Monroig O, Navarro JC, Amat F, Gonzalez P & Hontoria F (2007) Oxidative stability and changes in the particle size of liposomes used in the Artemia enrichment, *Aquaculture*, 266 (1-4), pp. 200-210.

This is the peer reviewed version of this article

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Manuscript Region of Origin:

Abstract: The oxidative stability and the particle size of several types of liposomes were assessed in order to characterize their behaviour when submitted to the aggressive conditions of the Artemia enrichments. Results show that all liposomes tested in this study were much more oxidatively stable than a commercial product based on fish oil emulsion. Whereas the initial thiobarbituric acid reactive substances (TBARS) concentration in the emulsion was only slightly higher than in liposomes, the concentration of TBARS in the emulsion increased up to values three orders of magnitude above those registered by liposomes after 21 h of incubation. Among the different liposome formulations, results indicate that vesicles composed of phospholipids containing long-chain highly unsaturated fatty acids (krill phospholipid extract) were generally less stable than those composed of shorter length-chain and more saturated acyl chains.

In regards to the particle size changes during enrichment, all liposomes maintained their original size during the experimental period when incubated without nauplii. In the presence of nauplii, liposomes did not exhibit notable changes in their size, except for unilamellar vesicles prepared by the extrusion methodology and formulated with soybean phosphatidylcholine. The implications of the results on the capability of liposomes to be used in Artemia nauplii enrichments are discussed.

1   Title:

2   Oxidative stability and changes in the particle size of liposomes used in the *Artemia*

3   enrichment

4

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18   **Abstract**

19   The oxidative stability and the particle size of several types of liposomes were

20   assessed in order to characterize their behaviour when submitted to the aggressive

21   conditions of the *Artemia* enrichments. Results show that all liposomes tested in this

22   study were much more oxidatively stable than a commercial product based on fish oil

23   emulsion. Whereas the initial thiobarbituric acid reactive substances (TBARS)

24   concentration in the emulsion was only slightly higher than in liposomes, the

25   concentration of TBARS in the emulsion increased up to values three orders of

26   magnitude above those registered by liposomes after 21 h of incubation. Among the

27   different liposome formulations, results indicate that vesicles composed of

28   phospholipids containing long-chain highly unsaturated fatty acids (krill phospholipid

29   extract) were generally less stable than those composed of shorter length-chain and

30   more saturated acyl chains.

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32   maintained their original size during the experimental period when incubated without

33   nauplii. In the presence of nauplii, liposomes did not exhibit notable changes in their

34   size, except for unilamellar vesicles prepared by the extrusion methodology and

35   formulated with soybean phosphatidylcholine. The implications of the results on the

36   capability of liposomes to be used in *Artemia* nauplii enrichments are discussed.

37   Keywords: *Artemia* enrichment; liposomes; oxidative stability; particle size; larviculture

38   **1. Introduction**

39   Deficiencies in the nutritional value of live preys used in the rearing of marine

40   fish larvae has been solved through enrichment procedures with products containing

41   high levels of essential nutrients. During this boosting process, live preys such as

42   rotifers and *Artemia* nauplii are incubated in aqueous media where the enrichment diet

43   is dispersed, thereby facilitating its incorporation through the passive filtering behaviour

44   of these organisms (Léger et al., 1986). A broad variety of enrichment products are

45   available and can be classified as those based on microalgae (Wickins, 1972; Watanabe

46   et al., 1978; Watanabe et al., 1982), spray-dried cells of *Schizochytrium* sp. (Barclay and

47   Zeller, 1996), microcapsules (Southgate and Lou, 1995) and oil emulsion products

48   (Léger et al., 1986; Han et al., 2000; Copeman et al., 2002). All of them contain

49   significant levels of highly polyunsaturated fatty acids (HUFA) as eicosapentaenoic

50   (EPA, 20:5n-3), docosahexaenoic (DHA, 22:6n-6) and arachidonic acids (ARA, 20:4n-

51   6) which are essential for marine fish larvae (Sargent et al., 1997; Izquierdo et al.,

52   2000). In addition to these compounds, several studies have focused their attention on

53   the importance of other nutrients such as vitamins (Merchie et al., 1997; Halver, 2002)

54   and free amino acids (Rønnestad et al., 1999; Tonheim et al., 2000; Rønnestad et al.,

55   2003) on the early development of marine fish larvae. Although some effort has been

56   made on the formulation of novel products which provide simultaneously all essential

57   nutrients (Dhert et al., 2005), the different water or lipid solubility of such essential

58   compounds has limited the success.

59   Since liposomes can include both hydrophilic and lipophilic substances, some

60   authors have proposed their use as an alternative tool to deliver essential nutrients to

61   marine fish larvae through their bioencapsulation in live preys (Ozkizilcik and Chu,

62   1994; Hontoria et al., 1994; McEvoy et al., 1996; Tonheim et al., 2000; Monroig et al.,

63   2003; Monroig et al., 2006a, b). Simply stated, liposomes can be defined as

64   phospholipid vesicles that enclose an aqueous internal compartment and with variable

65   sizes ranging from some dozens of nanometres to several microns, (New, 1990). The

66   previously described characteristics (i.e., the existence of an internal space, their

67   phospholipid based composition and the small particle size) differentiate liposomes

68   from other commercially available enrichment products, and hence determine their

69   behaviour as enrichment diets.

