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1 **Binding analysis between monomeric  $\beta$ -casein and hydrophobic**  
2 **bioactive compounds investigated by surface plasmon resonance and**  
3 **fluorescence spectroscopy**

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5 Abbreviated running title:  $\beta$ -casein-hydrophobic **bioactive compound** binding

6

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26 **Abstract**

27  $\beta$ -casein, a phosphoprotein representing 37 % of the bovine milk caseins, has specific  
28 features promoting its application as a nanocarrier for hydrophobic bioactives. In this  
29 study, the interactions of  $\beta$ -casein with curcumin and vitamin D3 under the same  
30 physico-chemical conditions were investigated. The interaction kinetics have been  
31 studied by surface plasmon resonance (SPR) and fluorescence spectroscopy. The  $K_D$   
32 value for curcumin- $\beta$ -casein interaction has been successfully evaluated ( $4.1 \pm 0.7 \times 10^{-4}$   
33 M) using SPR by fitting data to a 1:1 Langmuir interaction model. Conversely, the  
34 SPR responses obtained for vitamin D3 show that the interactions between this  
35 hydrophobic compound and the  $\beta$ -casein immobilized on the sensor chip were below  
36 the sensitivity of the SPR apparatus. Moreover, the fluorescence quenching data show  
37 that curcumin has higher affinity to  $\beta$ -casein ( $K_A = 23.5 \pm 1.9 \times 10^4 \text{ M}^{-1}$ ) than vitamin  
38 D3 ( $K_A = 5.8 \pm 1.1 \times 10^4 \text{ M}^{-1}$ ).

39

40 **Highlights:**

- 41 • The interactions between monomeric  $\beta$ -casein and two hydrophobic bioactive  
42 molecules, curcumin and vitamin D3, were evaluated.
- 43 • These interactions were investigated by surface plasmon resonance and  
44 fluorescence quenching.
- 45 • Curcumin has a higher binding affinity to  $\beta$ -casein compared to vitamin D3.

46

47 **Keywords:**  $\beta$ -casein, curcumin, vitamin D3, surface plasmon resonance, fluorescence  
48 quenching, binding parameters.

49

50 **Chemical compounds studied in this article**

51 Curcumin (PubChem CID: 969516); Vitamin D3 (PubChem CID: 5280795)

52

### 53 **1. Introduction**

54 Over the past years, there has been a high demand for incorporation of biologically  
55 active natural ingredients into functional food to contribute to consumer's well-being  
56 (Vieira da Silva, Barreira, & Oliveira, 2016). However, most of these bioactives display  
57 low solubility, poor stability during processing, and low permeability in the  
58 gastrointestinal tract. Therefore, different encapsulation strategies have been developed  
59 in order to avoid these limitations and protect these molecules during processing and  
60 storage, maintaining their active molecular form until consumption and delivery to the  
61 physiological target within the organism (Okuro, Furtado, Sato, & Cunha, 2015). Milk  
62 proteins, especially caseins, have unique and interesting functional properties making  
63 them effective nanocarriers for bioactives (Ranadheera, Liyanaarachchi, Chandrapala,  
64 Dissanayake, & Vasiljevic, 2016). As casein micelles is a natural self-assembly, some  
65 studies investigated the binding of hydrophobic compounds such as curcumin to native  
66 casein micelles (Sahu, Kasoju, & Bora, 2008) or to modified casein micelles (Benzaria,  
67 Maresca, Taieb, & Dumay, 2013). Among the four different caseins,  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -  
68 caseins, a particular interest is paid to  $\beta$ -casein. This phosphoprotein, composed of 209  
69 amino acids and with a molecular weight  $\sim$  24 kDa, constitutes about 37% of the  
70 caseins in bovine milk and is the most hydrophobic casein thanks to its hydrophobic C-  
71 terminal tail rich in proline residues. Consequently, several studies have shown that  $\beta$ -  
72 casein can be used as a natural nanocarrier for hydrophobic bioactive molecules in order  
73 to increase their bioavailability (Semo, Kesselman, Danino, & Livney, 2007; Shapira,  
74 Davidson, Avni, Assaraf, & Livney, 2012). Particularly, the separation between its  
75 hydrophobic C-terminal domain and its hydrophilic highly charged N-terminal domain

76 containing an anionic phosphoserine cluster makes it highly amphiphilic allowing the  $\beta$ -  
77 casein self-association in small oblate micelles (Holt & Sawyer, 1993; Swaisgood,  
78 2003). The intermolecular interactions to form  $\beta$ -casein micelles occur at concentrations  
79 above the critical micellization concentration (CMC) of  $\sim 0.5 - 2$  mg/mL at milk pH  
80 (6.8) and room temperature (O'Connell, Grinberg, & De Kruif, 2003). Several studies  
81 have investigated the encapsulation of hydrophobic molecules inside the hydrophobic  
82 core of  $\beta$ -casein micelles and have shown an improvement of the efficiency of poorly  
83 water-soluble compounds using this nanocarrier system (Esmaili et al., 2011;  
84 Mehranfar, Bordbar, Fani, & Keyhanfar, 2013). At the same time, some studies have  
85 focused on the investigation of the binding between  $\beta$ -casein and hydrophobic bioactive  
86 molecules such as lipophilic vitamins (Forrest, Yada, & Rousseau, 2005), flavan-3-ol  
87 derivatives (Bohin, Vincken, Van Der Hijden, & Gruppen, 2012) or naringenin  
88 (Moeiniafshari, Zarrabi, & Bordbar, 2015). The fluorescence spectroscopy is a  
89 commonly method used in these studies to evaluate the interaction between the  
90 hydrophobic bioactive compound and the protein. Indeed, fluorescence quenching  
91 analysis, recognized as a highly sensitive methodology with a fast and easy  
92 implementation, evaluates the changes in the local environment of the fluorophore and  
93 tryptophan residues, as a consequence of molecular interaction (Lakowicz, 2006).

94 Molecular interaction affinities can also be investigated using surface plasmon  
95 resonance (SPR). SPR is recognized as a technique giving the ability to detect highly  
96 dynamic complexes being difficult to investigate using other techniques (Douzi, 2017).

97 This well-suited label-free optical technique for affinity characterization provides real-  
98 time monitoring of binding kinetics. The method is based on the immobilization of one  
99 of the binding partners on a functionalized sensor surface. The other partner, free in  
100 solution, is then injected over the surface containing the immobilized molecule. The

101 binding is monitored by subsequent changes in the refractive index at the biosensor  
102 surface (Homola, Yee, & Gauglitz, 1999).

