

Binding analysis between monomeric β -case in and hydrophobic bioactive compounds investigated by surface plasmon resonance and fluorescence spectroscopy

Asma Bahri, Corinne Henriquet, Martine Pugnière, Sylvie Marchesseau,

Dominique Chevalier-Lucia

▶ To cite this version:

Asma Bahri, Corinne Henriquet, Martine Pugnière, Sylvie Marchesseau, Dominique Chevalier-Lucia. Binding analysis between monomeric β -casein and hydrophobic bioactive compounds investigated by surface plasmon resonance and fluorescence spectroscopy. Food Chemistry, 2019, 286, pp.289-296. 10.1016/j.foodchem.2019.01.176. hal-02048380

HAL Id: hal-02048380 https://hal.umontpellier.fr/hal-02048380

Submitted on 22 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1	Binding analysis between monomeric β -casein and hydrophobic
2	bioactive compounds investigated by surface plasmon resonance and
3	fluorescence spectroscopy
4	
5	Abbreviated running title: β -casein-hydrophobic bioactive compound binding
6	
7	Asma Bahri ^a , Corinne Henriquet ^b , Martine Pugnière ^b , Sylvie Marchesseau ^a ,
8	Dominique Chevalier-Lucia ^{a*}
9	
10	^a IATE, Université de Montpellier, CIRAD, INRA, Montpellier SupAgro, Montpellier,
11	France
12	^b IRCM, Université de Montpellier, ICM, INSERM, Montpellier, France
13	
14	* Corresponding author: dominique.chevalier-lucia@umontpellier.fr
15	
16	
17	
18	
19	
20	
21	
22	
23	
24	
25	

26 Abstract

27 β -casein, a phosphoprotein representing 37 % of the bovine milk caseins, has specific features promoting its application as a nanocarrier for hydrophobic bioactives. In this 28 29 study, the interactions of β -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- β -case in interaction has been successfully evaluated (4.1 ± 0.7 × 10⁻ ⁴ M) using SPR by fitting data to a 1:1 Langmuir interaction model. Conversely, the 33 34 SPR responses obtained for vitamin D3 show that the interactions between this 35 hydrophobic compound and the β -case in immobilized on the sensor chip were below 36 the sensitivity of the SPR apparatus. Moreover, the fluorescence quenching data show that curcumin has higher affinity to β -case (K_A = 23.5 ± 1.9 × 10⁴ M⁻¹) than vitamin 37 38 D3 (K_A = $5.8 \pm 1.1 \times 10^4 \,\mathrm{M}^{-1}$).

39

40 **Highlights:**

- 41 The interactions between monomeric β-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 β -case in compared to vitamin D3.
- 46
- 47 Keywords: β-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, Maresca, Taieb, & Dumay, 2013). Among the four different caseins, α_{s1} -, α_{s2} -, β - and κ -67 68 case ins, a particular interest is paid to β -case in. This phosphoprotein, composed of 209 69 amino acids and with a molecular weight ~ 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 β -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 β-77 casein self-association in small oblate micelles (Holt & Sawyer, 1993; Swaisgood, 78 2003). The intermolecular interactions to form β -case in micelles occur at concentrations above the critical micellization concentration (CMC) of ~ 0.5 - 2 mg/mL at milk pH 79 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 β -case in 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 β -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 binding is monitored by subsequent changes in the refractive index at the biosensor
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, & Pugnière, 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 x 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.

126 The objective of the present work was to study the interactions of β -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 β -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 β -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)-1piperazineethanesulfonic acid (HEPES, PubChem CID: 23831) were purchased from 138 139 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

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

157 2.2.1 Covalent immobilization of β -casein on CM5 sensor chip

158 β-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 μM β-161 casein stock solution was prepared in HBS-EP buffer (pH 7.4). The ß-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, β -casein 166 stock solution was diluted at 20-30 µg/mL in 10 mM acetate buffer at pH 3.6 and was 167 injected at 10 µl/min on two flow cells to reach two different immobilization levels of 168 2700 RU and 6900 RU, respectively. These two different β-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 β -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 µm 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 (ɛ) 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 (ϵ) 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 µM. 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 β -casein coated flow cells, in the injection of the 194 running buffer at a flow rate of 30 µL/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 ß-casein flow cells. Chloramphenicol, a negative control, was also injected 198 at 500 and 1000 µM 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 ß-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 ß-casein ([ß-casein]) as well as the stability of the 206 formed complex ([Bioactive-ß-casein]) and can be described by the Langmuir binding 207 model equation (Eq. 1) (Myszka, Jonsen, & Graves, 1998):

