * 100

2.5. Preparation of squid surimi gels

Prior to use (24 h), the surimi was thawed in a 4 °C refrigerator for easy cutting into smaller pieces. Then the surimi was homogenized at 2 °C in a vacuum homogenizer (FD112M10-72D, Stephan Machinery GmbH, Hameln, Germany) with 1 % NaCl and the amount of water (as ice) needed to obtain a moisture of 80.5 % in the final gels. Freeze-dried liposomal pastes were then incorporated to obtain a final concentration of 10.5 % w/w in the resulting gel (28 % on a dry basis, db), and the mixture was again homogenized for 5 min. Final concentrations of antioxidant extract, PC and glycerol in the surimi gels containing the freeze-dried liposomes were then 0.15 % (0.6 %, db), 3.8 % (15.6 %, db) and 2.9 % (11.8 %, db), respectively. To allow proper comparisons, the concentrations of each liposome constituent (antioxidant compound, PC and glycerol) were also provided on a dry basis in order to ignore the presence of water in both the surimi and the dried liposomal pastes. An alternative functional formulation without

195 PC and glycerol was prepared by the sole addition of the corresponding extracts to the surimi
196 at a concentration of 2 % (9 %, db). The resulting pastes were stuffed into 35 mm plastic
197 cellulose casings (Viscase SA, Bagnold Cedex, France) and heated in an Eller oven (Unimatic
198 1000, Micro 40, Eller, Merano) at 90 °C for 40 min. Then the gels were dipped into ice water to
199 cool them quickly and stored overnight at 4 °C. For long-term storage, gels were frozen and
200 stored at –20 °C for seven months.

201 2.6. Characterization of gels

202 2.6.1. Colour

203 A Konica Minolta CM-3500d colorimeter (Konica Minolta, Madrid, Spain) was used to
204 determine L*a*b* parameters using D65 illuminant (daylight) and D10 standard observer.
205 Results are the average of at least 10 replicates and are expressed as Hue angle and Chroma
206 values.

207 2.6.2. Mechanical properties

208 A puncture test was performed to determine the breaking force (in N) and breaking
209 deformation (in mm) of gels using a TA-XT plus Texture Analyser (Texture Technologies Corp.,
210 Scarsdale, NY, USA) with a cylindrical stainless steel plunger (5 mm diameter) attached to a 5
211 kg load cell, at 1.0 mm/s crosshead speed. Results were the mean values of at least five
212 measurements. Gel strength (N·mm) was the product of multiplying the breaking force by the
213 breaking deformation.

214 2.6.3. Soluble protein content

215 Surimi gels (1 g) were mixed with 25 mL of sodium chloride (5 % w/v) using an Omni-Mixer
216 model 17106 homogenizer (Omni Intl., Waterbury, Conn., USA). The resulting homogenates
217 were stirred for 30 min at 4 °C and then centrifuged at 6000 g for 30 min in a Sorvall RT60008
218 centrifuge. The protein content in the supernatant (soluble protein) and in the whole surimi

gel (total protein) was determined with a LECO-FP 2000 nitrogen/protein analyser (LECO Corp., St. Joseph, MI, USA) using a nitrogen-to-protein conversion factor of 6.25. Soluble protein content was expressed as the percentage of protein solubilized with respect to total protein in the surimi gel.

2.6.4. Thiobarbituric acid reactive substances (TBARS)

Surimi gels were solubilized with 7.5 % trichloroacetic acid (1:3 w/v) and centrifuged at 5100 g for 5 min at 4 °C. The supernatant was mixed with 0.02 M thiobarbituric acid (1:2 v/v) and kept at 20 °C for 15 hours in darkness. Finally, absorbance was measured at 532 nm. Results were expressed as µg of malondialdehyde (MDA) equivalents per kg of gel, based on a 1,1,3,3-tetraethoxypropane (TEP) standard curve.

2.7. *In vitro* simulated gastrointestinal digestion (sGID)

In vitro simulated gastrointestinal digestion of surimi gels was carried out in a water bath at 37 °C, according to Aleman et al. (2013), using pepsin solution at pH 2 for 2 h, followed by incubation with pancreatin and biliary salts at pH 6.5 for 2 h. Enzyme activity was stopped by cooling in ice. Final pH was adjusted to pH 7.2 and samples were centrifuged at 13000 g and 4 °C for 30 min. The resulting supernatants were freeze-dried and stored at −20 °C for further assays.

2.8. Determination of antioxidant activity

The ABTS radical scavenging capacity (ABTS assay, for hydrophilic compounds) and Superoxide ( ) free radical scavenging capacity (photochemiluminiscence, PCL assay, for lipophilic compounds) were used to measure the antioxidant activity as described previously (Alemán et al., 2011; Pérez-Santín et al., 2013). The results of the ABTS assay were expressed as mg Vitamin C equivalent antioxidant capacity (VCEAC) per g of sample, based on a standard curve

243 of vitamin C. The results for the PCL assay were expressed as mg Trolox equivalent antioxidant
244 capacity (TEAC) per g of sample, based on a standard curve of Trolox.
245
246 2.9. Statistical analysis
247 All determinations were carried out at least in triplicate. Statistical tests were performed using
248 the SPSS® computer program (IBM SPSS Statistics 22 Software, Inc., Chicago, IL, USA). One-way
249 analysis of variance was carried out. Differences between pairs of means were assessed on the
250 basis of confidence intervals using the Tukey test, with a significance level set at $p \leq 0.05$.
251
252 **3. Results and discussion**
253 3.1. Chemical properties of antioxidant extracts
254 The peptide molecular weight distribution (expressed in Dalton, Da) and amino acid
255 composition (expressed as number of residues per 1000 residues, ‰) of the acid-soluble
256 collagen hydrolysate (HC) are shown in Figure 1. According to the SEC molecular profile, HC
257 was mostly composed of peptide fractions with molecular weights ranging from 6.5 to 1.3 kDa
258 (≈11 %), 1.3 to 0.5 kDa (≈47 %) and lower than 0.5 kDa (≈42 %). Thus the predominant
259 molecular weight of the HC peptides is lower than 1.3 kDa (Figure 1a). The major constituent
260 amino acid residues were glycine (253 ‰), glutamic acid (117 ‰), alanine (98 ‰) and aspartic
261 acid (92 ‰) (Figure 1b). A noticeable proportion of hydroxylysine (20 ‰) was also detected.
262 Total imino acid content (proline + hydroxyproline) was 106 ‰, considerably lower than that
263 reported in a <3 kDa peptide fraction from fish sea bream scale collagen (Mosquera et al.,
264 2014). The total proportion of hydrophobic amino acids in HC was low (≈28 %), as compared to
265 the ≈62 % reported in the previous work (Mosquera et al., 2014).