70   As a preliminary attempt to study how liposomes behave under the *Artemia*

71   enrichment conditions, Monroig et al. (2003) assessed the capacity of several types of

72   vesicles to retain water soluble compounds dissolved in the internal aqueous space. It

73   was concluded that lamellarity, the incubation time and the membrane formulation

74   determine the leakage rate of water soluble compounds from the aqueous compartment

75   to the external space out of the membrane. However, other parameters are necessary to

76   be studied for the proper assessment of the liposome capability for the live prey

77   enrichment.

78   Lipid peroxidation, as an estimator of the oxidative stability, has been described

79   to be an important degradation process of polyunsaturated fatty acids (Gurr and

80   Harwood, 1991; Frankel, 1998). As aforementioned, most of the enrichment diets are

81   basically composed of polyunsaturated fatty acids and hence are susceptible to

82   peroxidize when exposed to the aggressive conditions of live prey enrichments i.e.

83   illumination, high temperature and dissolved oxygen (McEvoy et al., 1995). Therefore,

84   the oxidation process produces terminal toxic compounds such as aldehydes and

85   ketones which can be accumulated in the live preys through their passive filtering

86   behaviour. Finally, consumption of such harmful diets can lead to detrimental effects

87   (Hipkiss et al., 1997; Tesoriere et al., 2002; Del Rio et al., 2005) on larvae.

88   Alternatively, the phospholipid composition of liposomes can preserve the oxidative

89   stability during enrichment since several studies have demonstrated the antioxidant

90   properties of these compounds in comparison to other lipid classes such as triglycerides

91   and fatty acid ethyl esters (King et al., 1992a, b; Song et al., 1997).

92   Particle size is an important property for the enrichment products as it can

93   determine the bioencapsulation efficiency in the live prey (Gelabert-Fernández, R.,

94   2002; Han et al., 2005). Generally speaking, commercial enrichment diets produce

95   several micron-sized particles when dispersed in the enrichment medium (Léger et al.,

96   1987; Southgate and Lou, 1995; Han et al., 2005). On the other hand, the unilamellar

97   liposomes used in the present study are smaller with diameters varying around 150 nm.

98   Likewise, incubation of liposomes in the enrichment medium itself can favour the

99   occurrence of different phenomena such as fusion and aggregation, which can modify

100   the original size distribution (New, 1990), and therefore the bioencapsulation efficiency

101   in the nauplii.

102   In order to better characterize the behaviour of liposomes during the *Artemia*

103   enrichment, we studied the oxidative stability of several liposome formulations when

104   submitted to standard enrichment conditions, together with the changes in the vesicle

105   size throughout the process. For this reason, the concentration of TBARS formed during

106   the incubation of liposomes was used as an estimator of oxidative stability. In a second

107   experiment, changes in the particle size of liposomes were monitored throughout the

108   incubation period by means of a light scattering laser technique.

109   **2. Materials and methods**

110   *2.1. Preparation of liposomes*

111   The oxidative stability and the changes in the vesicle size were assessed in five

112   liposome formulations submitted to standard *Artemia* enrichment conditions. Three

113   different phospholipid sources (see Table 1 for fatty acid and lipid class composition)

114   were used in the preparation of liposomes: krill phospholipid extract (KPE), dipalmitoyl

115   phosphatidylcholine (DPPC), and soybean phosphatidylcholine (SPC), all from Avanti

116   Polar Lipids Inc. (Alabaster, AL, USA).

117   All five liposomes were unilamellar vesicles and were prepared using two

118   different methodologies. Firstly, multilamellar liposome suspensions obtained by simple

119   hydration of a phospholipid film with seawater (Monroig et al., 2003) were extruded

120   using a LiposoFast engine (Avestin Inc., Ottawa, ON, Canada) equipped with 100 nm

121   polycarbonate membranes thereby yielding unilamellar vesicles (Lext). A second type

122   of unilamellar liposomes was prepared using a detergent solubilization methodology

123   (Ldet). Two solutions, a micelle suspension composed by a mixture of phospholipid and

124   the detergent sodium cholate (7:10, mol/mol), and a saline solution (0.9 % NaCl), were

125 mixed and dialyzed allowing the removal of detergent from the micelle solution. The

126 continuous and very rapid detergent removal spontaneously leads to the formation of

127 liposomes. More details about these liposome preparation methodologies can be found

128 in New (1990). In both methodologies, original vesicle size was around 150 nm.

129   Membrane composition of all five liposomes used in the present study was as

130 follows:

131   1.Ldet KPE: liposomes composed of krill phospholipid extract.

132   2.Ldet KPE:vitA: liposomes composed of krill phospholipid extract and

133   retinyl palmitate (100:2, w/w).

134   3.Ldet SPC: liposomes composed of soybean phosphatidylcholine.

135   4.Lext SPC:CHO: liposomes composed of soybean phosphatidylcholine and

136   cholesterol (4:1, w/w).

137   5.Lext DPPC:CHO: liposomes composed of dipalmitoyl phosphatidylcholine

138   and cholesterol(4:1, w/w).

139 Cholesterol (CHO) was included as a membrane stabilizer (New, 1990, p. 22) in

140 liposomes prepared by the extrusion methodology (Lext) since it has previously seen to

141 reduce the leakage of water soluble substances in extrusion suspensions (Monroig et al.,

142 2003). Another type of liposomes (Ldet KPE:vitA) was formulated by combining the

143 antioxidant compound retinyl palmitate (Sigma, Alcobendas, Spain) with krill

144 phospholipid (KPE) in order to check the effect on the oxidative stability of HUFA-rich

145 KPE. Relevant features of the five liposome suspensions used in this study are gathered

146 in Table 2.

147 *2.2. Oxidative stability of liposomes during enrichment*

148   This experiment assessed the oxidative stability of the five liposome

149 formulations (Table 2) together with the self-emulsifying enrichment product Super

150 Selco (Inve, Ghent, Belgium) used as a reference product in previous studies (Monroig

151 et al., 2003, 2006a, b). The enrichment diets (liposomes or lipid emulsion) were

152 incubated in three replicate 1 litre cylinder-conical enrichment tubes filled with 700 ml

153 of filtered (0.45 μm) and UV light-sterilized sea water. During the incubation period (21

154 h), the enrichment tubes were placed in a thermostated bath at $28 \pm 1^{\circ}C$, illuminated

155 with fluorescent light tubes (1500-2000 lux), and aerated by means of 0.5 cm diameter

156 glass tubes giving an air flow of 1.0 litre per minute. Control of air flow was achieved

157 using rotameters (Key Instruments, Trevose, PA, USA). The experiment was carried out

158 in absence of nauplii to eliminate any effects of nauplii on the TBARS determination. In

159 this manner, the final lipid quantity would not be conditioned by the liposome type-

160 dependent filtratory efficiency of the nauplii. Furthermore, the inclusion of nauplii

161 implies the entry of microorganisms which could lead to false peroxide determinations

162 (McEvoy et al., 1995).

163   Sample collection was performed by taking an aliquot of the enrichment medium

164 at the beginning (t_o) and the end of the incubation period (t_o+21 h) under the standard

165 enrichment conditions detailed above. Samples of the enrichment medium were

166 immediately frozen and kept at -20 °C until further analyses.

167   The oxidative stability was measured by determining the concentration of the

168 thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et

169 al. (1979) with the following modifications. Enrichment medium aliquots (160 μl) were

170 reacted with 40 μl of 8.1% (w/v) sodium dodecyl sulphate containing 0.05% (w/v)

171 butylated hydroxytoluene (BHT), 300 μl of 20% acetic acid (pH=3.5) and 300 μl of

172 0.8% (w/v) thiobarbituric acid (TBA). A volume of sodium phosphate buffer was added

173 to complete a final volume in the test tubes of 800 μl. The reagents were mixed in

174 stoppered test tubes and incubated in a thermostated bath for 60 min at 95 °C. After

175 cooling in an ice bath, 200 µl of distilled water and 1 ml of n-butanol:pyridine (1:1, v/v)

176 were added, and the mixture was shaken vigorously. After centrifugation at 10000 x g

177 for 3 min, the fluorescence of the organic layer was determined, setting excitation and

178 emission wavelengths at 515 nm and 553 nm, respectively. TBARS concentrations were

179 derived from a standard curve using 1,1,3,3-tetraethoxypropane (TEP) and values

180 expressed in terms of TEP µmolar equivalents per litre. All chemicals used in the

181 TBARS measurements were obtained from Sigma (Alcobendas, Spain).

182 *2.3. Changes in the liposome size during enrichment*

183 The size (diameter) of liposome vesicles was estimated throughout a 22 h period

184 in three replicate cylinder-conical tubes. In comparison to the oxidative stability

185 experiment, smaller enrichment tubes were used (200 ml) filled with 100 ml of filtered

186 sea water (0.45 µm). Because of the smaller capacity of the tubes, the air flow was

187 decreased to give a similar hydrodynamic regime in both experiments. Lipid

188 concentration, illumination and temperature conditions were the same described in the

189 peroxide formation experience. In this case, changes in the liposome size distribution

190 were evaluated both in the presence and absence of nauplii in the enrichment medium to

191 precisely imitate the real enrichment conditions. For treatments containing *Artemia*

192 nauplii, newly hatched nauplii (Inve, Ghent, Belgium) were stocked at the beginning of

193 the trial (t_0) at naupliar densities of 300 individuals ml^{-1}

194 Medium samples were collected at the beginning (t_0) and after 12, 17 and 22 h of

195 incubation. When nauplii were present, the enrichment medium aliquot was filtered

196 through a 100 µm pore plankton mesh to eliminate the *Artemia* nauplii. About 4 ml of

197 deionized water and a portion of the enrichment medium were transferred into a 4.5 ml

198 polymethyl methacrylate cuvette, in order to obtain an intensity between 10^4 and 10^6

199 particles counted per second. Measurements of vesicle diameter were performed with a

200 Nanosizer Coulter N4 Plus instrument (Coulter Coultronics, Margency, France), which

201 uses the principle of light scattering applied to the Brownian motion of the particles.

202 Essentially, a laser light source is emitted through a sample producing a scattering

203 pattern which is then analysed by a personal computer system equipped with N4 Plus

204 software. Taking into account the parameters of temperature (20ºC), viscosity and

205 refractive index of the suspending medium (1.33 for deionized water), the mixture was

206 left to achieve thermostatic equilibrium for 5 min. After this period, three measures

207 were carried out using the following parameters: acquisition angle 90º and analysis time

208 of 180 s. The final diameter is calculated as the mean of the three measurements taken

209 during 180 s in every sample. The particle size of the lipid globules formed by the

210 commercial emulsion was not analysed because they are above the upper threshold of

211 the Nanosizer Coulter N4 Plus instrument.

212 *2.4. Lipid classes and fatty acid determinations*

213 The lipid class and fatty acid analyses of the phospholipid sources used in the

214 liposome preparation were analysed after a lipid extraction procedure carried out by the

215 method described by Folch et al. (1957). Lipid classes were determined using a high-

216 performance thin-layer chromatography (HPTLC) method as proposed Olsen and

217 Henderson (1989). Analyses of fatty acids were carried by gas chromatography as

218 described in Monroig et al. (2006a).

219 *2.5. Statistical analyses*

220 Analytical data are expressed as means ± standard deviations. Homogeneity of

221 variances was checked by Barlett's test. In cases of homoscedasticity, a Student's *t*-test

222 (Sokal and Rohlf, 1981) determined the differences in the TBARS concentration at t_o

223 and $t_o + 21$ h in all the enrichment products (liposomes or the commercial emulsion). In

224 the particle size experiment, a Student's *t*-test evaluated differences between the

225 presence and absence of nauplii in each sample point. If heterogeneity of variances

226 existed, a Welch's test was used to check differences between treatments. Comparisons

227 of the means with P values less or equal than 0.05 were considered significantly

228 different. All the statistical analyses were carried out using the SPSS statistical package

229 (SPSS Inc., Chicago, IL, USA).

230 **3. Results**

231 *3.1. Oxidative stability of liposomes during enrichment*

232 Fig. 1 illustrates the concentration of TBARS measured in the enrichment media

233 of all five liposomes and the commercial emulsion. The results indicate a significant

234 increase in the concentration of lipid peroxides in three liposome formulations

235 incubated during the 21 h period (Ldet KPE, Ldet KPE:vitA, and Lext SPC:CHO). On

236 the contrary, both Ldet SPC and Lext DPPC:CHO did not display significant increase in

237 the peroxide concentration after their incubation under the experimental conditions. In

238 spite of the formation of lipid peroxides in some formulations, the liposome treatments

239 showed a higher oxidative stability in comparison to the commercial emulsion whose

240 peroxide levels increased up to three orders of magnitude beyond liposome oxidation

241 rates. In the case of liposomes, little differences were observed among formulations and

242 final TBARS concentration registered values of the same order of magnitude in all

243 treatments.

244 *3.2. Changes in the liposome size during enrichment*

245 Diameters of liposomes throughout the enrichment period are represented in Fig.

246 2. All five liposome formulations showed minor changes in their size during the

247 incubation time when exposed to the experimental conditions in absence of *Artemia*

248 nauplii. In these circumstances, all formulations maintained their original size of around

249   150 nm. However, the presence of *Artemia* nauplii in the medium gave rise to an

250   increase in the particle diameter of liposomes. Except for Ldet SPC and Ldet KPE, a

251   significant increase ($P \leq 0.05$) in the liposome size in the presence of *Artemia* was

252   observed at 17 h of incubation and onwards. Thus, Ldet KPE did not show differences

253   between the absence and presence of *Artemia* nauplii at any sampling point (Fig. 2a),

254   whereas Ldet SPC liposomes (Fig. 2c) exhibited a significant increase ($P \leq 0.05$) only at

255   the end of incubation ($t_o + 22$ h). The inclusion of retinyl palmitate in krill liposomes

256   (Ldet KPE:vitA) was associated to a slight increase in the liposome diameters in the

257   presence of *Artemia* nauplii (Fig. 2b).

258   The most obvious augment in the particle diameter was seen in Lext SPC:CHO

259   liposomes (Fig. 2d). In this formulation, the presence of nauplii caused a marked

260   increase in particle sizes, which was associated with high variability at 12 h and

261   onwards. Another remarkable result is the different behaviour showed by Lext

262   SPC:CHO (Fig. 2d) and Lext DPPC:CHO (Fig. 2e). Despite the fact that both

263   suspensions were formulated with purified phospholipid sources mixed with CHO at the

264   same phospholipid:CHO ratio, DPPC liposomes showed much lower increase in size in

265   the presence of nauplii compared to SPC vesicles (Fig. 2e).

266   **4. Discussion**

267   The use of liposomes as an enrichment diet implies their submission to

268   aggressive conditions which can modify their original properties. In this context,

269   Monroig et al. (2003) studied the leakage shown by several types of liposomes

270   submitted to enrichment conditions. It was concluded that liposomes become leaky

271   under the aggressive environment of enrichment, contrarily to their stable behaviour

272   when stored at room conditions (i.e., no agitation, room temperature, etc.) (Hontoria et

273 al., 1998). In a second approach, the study of the oxidative stability and the changes in

274 the size distribution of liposomes during the *Artemia* enrichment process contribute to a

275 better global assessment of the capacity of liposomes to be used in *Artemia* enrichment

276 procedures.

277 McEvoy et al. (1995) reported the oxidative stability of several enrichment diets

278 submitted to standard *Artemia* enrichment conditions. These authors found that all the

279 enrichment products tested, including the commercial product Super Selco used as

280 reference in the present study, showed autoxidation of polyunsaturated fatty acids after

281 23 h of incubation. The results of the present study partially confirm the occurrence of

282 this degradation process when liposomes are employed as *Artemia* enrichment diets

283 during a period of 21 h. However, the oxidation rates of liposomes were clearly lower

284 than that of the lipid emulsion Super Selco despite the fact that this product contains

285 antioxidant substances (McEvoy et al., 1995). The difference in the oxidative stability

286 between liposomes and the commercial emulsion might be related to their lipid class

287 composition. Whereas the commercial emulsion is essentially composed of

288 triacylglycerides (TAG) and fatty acid ethyl esters (FAEE) (see Table 1), liposomes are

289 vesicles mostly comprised of phospholipids. Consequently, fatty acids included in

290 liposome phospholipids would be efficiently protected since these compounds have

291 been described to possess antioxidant properties in comparison to other lipid classes

292 such as TAG and FAEE (King et al., 1992a, b; Song et al., 1997). In order to explain the

293 antioxidant mechanisms acting on phospholipids, King et al. (1992a) cited several

294 postulates which included synergism between phospholipids and tocopherol, chelation

295 of pro-oxidant metals by the phospholipid phosphate groups, formation of Maillard-type

296 products between phospholipids and oxidation products, and, finally, their action as an

297 oxygen barrier through the oil/air interfaces. More recently, Lyberg et al. (2005) studied

298   the oxidative stability of free DHA, DHA mixed with phosphatidylcholine (PC) and

299   DHA incorporated in PC, phosphatidylethanolamine (PE) and TAG. It was found that

300   DHA included in the structure of the phospholipid (PC and PE) was the most protected

301   alternative against oxidation, and proposed a key role of the polar group in the

302   protection mechanism. These authors hypothesized that the best protection was

303   achieved in those parts of the fatty acid chain that were close to the polar group. Thus,

304   in addition to carbons close to the carboxyl group, DHA terminal carbons are also

305   protected due to the curve exhibited by DHA as a result of its high degree of

306   unsaturation. Consequently, the oxidative stability of liposomes would reduce the

307   formation of lipid peroxides during enrichment, its subsequent accumulation in live

308   preys and, finally, the potential harmful effects (Hipkiss et al., 1997; Tesoriere et al.,

309   2002; Del Rio et al., 2005) on larvae fed with those nauplii. In a larval feeding trial,

310   Monroig et al. (2006a) obtained higher concentrations of lipid peroxides in larvae fed

311   *Artemia* nauplii enriched with the same commercial emulsion employed in the present

312   study when compared to larvae fed liposome enriched nauplii.

313   As aforesaid, McEvoy et al. (1995) assessed the autoxidation of several

314   enrichment products including the commercial product Super Selco also tested in the

315   present study. Assuming that the concentration of total lipid present in the enrichment

316   medium at the beginning and at the end of incubation was 0.5 g l^{-1}, our data can be

317   converted into the same units used by these authors (moles of TBARS per lipid weight

318   unit) and, hence, data from both studies can be compared. At the beginning of the trials,

319   the concentration of TBARS was 1420±300 nmol g $lipid^{-1}$ in our experiment (Fig. 1)

320   and 2300 nmol g $lipid^{-1}$ in study of McEvoy et al (1995) (data estimated from Fig. 6).

321   However, the incubation of Super Selco under the enrichment conditions and in the

322   absence of nauplii produced a much higher increase in the TBARS concentration in the

323   present study (196240 ± 13060 nmol g lipid^{-1}) in comparison to data obtained by

324   McEvoy et al. (1995) (approximately 3200 nmol g lipid^{-1}), even if the incubation period

325   in this latter experiment was 2 h longer. Some hypotheses arise to explain the high lipid

326   peroxide production in our assay. Firstly, McEvoy et al. (1995) administered the

327   emulsion Super Selco in two separate doses. This strategy is recommended by the

328   manufacturers to minimize the degradation of the enrichment product given that it is

329   exposed to a damaging environment during a shorter period, and it has been used in

330   numerous studies concerning live prey enrichment trials (McEvoy et al., 1995, 1996;

331   Han et al., 2005). Another possibility explaining the high degradation of the commercial

332   emulsion in our study may be due to the depletion of the antioxidant compounds present

333   in our product stock. Although maintained at the manufacturer's recommended

334   temperature of 4 ºC and stored under nitrogen, the antioxidant compounds of the stock

335   could be depleted and, despite the fact the initial TBARS concentration was similar to

336   that of McEvoy et al. (1995), antioxidant protection may not have acted once the

337   product is exposed to the aggressive conditions of the enrichment process. One of the

338   antioxidant substances included in the formulation of Super Selco is retynil palmitate.

339   This compound, incorporated in the Ldet KPE:vitA liposomes, did not enhance the

340   oxidative stability of krill pure liposomes (Ldet KPE). Pro-oxidant conditions of

341   enrichment procedures (temperature, oxygenation, and illumination) could partially

342   degrade retynil palmitate of liposomes thus compromising its protection role against

343   autoxidation of unsaturated fatty acid chains of krill phospholipid (Woollard and Inkyk,

344   2003). Indeed, Moren et al. (2005) were not able to detect vitamin A after 12 h of

345   exposure of a commercial product similar to Super Selco (DC-DHA Selco, Inve, Ghent,

346   Belgium) in simulated enrichment conditions.

347   Additionally to the lipid class composition, autoxidation of lipids depends on the

348   acyl chain length and the unsaturation degree of fatty acids (Holman and Elmer, 1947;

349   Cosgrove et al., 1987). This could explain differences in the oxidative stability among

350   liposome formulation assessed in this study. In fact, krill liposomes (Ldet KPE and Ldet

351   KPE:vitA), which contain both the longest and the highest unsaturated fatty acids (note

352   HUFA levels in Table 1), underwent autoxidation during the incubation period.

353   Conversely, soybean liposomes (Lext SPC:CHO and Ldet SPC) showed lower lipid

354   peroxide concentrations than krill vesicles, and even Ldet SPC did not suffer any

355   significant increase in the peroxide concentration throughout the assay. This high

356   oxidative stability was also observed in Lext DPPC:CHO whose fatty acids are totally

357   saturated (palmitic acid, 16:0). All these results are in agreement with the general

358   acceptance that the oxidative stability of fatty acids decreases with increasing degrees of

359   unsaturation. However, Nara et al. (1998) and Araseki et al. (2002) found that PC

360   liposomes containing HUFAs were more stable than liposomes formulated with more

361   saturated PCs. These authors suggested that the degree of unsaturation was not the main

362   factor affecting the oxidative stability of PC in liposomes and hypothesized that DHA in

363   liposomes would adopt a tightly packed conformation. The higher reactivity of DHA

364   derived from the higher degree of unsaturation may compensate for the difficulty of free

365   radicals and/or oxygen to attack this tighter conformation of DHA in liposomes.

366   Nevertheless, the results of these authors were obtained under very well-defined

367   conditions (temperature of 37 °C, darkness, no agitation, etc.) in which liposomes were

368   formulated with pure PCs containing fatty acid chains in known positions of the

369   glycerol moiety. All these differences could limit the extrapolation of the results to

370   those obtained in the present study where pro-oxidative conditions are most likely to be

371   drastic.

372   The changes in liposome size during the enrichment process showed different

373   results depending on the absence or presence of *Artemia* nauplii in the enrichment

374   medium. Despite the habitual occurrence of physical phenomena such as fusion and

375   aggregation observed in liposome suspensions which originate a final increase in the

376   particle size of vesicles (New, 1995, p. 17), no liposome formulation displayed visible

377   changes in their vesicle diameter in the absence nauplii. Simply stated, fusion can be

378   defined as the interaction of two separate membrane sheets such that they combine to

379   form a single sheet in which membrane lipids are shared. Additionally, aggregation

380   occurs when neutral planar phospholipid membranes establish weak interactions such as

381   van der Waals forces. However, both mechanisms do not seem to occur in liposomes

382   submitted to our experimental conditions according to the minor changes observed

383   when liposomes were incubated in seawater without nauplii. This result would disagree

384   with those obtained by Hontoria et al. (1994), who corroborated that extruded liposomes

385   maintained under no agitation conditions aggregate after their preparation. In addition,

386   Koven et al. (1999) point out the presence of divalent ions in seawater to explain

387   aggregation of extruded liposomes to form particles of 20-30 microns. The apparent

388   discrepancy can be explained by means of several mechanisms. Firstly, the turbulent

389   hydrodynamic regime of the enrichment process might handicap the establishment of

390   the above mentioned interactions between vesicles so that liposomes remain in the

391   single original state. In the case of liposomes prepared by the detergent solubilization

392   methodology (Ldet), a second antifusogenic mechanism may be acting. Given that the

393   detergent removal during the preparation of Ldet is not complete (Allen et al., 1980),

394   along with the negative charge of sodium cholate, Ldet would exhibit a decreased fusion

395   rate because of the repulsion forces between charged vesicles. A similar mechanism was

396   proposed by Lasic (1989) to explain the reduced size of Ldet prepared with sodium

397   cholate instead of the zwitterionic detergent CHAPS. In these latter liposomes, the

398   simultaneous co-existence of positive and negative charges in the CHAPS molecule

399   would facilitate fusion of vesicles and thus their bigger size in comparison to charged

400   sodium cholate liposomes. On the other hand, liposomes prepared by extrusion do not

401   contain detergent and, consequently, would be more prone to aggregate.

402   The presence of *Artemia* nauplii in the enrichment medium would affect in a

403   different way liposomes prepared by the extrusion (Lext) or detergent solubilization

404   (Ldet) methodologies. In the case of Ldet, the above mechanisms seem to remain

405   avoiding vesicle condensation. On the contrary, a growth in size was observed in Lext

406   which was especially visible in Lext containing SPC. Although not presented in this

407   study, other liposomes prepared by extrusion and composed of CHO and other

408   phospholipid sources (krill phospholipid extract and egg PC) have been observed to

409   undergo a similar increase in their size in the presence of nauplii (unpublished data).

410   The inclusion of *Artemia* could bring some shedding metabolites, degradation by-

411   products, etc., which may facilitate the aggregation of the Lext formulations contrarily

412   to Ldet. On the other hand, Lext DPPC:CHO liposomes would represent the exception

413   among other Lext formulations since they did not display important changes in their

414   size, as seen in Ldet . The result is probably due to the fact that DPPC molecules would

415   remain in a tightly ordered solid phase at 28 ºC (New, 1990, p. 8), whereas more

416   unsaturated phospholipids (SPC and KPE) would be in a liquid-crystalline phase.

417   Consequently, the membrane fluidity of DPPC liposomes would be reduced and

418   phenomena such as fusion hindered. This hypothesis, valid for pure DPPC liposomes,

419   can also be applicable to the formulation DPPC:CHO because the presence of CHO at

420   molar ratio DPPC:CHO of 2:1 would only displace the phase transition temperature

421   from 41 ºC to 44 ºC (New, 1990, p. 20). Consequently, Lext DPPC:CHO would remain

422   in a solid stable phase at the *Artemia* enrichment temperature of 28 ºC.

423   Despite the fact that some liposomes can exhibit an increase in size during the

424   enrichment process, this cannot be considered as a disadvantage for their use as live

425   prey enrichment diets. Indeed, we studied the efficiency of different types of krill

426   liposomes to enhance the HUFA content in *Artemia* nauplii and found that Ldet

427   enriched nauplii showed the maximal HUFA contents among liposome treatments

428   (Monroig et al., 2006a). These latter results confirmed the usefulness of these small

429   vesicles despite the fact that other studies had proposed that the optimal size of

430   enrichment products has to be higher than that of liposomes assessed in this study

431   (Caers et al., 2000; Gelabert-Fernández, 2002; Han et al., 2005).

432   In summary, the results indicate that liposomes are more stable against oxidation

433   than other commercial products based on fish oil emulsion. Whereas the initial lipid

434   peroxide levels of liposomes and emulsion were similar, their incubation under standard

435   *Artemia* enrichment conditions produced an increase in TBARS in the emulsion up to

436   values three orders of magnitude above those registered by liposomes after 21 h of

437   incubation. Differences of oxidation rates among liposomes are related to the chain

438   length and the unsaturation degree of their fatty acids. Thus, liposomes composed of

439   phospholipids containing long-chain highly unsaturated fatty acids (krill phospholipid

440   extract) were normally less stable than those composed of shorter length-chain and more

441   saturated liposomes.

442   The study of the vesicle size throughout enrichment showed minor changes in

443   the liposome diameter when the incubations were carried out without nauplii. In the

444   presence of nauplii, liposomes do not exhibit notable changes in their size and only SPC

445   extruded liposomes displayed an evident size increase.

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596   Tables

597

598   Table 1. Selected fatty acid (percent of total fatty acids) and lipid class composition

599   (percent of total lipids) of the enrichment products.

	Commercial emulsion	KPE	SPC	DPPC
Fatty acid				
16:0	3.3	25.5	15.1	100.0
18:0	3.0	1.0	3.0	0.0
18:1n-9	10.8	5.4	13.6	0.0
18:2n-6	6.1	1.8	60.6	0.0
18:3n-3	1.4	1.4	3.6	0.0
20:4n-6	1.6	0.7	0.0	0.0
20:5n-3	19.8	29.7	0.0	0.0
22:5n-3	4.8	0.8	0.0	0.0
22:6n-3	25.4	18.4	0.0	0.0
Saturates	8.0	28.6	18.7	100.0
Monounsaturates	25.4	13.2	15.8	0.0
Polyunsaturates	63.0	55.8	64.5	0.0
Total n-3	53.8	52.5	3.8	0.0
Total n-6	9.7	3.2	60.7	0.0
HUFA n-3	51.4	49.3	0.2	0.0
HUFA n-6	2.8	1.4	0.1	0.0
DHA/EPA	1.3	0.6	0.0	0.0
Lipid class				
FAEE/TAG	91.1	0.0	0.0	0.0
PC	0.4	67.0	95.0	100.0
PE	0.0	9.0	0.0	0.0
Other	8.4	24.0	5.0	0.0

600   KPE: krill phospholipid extract; SPC: soybean phosphatidylcholine; DPPC: dipalmitoyl

601   phosphatidylcholine; HUFA n-3: $\geq$20:3n-3; HUFA n-6: $\geq$20:2n-6; DHA/EPA:

602   docosahexaenoic and eicosapentaenoic fatty acid ratio; FAEE/TAG: combined fraction

603   of fatty acid ethyl esters and triacylglycerols; PC: phosphatidylcholine; PE:

604   phosphatidylethanolamine.

605

606   Table 2. Characteristics of liposomes used in the present study.

Name	*Lamellarity*	*Preparation method*	*Composition (w/w)*
Ldet KPE	Unilamellar	Detergent solubilization	KPE 100%
Ldet KPE:vit A	Unilamellar	Detergent solubilization	KPE:retinyl palmitate (100:2)
Ldet SPC	Unilamellar	Detergent solubilization	SPC 100%
Lext SPC:CHO	Unilamellar	Extrusion	SPC:CHO (4:1)
Lext DPPC:CHO	Unilamellar	Extrusion	DPPC:CHO (4:1)

607   KPE: krill phospholipid extract; SPC: soybean phosphatidylcholine; DPPC: dipalmitoyl

608   phosphatidylcholine; CHO: cholesterol; vitA: retinyl palmitate; Ldet: unilamellar

609   vesicles prepared by a detergent solubilization methodology; Lext: unilamellar vesicles

610   prepared by extrusion through a polycarbonate membranes.

611

612   Figure legends

613

614   Figure 1

615   Concentration of TBARS in the *Artemia* enrichment media containing different

616   products. Data correspond to three replicate samples measured at the beginning (t_o) and

617   after 21 h of incubation ($t_o + 21$). Error bars are standard deviations. Significant

618   differences in the TBARS concentration at t_o and $t_o + 21$ h in all the enrichment

619   products are indicated with the symbol "*" ($P \leq 0.05$).

620   Ldet: unilamellar vesicles prepared by a detergent solubilization methodology; Lext:

621   unilamellar vesicles prepared by extrusion through a polycarbonate membranes; KPE:

622   krill phospholipid extract; SPC: soybean phosphatidylcholine; DPPC: dipalmitoyl

623   phosphatidylcholine; CHO: cholesterol; vitA: retinyl plamitate.

624

625   Figure 2

626   Particle size from several liposome formulations Ldet (a, b, and c) and Lext (d and e)

627   incubated under enrichment conditions in the presence and absence of *Artemia* nauplii.

628   Data represent means and error bars are standard deviations (n=3). Treatments with

629   different letters in a sampling point are significantly different from each other ($P \leq 0.05$).

630   Ldet: unilamellar vesicles prepared by a detergent solubilization methodology; Lext:

631   unilamellar vesicles prepared by extrusion through a polycarbonate membranes; KPE:

632   krill phospholipid extract; SPC: soybean phosphatidylcholine; DPPC: dipalmitoyl

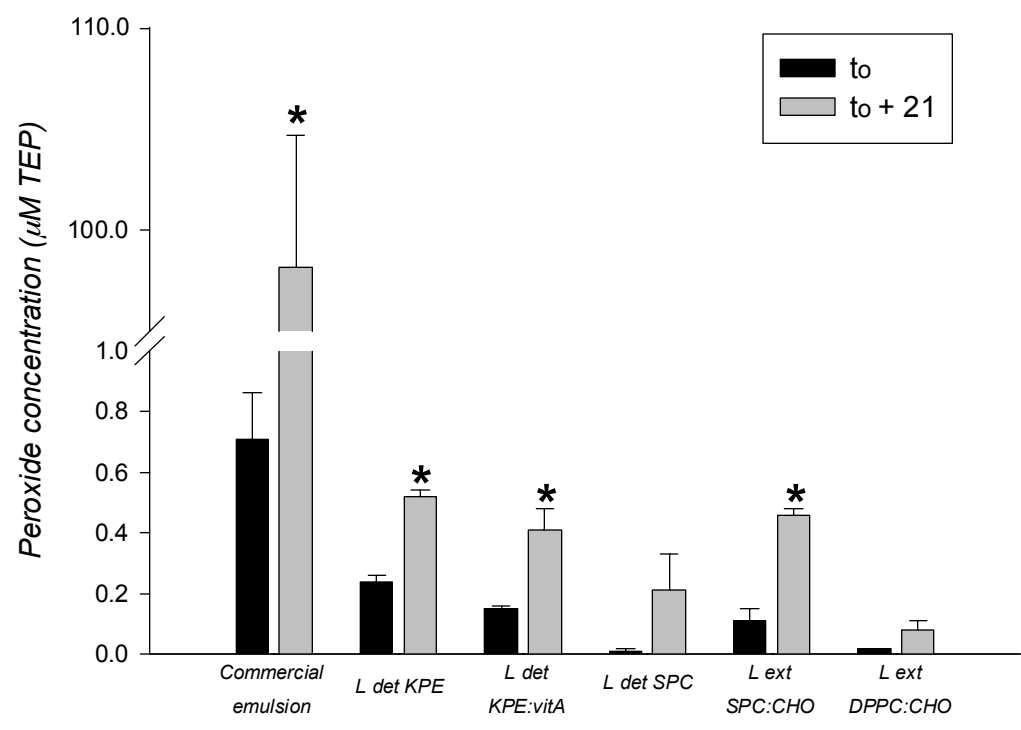633   phosphatidylcholine; CHO: cholesterol; vitA: retinyl plamitate.

634

Figure 1

Figure 2