103 SPR has been used more and more in the last decade to characterize the interactions  
104 between proteins and low molecular weight molecules in biochemical, biophysical and  
105 biomedical fields (Vachali, Li, Nelson, & Bernstein, 2012; Watrelot et al., 2016; Xiao,  
106 Jiang, Zhou, Li, & Liu, 2011). Some studies investigated SPR interactions between  
107 caseins (Marchesseau, Mani, Martineau, Roquet, Cuq, & Pugnère, 2002) or between  
108 caseins and polysaccharides (Thompson, Singh, & Dalgleish, 2010). However, up to  
109 now, SPR has not been used to study and quantify the interaction between caseins and  
110 hydrophobic low molecular weight biomolecules.

111 Curcumin is a low-molecular weight, natural polyphenolic phytoconstituent isolated  
112 from the dried rhizome of the plant *Curcuma Longa*. It has been used for centuries as a  
113 naturally occurring medicine for disease treatment and displays potent anti-  
114 inflammatory, antitumor and antioxidant activities (Hewlings & Kalmen, 2017).  
115 However, curcumin bioavailability is limited due to its low solubility in aqueous  
116 solution ( $2.99 \times 10^{-8}$  M) and it is characterized by a poor stability towards oxidation and  
117 light. Vitamin D3, one of the major forms of vitamin D, is hydrophobic in nature and  
118 highly susceptible to environmental conditions such as temperature and light. It is  
119 implicated in active intestinal calcium absorption but it was also shown recently that its  
120 role also includes immune function, cardiovascular health and cancer prevention  
121 (Gupta, Behera, Pawdal, Rawat, Baldi & Gupta, 2019). Nowadays, vitamin D 3  
122 deficiency is a public health problem in many countries. Therefore, the supplementation  
123 of food products with curcumin or vitamin D3 could offer additional source of these  
124 essential hydrophobic bioactive compounds.

125

126 The objective of the present work was to study the interactions of  $\beta$ -casein with  
127 curcumin and vitamin D3 using surface plasmon resonance and fluorescence quenching  
128 spectroscopy. The binding kinetics were investigated using both methodologies by  
129 gradually adding higher concentrations of hydrophobic bioactives to  $\beta$ -casein. A  
130 comparison of binding affinities was then performed for the two different hydrophobic  
131 molecules.

132

## 133 **2. Materials and methods**

### 134 **2.1 Materials and chemicals**

135  $\beta$ -casein from bovine milk (> 98%), curcumin (PubChem CID: 969516,  $\geq$  65%),  
136 chloramphenicol (PubChem CID: 5959), absolute ethanol (PubChem CID: 702),  
137 dimethyl sulfoxide (DMSO, PubChem CID: 679) and 4-(2-hydroxyethyl)-1-  
138 piperazineethanesulfonic acid (HEPES, PubChem CID: 23831) were purchased from  
139 Sigma-Aldrich (Saint-Quentin Fallavier, France). Vitamin D3 (PubChem CID:  
140 5280795, 99%) was obtained from Thermofisher Acros Organics (Geel, Belgium).  
141 Sodium acetate was purchased from Merck (Darmstadt, Germany). CM5 sensor chips,  
142 HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% v/v  
143 surfactant P20), HBS-EP+ buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA  
144 and 0.5% v/v surfactant P20), HBS-N buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl), N-  
145 ethyl-N'-(dimethylaminopropyl)carbodiimide hydrochloride (PubChem CID: 2723939,  
146 EDC), N-hydroxysuccinimide (PubChem CID: 80170, NHS) and 1M ethanolamine-HCl  
147 pH 8.5 were all sourced from GE Healthcare Life Sciences (Velizy-Villacoublay,  
148 France). All solutions were prepared using Milli-Q water (Millipore®).

### 149 **2.2 Surface plasmon resonance (SPR) studies**

150 SPR analyses to evaluate the interaction of curcumin or vitamin D3 (analyte) with  $\beta$ -  
151 casein (ligand) immobilized onto a carboxymethylated dextran sensor surface (CM5  
152 sensor chip) were performed using a Biacore T200 apparatus at 25°C (GE Healthcare  
153 Life Sciences, Uppsala, Sweden). During the analyte-ligand interaction, the change of  
154 the refractive index was measured in real time that allowed to plot the results of  
155 interaction as response units (RU) versus time (one RU represents the binding of 1 pg of  
156 protein per square mm).

### 157 *2.2.1 Covalent immobilization of $\beta$ -casein on CM5 sensor chip*

158  $\beta$ -casein was covalently immobilized on the CM5 sensor chip by a standard amine-  
159 coupling chemistry according to the manufacturer's protocol (GE Healthcare). Briefly,  
160 the surface was functionalized with 0.4 M EDC and 0.1 M NHS mixture. 40  $\mu$ M  $\beta$ -  
161 casein stock solution was prepared in HBS-EP buffer (pH 7.4). **The  $\beta$ -casein monomeric**  
162 **state in the experimental conditions was checked by dynamic light scattering analysis**  
163 **(data not shown)**. The solution was vortexed and placed in an ultrasonic bath for 10  
164 min. Prior to covalent immobilization, a preconcentration test at different pH values was  
165 performed to obtain the optimum pH for the immobilization procedure. Thus,  $\beta$ -casein  
166 stock solution was diluted at 20-30  $\mu$ g/mL in 10 mM acetate buffer at pH 3.6 and was  
167 injected at 10  $\mu$ l/min on two flow cells to reach two different immobilization levels of  
168 2700 RU and 6900 RU, respectively. These two different  $\beta$ -casein immobilization  
169 levels were explored in order to monitor the effect of mass transport hindrance caused  
170 by the surface binding sites located at different points (Schuck & Zhao, 2010). The  
171 unreacted carboxyl groups of the dextran were blocked with 1 M ethanolamine-HCl, pH  
172 8.5 and the surface was washed with HBS-N buffer. One other flow cell, treated with  
173 the same procedure but without protein was used as a control surface. Coated surfaces  
174 were stable for at least 2 days.



175 **2.2.2 Kinetic analysis of  $\beta$ -casein-hydrophobic bioactive compound interaction**