The pomegranate peel and albedo extract (PG) presented high total phenolic content (166 mg gallic acid eq./g dry extract). The main compounds identified in the pomegranate extract (PG) were, in descending order, β-punicalagin, ellagic acid, α-punicalagin, rutin and epigallocatechin (Figure 2). The predominant presence of ellagitannins (punicalagin anomers and ellagic acid) in pomegranate peel extract has been documented previously (Masci et al., 2016).

The shrimp lipid extract (SL) was composed of 804 mg/g fatty acids (saturated = 31 %, monounsaturated = 25 % and polyunsaturated = 44 %). Other compounds present in the lipid extract were α-tocopherol (126 mg/g), cholesterol (65 mg/g) and astaxanthin (7 mg/g, in the form of free astaxanthin, and also as mono- and diesters, which were tentatively identified by HPLC-MS). The DHA (C22:6) astaxanthin monoester was found to be the most abundant (Gómez-Estaca et al., 2017).

3.2. Vesicle characterization

3.2.1. Fresh liposomal dispersions

The particle size of freshly prepared liposomes loaded with squid collagen hydrolysate (L-HC), pomegranate peel polyphenolic extract (L-PG) and shrimp lipid extract (L-SL) yielded unimodal distributions with particle sizes in all cases close to the range of about 100 nm. The results of z-average (mean particle size), polydispersity index (particle size distribution) and zeta potential (membrane surface charge) of fresh and rehydrated freeze-dried liposomes are summarized in Table 1. Fresh liposomes containing the polyphenolic extract (L-PG) and the shrimp lipid extract (L-SL) showed similar z-average values, 81.0 and 80.3 nm, respectively, while the collagen-hydrolysate-loaded liposomes (L-HC) were slightly smaller ($p \leq 0.05$), 75.7 nm. Similar vesicle size was reported previously for PC-liposomes loaded with a <1 kDa squid tunic peptide fraction obtained by the film hydration method (Mosquera, Giménez, Montero, & Gómez-Guillén, 2016). Also α-tocopherol-loaded liposomes in the nanometre range were reported by Charcosset, Juban, Valour Urbaniak, and Fessi (2015) using the ethanol injection method, and

291 by Memoli, Palermiti, Travagli, and Alhaique (1995) for liposomes of soya phospholipids
292 prepared by sonication. In the present study, working with the same encapsulating material
293 and liposome production method, only slight differences in mean particle size were detected,
294 despite the differences in the hydrophobic nature of the loaded material. No significant
295 differences ($p>0.05$) were observed regarding the polydispersity index (0.218–0.238) in the
296 three liposomal dispersions, and it was well within the range between 0.2 and 0.3 expected for
297 systems prepared from biological materials (Malheiros, Sant'Anna, Micheletto, da Silveira, &
298 Brandelli, 2011). The zeta potential in all the fresh liposomal preparations showed strong
299 electronegative values, especially in the case of L-SL (–88.2 mV). The zeta potential is normally
300 used as an indicator of particle stability, and when values are above 30 mV or below –30 mV
301 the dispersions are considered physically stable (Müller, Jacobs, & Kayser, 2001).

302 The entrapment efficiency (EE) varied depending on the encapsulated material. The high EE
303 shown by the collagen hydrolysate (≈95 %) could be explained by the low average molecular
304 weight of HC (predominantly <1.3 kDa) and the relatively low amount of proline,
305 hydroxyproline and total hydrophobic residues. The more abundant hydrophilic peptides
306 would therefore be located preferentially in the inner aqueous phase of the liposome and also
307 attached to the surface membrane polar heads. In the case of L-SL the EE was higher than 90
308 %, in agreement with Tan et al. (2013) for phosphatidylcholine-liposomes incorporating lutein
309 (82.64–91.98 %). In this case, since the material is predominantly of hydrophobic nature
310 (water-insoluble), it is expected to be located mostly inside the bilayer. L-PG liposomes
311 showed noticeably lower EE (63 %) than L-HC and L-SL, indicating that a considerable amount
312 of PG would remain non-encapsulated. Other authors showed 89 % EE for a pomegranate peel
313 extracted procyanidine incorporated in liposomes of soybean lecithin and cholesterol (Hu, Yao,
314 & Liu, 2010). In the present work, the main polar phenolic compounds present in PG, such as
315 punicalagin anomers and rutin, would be preferentially located in the liposome aqueous core.
316 However, ellagic acid, which is poorly soluble in polar solvents, would intercalate into the

317 aliphatic chain zone of the membrane lipids, presumably with relatively low efficiency.
318 Phenolic compounds are known to interact with the polar head groups and also intercalate
319 into the bilayer lipid membrane (Pawlikowska-Pawlęga et al., 2014). The EE depends greatly on
320 the composition of the encapsulating material, the preparation method, the presence of
321 cryoprotectants, and the chemical properties and concentration of the loaded material (Tan et
322 al., 2014; Xia, Cheng, Cheng, & Xu, 2016). In the present study, working under identical
323 conditions, the PG extract presented considerably lower entrapment capacity than HC and SL,
324 which was mainly attributed to the particular chemical composition of the polyphenolic
325 extract.

326 3.2.2. Freeze-dried liposomes

327 Residual moisture in all dried samples was almost the same ($p>0.05$), ranging between 34 and
328 36 %. This water content is much higher than values reported previously, such as 0.4–0.7 %
329 (Van Winden, Zhang, & Crommelin, 1997). In order to protect the liposomal stability during
330 freeze-drying, glycerol (42 %, dw) was included in the formulation to maintain vesicle integrity
331 and prevent sedimentation and leakage of the entrapped bioactive materials (Mozafari, 2005).
332 Glycerol has been reported to play a plasticizer role by inducing increased hydration of the
333 lipid bilayers (Manca et al., 2013). The relatively high water content in the present lyophilized
334 liposomes could therefore be attributed to the cryoprotectant added. The addition of glycerol
335 inhibited the formation of a dry lyophilized powder, providing samples with a gluey and
336 appearance, as observed before by Stark, Pabst, and Prassl (2010). Despite this, the lyophilized
337 liposomal pastes could be easily rehydrated in water. The water solubility at 20 °C showed no
338 significant differences ($p>0.05$) between the three liposomal pastes studied, with values
339 ranging from 82.15 % in L-HC to 84.88 % in L-PG. Similar high water solubility was obtained for
340 the L-SL preparation (83.11 %), despite the strongly lipophilic nature of the shrimp lipid
341 extract. The poor bioavailability of astaxanthin has been shown to be improved by liposomal

342 encapsulation as a result of enhanced solubility in an aqueous system (Peng, Chang, Peng, &
343 Chyau, 2010).