208 d[Bioactive- β -casein]/dt = K_a [Bioactive] [β -casein] - K_d [Bioactive- β -casein] (1)

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:

$$dR/dt = K_a C (Rmax-R) - K_d R \quad (2)$$

where the bound bioactive concentration ([Bioactive-β-casein]) is directly proportional
to the SPR response (R), the concentration of the analyte ([Bioactive]) is the injected
concentration (C) and the free β-casein concentration ([β-casein]) is obtained as the
difference between Rmax, maximum response due to analyte binding directly
proportional to the total β-casein concentration, and R.

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

219 $C (Rmax-R) = K_D R (3)$

220 where $K_D = k_d/k_a$.

221 The effectiveness of the fitting is evaluated by the χ^2 value.

222 2.3 Evaluation of β-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 μ m 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 -case in intrinsic fluorescence was 238 measured at a concentration of 8 µM in the presence of increasing concentrations of 239 curcumin and vitamin D3by adding 1 to 100 μ L 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 -case in 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**

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

250

251 **3. Results and discussion**

252 **3.1 Surface plasmon binding experiments**

253 3.1.1 β-Casein immobilization on CM5 sensor chip

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

261 Two experimental conditions require special attention to optimize the immobilization of 262 β -caseins on CM5 sensor chip. Firstly, the β -casein dispersion has to be prepared at a 263 low concentration (20-30 µg/mL) in order to prevent its self-association occurring at 264 concentrations above 0.5 to 2 mg/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 β -case at milk pH (~6.8). Thus, the pH was lowered to 3.6 since the β

273 casein pI is 4.8 (Walstra, Wouters, & Geurts, 2006). These optimized conditions have 274 enabled high β-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 β 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 β -case in, 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 β casein should be more available 283 for hydrophobic interactions with hydrophobic molecules.

284 3.1.2 Surface plasmon resonance kinetic analysis

The sensorgrams and the concentration-dependence of the specific binding of curcumin to immobilized β -casein after DMSO and double blank correction are presented in Fig. 1A-B and Fig. 1C-D, respectively. These results are shown for two β -casein immobilization levels, 6900 RU for Fig. 1A and C and 2700 RU for Fig. 1B and D. No association was observed for either the control flowcell or the negative control. As expected for small molecular weight molecules, the box-shape of the sensorgram revealed fast association and dissociation rates, which makes difficult to quantify them.

As curcumin is poorly soluble in aqueous solutions $(2.99 \times 10^{-8} \text{ M})$ (Letchford, Liggins, & Burt, 2008), different organic solvents like dimethyl sulfoxide (DMSO) and ethanol were considered. As the solubility information for curcumin according to the supplier was of 10 mg/mL and 25 mg/mL in ethanol and DMSO, respectively, DMSO was chosen as solvent in order to reach higher concentrations of curcumin in the diluted 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 β -casein immobilized on 304 CM5 chip and vitamin D3 under these SPR conditions. According to the manufacturer (GeHealthcare), the sensitivity of Biacore T200 apparatus enables precise affinity 305 306 analysis of low molecular weight molecules with a maximal dissociation constant (KD) 307 value in the millimolar range.

308 Concerning curcumin binding to β -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 β-314 casein fitted to 1:1 Langmuir interaction model. The K_D value obtained from the SPR analyses is $4.1 \pm 0.7 \times 10^{-4}$ M for curcumin in DMSO. The calculated χ^2 value (χ^2 = 315 316 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 curcumin dissolved in ethanol reveals similar k_D value 6.9 ± 0.9 × 10⁻⁴ M as that 318 319 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

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

326 3.2. Fluorescence spectroscopy analysis of β-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 β -casein, the intrinsic fluorescence emission of β -casein solution excited at 280 nm wavelength was 332 333 investigated after a progressive addition of each bioactive compound (vitamin D3 and 334 curcumin). As for SPR study, the β -case in concentration is below its CMC in order to 335 prevent its self-association. Fluorescence emission spectra of β -case in 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 λ_{max} of 345 nm was observed 339 for the single tryptophan of individual β -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 β case in 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 β-casein 344 fluorescence intensity. This fluorescence quenching is attributed to curcumin-β-casein 345 or vitamin D3-β-casein complex formation reducing the fluorescence intensity of the 346 tryptophan residues residue. The maximum of the fluorescence emission spectra (λ_{max}) 347 was plotted against curcumin and vitamin D3 concentration in Fig 3C and 3D,