176 **Bioactive compound** stock solutions were prepared by dissolving curcumin (34 mM) in  
177 DMSO and vitamin D3 (53 mM) in ethanol. For each **bioactive compound**, a specific  
178 solvent was selected to ensure a high solubility of the **bioactive compound**; curcumin  
179 solubility in DMSO is 68 mM and vitamin D3 solubility in ethanol is 78 mM. Stock  
180 solutions were filtered through a 0.2  $\mu$ m cellulose-acetate membrane (Sartorius,  
181 Goettingen, Germany) and protected from light during experiments. The **bioactive**  
182 **compound** stock solution concentrations were spectrophotometrically determined at 420  
183 nm for curcumin using a molar absorption coefficient ( $\epsilon$ ) of 49,000 AU/mol/cm in  
184 DMSO (Khumsupan, Ramirez, Khumsupan, & Narayanaswami, 2011) and at 265 nm  
185 for vitamin D3 using a molar absorption coefficient ( $\epsilon$ ) of 18,300 AU/mol/L in ethanol  
186 (Vieth, Kimball, Hu, & Walfish, 2004). Prior to SPR analysis, **bioactive compound**  
187 stock solutions were diluted using HBS-EP+ buffer to obtain **bioactive compound**  
188 concentrations between 3 and 1000  $\mu$ M. The final concentration of DMSO and ethanol  
189 did not exceed 3% (v/v) in diluted **bioactive compound** solutions injected in flow cells.  
190 HBS-EP+ containing 3% (v/v) DMSO or 3% ethanol was used as running buffer. The  
191 diluted **bioactive compound** solutions at various increasing concentrations were  
192 successively injected. The analysis cycle for each **bioactive compound** concentration  
193 consisted, for the control cell and both  $\beta$ -casein coated flow cells, in the injection of the  
194 running buffer at a flow rate of 30  $\mu$ L/min, then the diluted **bioactive compound**  
195 solution for 60 s (association phase) and finally the running buffer for 100 s  
196 (dissociation phase). The signal of the control surface flow cell was subtracted from the  
197 signal of the  $\beta$ -casein flow cells. Chloramphenicol, a negative control, was also injected  
198 at 500 and 1000  $\mu$ M in HBS-EP+ containing 3% (v/v) DMSO. Each experiment series  
199 included blanks (running buffer), injection of **bioactive compound**, negative control, and

200 solvent corrections. Experiments were repeated three times. Sensorgrams were analyzed  
201 using BIA evaluation 3.0 software by steady-state fitting with the one-to-one Langmuir  
202 binding model to obtain  $K_D$  value. When a bioactive compound, injected in the flow  
203 system as the analyte, reacts with  $\beta$ -casein, immobilized as the ligand, to form a  
204 complex, the rate of the complex formation depends on the free concentrations of the  
205 bioactive compound ([Bioactive]) and  $\beta$ -casein ( $[\beta\text{-casein}]$ ) as well as the stability of the  
206 formed complex ( $[\text{Bioactive-}\beta\text{-casein}]$ ) and can be described by the Langmuir binding  
207 model equation (Eq. 1) (Myszka, Jonsen, & Graves, 1998):

$$208 \quad \frac{d[\text{Bioactive-}\beta\text{-casein}]}{dt} = K_a [\text{Bioactive}] [\beta\text{-casein}] - K_d [\text{Bioactive-}\beta\text{-casein}] \quad (1)$$

209 where  $K_a$  is the association rate constant and  $K_d$  is the dissociation rate constant.

210 In SPR experiment, the Langmuir binding model equation (Eq. 1) can be written as Eq.  
211 2:

$$212 \quad \frac{dR}{dt} = K_a C (R_{\text{max}} - R) - K_d R \quad (2)$$

213 where the bound bioactive concentration ( $[\text{Bioactive-}\beta\text{-casein}]$ ) is directly proportional  
214 to the SPR response ( $R$ ), the concentration of the analyte ( $[\text{Bioactive}]$ ) is the injected  
215 concentration ( $C$ ) and the free  $\beta$ -casein concentration ( $[\beta\text{-casein}]$ ) is obtained as the  
216 difference between  $R_{\text{max}}$ , maximum response due to analyte binding directly  
217 proportional to the total  $\beta$ -casein concentration, and  $R$ .

218 At steady state,  $dR/dt = 0$  and Eq. 2 can be written as Eq. 3:

$$219 \quad C (R_{\text{max}} - R) = K_D R \quad (3)$$

220 where  $K_D = k_d/k_a$ .

221 The effectiveness of the fitting is evaluated by the  $\chi^2$  value.

222 **2.3 Evaluation of  $\beta$ -casein-hydrophobic bioactive compound interaction by**  
223 **fluorescence spectroscopy**

224 Fluorescence measurements were performed on a Cary Eclipse spectrofluorimeter at  
225 25°C using a 1 cm quartz cuvette (101-QS, Hellma Analytics). A 20 µM β-casein **stock**  
226 solution was freshly prepared in a 10 mM HEPES buffer (pH 7.1). The solution was  
227 then vortexed and placed in an ultrasonic bath for 1 h before being filtered through a 0.2  
228 µm cellulose-acetate membrane (Sartorius, Goettingen, Germany). The β-casein  
229 concentration was checked by absorbance measurement at 280 nm using an extinction  
230 coefficient E (1%/1 cm) of 4.65 (De Kruif & Grinberg, 2002). Concurrently, curcumin  
231 and vitamin D3 stock solutions (3 mM) were prepared in absolute ethanol then filtered  
232 through a 0.2 µm cellulose-acetate membrane (Sartorius, Goettingen, Germany). The  
233 **bioactive compound** concentrations in ethanol were evaluated by measuring the  
234 absorbance at 427 nm for curcumin solution and at 265 nm for vitamin D3 solution with  
235 a molar extinction coefficient of 61,864 AU/mol/L/cm (Majhi, Rahman, Panchal, &  
236 Das, 2010) and 18,300 AU/mol/L/cm (Vieth et al., 2004) for curcumin and vitamin D3,  
237 respectively. For both **bioactive compounds**, **β-casein intrinsic fluorescence was**  
238 **measured at a concentration of 8 µM in the presence of increasing concentrations of**  
239 **curcumin and vitamin D3 by adding 1 to 100 µL of bioactive compound solution to β-**  
240 **casein dispersion**. The mixture was immediately vortexed for 15 s and allowed to  
241 equilibrate for 5 min before fluorescence measurements. The emission spectra were  
242 recorded from 290 to 500 nm with an excitation wavelength of 280 nm. Both the  
243 excitation and emission slit widths were set at 5 nm. A control experiment was  
244 performed on β-casein without an addition of **bioactive compound** and treated exactly as  
245 the sample containing **bioactive compound**. The measurements have been repeated three  
246 times and the mean values were reported.

#### 247 **2.4 Statistical analysis**

248 All measurements were made in triplicates at least. The differences were considered  
249 significant at  $p < 0.05$  level.

250

### 251 **3. Results and discussion**

#### 252 **3.1 Surface plasmon binding experiments**

##### 253 ***3.1.1 $\beta$ -Casein immobilization on CM5 sensor chip***

254 To minimize the effects of mass transport, steric hindrance, crowding and aggregation  
255 of high capacity surface, it is recommendable to have low protein density surface and  
256 high running flow rate for kinetics evaluation by SPR (Xiao et al., 2011). Nevertheless,  
257 for the binding analysis with low molecular weight molecules, such as curcumin and  
258 vitamin D3, the mass transport effect is minimized and a high density of protein  
259 immobilization is necessary to enhance the binding between  $\beta$ -casein and hydrophobic  
260 compounds in order to obtain a maximum of response in RU.

261 Two experimental conditions require special attention to optimize the immobilization of  
262  $\beta$ -caseins on CM5 sensor chip. Firstly, the  $\beta$ -casein dispersion has to be prepared at a  
263 low concentration (20-30  $\mu\text{g}/\text{mL}$ ) in order to prevent its self-association occurring at  
264 concentrations above 0.5 to 2  $\text{mg}/\text{mL}$  at **milk pH (6.8)** and **25°C** prior to immobilization  
265 (O'Connell et al., 2003). Besides, the highest level of immobilization of a protein on a  
266 carboxymethyl dextran chip (CM5) is obtained when the running buffer has a pH  
267 approximately one unit below the protein isoelectric point (pI). At this pH, the protein  
268 has a net positive charge and, consequently, electrostatic interactions between the  
269 positively charged protein and the negatively charged carboxymethyl dextran matrix  
270 increase the pre-concentration of the protein at the chip surface for the amine coupling  
271 establishment (Johnsson, Löfås, & Lindquist, 1991). Consequently, it is impossible to  
272 immobilize  $\beta$ -casein at **milk pH (~6.8)**. Thus, the pH was lowered to 3.6 since the  $\beta$

273 casein pI is 4.8 (Walstra, Wouters, & Geurts, 2006). These optimized conditions have  
274 enabled high  $\beta$ -casein immobilization densities (i.e.6900 RU and 2700 RU, the two  
275 different levels tested) on CM5 sensor chip. The N-hydroxy succinimide esters  
276 activated by the functionalization of the CM5 carboxyl groups by EDC/NHS have  
277 enabled protein binding to the surface by amine coupling. As primary amines are highly  
278 reactive, there is a strong probability that  $\beta$  casein was covalently linked to the CM5  
279 chip thanks to the N terminus hydrophilic region. Besides, according to the amino acid  
280 sequence of  $\beta$ -casein, the other primary amines from lysine are mostly located on the  
281 hydrophilic region close to the N-terminus region (Fischer, 2010; Holt & Sawyer,  
282 1993). Consequently, the hydrophobic fragment of  $\beta$  casein should be more available  
283 for hydrophobic interactions with hydrophobic molecules.

### 284 ***3.1.2 Surface plasmon resonance kinetic analysis***

285 The sensorgrams and the concentration-dependence of the specific binding of curcumin  
286 to immobilized  $\beta$ -casein after DMSO and double blank correction are presented in Fig.  
287 1A-B and Fig. 1C-D, respectively. These results are shown for two  $\beta$ -casein  
288 immobilization levels, 6900 RU for Fig. 1A and C and 2700 RU for Fig. 1B and D. No  
289 association was observed for either the control flowcell or the negative control. As  
290 expected for small molecular weight molecules, the box-shape of the sensorgram  
291 revealed fast association and dissociation rates, which makes difficult to quantify them.  
292 As curcumin is poorly soluble in aqueous solutions ( $2.99 \times 10^{-8}$  M) (Letchford, Liggins,  
293 & Burt, 2008), different organic solvents like dimethyl sulfoxide (DMSO) and ethanol  
294 were considered. As the solubility information for curcumin according to the supplier  
295 was of 10 mg/mL and 25 mg/mL in ethanol and DMSO, respectively, DMSO was  
296 chosen as solvent in order to reach higher concentrations of curcumin in the diluted  
297 solution injected during the association phase. Unlike curcumin, vitamin D3 shows

298 higher solubility in ethanol (30 mg/mL) than in DMSO. Therefore, ethanol was used as  
299 a solvent for vitamin D3 prior to SPR study. However, vitamin D3 was unsuccessfully  
300 analyzed using the same procedure as for curcumin tested in HBS EP+ containing 3%  
301 ethanol (Fig. 2) and in 3% DMSO (v/v) (data not shown). The responses of vitamin D3  
302 under these both conditions were under the limit of T200 Biacore apparatus sensitivity.  
303 It clearly means that no interaction was observed between  $\beta$ -casein immobilized on  
304 CM5 chip and vitamin D3 under these SPR conditions. **According to the manufacturer**  
305 **(GeHealthcare), the sensitivity of Biacore T200 apparatus enables precise affinity**  
306 **analysis of low molecular weight molecules with a maximal dissociation constant (KD)**  
307 **value in the millimolar range.**

308 Concerning curcumin binding to  $\beta$ -casein and in order to obtain accurate estimations of  
309 the binding constants, SPR data have been analyzed by fitting the SPR sensorgrams  
310 using non-linear fitting of the SPR signal at the steady state with a Langmuir binding  
311 isotherm model (De Mol & Fischer, 2008). The response in RU is then considered as  
312 directly proportional to the injected analyte concentration (C) (Fig. 1 A-B). The curves  
313 shown in Fig. 1 C-D represent steady-state affinity analysis of curcumin binding to  $\beta$ -  
314 casein fitted to 1:1 Langmuir interaction model. The  $K_D$  value obtained from the SPR  
315 analyses is  $4.1 \pm 0.7 \times 10^{-4}$  M for curcumin in DMSO. The calculated  $\chi^2$  value ( $\chi^2 =$   
316 4.54) indicates a good accuracy of the fitting. Figure 2 shows a comparison of dose-  
317 effect of curcumin and vitamin D3 in different solvents. The kinetic study obtained for  
318 curcumin dissolved in ethanol reveals similar  $k_D$  value  $6.9 \pm 0.9 \times 10^{-4}$  M as that  
319 obtained in DMSO. However, vitamin D3 gave too low responses to be analyzed in 3%  
320 ethanol. Although the study of interactions between low molecular weight molecules  
321 and bovine proteins starts to be carried out by SPR technique (Park, Kim, Lim, & Lee,  
322 2015; Watrelot et al., 2016), this analytical technique has not been used previously to

323 study interactions between  $\beta$ -casein and hydrophobic low molecular weight molecules  
324 such as curcumin. To further explore the binding of  $\beta$ -casein to curcumin and vitamin  
325 D3, fluorescence spectroscopy was also performed to complement the SPR analyses.

### 326 *3.2. Fluorescence spectroscopy analysis of $\beta$ -casein interactions with curcumin and* 327 *vitamin D3*

328 Fluorescence spectroscopy dealing with transitions from the excited state to the ground  
329 state has a widespread application in the field of biophysics, pharmaceuticals but also  
330 food science (Williams & Daviter, 2013). In order to get an additional insight on the  
331 interaction between small hydrophobic molecules and  $\beta$ -casein, the intrinsic  
332 fluorescence emission of  $\beta$ -casein solution excited at 280 nm wavelength was  
333 investigated after a progressive addition of each bioactive compound (vitamin D3 and  
334 curcumin). As for SPR study, the  $\beta$ -casein concentration is below its CMC in order to  
335 prevent its self-association. Fluorescence emission spectra of  $\beta$ -casein display a peak at  
336 345 nm as shown in Fig. 3. The protein excitation at 280 nm caused the emission of  
337 mainly tryptophan residues and, in aqueous solution, the emission maximum  
338 wavelength of free tryptophan is close to 352 nm. A lower  $\lambda_{\max}$  of 345 nm was observed  
339 for the single tryptophan of individual  $\beta$ -casein located in its hydrophobic fragment.  
340 This blue shift could be attributed to the apolar environment location of this residue  
341 (Benzaria et al., 2013). The emission spectra of the  $\beta$  casein solution containing  
342 increasing content of curcumin (Fig. 3A) or vitamin D3 (Fig. 3B) show that the increase  
343 of bioactive compound concentration induced a gradual decrease of  $\beta$ -casein  
344 fluorescence intensity. This fluorescence quenching is attributed to curcumin- $\beta$ -casein  
345 or vitamin D3- $\beta$ -casein complex formation reducing the fluorescence intensity of the  
346 tryptophan residues residue. The maximum of the fluorescence emission spectra ( $\lambda_{\max}$ )  
347 was plotted against curcumin and vitamin D3 concentration in Fig 3C and 3D,

348 respectively. Curcumin- $\beta$ -casein binding generates a blue shift of fluorescence emission  
349 maximum wavelength by  $\sim 12$  nm in the presence of curcumin (Fig. 3C). This  $\lambda_{\max}$  shift  
350 points out the change in the polarity of the  $\beta$ -casein tryptophan residue  
351 microenvironment, as consequence of curcumin binding which strongly suggests the  
352 hydrophobic character of curcumin and  $\beta$ -casein interactions. This hydrophobic contact  
353 of curcumin with hydrophobic domain of  $\beta$ -casein was also reported by [Bourassa,](#)  
354 [Bariyanga, & Tajmir-Riahi \(2013\)](#). Conversely, no shift was observed for vitamin D3- $\beta$ -  
355 casein complexation (Fig. 3B) suggesting that the transfer of vitamin D3 from the polar  
356 aqueous environment to the apolar hydrophobic region of  $\beta$ -casein does not modify the  
357 hydrophobicity region close to the tryptophan residue as concluded for curcumin. This  
358 was in accordance with a previous study showing that the interactions of  $\beta$ -casein with  
359 vitamin D3 are mainly hydrophobic inducing a disturbance in the phenylalanine  
360 residues located in the hydrophobic domain of  $\beta$ -casein ([Forrest et al., 2005](#)). Besides,  
361 as the phenolic hydrogens of curcumin have pKa values of 8.38, 9.88, and 10.51 in  
362 aqueous solutions, at experimental pH ( $\sim 7.1$ ), curcumin is in a neutral form and its  
363 binding due to charge interactions can be neglected ([Sahu et al., 2008](#)).

364 The binding constants were evaluated from the fluorescence quenching of  $\beta$ -casein in  
365 presence of **bioactive compound**. In order to check the presence of static or dynamic  
366 quenching in **bioactive compound**- $\beta$ -casein complexes, Eq. 4 was used to evaluate the  
367 Stern-Volmer constant ([Lakowicz, 2006](#)):

$$368 \quad S_0/S = 1 + K_{sv}[Q] \quad (4)$$

369 where  $S_0$  and  $S$  are the fluorescence areas in the absence and presence of **bioactive**  
370 **compound**, respectively,  $[Q]$  is the **bioactive compound** concentration and  $K_{sv}$  is the  
371 Stern-Volmer quenching constant. The Stern-Volmer plots for  $\beta$ -casein quenching by  
372 curcumin and vitamin D3 are presented in Fig. 4A and 4B, respectively. A linear



373 dependence at **bioactive compound** concentrations lower than 10  $\mu\text{M}$  clearly appeared  
374 for the two hydrophobic **bioactive compounds** indicating that the quenching is mainly  
375 static in nature with some degree of dynamic quenching at high **bioactive compound**  
376 concentrations. The Stern-Volmer quenching constants ( $K_{SV}$ ) for curcumin and vitamin  
377 D3 were evaluated to  $13.5 \pm 2.0 \times 10^4 \text{ M}^{-1}$  and  $3.4 \pm 0.3 \times 10^4 \text{ M}^{-1}$ , respectively.

378 When small molecules are bound independently to a set of equivalent sites on a  
379 macromolecule, the binding constant and the number of binding sites can be  
380 comparatively calculated assuming the static quenching by Eq. 5:

$$381 \quad \ln[(S_0-S)/S] = \ln K_A + n \ln[Q] \quad (5)$$

382 Here, S,  $S_0$ , and [Q] are the same as the parameters in the Stern-Volmer equation (Eq.  
383 4),  $K_A$  is the association constant and n is the number of binding sites. The plots of  
384  $\ln[(S_0-S)/S]$  versus  $\ln[\text{curcumin}]$  and  $\ln[\text{vitamin D3}]$  are shown in Fig. 4C and Fig. 4D,  
385 respectively. The n,  $K_A$ , and  $K_D$  estimated values for the  $\beta$ -casein-**bioactive compound**  
386 complexes are summarized in Table 1. The obtained  $K_A$  and the number of binding sites  
387 value (n) for curcumin were  $23.5 \pm 1.9 \times 10^4 \text{ M}^{-1}$  and  $0.74 \pm 0.05$ , respectively. **This**  
388 **association constant is in good agreement with that reported for bisdemethoxycurcumin**  
389 **of about  $2.01 \pm 0.03 \times 10^5 \text{ M}^{-1}$  (Mehranfar, Bordbar, Keyhanfar & Behbahani, 2013).**  
390 **However, a four times lower  $K_A$  value of  $4.4 \pm 0.03 \times 10^4 \text{ M}^{-1}$  was obtained for**  
391 **diacetylcurcumin- $\beta$ -casein interactions evaluated by fluorescence quenching in another**  
392 **previous study (Mehranfar, Bordbar, Fani, & Keyhanfar, 2013). These results suggest**  
393 **that the phenolic OH groups of curcumin and bisdemethoxycurcumin play an important**  
394 **role in the interaction with  $\beta$ -casein (Mohammadi, Bordbar, Divsalar, Mohammadi, &**  
395 **Saboury, 2009). Lower  $K_A$  values in the order of  $1.10^3 \text{ M}^{-1}$  were obtained for other**  
396 **polyphenols (Hasni, Bourassa, Hamdani, Samson, Carpentier, & Tajmir-Riahi, 2011).**  
397 **The fluorescence quenching results obtained in a previous study investigating the**

398 interaction between curcumin and whole casein showed a higher binding constant  
399 ( $13.43 \times 10^5 \text{ M}^{-1}$ ) than that obtained in the present study (Nadi et al., 2014). This  
400 difference could be attributed to the presence of  $\alpha$  and  $\kappa$ -caseins in the whole casein  
401 dispersion tested. The thermodynamic parameters evaluated in this study confirmed the  
402 hydrophobic nature of casein-curcumin interactions. Besides, the fluorescence results  
403 suggested that curcumin was located at the vicinity of the caseins tryptophanyl side  
404 chains due to a fluorescence energy transfer from the fluorophores (tryptophans) to the  
405 quencher (curcumin).

406 Concerning vitamin D3, a previous study on vitamin D3 and bovine  $\beta$ -casein  
407 interactions (Forrest et al., 2005) reported significantly higher  $K_A$  values ( $3.8\text{-}7.7 \times 10^6$   
408  $\text{M}^{-1}$ ) compared to the  $K_A$  presented in Table 1 ( $5.8 \pm 1.1 \times 10^4 \text{ M}^{-1}$ ). This difference  
409 could be attributed to the ionic strength effect on the protein charges by the ions  
410 enhancing hydrophobic interactions due the decrease of the protein solubility.

411 Curcumin has clearly a significantly higher affinity to  $\beta$ -casein than vitamin D3. Firstly,  
412 the hydrophobicity of vitamin D3 ( $\log P = 7.9$ ) is significantly higher than that observed  
413 for curcumin ( $\log P = 3.6$ ), as evaluated by Costache, Sheihet, Zaveri, & Knight (2010),  
414 potentially limiting its flexibility in the aqueous medium during fluorescence  
415 measurements. Besides, the higher interaction obtained for curcumin could be attributed  
416 to its phenolic hydroxyl groups playing an important role in bioactive compound-  
417 protein interactions. In fact, the two phenyl rings of curcumin are involved most likely  
418 in  $\pi$ - $\pi$  stacking interaction with the aromatic side chains of  $\beta$ -casein (Hobani, Jerah, &  
419 Bidwai, 2017; Mcgaughey, Gagne, & Rappe, 1998).

420 Comparing the binding kinetic results obtained using SPR and fluorescence quenching,  
421 it clearly appears that the equilibrium association constant ( $K_A$ ) obtained from  
422 fluorescence quenching is significantly higher than that evaluated by SPR (Table 1).

423 Same observations were previously reported by studies comparing SPR and  
424 fluorescence quenching for bimolecular interaction determination highlighting also  
425 lower association constants for SPR (Plesa et al., 2011; Xiao et al., 2011). This  
426 difference may be attributed to the different experimental conditions of these two  
427 techniques. The fluorescence quenching method is based on quenching of tryptophan  
428 residues when the excitation wavelength is set at 280 nm. That is to say, this method  
429 focuses on the interaction between tryptophan and hydrophobic molecules. When the  
430 fluorescence intensity decreases with the bioactive molecule addition, it means that the  
431 microenvironment of the tryptophan changed and the binding occurred between  $\beta$ -  
432 casein and the hydrophobic bioactive. Thus, the fluorescence quenching method does  
433 not consider the interaction between bioactive compounds and other sites on  $\beta$ -casein.  
434 Concerning SPR method, it is based on refractive index change on the sensor chip  
435 surface. The entire signal is caused by any interaction recorded without consideration of  
436 the binding sites. However, in the case of our experiments, the  $\beta$ -casein monomers were  
437 immobilized on the sensor chip surface by amine coupling involving the binding of free  
438 amine groups mainly present on its hydrophilic tail which could favor the hydrophobic  
439 fragment available to interact with other molecules. The SPR allows the description of  
440 binding events of a number of different molecules to the immobilized  $\beta$ -casein, which  
441 can be monitored in real-time without labeling requirements. The major advantage of  
442 SPR is the evaluation of specific interactions since the unspecific binding is evaluated  
443 on a control channel and thus removed from the measured signal. Therefore, both  
444 methods can be used to evaluate the interactions between hydrophobic bioactive  
445 compounds and  $\beta$ -casein while bearing in mind that the fluorescence method is focused  
446 on binding sites around the fluorophore residues while SPR is more accurate for all  
447 kinds of binding sites investigation.

448

#### 449 **4. Conclusion**

450  $\beta$ -casein, the most hydrophobic casein in bovine milk, displays an open conformation  
451 which is easily accessible to gastric proteases. Hence, it may be used as an oral delivery  
452 vehicle to release hydrophobic bioactive molecules such as curcumin and vitamin D3.  
453 The investigation of the affinities and kinetics of the interaction between  $\beta$ -casein and  
454 two hydrophobic compounds was performed to understand how these bioactive  
455 compounds could be associated to  $\beta$ -casein prior to their delivery in the organism. We  
456 have evaluated the interactions between curcumin and vitamin D3, as model  
457 hydrophobic nutraceutical compounds, and  $\beta$ -casein by a multimethodological approach  
458 using surface plasmon resonance (SPR) biosensor in comparison with fluorescence  
459 spectroscopy as a traditional and classical method to evaluate the association constants.  
460 The fluorescence quenching data show that curcumin has higher affinity to  $\beta$ -casein ( $K_A$   
461  $= 23.5 \pm 1.9 \times 10^4 \text{ M}^{-1}$ ) than vitamin D3 ( $K_A = 5.8 \pm 1.1 \times 10^4 \text{ M}^{-1}$ ). The  $K_A$  value for  
462 curcumin- $\beta$ -casein interaction has been successfully evaluated ( $2.45 \pm 1.4 \times 10^3 \text{ M}^{-1}$ )  
463 using SPR by fitting data to a 1:1 Langmuir interaction model. Conversely, the SPR  
464 responses obtained for vitamin D3 show that the interactions between this hydrophobic  
465 compound and the  $\beta$ -casein immobilized on the sensor chip were below the sensitivity  
466 of the SPR apparatus. It clearly appears that the equilibrium association constant ( $K_A$ )  
467 obtained from fluorescence quenching is significantly higher than that evaluated by  
468 SPR. This difference may be attributed to the different principles of these two  
469 techniques. The fluorescence quenching method is based on quenching of tryptophan  
470 residues when they interact with hydrophobic molecules without consideration of the  
471 interactions with other sites on  $\beta$ -casein. Unlike fluorescence quenching, SPR, a  
472 powerful non-destructive and label-free technique, offers a direct and real-time

473 monitoring of the interaction kinetics regardless of the binding sites. The fluorescence  
474 quenching is more accurate to study the binding sites around the tryptophan residues  
475 while the SPR technique offers the opportunity to study all kinds of binding sites. In  
476 summary, both SPR and fluorescence results provided evidence about the higher  
477 affinity of curcumin with  $\beta$ -casein compared to vitamin D3. The SPR developed method  
478 is versatile for measuring the binding of  $\beta$ -casein to other bioactive molecules,  
479 promising applications in many other protein-small molecules interactions.

480

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484

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633

634

635 **Figure captions**

636 **Figure 1:** Binding sensorgrams for curcumin interaction with immobilized  $\beta$ -casein on  
637 CM5 biosensor at 25°C (A and B) and steady-state affinity analysis of curcumin  
638 binding to  $\beta$ -casein fitted to a 1:1 interaction model (C and D). Two  $\beta$ -casein  
639 immobilization levels of 6900 RU (A and C) and 2700 RU (B and D) were tested.  
640 Curcumin was prepared at 34 mM in DMSO then injected in the flow system at  
641 different concentrations (a) 0, (b) 3.9, (c) 7.8, (d) 15.6, (e) 31.2, (f) 62.5, (g) 125, (h)  
642 250, (i) 500 and (j) 1000  $\mu$ M. The experiment was repeated three times.

643

644 **Figure 2:** Steady-state affinity analysis of  $\beta$ -casein binding to curcumin and vitamin D3  
645 respectively, prepared in different solvents for stock solutions then diluted in HBS-EP+.  
646  $\beta$ -casein immobilization level was 2 700 RU.

647

648 **Figure 3:**  $\beta$ -casein **intrinsic fluorescence** emission spectra ( $T=25^{\circ}\text{C}$ ,  $\lambda_{\text{ex}}=280$  nm) and  
649 shift of the maximum peak **in the presence of increasing concentration of curcumin and**  
650 **vitamin D3** added to 8  $\mu$ M  $\beta$ -casein. Curcumin was prepared at 3 mM in absolute  
651 ethanol then added to  $\beta$ -casein solution at different concentrations (a) 0, (b) 0.7, (c) 1.4,  
652 (d) 2, (e) 2.8, (f) 3.5, (g) 5.5, (h) 6.9, (i) 10.4, (j) 13.9, (k) 20.8, (l) 27.7, (m) 34.7 and  
653 (n) 69  $\mu$ M. Vitamin D3 was prepared at 3 mM in absolute ethanol then added to  $\beta$ -  
654 casein solution at different concentrations (a) 0, (b) 0.8, (c) 1.7, (d) 2.5, (e) 3.4, (f) 4.2,  
655 (g) 5.1, (h) 6.8, (i) 8.5, (j) 12.7, (k) 17, (l) 25.5, (m) 34 and (n) 85  $\mu$ M. The experiment  
656 was repeated three times.

657

658 **Figure 4:** Sterne-Volmer plots of the fluorescence quenching of the  $\beta$ -casein by  
659 curcumin (A) and vitamin D3 (B) and the plot of  $\ln[(S_0-S)/S]$  versus  $\ln[\text{curcumin}]$  (A)  
660 and  $\ln[\text{Vitamin D3}]$  (B) for the binding parameter evaluation with  $\beta$ -casein at 25°C.  
661

Figure 1

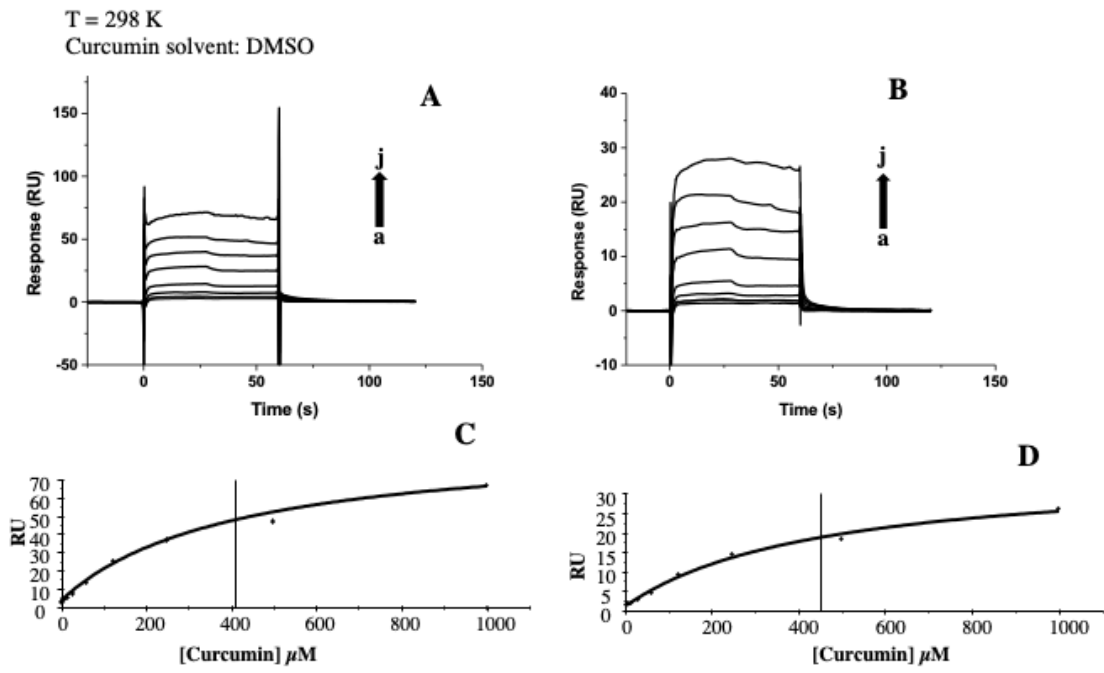


Figure 2

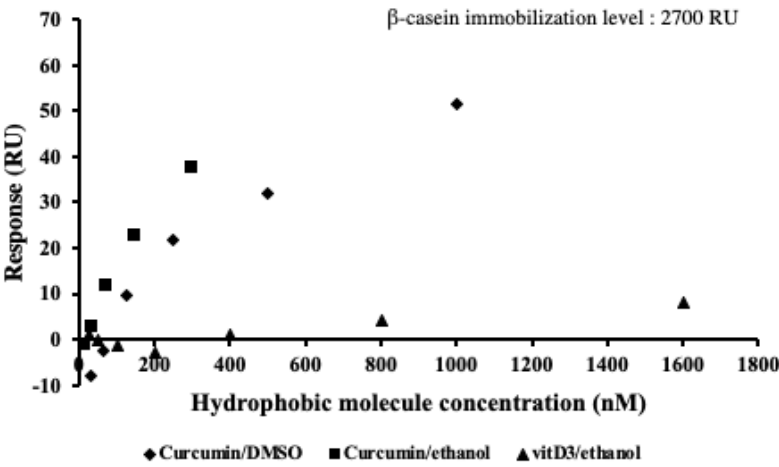


Figure 3

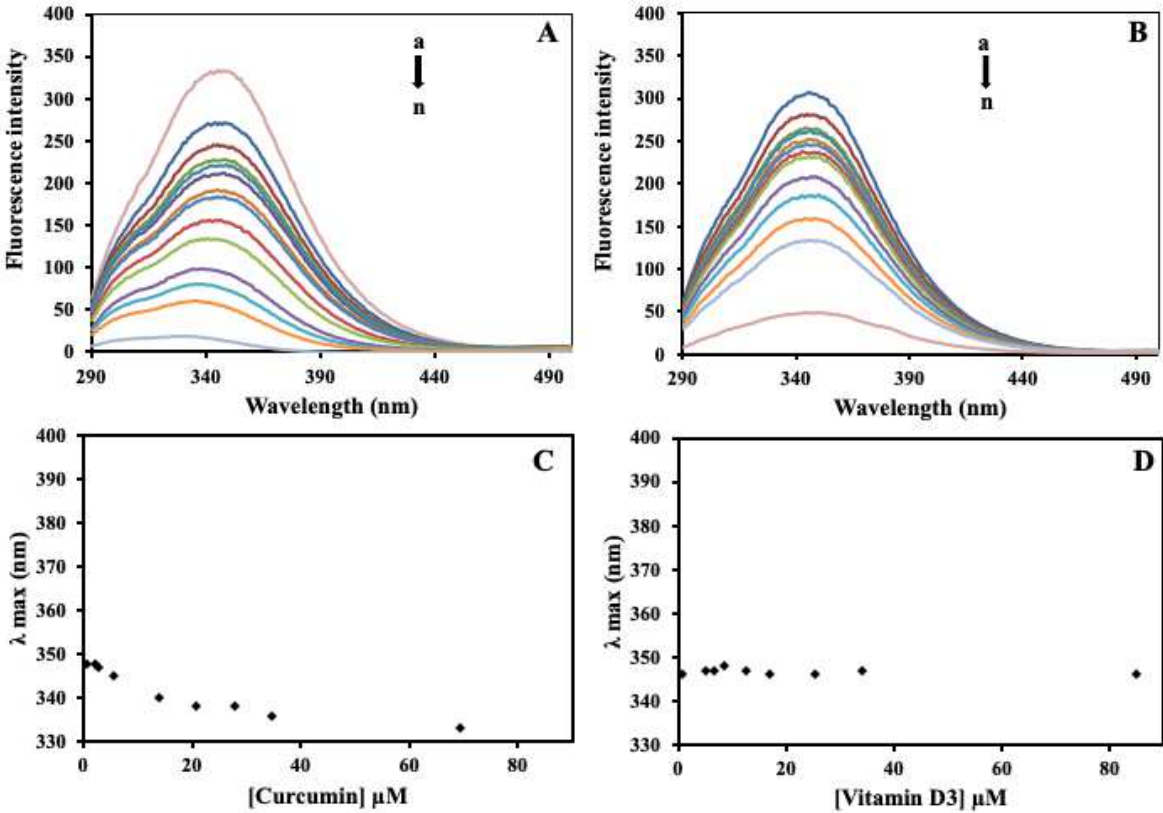
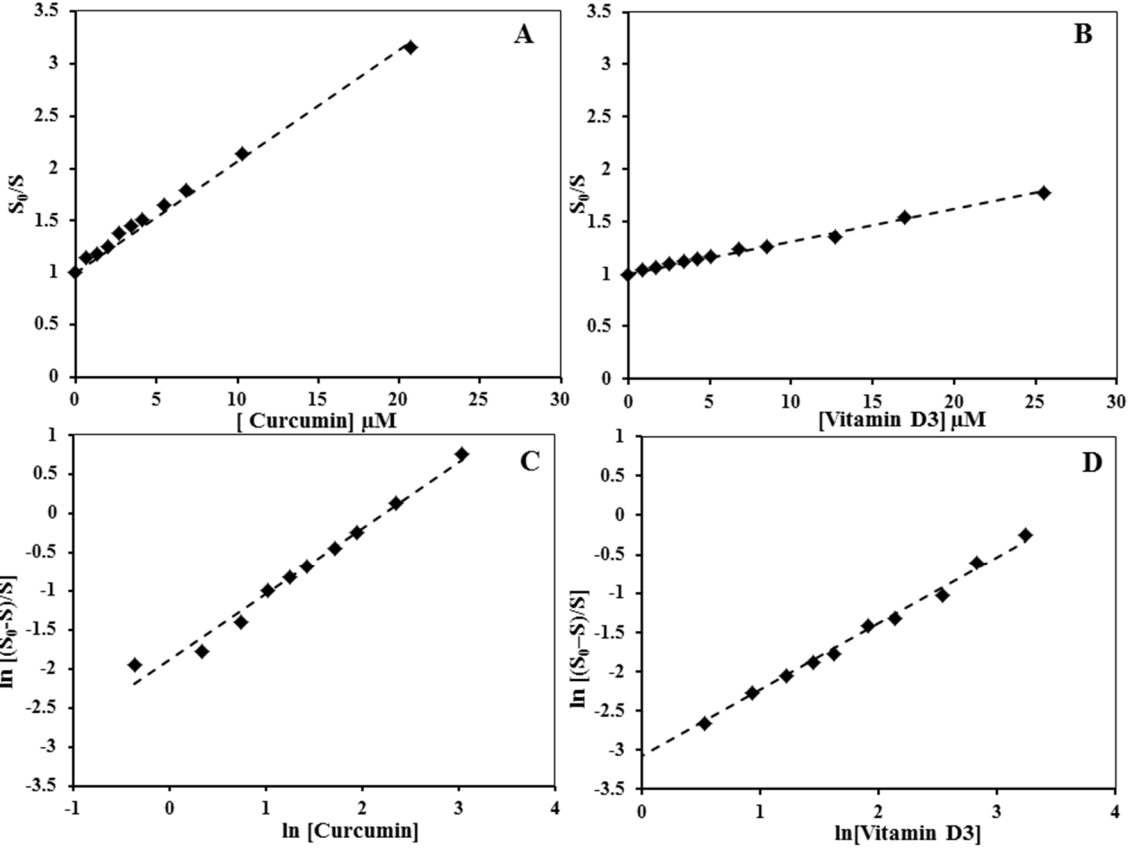




Figure 4



1 **Table 1:** Binding parameters of the interactions between curcumin or vitamin D3 and  $\beta$ -  
 2 casein at 25°C obtained by SPR and fluorescence quenching.

Method	SPR	Fluorescence quenching		
	$K_A$	$K_A$	n	$K_{sv}$
	( $\times 10^3 M^{-1}$ )	( $\times 10^4 M^{-1}$ )		( $\times 10^4 M^{-1}$ )
Curcumin	2.45 $\pm$ 1.42	23.5 $\pm$ 1.9 <sup>a</sup>	0.74 $\pm$ 0.05 <sup>a</sup>	13.5 $\pm$ 2.0
Vitamin D3	-	5.8 $\pm$ 1.1 <sup>b</sup>	0.82 $\pm$ 0.04 <sup>a</sup>	3.8 $\pm$ 0.3

3 Results are expressed as the means  $\pm$  standard deviations of three replicates.

4 Different superscript letters presented in the same column indicate significant differences

5 ( $p < 0.05$ )

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