344 Freeze-drying caused significant variation ($p \leq 0.05$) of liposomes upon rehydration in practically
345 all parameters (Table 1). This behaviour was typical for particle destabilization due to
346 aggregation. Thus, a significant increase in z-average was observed in the three liposomal
347 preparations, with L-HC yielding the smallest average vesicle size (198.9 nm). The highest
348 variation in particle size was produced in the case of L-SL (3.5-fold increase). The polydispersity
349 index also increased to values ranging from 0.46 to 0.52. Pronounced differences were
350 observed in particle size distribution among the three rehydrated liposomal dispersions, where
351 the initial unimodal distribution changed into bi- or trimodal distributions (Figure 3).
352 Interestingly, after rehydration L-HC kept 62 % (expressed in intensity) of particles measuring
353 162 ± 77 nm, in contrast to L-PG and L-SL, which registered, respectively, 30.5 % of 121 ± 37
354 nm and 26 % of 111 ± 36 nm vesicles. Freeze-drying-induced an increase in mean particle
355 diameter with respect to fresh liposomes, as has been previously reported, probably due to
356 the breaking of hydrogen bonding between the water molecules and phospholipid head
357 groups (Stark et al., 2010). During the process of freeze-drying, the liposomes are firstly
358 concentrated by the propagation of the ice front. Subsequently, during water removal, the
359 chance of membrane apposition increases, producing liposome fusion or aggregation (Chen et
360 al., 2010). The present results indicate that, despite the incorporation of glycerol, a certain
361 amount of large vesicles (0.5–1 μm) was produced, resulting from freeze-drying-induced fusion
362 or aggregation. In the present study, which tried to simulate a feasible industrial method for
363 preparing the freeze-dried liposomes, large vesicles were formed, but a significant percentage
364 still remained in the original nanoscale range (around 100 nm). In addition, although
365 rehydrated particles became less electronegative in all formulations (Table 1), zeta potential
366 values around –60 mV denoted very high vesicle stability. It should be noted that changes in
367 liposome properties may occur to some extent during the rehydration process. In the present

368 work, the freeze-dried pastes were directly homogenized with the surimi, which, despite
369 having 80.5 % moisture, presumably did not allow complete rehydration of the liposomes.

370 3.3. Surimi gel characterization

371 The freeze-dried liposomal pastes were homogenized with the salt-ground muscle at 10.5 %
372 and heated at 90 °C to obtain the corresponding thermally-induced surimi gels. Another type
373 of functional seafood product was also tested by direct addition of the bioactive extracts (non-
374 encapsulated) to the gel (Free) at a concentration of 2 %. All the liposome-containing gels, as
375 well as the control gel (without liposomes or bioactive extracts), were easy to remove from the
376 casings and presented a smooth, cohesive and somewhat glossy appearance. The gels with
377 liposomes showed protein solubility values of 7.81 ± 2.28 % in the control gel, 8.36 ± 1.50 % in
378 L-HC, 8.42 ± 0.40 % in L-SL, and slightly higher, 14.42 ± 2.91 % in L-PG. All these properties
379 were indicative of proper protein gelation. In contrast, gels made with non-encapsulated
380 bioactive extracts were weak and crumbly, and presented strong water exudate, which was
381 more intense with the pomegranate extract. Although the addition of the non-encapsulated
382 extracts was at a lower concentration than the dried liposomes (2 % *vs* 10.5 %), they caused
383 stronger interference in the formation of the gel network, probably by disturbing the
384 myofibrillar protein hydration state. The strong reactivity of the phenolic compounds in the
385 free mode of addition (Free-PG), or the amount which was not efficiently encapsulated in the
386 liposomal mode (L-PG), caused higher water loss (in Free-PG) or lower protein thermal
387 aggregation (with L-PG). Some properties of both types of functional formulations will be
388 compared, despite their noticeable differences in chemical composition, especially regarding
389 the concentration of bioactive extract. In this respect, it should be mentioned that the
390 effective concentration of the extract when added in the liposomal form to the gel was 0.15 %,
391 which means a 13-fold dilution effect with respect to the free form (2 %). From a technological
392 point of view, direct addition of the bioactive extracts caused a failure in texture and water

393 binding. In order to ameliorate this detrimental effect, gelling and water binding ingredients
394 would be necessary; however, their addition would result in dilution of the bioactive extract. In
395 contrast, in the liposome-containing gels, no other ingredients were needed for proper
396 gelation. In addition, the presence of soy phosphatidylcholine (comprising around 4 % of the
397 final gel composition) provides a natural source of polyunsaturated fatty acids, contributing to
398 the beneficial healthy properties of the functional product.

399 3.3.1. Colour

400 The colour polar plot of surimi gels containing the various freeze-dried liposomes is shown in
401 Figure 4. The colour parameters of gels containing the free active compounds (Free-HC, Free-
402 PG and Free-SL) are also represented. The addition of Free-HC and Free-PG caused a slight
403 change in hue value as compared to the control gel. In contrast, Free-SL moved the resulting
404 gel into the orange-reddish region, in accordance to the striking red colour of the astaxanthin-
405 rich shrimp lipid extract. The colours of surimi gels with added L-HC or L-PG liposomes were
406 very close to each other, however Free-PG led to significantly higher ($p<0.05$) chroma than L-
407 PG. Both hue and chroma values in the L-SL-containing gel were considerably attenuated when
408 compared with the corresponding gel with Free-SL, in part due to the considerably lower
409 concentration of SL in the former. No significant differences ($p>0.05$) in colour parameters
410 were observed in liposome-added surimi gels as a function of time over 7 months of frozen
411 storage (data not shown).

412 3.3.2. Gel strength

413 The puncture test was carried out only on the liposome-containing surimi gels, as well as on
414 the control gel without liposomes, since the formulations with free bioactive extracts did not
415 gel adequately (Table 2). All unfrozen liposome-incorporated gels showed a significant
416 decrease ($p\leq0.05$) in breaking force, breaking deformation and gel strength, as compared to
417 the gel with no liposome addition. This effect was attributed to some interference in protein-

418 protein interactions. Despite the different nature of the encapsulated extracts, no significant
419 differences ($p>0.05$) in gel strength were observed between L-HC and L-SL gels. In contrast, L-
420 PG gels showed lower ($p\leq0.05$) gel strength and breaking deformation values, coinciding with
421 the slightly higher percentage of soluble protein found in this gel. This finding indicates that
422 the addition of L-PG caused slightly more disturbance in the gel protein network, probably
423 owing to the contribution of the non-encapsulated phenolic compounds. With regard to the
424 stability of the gels during frozen storage, the control gel showed a strong tendency to harden
425 ($p\leq0.05$) at the end of the long-term frozen storage (7 months). In contrast, the gels with
426 liposomes proved to be more stable from month 3 to 7, by preventing further cold-induced
427 protein aggregation (Table 2). Frozen gels with L-PG exhibited a pronounced increase in both
428 breaking force and deformation during the first three months of storage, as compared to the
429 gels with L-HC and L-SL. This effect could be the result of the initial weaker gel structure in L-
430 PG gels. All gels with added liposomes remained noticeably softer than the control at the end
431 of the storage period, which indicates good tolerance to frozen storage.

432 3.3.3. Thiobarbituric acid reactive substances (TBARS)

433 The oxidative stability of surimi gels with liposomes was determined following the TBARS
434 method, which quantifies the total amount of malondialdehyde (MDA) equivalent
435 accumulation (Table 2). In general, very low values were recorded in all samples, largely due to
436 the lean composition of the squid surimi. The addition of liposomes significantly ($p\leq0.05$)
437 increased the TBARS content in the surimi gels, more markedly in the case of gels with added
438 L-HC, followed by, in descending order, L-SL and L-PG. The increase in TBARS was inversely
439 related to the antioxidative capacity. The fatty acid composition of phosphatidylcholine from
440 soybean lecithin is characterized by a high degree of unsaturation, which makes the resulting
441 liposomes susceptible to being oxidized (Wang & Wang, 2008). Lipid oxidation might have
442 occurred during the liposome production by the sonication step, and also during the thermal

443 gelation of the myofibrillar proteins. The three types of active compounds used in the present
444 study are well known to have antioxidant properties, as will be discussed later. In spite of this,
445 the TBARS of gels with the loaded liposomes was slightly higher than in the control gel
446 (without liposomes), and tended to increase after long-term frozen storage. This finding
447 suggests that the encapsulated extracts were not fully available to protect the gel matrix from
448 lipid oxidation. The lower TBARS accumulation in gels with added L-PG could be ascribed to the
449 lower encapsulation efficiency of PG in the liposomal preparation, and also to its higher
450 antioxidant capacity.

451 3.4. Antioxidant activity

452 3.4.1. Free and encapsulated active compounds

453 The antioxidant activity of the collagen hydrolysate (HC), the pomegranate phenolic extract
454 (PG) and the shrimp lipid extract (SL) is shown in Figure 5. The radical scavenging activities of
455 PG were much greater than those of HC and SL. The hydrophobic nature of the shrimp lipid
456 extract prevented the analysis of the ABTS assay in aqueous solution. However, the
457 encapsulation of SL in liposomes made it water-soluble, leading to measurable ABTS values
458 (Fig. 5a). Astaxanthin and α-tocopherol, both contained in a significant amount in SL, are well-
459 known antioxidants (Gómez-Estaca et al., 2016; Gómez-Estaca et al., 2017). According to Hama
460 et al. (2012), the terminal rings of astaxanthin, located preferentially on the liposomal
461 membrane surface, as well as the conjugated polyene moieties at the interface of the lipid
462 membranes, would be available to scavenge hydroxyl radicals. Given that the ABTS activity of
463 an empty liposome was 2.07 ± 0.03 mg VCEAC/g sample, the activity of the liposome loaded
464 with SL could be mostly attributable to the extract. Slight reduction in ABTS values in PG-
465 loaded liposomes was found with respect to the corresponding phenolic extract (PG in
466 aqueous solution). This loss of activity could be due to partial degradation of phenolic
467 compounds during liposome preparation or to less availability of reactive groups resulting

468 from interactions with the bilayer membrane (Lopes de Azambuja et al., 2015). In contrast, HC
469 became apparently more antioxidant when included in liposomes, with around a 4-fold
470 increase in ABTS value (from 20.36 to 90.40 VCEAC mg/g bioactive). Other authors have
471 attributed such an increase to the contribution of the encapsulating material (da Rosa
472 Zavareze et al., 2014). In the present study, the low ABTS values found in the empty capsules
473 suggests that this effect is probably attributable to enhanced peptide conformation for radical
474 scavenging at the polar membrane surface.

475 The PCL assay was performed to consider the antioxidant property of the entire lipophilic
476 material, including the encapsulating material (Fig. 5b). The results showed that the PCL
477 activity of the lipophilic fraction of HC was low and close to that measured in the
478 corresponding loaded liposomes (L-HC) (0.378 and 0.326 TEAC mg/g, respectively). The PCL
479 activity of an empty liposome was 0.02 ± 0.00 TEAC mg/g sample, so the activity of L-HC was
480 mostly attributable to the hydrolysate. The activity of SL was 60.89 TEAC mg/g, while L-SL
481 showed only 0.502 TEAC mg/g. Given the high encapsulation efficiency in L-SL (≈90 %), the low
482 PCL values therefore were mostly attributed to the encapsulating process, with a minimal
483 contribution of SL. This low contribution might suggest that the amount of the entrapped
484 material that could have leaked out as a result of freeze-drying was very low. These findings
485 indicate that encapsulation of the lipophilic compounds in PC-liposomes would be useful to
486 make them water-soluble in order to determine antioxidant properties in aqueous dispersions.
487 However, no great contribution was found when measuring the radical scavenging capacity in
488 a lipophilic medium, probably because a major part of the bioactive extract is entrapped inside
489 the bilayer. The PG extract showed the highest superoxide radical scavenging activity (644.54
490 TEAC mg/g), and PCL values were also considerably diminished in the corresponding PG-loaded
491 liposomes (L-PG) (38.474 TEAC mg/g). The higher antioxidant activities found in L-PG could be
492 explained by the amount of non-encapsulated highly active phenolic compounds (EE=63 %),
493 which remained partially available for radical scavenging in the liposomal dispersion. In

494 particular, ellagic acid has been reported as a main component in pomegranate peel extract
495 yielding high antioxidant capacity (Masci et al., 2016).

496

497 3.4.2. Simulated gastrointestinal digestion of gels

498 In order to gain an insight into the potential bioaccesibility, the surimi gels incorporating the
499 liposome-encapsulated extracts were subjected to simulated gastrointestinal digestion (sGID).
500 The residual radical scavenging (ABTS method) was determined in the digests, expressed per g
501 of surimi gel on a dry weight basis (Figure 6). For comparison purposes, formulations with free
502 added extracts (non-encapsulated) were also tested, although, as discussed above, they did
503 not show adequate gel properties. The radical scavenging capacity of the digests was lower
504 when the bioactive extracts were added encapsulated in liposomes as compared to the free
505 form. The ABTS value of the surimi gel digest without any extract was 6.53 ± 0.09 mg VCEAC/g,
506 which was noticeably lower than with the added liposomes (around 14.5 mg VCEAC/g). The
507 antioxidant activity found in the surimi gels after *in vitro* gastrointestinal digestion was related
508 to the release of short peptides from the thermally polymerized myosin in the gel (Ueki et al.,
509 2014). The ABTS values were increased by the addition of the various extracts, especially in the
510 free mode, but slight differences depending on the type of extract were observed. A possible
511 explanation for the lack of differences is that the radical scavenging activity attributable to the
512 extracts was considerably masked by the digestibility of the protein gel. Protein digestion could
513 have been favoured in the poorer and weaker gel matrices resulting from the distortion of
514 protein-protein interactions by the presence of bioactive extracts or liposomes. The effect was
515 more evident with the free-added extracts, which produced improper gels. The lower ABTS
516 values found in all the liposome-containing gels would be also the result of the dilution effect
517 produced by the liposome content (10.5 %) in the digestible myofibrillar protein. It should be
518 considered that the activity of the various extracts might also have been modified as a result of

519 the sGID. In this respect, an increase in radical scavenging activity was found after subjecting
520 HC to sGID, increasing the ABTS values from 22.36 to 33.17 VCEAC mg/g of dry extract. Similar
521 results were obtained by Alemán et al. (2013) for digested peptide fractions from squid
522 hydrolysate. This effect could contribute slightly to the ABTS values in the corresponding
523 digested surimi gels. In contrast, the activity of PG after sGID decreased significantly ($p \leq 0.05$)
524 from 360.01 to 188.30 VCEAC mg/g of dry extract, indicating noticeable inactivation of
525 phenolic compounds after digestion (Surek & Nilufer-Erdil, 2016). This could be a reason why,
526 although it had much more activity, the difference in the resulting PG-added gel was small in
527 comparison with the HC- and SL-added gels. In the gels with liposomes, no significant
528 differences as a function of extract were found, so it could be presumed that the encapsulated
529 material was protected inside the vesicle from interactions with the gel matrix during the sGID.

530

531 **4. Conclusions**

532 The three different waste materials were successfully encapsulated in soy phosphatidylcholine
533 nanoliposomes with high particle stability. Although freeze-drying caused an increase in the
534 average particle size and polydispersity of rehydrated liposomes, a significant percentage still
535 remained near the original nanoscale. Freeze-drying represents a feasible solution to
536 standardize the moisture content in surimi gels. The incorporation of freeze-dried liposomes
537 helped to provide gel integrity and to maintain stability during long-term frozen storage. The
538 surimi gels subjected to sGID showed noticeable radical scavenging capacity, attributed
539 predominantly to the digested myofibrillar protein. The encapsulated extracts were not
540 available to protect the gel matrix from lipid oxidation, which in any case was very low.
541 Therefore it was speculated that the active compounds were efficiently protected from
542 process degradation or interactions in the gel matrix by the liposome membrane. However,

543 further *in vivo* studies would be needed to ascertain the bioavailability of the encapsulated
544 bioactive extracts in the surimi gels.

545

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549

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669 FIGURE CAPTIONS

670

671 Figure 1. Molecular weight distribution (a) and amino acid composition (b) of the giant squid
672 acid-soluble-collagen hydrolysate (HC).

673 Figure 2. HPLC chromatographic profile of the phenolic compounds present in the
674 pomegranate peel and albedo extract (PG).

675 Figure 3. Particle size distribution of rehydrated freeze-dried liposomes loaded with (a)
676 collagen hydrolysate (L-HC), (b) pomegranate peel extract (L-PG) and (c) shrimp lipid (L-SL).

677 Figure 4. Polar plot representation of Hue and Chroma values of surimi gels with the addition
678 of non-encapsulated collagen hydrolysate (Free-HC), pomegranate peel extract (Free-PG) and
679 shrimp lipid extract (Free-SL), and of the respective freeze-dried liposomes (L-HC, L-PG and L-
680 SL). Control = gel without additives.

681 Figure 5. Antioxidant activity of non-encapsulated (Extract) and liposome-encapsulated
682 (Liposome) collagen hydrolysate (HC), pomegranate peel extract (PG) and shrimp lipid extract
683 (SL). (a) Results of the ABTS assay; (b) results of Photochemiluminiscence assay. Different
684 letters (a, b, c, …) indicate significant differences ($p \leq 0.05$) among samples.

685 Figure 6. Antioxidant activity (ABTS assay) of surimi gels with the addition of non-encapsulated
686 (Free) collagen hydrolysate (HC), pomegranate peel extract (PG) and shrimp lipid extract (SL),
687 and of the respective freeze-dried liposomes (Liposome). Different letters (a, b, c, …) indicate
688 significant differences ($p \leq 0.05$) among samples.

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692

693 Table 1.- Size (z-average), polydispersity and zeta potential of fresh and rehydrated freeze-dried
694 liposomes loaded with collagen hydrolysate (L-HC), pomegranate peel extract (L-PG) and shrimp lipid
695 (L-SL).

	Z-average (nm)		Polydispersity index		Zeta potential (mV)	
	Fresh	Freeze-dried	Fresh	Freeze-dried	Fresh	Freeze-dried
L-HC	$75.7 \pm 0.5^{a/x}$	$198.9 \pm 2.8^{a/y}$	$0.231 \pm 0.010^{a/x}$	$0.462 \pm 0.035^{a/y}$	$-68.4 \pm 1.8^{a/x}$	$-62.9 \pm 1.2^{b/x}$
L-PG	$81.0 \pm 0.6^{b/x}$	$274.2 \pm 3.3^{b/y}$	$0.238 \pm 0.012^{a/x}$	$0.518 \pm 0.029^{a/y}$	$-64.6 \pm 1.8^{a/x}$	$-58.5 \pm 1.6^{a/y}$
L-SL	$80.3 \pm 1.1^{b/x}$	$282.9 \pm 2.3^{c/y}$	$0.218 \pm 0.013^{a/x}$	$0.523 \pm 0.037^{a/y}$	$-88.2 \pm 2.7^{b/x}$	$-62.5 \pm 1.7^{b/y}$

696 Different letters (a, b, c) indicate significant differences ($p \leq 0.05$) among samples as a function of
697 loaded substance. Different letters (x, y) indicate significant differences ($p < 0.05$) between fresh and
698 freeze-dried samples.

699

Table 2.- Breaking force, breaking deformation, gel strength and TBARS of surimi gels without liposomes (G-control) and with liposomes loaded with collagen hydrolysate (L-HC), pomegranate peel extract (L-PG) and shrimp lipid extract (L-SL), determined after overnight storage at 4 °C (Unfrozen) and after 3 months and 7 months of frozen storage at –20 °C.

	Force N	Deformation mm	Gel strength N·mm	TBARS µg MDA/kg gel
Unfrozen				
G-control	$1.25 \pm 0.05^{C/X}$	$7.15 \pm 0.16^{C/X}$	$8.94 \pm 0.29^{C/X}$	$0.088 \pm 0.000^{A/X}$
L-HC	$0.41 \pm 0.01^{A,B/X}$	$6.39 \pm 0.12^{B,C/X,Y}$	$2.62 \pm 0.05^{B/X}$	$0.511 \pm 0.042^{D/X}$
L-PG	$0.35 \pm 0.03^{A/X}$	$5.21 \pm 0.75^{A/X}$	$1.82 \pm 0.20^{A/X}$	$0.276 \pm 0.027^{B/X}$
L-SL	$0.47 \pm 0.02^{B/X}$	$6.13 \pm 0.06^{B/X}$	$2.88 \pm 0.09^{B/X}$	$0.386 \pm 0.042^{C/X}$
3 months				
G-control	$1.71 \pm 0.13^{D/Y}$	$6.98 \pm 0.04^{A/X}$	$11.94 \pm 0.49^{D/Y}$	$0.015 \pm 0.000^{A/X}$
L-HC	$0.76 \pm 0.04^{B/Y}$	$6.91 \pm 0.39^{A/Y}$	$5.25 \pm 0.29^{B/Z}$	$0.508 \pm 0.059^{D/X}$
L-PG	$0.94 \pm 0.01^{C/Y}$	$7.76 \pm 0.12^{A/Y}$	$7.29 \pm 0.08^{C/Y}$	$0.220 \pm 0.050^{B/X}$
L-SL	$0.57 \pm 0.08^{A/X}$	$6.21 \pm 1.17^{A/X}$	$3.54 \pm 0.59^{A/X}$	$0.380 \pm 0.072^{C/X}$
7 months				
G-control	$2.47 \pm 0.03^{D/Z}$	$8.00 \pm 0.00^{D/Y}$	$19.76 \pm 0.10^{D/Z}$	$0.333 \pm 0.074^{A/Y}$
L-HC	$0.74 \pm 0.02^{A/Y}$	$6.01 \pm 0.07^{A/X}$	$4.45 \pm 0.08^{A/Y}$	$1.090 \pm 0.048^{C/Y}$
L-PG	$1.02 \pm 0.01^{C/Z}$	$7.23 \pm 0.05^{C/Y}$	$7.37 \pm 0.05^{C/Y}$	$0.763 \pm 0.085^{B/Y}$
L-SL	$0.87 \pm 0.02^{B/Y}$	$6.81 \pm 0.13^{B/X}$	$5.92 \pm 0.13^{B/Y}$	$0.848 \pm 0.052^{B/Y}$

Different letters (A, B, C, ….) indicate significant differences ($p \leq 0.05$) between samples for the same time of analysis.

Different letters (X, Y, Z) indicate significance differences ($p < 0.05$) between the same sample for different times of analysis and the same parameter.

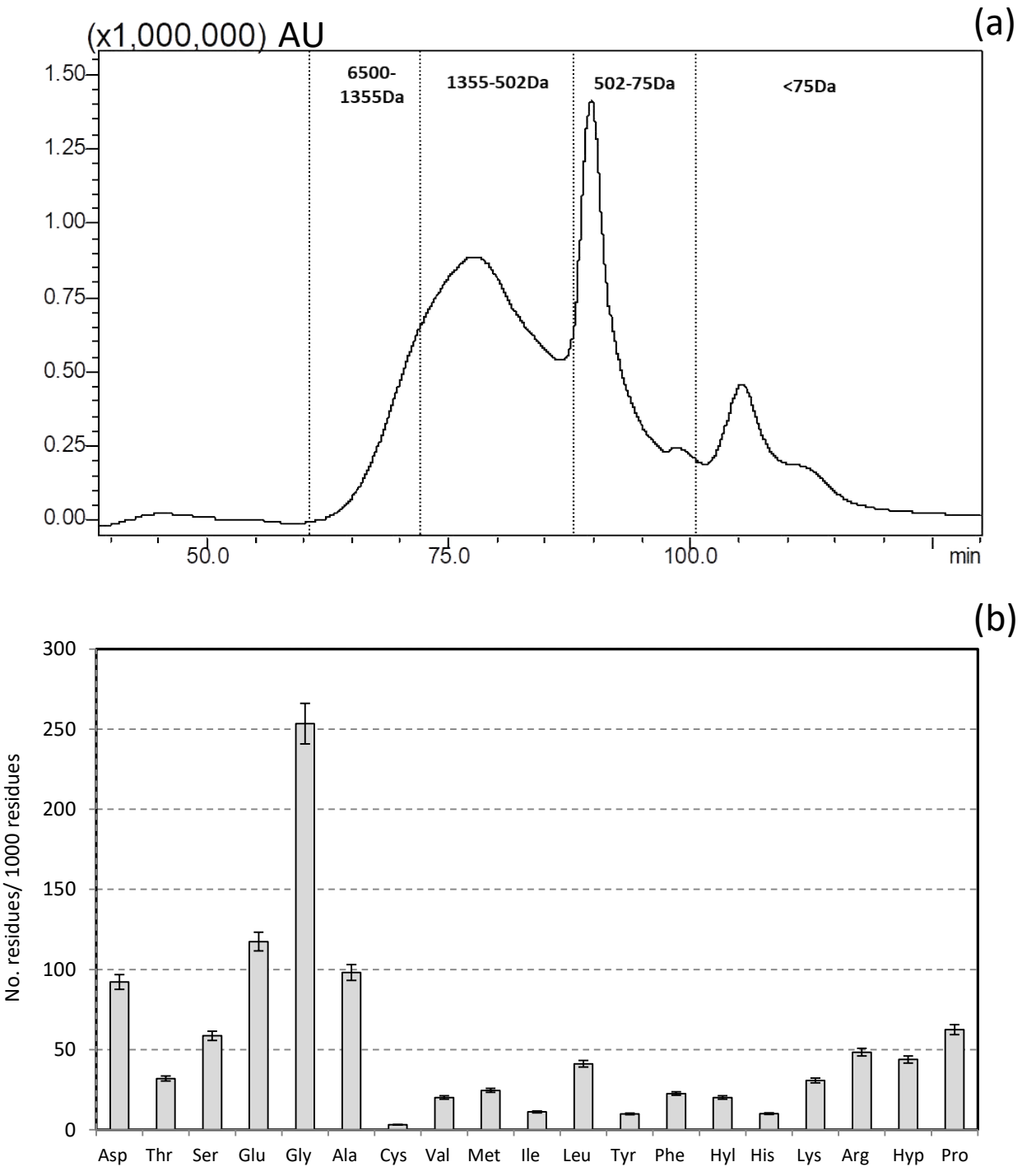

(a)
(x1,000,000) AU
1.50
1.25
1.00
0.75
0.50
0.25
0.00
6500-
1355Da
1355-502Da
502-75Da
<75Da
50.0
75.0
100.0
min
(b)
No. residues/ 1000 residues
300
250
200
150
100
50
0
Asp
Thr
Ser
Glu
Gly
Ala
Cys
Val
Met
Ile
Leu
Tyr
Phe
Hyl
His
Lys
Arg
Hyp
Pro

Figure 1

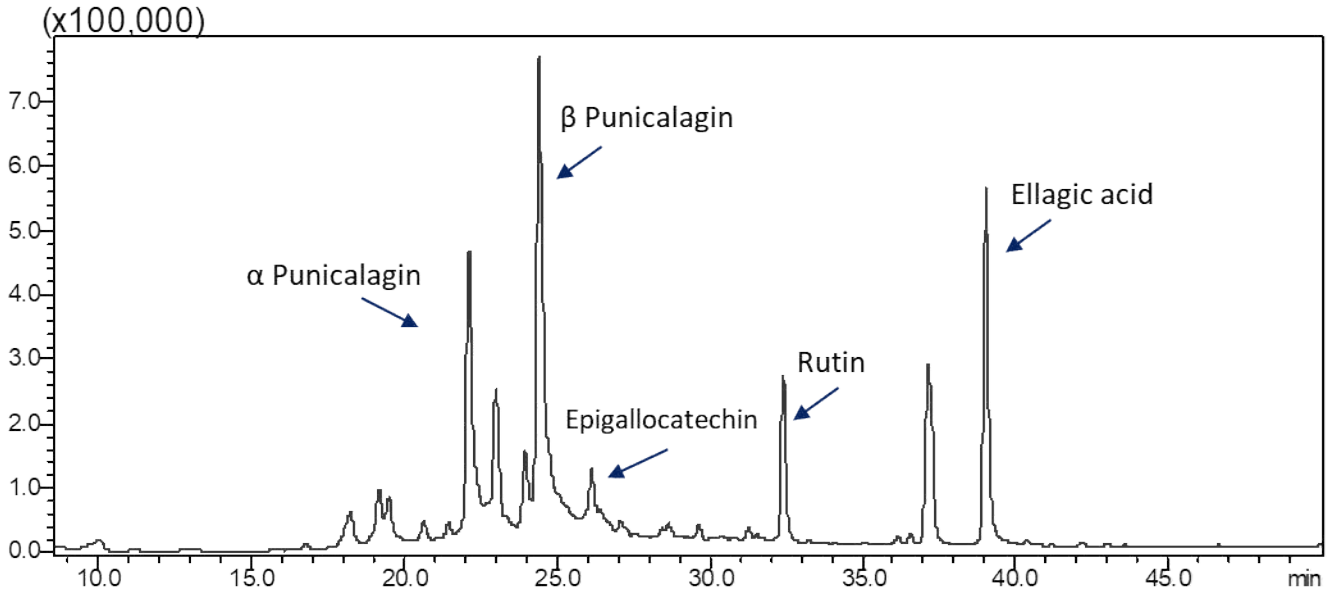

(x100,000)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
β Punicalagin
Ellagic acid
α Punicalagin
Rutin
Epigallocatechin
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
min

Figure 2

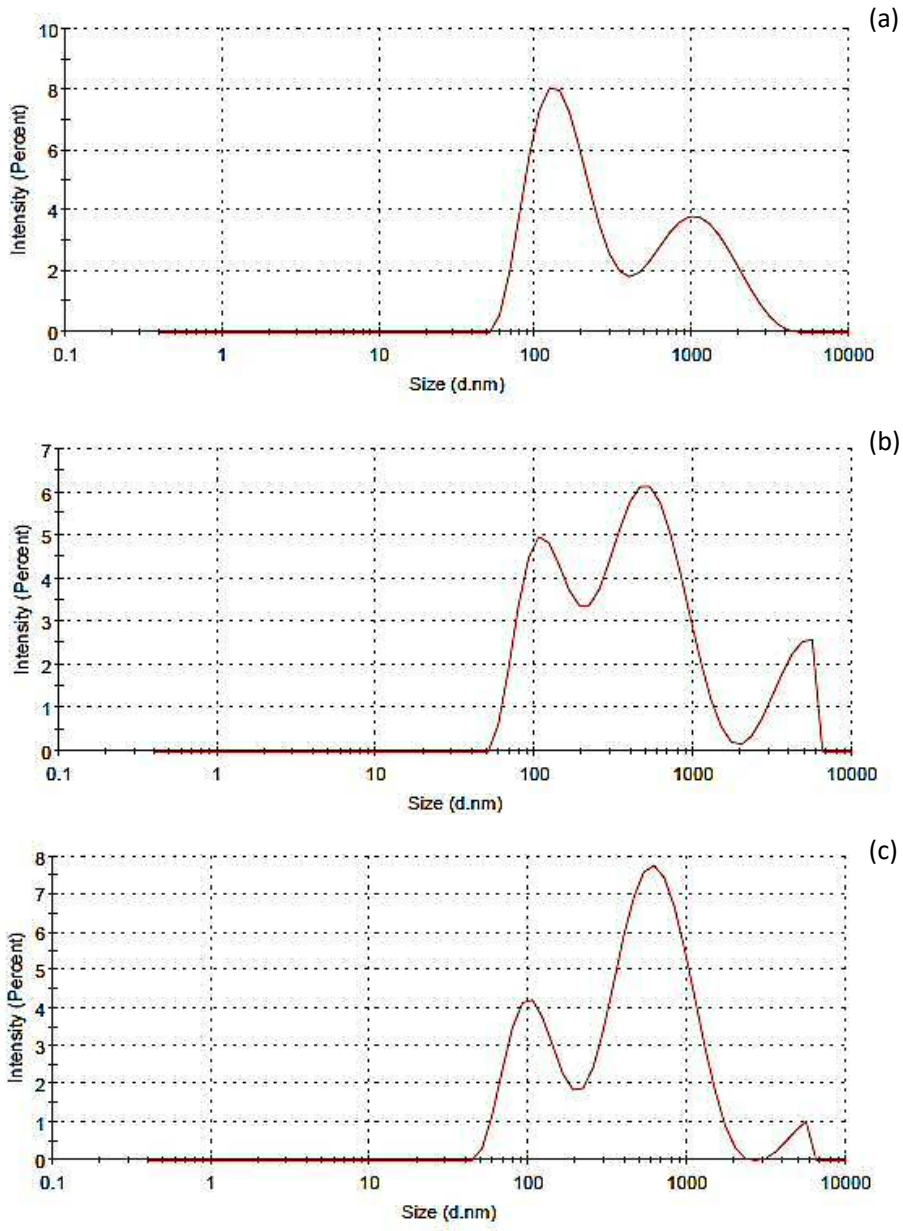

(a)
Intensity (Percent)
Size (d.nm)
(b)
Intensity (Percent)
Size (d.nm)
(c)
Intensity (Percent)
Size (d.nm)

Figure 3

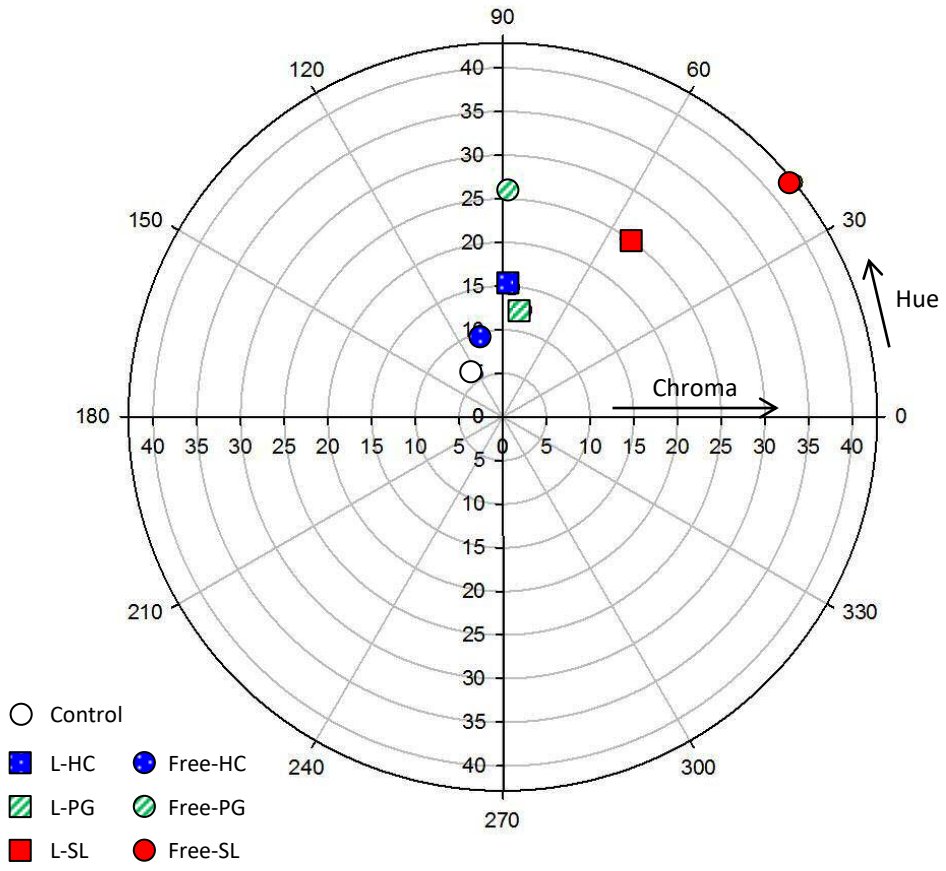
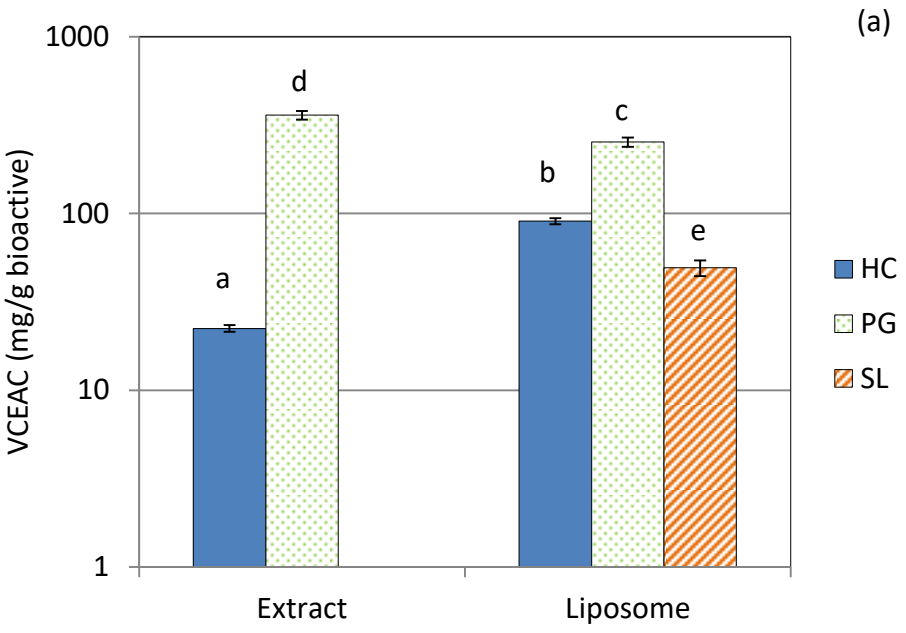
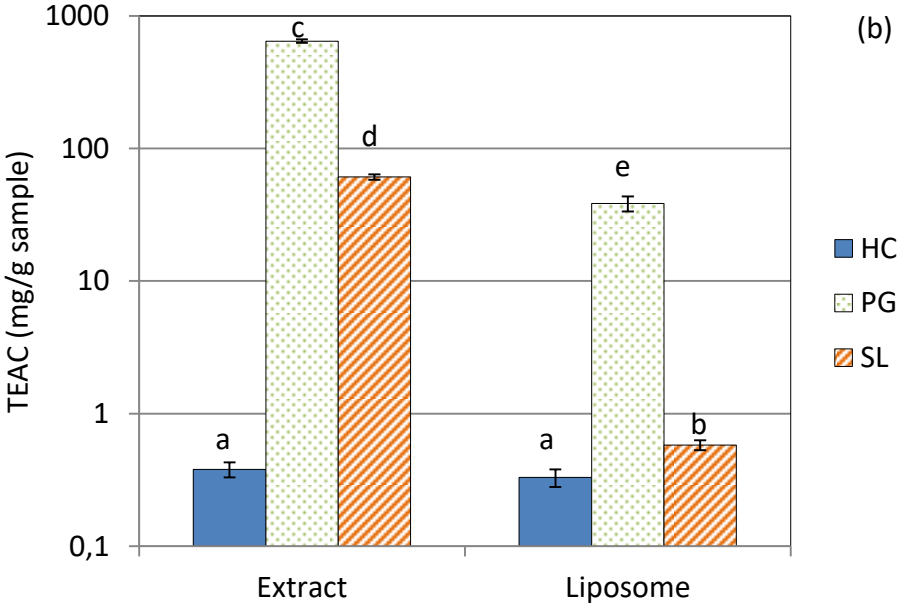

90
120
60
150
30
180
0
210
330
240
300
270
Hue
Chroma
Control
L-HC
Free-HC
L-PG
Free-PG
L-SL
Free-SL

Figure 4


(a)
1000
100
10
1
VCEAC (mg/g bioactive)
a
d
b
c
e
Extract
Liposome
HC
PG
SL


(b)
1000
100
10
1
0,1
TEAC (mg/g sample)
a
c
d
a
e
b
Extract
Liposome
HC
PG
SL

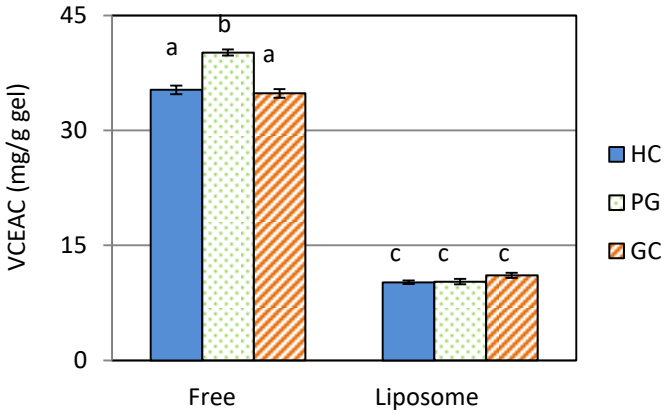


Figure 6