348 respectively. Curcumin-*β*-casein binding generates a blue shift of fluorescence emission 349 maximum wavelength by ~12 nm in the presence of curcumin (Fig. 3C). This λ_{max} shift 350 points out the change in the polarity of the β -case tryptophan residue 351 microenvironment, as consequence of curcumin binding which strongly suggests the 352 hydrophobic character of curcumin and β -case in interactions. This hydrophobic contact 353 of curcumin with hydrophobic domain of β -casein was also reported by Bourassa, 354 Bariyanga, & Tajmir-Riahi (2013). Conversely, no shift was observed for vitamin D3-B-355 casein complexation (Fig. 3B) suggesting that the transfer of vitamin D3 from the polar 356 aqueous environment to the apolar hydrophobic region of β -case 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 β -casein with 359 vitamin D3 are mainly hydrophobic inducing a disturbance in the phenylalanine 360 residues located in the hydrophobic domain of β -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 aqueous solutions, at experimental pH (~7.1), curcumin is in a neutral form and its 362 363 binding due to charge interactions can be neglected (Sahu et al., 2008).

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

368

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

where S_0 and S are the fluorescence areas in the absence and presence of bioactive compound, respectively, [Q] is the bioactive compound concentration and K_{sv} is the Stern-Volmer quenching constant. The Stern-Volmer plots for β -casein quenching by curcumin and vitamin D3 are presented in Fig. 4A and 4B, respectively. A linear 373 dependence at bioactive compound concentrations lower than 10 μ 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 ± 2.0 × 10⁴ M⁻¹ and 3.4 ± 0.3 × 10⁴ M⁻¹, respectively.

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

381
$$Ln[(S_0-S)/S] = lnK_A + n ln[Q]$$
 (5)

382 Here, S, S₀, 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[(S₀-S)/S] versus ln[curcumin] and ln[vitamin D3] are shown in Fig. 4C and Fig. 4D, 385 respectively. The n, K_A, and K_D estimated values for the β-casein-bioactive compound 386 complexes are summarized in Table 1. The obtained KA and the number of binding sites value (n) for curcumin were $23.5 \pm 1.9 \times 10^4 \,\mathrm{M}^{-1}$ and 0.74 ± 0.05 , respectively. This 387 388 association constant is in good agreement with that reported for bisdemethoxycurcumin of about $2.01 \pm 0.03 \times 10^5$ M⁻¹ (Mehranfar, Bordbar, Keyhanfar & Behbahani, 2013). 389 However, a four times lower K_A value of 4.4 \pm 0.03 \times 10⁴ M⁻¹ was obtained for 390 391 diacetylcurcumin-ß-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 role in the interaction with ß-casein (Mohammadi, Bordbar, Divsalar, Mohammadi, & 394 Saboury, 2009). Lower K_A values in the order of 1.10³ M⁻¹ were obtained for other 395 396 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 $(13.43 \times 10^5 \text{ M}^{-1})$ than that obtained in the present study (Nadi et al., 2014). This 399 400 difference could be attributed to the presence of α and κ -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 β -casein 407 interactions (Forrest et al., 2005) reported significantly higher K_A values (3.8-7.7 × 10⁶ 408 M⁻¹) compared to the K_A presented in Table 1 (5.8 ± 1.1 × 10⁴ 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 β -case than vitamin D3. Firstly, 412 the hydrophobicity of vitamin D3 (logP = 7.9) is significantly higher than that observed 413 for curcumin (logP = 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 π - π stacking interaction with the aromatic side chains of β -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 β -432 casein and the hydrophobic bioactive. Thus, the fluorescence quenching method does 433 not consider the interaction between bioactive compounds and other sites on β -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 β -case 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 tale 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 ß-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 β -case in 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 β-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 ß-casein and 454 two hydrophobic compounds was performed to understand how these bioactive 455 compounds could be associated to β-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 ß-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 β -casein (K_A) = $23.5 \pm 1.9 \times 10^4 \text{ M}^{-1}$) than vitamin D3 (K_A = $5.8 \pm 1.1 \times 10^4 \text{ M}^{-1}$). The K_A value for 461 curcumin- β -case in interaction has been successfully evaluated (2.45 ± 1.4 × 10³ M⁻¹) 462 463 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 β -case in 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 β -casein. Unlike fluorescence quenching, SPR, a powerful non-destructive and label-free technique, offers a direct and real-time 472

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 β -casein compared to vitamin D3. The SPR developed method 478 is versatile for measuring the binding of β -casein to other bioactive molecules, 479 promising applications in many other protein-small molecules interactions.

480

481 Acknowledgements

482 The authors would like to acknowledge the French Ministry of Higher Education and483 Research for financial support.

484

485 **References**

- 486 Benzaria, A., Maresca, M., Taieb, N., & Dumay, E. (2013). Interaction of curcumin
 487 with phosphocasein micelles processed or not by dynamic high pressure. *Food*488 *Chemistry*, 138, 2327-2337.
- 489 Bohin, M. C., Vincken, J. P., Van Der Hijden, H. T. W. M., & Gruppen, H. (2012).
- 490 Efficacy of food proteins as carriers for flavonoids. *Journal of Agricultural and*491 *Food Chemistry*, 60(16), 4136–4143.
- Bourassa, P., Bariyanga, J., & Tajmir-Riahi, H. A. (2013). Binding sites of resveratrol,
 genistein, and curcumin with milk α- and β-caseins. *Journal of Physical Chemistry B*, *117*(5), 1287–1295.
- 495 Costache, A. D., Sheihet, L., Zaveri, K., & Knight, D. (2010). Polymer-drug
 496 interactions in tyrosine-derived triblock copolymer nanospheres: a computational
 497 modeling approach. *Molecular Pharmaceutics*, 6(5), 1620–1627.

- 498 De Kruif, C. G., & Grinberg, V. Y. (2002). Micellisation of β-casein. *Colloids and*499 Surfaces, A: Physicochemical and Engineering Aspects, 210(2–3), 183–190.
- De Mol, N. J., & Fischer, M. (2008). Kinetic and thermodynamic analysis of ligandreceptor interactions: SPR applications in drug development. In *Handbook of Surface Plasmon Resonance* (pp. 123–172).
- 503 Douzi, B. (2017). Protein-protein interactions: Surface Plasmon Resonance. In L.
- Journet & E. Cascales (Eds.), *Bacterial Protein Secretion Systems* (Vol. 1615, pp.
 257–275). New York: Springer.
- 506 Esmaili, M., Ghaffari, S. M., Moosavi-Movahedi, Z., Atri, M. S., Sharifizadeh, A.,
- 507 Farhadi, M., Moosavi-Movahedi, A. A. (2011). β-casein-micelle as a nano vehicle
 508 for solubility enhancement of curcumin; food industry application. *LWT Food*509 *Science and Technology*, 44(10), 2166–2172.
- 510 Fischer, M. J. E. (2010). Amine Coupling Through EDC/NHS: A Practical Approach.
- 511 In N. Mol & M. Fischer (Eds.), *Surface Plasmon Resonance* (Vol. 627, pp. 55–74).
 512 Humana Press.
- 513 Forrest, S. A., Yada, R. Y., & Rousseau, D. (2005). Interactions of vitamin D3 with
- 514 bovine β -lactoglobulin A and β -casein. *Journal of Agricultural and Food* 515 *Chemistry*, 53(20), 8003–8009.
- 516 Gupta, R., Behera, C., Paudwal, G., Rawat, N., Baldi, A., & Gupta, P.N. (2019). Recent
 517 advances in formulation strategies for efficient delivery of vitamin D. AAPS
- 518 *PharmSciTech.*, 20, 11.
- 519 Hasni, I., Bourassa, P., Hamdani, S., Samson, G., Carpentier, R., & Tajmir-Riahi, H. A.
- 520 (2011). Interaction of milk α- and β-caseins with tea polyphenols. *Food Chemistry*,
 521 *126*(2), 630–639.
- 522 Hewlings, S.J., & Kalmen, D.S. (2017). Curcumin: a review of its' effects on human

523 health. *Foods*, *6*, 92.

- Hobani, Y., Jerah, A., & Bidwai, A. (2017). A comparative molecular docking study of
 curcumin and methotrexate to dihydrofolate reductase. *Bioinformation*, *13*(03), 63–
 66.
- 527 Holt, C., & Sawyer, L. (1993). Caseins as rheomorphic proteins: interpretation of 528 primary and secondary structures of the α_{s1} -, β - and κ -caseins. *Journal of the* 529 *Chemical Society, Faraday Transactions*, 89(15), 2683–2692.
- Homola, J., Yee, S. S., & Gauglitz, G. (1999). Surface plasmon resonance sensors:
 review. *Sensors and Actuators B: Chemical*, 54(1), 3–15.
- Johnsson, B., