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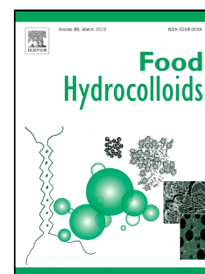

Interaction of vitamin D3 with beta-lactoglobulin at high vitamin/protein ratios: characterization of size and surface charge of nanoparticles

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1 **Interaction of vitamin D3 with beta-lactoglobulin at high vitamin/protein ratios:**
2 **characterization of size and surface charge of nanoparticles**

3

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17 **Abstract**

18 Interaction between vitamin D_3 with beta-lactoglobulin (β-LG) was studied by turbidimetric
19 measurements covering vitamin concentrations up to 500 μM, within a wide range of vitamin
20 concentration and at high vitamin/protein ratios, both conditions that have not been assayed in
21 previous studies. Turbidity of vitamin D_3 in the absence and presence of β-LG (20, 40 and 100 μM)
22 in 20 mM phosphate buffer at pH 7.0 proved the expected vitamin-protein interaction as well as the
23 effect of protein concentration. In order to estimate the proportion of bound vitamin in the vitamin-
24 protein complex, a binding parameter (BP) was defined and its dependence on protein concentration
25 was analysed. Fluorescence quenching with acrylamide for 100 μM vitamin D_3 and 20 μM β-LG in
26 20 mM phosphate buffer at pH 7.0, suggested vitamin D_3 interact in the hydrophobic calix in the
27 protein. Circular dichroism experiments showed the binding of the vitamin causes conformational
28 changes in the secondary protein structure. Particle size and zeta potential determinations were also
29 carried out in order to establish possible conformational models of interaction vitamin-protein. The
30 higher the vitamin concentration, the greater the bound vitamin proportion was; which could be due
31 to a cooperative phenomenon and/or a stacking process. These studies would be useful for a better
32 understanding of the β-LG properties as a carrier of hydrophobic vitamins or other hydrophobic
33 nutraceuticals in order to enrich non-fat foods.

34

35 **Keywords**

36 beta-lactoglobulin; vitamin D_3; binding site; conformational change; stacking process; nanoparticle
37 size

38

39 **1. Introduction**

40 Vitamin D_3 (cholecalciferol) fulfills a primary biological function in human metabolism,
41 maintaining blood calcium and phosphorus concentrations within a physiological range. Recent

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42 studies suggest that this hormone-like vitamin has also other roles, from cells development to
43 immunity mechanisms and protective effect against a variety of diseases, including multiple
44 sclerosis, diabetes or cancer (Delavari et al., 2015; Forrest, Yada, & Rousseau, 2005; Maalouf et al.,
45 2008); Vieth, 2001). Although this vitamin can be physiologically synthesized in the human skin,
46 from 7-dehydrocholesterol via exposure to sunlight, its deficit is not infrequent especially among
47 people who live in high latitudes with reduced sun exposure, dark-skinned people, or those who
48 have a poor dietary intake (Vieth, Cole, Hawker, Trang, & Rubin, 2002). Vitamin D_3 deficit may
49 lead to unhealthy conditions, such as bone or cardiovascular diseases, microbial infections, etc.
50 (Vieth, 2001). Only very few foods are naturally rich in vitamin D, such as cod-liver oil, and small
51 amounts of this vitamin are found in mammalian liver, eggs or dairy products (Diarrassouba et al.,
52 2013; van den Berg, 1997). Therefore, fortified foods and dietary supplements with vitamin D_3 are
53 needed to supply the daily nutritional requirements.
54 However, the incorporation of fat-soluble vitamins like D_3 is a challenge for food industry,
55 especially for those foods with high water content (Diarrassouba et al., 2013). Moreover, vitamin D_3
56 is damaged by light, pressure or oxidation; therefore it should be protected in order to maintain its
57 stability. Milk proteins can be used as nanovehicles for vitamin D_3 by forming hydrophilic vitamin-
58 protein complexes, improving the processes of protection and delivery of this hydrophobic vitamin.
59 Many authors have studied different whey proteins as carriers for vitamin D_3: β-lactoglobulin (β-
60 LG) (Diarrassouba et al., 2013; Forrest et al., 2005; Wang, Allen, & Swaisgood, 1997; Yang, Chen,
61 Chen, Wu, & Mao, 2009), and α-lactalbumin (Delavari et al., 2015); as well as β-casein (Forrest et
62 al, 2005), and whey protein isolate (Abbasi, Emam-Djomeh, Mousabi, & Davoodi, 2014). Several
63 authors have also studied the interactions between the major whey protein β-LG and other
64 hydrophobic vitamins: retinol (Cho, Batt, & Sawyer, 1994), α-tocopherol, (Liang, Tremblay-Hébert,
65 & Subirade, 2011) and vitamin D_2 (Ron, Zimet, Bargarum, & Livney, 2010; Wang et al., 1997).

66 Thus, the role of the main whey protein in milk, β-LG, as a carrier of bioactive molecules has long
67 been established.

68 β-LG is a globular protein with a monomer molecular weight of about 18.3 kDa, with a
69 predominantly β-sheet conformation containing nine antiparallel β-strands from A to I. Eight of
70 these strands (A-H) are twisted into a cone-shaped barrel forming a hydrophobic pocket, with a
71 Trp_{19} at the bottom of this calyx, while the EF loop (residues 85-90) acts as a door at the open end,
72 which is closed or open depending on the pH (Forrest et al., 2005; Sawyer, Kontopidis, & Wu,
73 1999). A second hydrophobic area is located on the protein surface between the α-helix portion
74 (residues 130-140) and β-strand H (residues 121-116). At neutral pH, β-LG exists as a stable dimer,
75 in which β-strand I (residues 146-151) of two monomers is involved in dimer association, and tends
76 to dissociate into monomers at acid pH. Each monomer has two disulfide bonds (Cys66-Cys160 and
77 Cys106-Cys119), which stabilize the protein tertiary structure, and one free sulfhydryl group at
78 position Cys121 (Busti, Scarpeci, Gatti, & Delorenzi, 2005; Kontopidis, Holt, & Sawyer, 2002). It
79 has been reported several sites in β-LG which can bind hydrophobic ligands: a primary binding site
80 in the internal cavity of the β-barrel (central calyx), when pH conditions allow the EF loop to
81 remain open; a surface hydrophobic patch, between the α-helix and the β-barrel; an outer surface
82 hydrophobic patch near Trp_{19}-Arg_{124}, close to the entrance of the β-barrel; and a site shared between
83 the monomers when protein dimers are present (Forrest et al., 2005; Liang & Subirade, 2012;
84 Liang, Tajmir-Riahi, & Subirade, 2008; Narayan, & Berliner, 1997; Wang et al., 1997).

85 Vitamin D_3-β-LG interaction has generally been studied using steady-state protein fluorescence
86 measurements, based on the quenching of vitamin D_3 over Trp and Tyr fluorescence at 280 nm. All
87 these studies had to be carried out at low concentrations of both vitamin and protein, since inner
88 filter and turbidity effects interfere with fluorescence measurements when higher vitamin
89 concentrations are assayed (van de Weert & Stella, 2011). Meredith, Bolt, and Rosenberg (1984)
90 presented evidence that vitamin D_3 forms aggregates with micelle-like organization in aqueous

91 media, but proposed the use of the neutral term “aggregate” rather than “micelle” to describe the
92 supramolecular structure of the vitamin in water. The hydrophobic characteristics of the vitamin
93 promote the formation of dispersed aggregates in water, with sizes of nanoparticles. Several works
94 (Abbasi et al., 2014; Delavari et al., 2015; Ron et al., 2010) have reported sizes of vitamin D_3-
95 protein complexes, but there are not reported results for vitamin particles sizes, in absence of
96 protein.
97 Therefore, in spite of being a known subject, some aspects of the vitamin D_3-β-LG interaction
98 might be still a controversial topic. For instance, high vitamin concentrations have rarely been
99 assayed, and the reported sizes of the complex vitamin D_3-β-LG might be approached from other
100 perspective with a set of new results. In the present work, the interaction between vitamin D_3 and β-
101 LG was studied covering a wider range of vitamin and protein concentrations, with the aim of
102 getting a further knowledge on binding characteristics. For this purpose, turbidimetry, circular
103 dichroism, fluorescence quenching, particle size and ζ-potential measurements were carried out for
104 vitamin D_3 in absence and in presence of the protein. A new approach on the model of interaction
105 between vitamin D_3 and β-LG can be useful to incorporate more efficiently this vitamin to the daily
106 diet by new procedures and may open a new scope of investigation.
107

108 **2. Materials and methods**

109 **2.1. Materials**

110 β-LG AB was purchased from Sigma-Aldrich Chemical Co. (St-Louis, MO, USA). Solid vitamin
111 D_3 (cholecalciferol) was purchased from Zhejiang Garden Biochemical High-Tech, Co., Ltd.
112 (Dongyan, China). These reagents were used without further purification. All other chemical
113 reagents were pro analysis grade.

114 **2.2. Sample preparation**

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115 3 mM β-LG stock solution was made by dissolving the protein in 20 mM phosphate buffer at pH
116 7.0. Different concentrations of β-LG solutions were prepared by diluting the stock solution with
117 the same phosphate buffer. Concentrations were determined by spectrophotometry with a molar
118 extinction coefficient of 13,900$M^{-1}cm^{-1}$ at 280 nm. Vitamin D_3 was dissolved in ethanol to prepare
119 10 mM stock solution. Vitamin concentration was determined by spectrophotometry at 265 nm with
120 molar extinction coefficients of 18,900$M^{-1}cm^{-1}$.

121 **2.3. Turbidimetric measurements**

122 Turbidity (τ) in suspensions of particles is a measure of the reduction of intensity of the transmitted
123 beam, due to the scattering phenomenon, and it can be defined as: $\tau = 2.303\ Abs_{\lambda}$, where Abs_{λ} is the
124 absorbance of the sample at the incident wavelength λ.

125 Consecutive aliquots of vitamin D_3 stock solution were added to 3.0 mL of 20, 40 and 100 µM β-
126 LG solutions in 20 mM phosphate buffer at pH 7.0. After each addition, turbidity were estimated by
127 measuring absorbance at 500 nm (A_{500}), covering a vitamin concentration range up to 500 µM.
128 Turbidity values of vitamin solution in the same concentration range were also estimated in the
129 absence of the protein. Turbidity values were plotted versus vitamin concentration and fitted using
130 linear or nonlinear regressions. Spectrophotometric measurements were determined using a Jasco
131 V-550 (Jasco International Co., Tokyo, Japan) spectrophotometer. Triplicate measurements were
132 taken for all samples, at a controlled temperature of 25 °C.

133 **2.4. Particle size and zeta potential measurements**

134 Particle size analysis experiments were carried out using a Dynamic Laser Light Scattering (DLS)
135 instrument (Horiba, Nano Particle Analyzer SZ-100, Kyoto, Japan). The hydrodynamic diameter is
136 the diameter of an equivalent sphere that diffuses at the same average rate as the particle under
137 examination. The mean hydrodynamic diameter was obtained from the peak of scattered light
138 intensity distribution (Arzenšek, Podgornik, & Kuzman, 2010; Hiemenz & Rajagopalan, 1997).
139 Electrical charge (ζ-potential) of protein solutions, vitamin solutions and vitamin-protein systems

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140 were determined using micro-electrophoresis measurements. Before measurements, samples were
141 filtered through 1 µm microfilters (Whatman International Ltd., Maidstone, Kent, UK) and
142 immediately used. DLS and ζ-potential determinations were carried out for suspensions of vitamin
143 D_3 at different concentrations, from 20 µM to 500 µM, in absence and in presence of 20, 40 and 100
144 µM β-LG solutions in 20 mM phosphate buffer at pH 7.0. Measurements were carried out in
145 triplicate. The temperature was set at 25 °C.

146 **2.5. Binding parameter**

147 In order to estimate the proportion of bound vitamin in the complex vitamin-protein, a binding
148 parameter (BP) can be defined by the following equation (Sponton, Perez, Carrara, & Santiago,
149 2015a):

150 $$BP\ (\%) = [(\tau_V - \tau_{VP}) / \tau_V] \times 100 \quad (1)$$

151 where τ_V is turbidity of vitamin D_3 in buffer and τ_{VP} is turbidity of vitamin D_3 in presence of β-LG.
152 From turbidimetric results one different BP for each vitamin concentration can be calculated,
153 considering values of corresponding linear and nonlinear fitting curves.

154 **2.6. Circular dichroism**

155 In order to study the effects on protein secondary structure, caused either by ethanol or by the
156 vitamin itself, far UV-CD (195-240 nm) spectra were obtained for 20 µM protein solutions in
157 buffer, in a 0.1 cm path length cell, using a Jasco J-810 spectropolarimeter (Jasco International Co.,
158 Tokyo, Japan). Results were reported as mean residue ellipticity ($[\theta]_{MRW}$) in units of deg cm^2 $dmol^{-}$
159 ^{1}, using a mean residue weight of 113 g.

160 Possible solvent effects on β-LG were studied in the absence and presence of 4% and 8% (v/v)
161 ethanol and the changes on protein structure that the vitamin could have induced were investigated
162 at 100 µM vitamin D_3 concentration. Each spectrum was the accumulation of five successive

163 measurements and the baseline was corrected by subtracting buffer spectrum. All experiments were
164 carried out three times.

165 **2.7. Fluorescence quenching**

166 Quenching of protein intrinsic fluorescence by acrylamide was studied for a vitamin-protein
167 solution (100 μM vitamin D_3, 20 μM β-LG, 20 mM phosphate buffer at pH 7.0) by addition of
168 aliquots of acrylamide 5 M, with an acrylamide concentration up to 0.2 mM. Intrinsic fluorescence
169 intensity (F) at 336 nm was measured after each addition of quencher aliquots in a Jasco FP 770
170 spectrofluorometer (Jasco International Co., Tokyo, Japan) using excitation at 295 nm (Moro, Gatti,
171 & Delorenzi, 2001). All experiences were carried out three times, at 25 °C.
172 Stern-Volmer constants (Kapp) were calculated from the slopes of the graphs F_0 / F vs. acrylamide
173 concentration, based on the equation:

174 $$F_0 / F = 1 + K_{app} [acrylamide] \qquad (2)$$

175 where Kapp is an apparent constant for proteins with more than one tryptophanyl residue that can
176 be quenched by acrylamide.

177 **2.8. Statistical analysis**

178 Statistical analysis was performed using Statgraphics Plus for Windows (Manugistics Inc,
179 Rockville, MA, USA). Analysis of variance (ANOVA) was used and when the effect of the factors
180 was significant (*p < 0.05*), the test of multiple ranks honestly significant difference (HSD) of Tukey
181 was applied (95% of confidence level).
182

183 **3. Results and discussion**

184 **3.1. Turbidimetric measurements: interaction between vitamin D_3 and β-LG**

185 The interaction of vitamin D_3 with β-LG was studied by turbidimetry for a wider range of
186 concentration than those used in the cited previous work (Forrest et al., 2005; Wang et al., 1997),
187 covering vitamin concentrations up to 500 μM and protein concentrations up to 100 μM. It was

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188 observed that turbidity continuously increased when successive aliquots of vitamin D_3 solution were
189 added, both in the absence and presence of β-LG (Figure 1). The nanoparticles of vitamin
190 aggregates (Meredith et al., 1984) are responsible for this increment of turbidity, showing a direct
191 relationship between vitamin concentration and the values of turbidity.
192 As can also be seen in Figure 1, the turbidity of vitamin D_3 solutions diminished when β-LG was
193 present, for all the assayed range of vitamin concentration. Moreover, the higher the protein
194 concentration, the more important this reduction was. Similar results from turbidimetric
195 measurements were reported by other authors for α-tocopherol-β-LG interactions (Liang et al.,
196 2011), or for linoleic acid-ovoalbumin interactions (Sponton, Perez, Carrara, & Santiago, 2015b).
197 All these results prove the interaction between vitamin D_3 and β-LG, where the protein captures
198 some proportion of vitamin to form hydro-soluble complexes (Forrest et al., 2005; Wang et al.,
199 1997; Yang et al., 2009) which have no significant contribution to the turbidity, and then lead to the
200 turbidity decrease.
201 Several species are present in the studied systems, such as: single molecules of the hydrophobic
202 vitamin, nanoparticles of aggregated vitamin, monomers of β-LG, dimers of β-LG, and particles of
203 vitamin D_3-protein complexes (Cogan, Kopelman, Mokady, & Shinitzky, 1976; Liang et al., 2011).
204 For suspensions like these, in which particles are different in size and shape, turbidity is the result
205 of contributions from each type of particle (Hall et al., 2016). Particle size and shape should be
206 taken into account and then, further studies on size and zeta potential were determined in order to
207 better understand these turbidimetric results.

208 **3.2. Particle size and zeta potential measurements**

209 All the obtained curves of intensity distribution were monomodal. Table 1 shows the values of
210 mean hydrodynamic diameter from DLS experiments for suspensions of vitamin D_3 at different
211 concentrations up to 500 μM, in the absence and presence of different concentrations of β-LG (20,
212 40 and 100 μM). All assayed vitamin samples presented mean hydrodynamic diameter higher than

213 100 nm, confirming the vitamin aggregates present nanoparticle dimensions. Mean hydrodynamic
214 diameters significantly increased with the concentration of vitamin D_3, independently of the
215 presence of β-LG or its concentration ($p < 0.05$). Therefore, the size of the vitamin aggregates
216 seems to be determined only by the vitamin concentration and the presence of the protein does not
217 affect it. Thus, the observed turbidity decrease in the presence of the protein may be due to a lower
218 number of nanoparticles of vitamin aggregates, since their size remains constant.
219 Figure 2 shows the intensity distribution of particle sizes for vitamin D_3, β-LG, and vitamin D_3-β-
220 LG samples. While β-LG curve presented a peak at approximately 7 nm, both vitamin D_3 and
221 vitamin D_3-β-LG samples showed the same particle size with peaks at approximately 130 nm.
222 Several authors reported sizes of different vitamin D_3-protein complexes, with values from 100 nm
223 to over 200 nm (Abbasi et al., 2014; Delavari et al., 2015). However, it should be noted that the size
224 of the nanoparticles of vitamin aggregates, in the absence of protein, has not been considered in
225 these previous works. In DLS technique, small particles may be underestimated due to the fact that
226 large particles scatter light with a greater intensity than the small ones (Hiemenz & Rajagopalan,
227 1997). According to the results of the present work (Table 1, Figure 2), the nanoparticles of vitamin
228 aggregates, very much larger than the protein molecule, could make the smaller particles remain
229 undetected, and DLS reported size values of 100 nm or 200 nm would correspond to the
230 nanoparticles of vitamin aggregates, rather than to the vitamin-protein complexes.
231 Forrest et al. (2005) worked with lower concentrations of vitamin D_3 (from 5 μM to 20 μM), and
232 7.38 μM β-LG, and they reported apparent mole ratios of ligand to protein (n) of about 1.3 for the
233 complex vitamin D_3-β-LG at pH 6.6. Although particle size determination was not an aim of this
234 work, the informed vitamin/protein proportion implies that both the complex and the protein must
235 have similar sizes. In the present work, these experimental conditions were reproduced in order to
236 determine nanoparticles sizes. The size values obtained were 104 ± 31 nm and 113 ± 34 nm for 5
237 μM and 20 μM vitamin solutions, respectively. These results strengthen the interpretation that

238 nanoparticle dimensions like these correspond to the vitamin aggregates and not to the vitamin-
239 protein complex.
240 Table 2 shows ζ-potential of suspensions of vitamin D_3, at different concentrations, from 20 μM to
241 500 μM, in the absence and presence of different β-LG concentrations. All ζ-potential presented
242 negative values and showed no significant changes when either vitamin or protein concentrations
243 increased ($p<0.05$). Although the vitamin does not have charge, these negative ζ-potentials can be
244 due to the presence of a negative surface charge at the interface water-nanoparticle of vitamin
245 aggregates, caused by the rapid adsorption of hydroxyl ions from the aqueous environment
246 (Marinova et al., 1996). Thus, nanoparticles of vitamin D_3 seem to be present as a supramolecular
247 structure stabilized by electrostatic repulsion and Brownian movement. On the other hand, ζ-
248 potential of β-LG was also negative (-13.5 ± 2.1 mV), hindering the interaction with negatively
249 charged nanoparticles of vitamin. Since vitamin nanoparticle sizes have not changed in the presence
250 of the protein, interactions between β-LG and vitamin D_3 to form vitamin-protein complexes would
251 occur as an independent and simultaneous process when vitamin is added, between the protein and
252 the non charged vitamin molecules, rather than one in which the protein removes vitamin from the
253 previously formed supramolecular vitamin structures.

254 **3.3. Binding parameter**

255 Turbidimetry and DLS are both light scattering techniques, in which the light scattered by larger
256 particles is more important than the one scattered by smaller ones (Hiemenz & Rajagopalan, 1997).
257 For the systems assayed in this work, vitamin D_3 nanoparticles are responsible for turbidity and for
258 any changes in turbidity (Figure 1). Being λ the wavelength of the incident beam, particles of sizes
259 larger than λ/20, follow a non-Rayleigh behaviour and shape and size correction factors must be
260 introduced (Camerini-Otero & Day, 1978; Camerini-Otero, Franklin, & Day, 1974; Doty & Edsall,
261 1951; Doty & Steiner, 1950; Hall et al., 2016; Yang & Hogg, 1979). The DLS experiences carried
262 out in the present work showed that vitamin nanoparticles were large enough to add those

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263 correction factors. Since hydrodynamic diameters were approximately constant for each vitamin
264 concentration, in the absence or presence of β-LG (Table 1), at each vitamin concentration the
265 shape and size correction factors would be the same and can be cancelled. Then, τ is directly
266 proportional to N (number of nanoparticles) for each vitamin concentration, and Equation 1
267 becomes Equation 3:

268 $$BP\ (\%) = [(N_V - N_{VP}) / N_V] \times 100 \quad (3)$$

269 where N_V and N_{VP} are the number of nanoparticles of vitamin D_3 /volume unit in the absence and
270 presence of β-LG, respectively.

271 For a proper understanding of BP and BP changes, vitamin concentration, vitamin/protein ratio and
272 the rates of increase of each type of N should be considered. BP vs. vitamin D_3 concentration
273 (Figure 3) shows different curves depending on protein concentration and all of them should be
274 explained through Equation 3.

275 Forrest et al. (2005) reported that vitamin/protein ratio is ~1.5, between pH 6.6 and 8.0, and at the
276 saturation point. They also proposed there are different sites to bind vitamin molecules in the
277 protein: within the central cavity of β-LG, at the surface patch, and/or in the protein dimer. In the
278 present work, that vitamin/protein saturation ratio was verified for β-LG 100 μM and vitamin
279 concentration up to 200 μM (Figure 3), where BP diminished with vitamin concentration. This can
280 be explained by supposing a saturation state, where the binding sites have already been occupied
281 and subsequent additions of vitamin contribute to increase the number of non-bound vitamin
282 nanoparticles (N_{VP}), leading to a decrease of BP (Figure 3 and Equation 3), for this range of vitamin
283 concentration.

284 For the two lower protein concentrations (20 μM and 40 μM), vitamin/protein ratios were always
285 higher than 1.5 and saturation state was verified for all vitamin concentrations. The lower the
286 protein concentration, the higher the non-bound vitamin concentration and the rate of increase of
287 N_{VP} are. Thus, in the same range of 100-200 μM vitamin, BP values remained approximately

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288 constant for 40 μM β-LG, suggesting N_{VP} and N_V increase at the same rate, while BP increased for
289 20 μM β-LG due to a higher rate of increase for N_{VP} than for N_V (Figure 3 and Equation 3).
290 At vitamin concentrations higher than 200 μM, and for all three assayed protein concentrations, BP
291 values increased with vitamin concentration suggesting a different process of binding, which would
292 allow β-LG to bind of an increasing number of vitamin molecules.

293 **3.4. Circular dichroism: influence of solvent and vitamin on β-LG structure**

294 In this work, ethanol concentrations were < 5% (v/v) at the end of each titration. Figure 4 shows no
295 significant changes between protein far UV-CD spectra in the absence and the presence of ethanol
296 up to 8% (v/v), suggesting that the solvent added with the vitamin has no effect on the secondary
297 structure of the protein.
298 The far UV-CD spectrum of native β-LG is typical of a protein with an anti-parallel β-structure with
299 a broad negative minimum at 215 nm, as it has been reported by several authors (Mehraban,
300 Yousefi, Taheri-Kafrani, Panahi, & Khalafi-Nezhad, 2013; Mohammadi, Sahihi, & Khalegh
301 Bordbar, 2015). On the other hand, proteins which are mostly α-helix structure show two minima at
302 208 and 222 nm. The interaction between vitamin D_3 and some α-helix proteins such as insulin
303 leads to a decrease in the size of the negative CD signal within 200-230 nm (Soleymani et al.,
304 2016).
305 Figure 5 shows changes in the far UV-CD spectra when native β-LG is in presence of vitamin D_3.
306 Since the major change occurred between 210-215 nm and no modifications were observed near
307 222 nm, it can be assumed that the binding of the vitamin caused perturbations in the protein β-
308 sheet structure rather than in the α-helix portion. To sum up, although ethanol has not caused any
309 significant conformational change on the protein, the vitamin itself has.

310 **3.5. Fluorescence quenching: vitamin binding effect**

311 Fluorescence quenching by acrylamide is an accepted method to estimate protein surface
312 hydrophobicity and it is related to the tryptophan accessibility to the quencher (Moro et al., 2001).

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313 The more flexible and accessible the protein structure, the more pronounced the slope in the Stern-
314 Volmer plot and the greater the K_{app} (Moro et al., 2001; Moro, Báez, Busti, Ballerini, & Delorenzi,
315 2011; Palazolo, Rodríguez, Farruggia, Picó, & Delorenzi, 2000). It has been reported (Weber, &
316 Senior, 2000; Wells, Nakazawa, Manabe, & Song, 1994) changes in tryptophan accessibility due to
317 the conformational changes when a ligand binds a protein.

318 Trp_{19} is the main responsible amino acid residue of bovine β-LG fluorescence (Albani, Vogelaer,
319 Bretesche, & Kmiecik, 2014). Fluorescence quenching by acrylamide was carried out at the
320 wavelengths where the intrinsic fluorescence of native β-LG depends on Trp_{19} accessibility. Figure
321 6 shows the Stern-Volmer plots for native β-LG in the absence and presence of vitamin D_3, with
322 K_{app} values were 1.67 ± 0.02 M^{-1} and 1.34 ± 0.02 M^{-1}, respectively. The decrease in K_{app} value
323 suggests the bound vitamin produces some protective effect on the fluorophore, maybe due to a
324 conformational rearrangement around Trp_{19} in the hydrophobic cavity, which hinders the
325 fluorescence quenching process.

326 **3.6. Interaction vitamin-protein**

327 At low vitamin concentrations, CD experiments showed vitamin binding affected the secondary
328 structure of protein and fluorescence quenching showed the vitamin interacts with Trp19, which
329 suggest a relatively conserved bottom calyx structure.

330 On the other hand, at higher vitamin concentrations and higher vitamin/protein ratios, the
331 continuous increase of BP showed that the interaction vitamin-protein allowed β-LG to bind an
332 increasing number of vitamin molecules. This could be due to an increasing number of available
333 hydrophobic sites on the protein surface, suggesting a cooperative phenomenon caused by the
334 bound vitamin. Some authors have reported cooperative binding between different ligands and β-
335 LG (Zhang, Liu, Subirade, Zhou, & Liang, 2014). An alternative explanation could be a stacking
336 process of the planar vitamin molecules on a limited and constant number of hydrophobic sites on
337 the protein. In this regard, some authors have reported that a great number of planar molecules, such

338 as polyphenols or others amphiphilic molecules, bind β-LG by a stacking process (Charlton et al.,
339 2002; von Staszewski, Pizones Ruiz-Henestrosa, & Pilosof, 2014). A third possibility could be that
340 the cooperative processes as well as the stacking processes were both responsible for the high BP
341 values.
342

343 **4. Conclusions**

344 When vitamin D3 is added to an aqueous environment, such as a β-LG solution in buffer, the
345 vitamin molecules follow two independent and simultaneous processes: an aggregation process,
346 forming nanoparticles of vitamin aggregates, a supramolecular structure which remains in
347 suspension; and a process of binding to the protein, producing a hydrophilic vitamin-protein
348 complex. Vitamin D3 molecules, with a non charged structure, interact with the hydrophobic site in
349 the calyx of β-LG as well as with some hydrophobic surface patches of the protein. On the other
350 hand, both nanoparticles of aggregated vitamin and protein molecules have a negative surface
351 charge, measured by ζ-potential, hindering the interaction between them. Thus, the formation of the
352 vitamin-protein complex would be result of this independent process of binding, rather than one in
353 which the protein removes vitamin from the nanoparticles of vitamin aggregates.

354 In presence of the protein, the size of the vitamin nanoparticles in suspension does not change, but
355 the number of them is lower and therefore, the turbidity decreased. The higher the protein
356 concentration, the more important this decrease is. The sizes of nanoparticles aggregated vitamin
357 are independent of the presence of the protein and much larger than the vitamin-protein complexes,
358 which remain undetectable for certain techniques, such as DLS.

359 The binding of the vitamin causes conformational changes in the secondary structure of the protein,
360 which would lead to an increasing number of bound vitamin molecules. Different models of
361 vitamin-protein interaction could explain these results: a cooperativity process caused by the bound
362 vitamin which produces an increasing number of available hydrophobic sites on the protein surface;

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363 and/or a model of stacking of the vitamin molecules on a constant number of hydrophobic sites in
364 the protein.
365 These studies would be useful for a better understanding of the β-LG properties as a carrier of
366 hydrophobic vitamins or other hydrophobic nutraceuticals in order to enrich foods.
367

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375

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493

494 **Figure captions**

495

496 **Figure 1.** Turbidity vs. vitamin D_3 concentration in the absence (●) and presence of β-LG: 20 μM
497 (Δ), 40 μM (□) and 100 μM (■). Error bars were calculated from the standard error of three
498 replicates. Lines represent the nonlinear and linear fits.

499

500 **Figure 2.** Intensity distribution for: 100 μM β-LG (solid line), 500 μM vitamin D_3 (dashed line) and
501 500 μM vitamin D_3 in presence of 100 μM β-LG (dotted line) at 25 ºC and pH 7.0.

502

503 **Figure 3.** Binding parameter (BP) vs. vitamin D_3 concentration for different β-LG concentrations:
504 20 μM (Δ), 40 μM (□) and 100 μM (■).

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506 **Figure 4.** Far UV-CD spectra of 20 μM β-LG in the absence of ethanol (solid line) and presence of
507 4% (v/v) ethanol (dashed line) and 8% (v/v) ethanol (dotted line).
508
509 **Figure 5.** Far UV-CD spectra of 20 μM β-LG in the absence of vitamin D_3 (solid line) and presence
510 of 100 μM vitamin D_3 (dashed line).
511
512 **Figure 6.** Stern-Volmer plots of 20 μM β-LG fluorescence quenching by acrylamide, in the absence
513 (●) and presence (○) of 100 μM vitamin D_3. Error bars were calculated from the standard error of
514 three replicates.
515
516

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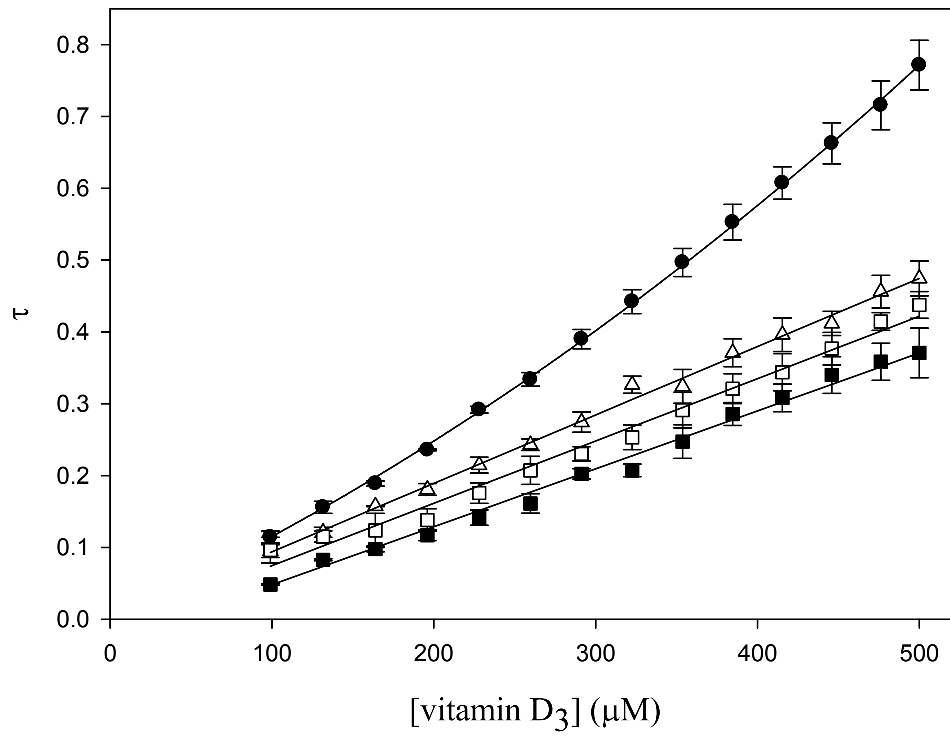

Figure 1

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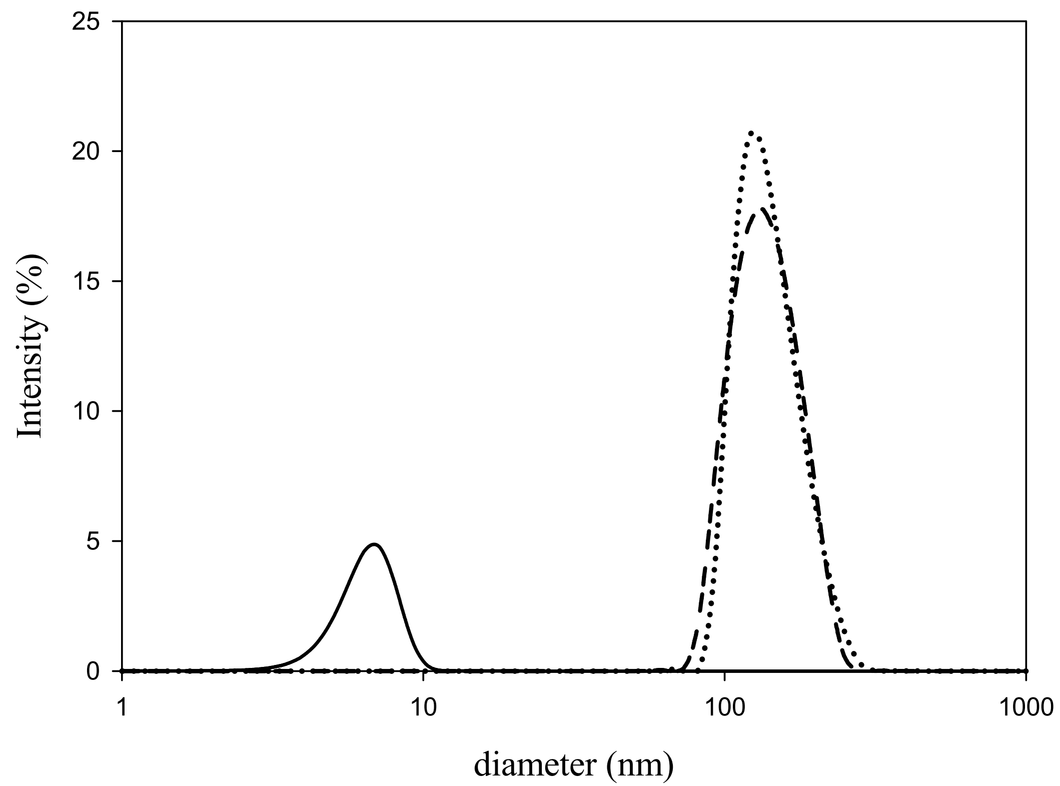

Figure 2.

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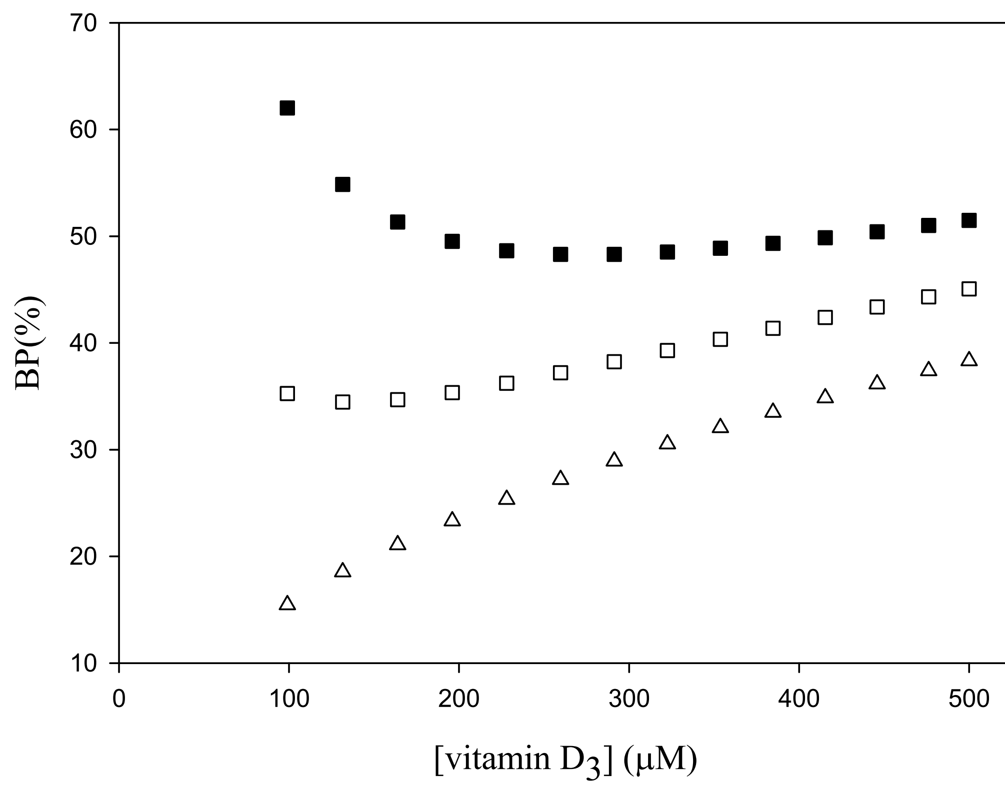

Figure 3.

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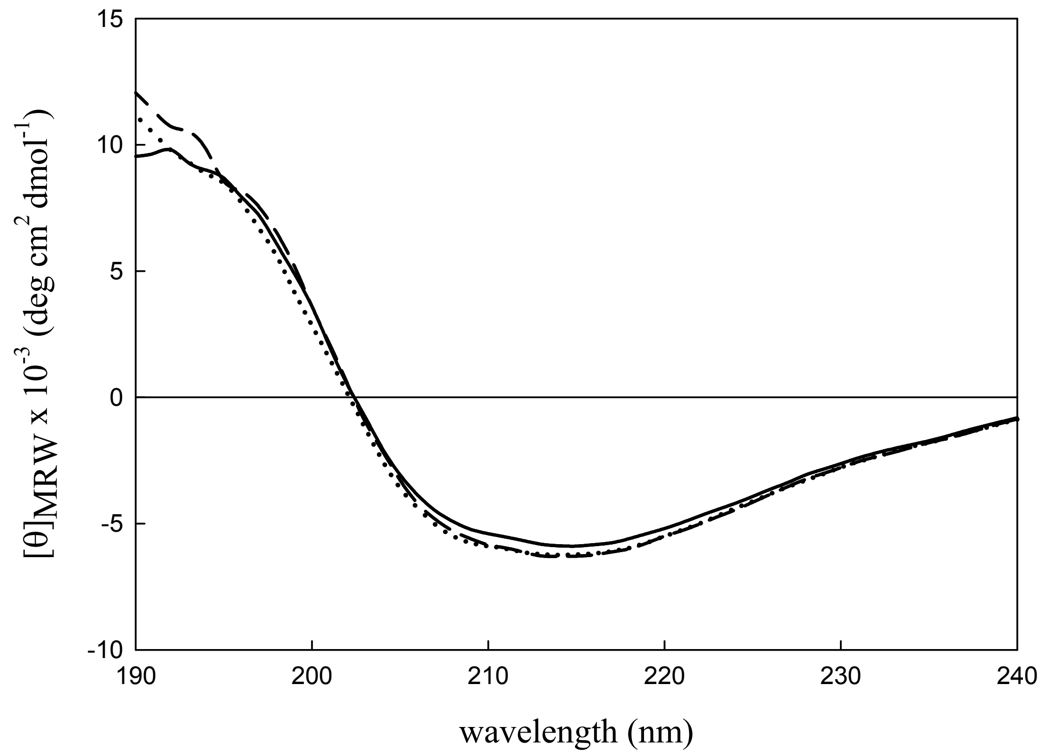

Figure 4.

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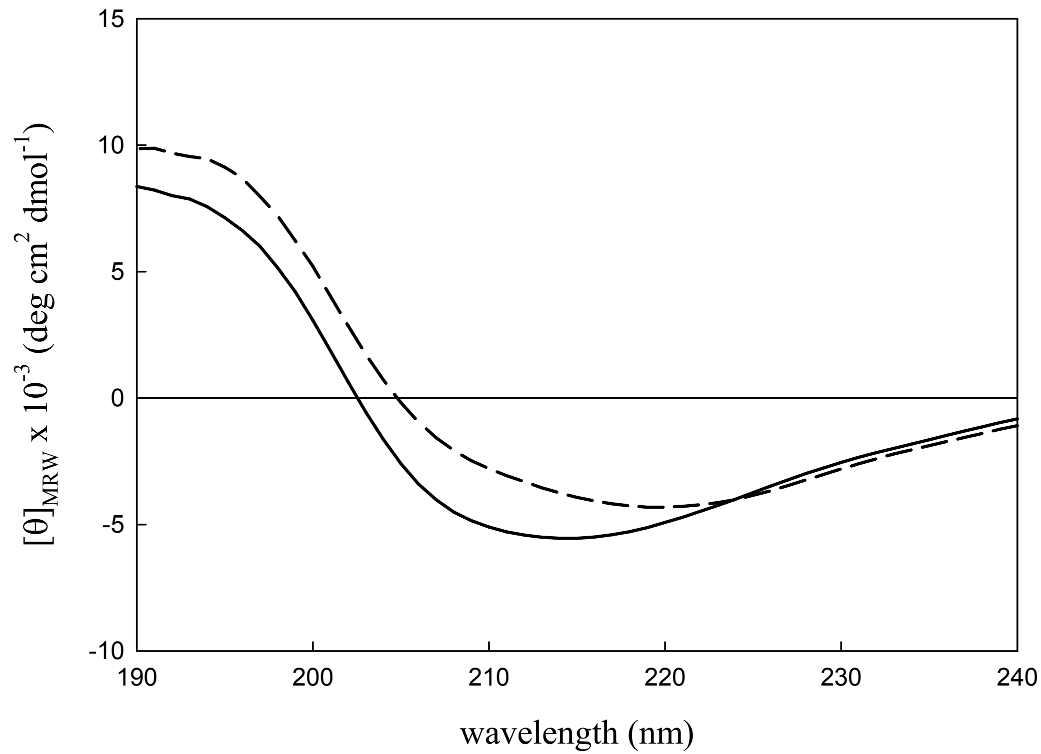

Figure 5.

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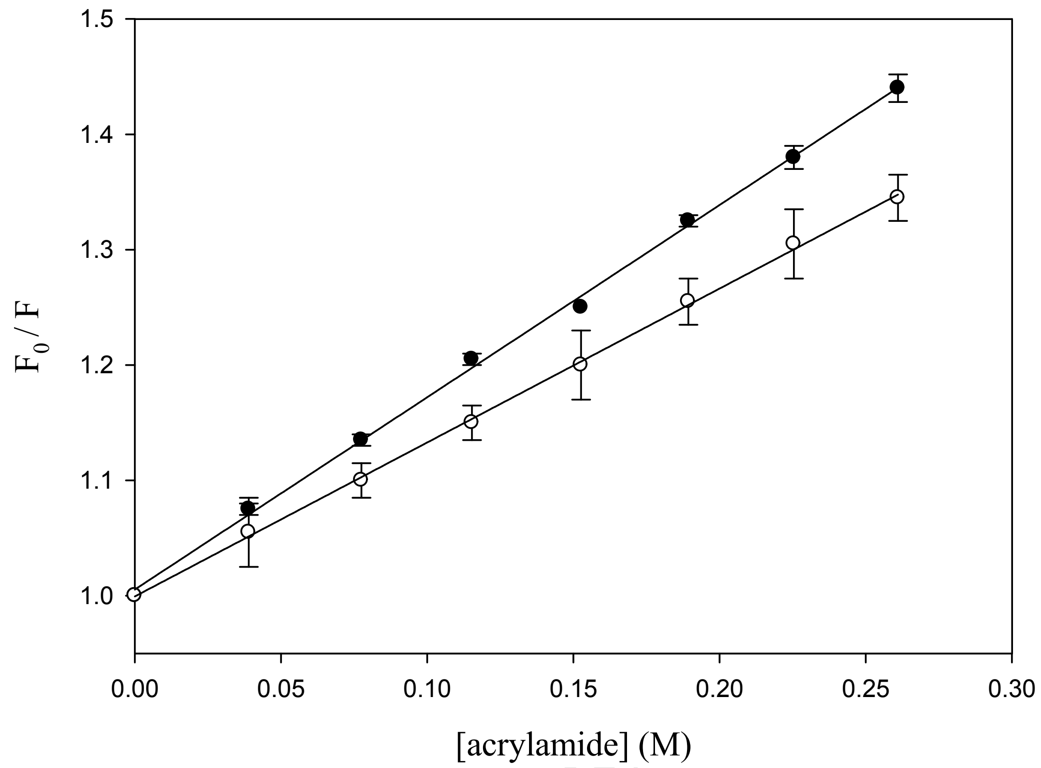

Figure 6.

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Nanoparticles of vitamin and vitamin-protein complex are formed independently.

Turbidity decrease is due to a lower number of nanoparticles of vitamin aggregates.

The higher the vitamin concentration, the more important the binding is.

Vitamin-protein complexes remain undetectable for DLS technique.

Stacking and/or cooperative phenomena may occur at high vitamin concentrations.

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β-lactoglobulin

vitamin D_3

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Table 1. Mean hydrodynamic diameter (nm) for vitamin D_3 nanoparticles in presence of different concentrations of β-LG.

vitamin D_3 (μM)	β-LG (μM)			
	0	20	40	100
20	100 ± 10^a	100 ± 9^a	101 ± 5^a	102 ± 7^a
100	135 ± 13^b	138 ± 21^b	137 ± 18^b	133 ± 13^b
200	140 ± 7^b	140 ± 26^b	141 ± 23^b	141 ± 11^c
300	155 ± 3^c	161 ± 4^c	160 ± 5^c	154 ± 3^d
500	161 ± 20^c	163 ± 29^c	160 ± 27^c	157 ± 9^d

Values are the mean ± standard deviation. Different letters in the same column indicate significant differences ($p<0.05$).

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Table 2. ζ-potential (mV) for vitamin D_3 nanoparticles in function of β-LG concentration.

vitamin D_3 (μM)	β-LG (μM)			
	0	20	40	100
20	-18.0 ± 0.6[a]	-18.9 ± 3.0[a]	-19.1 ± 2.9[a]	-20.1 ± 0.9[a]
100	-18.3 ± 3.4[a]	-18.2 ± 4.8[a]	-18.0 ± 5.1[a]	-17.7 ± 4.3[a]
200	-17.1 ± 2.3[a]	-17.4 ± 2.3[a]	-18.0 ± 4.0[a]	-21.6 ± 5.4[a]
300	-17.5 ± 2.5[a]	-17.8 ± 3.1[a]	-17.5 ± 3.5[a]	-18.0 ± 2.1[a]
500	-18.8 ± 2.0[a]	-18.2 ± 5.4[a]	-17.0 ± 6.0[a]	-15.3 ± 4.0[a]

Values are the mean ± standard deviation. Different letters in the same column indicate significant differences ($p<0.05$).

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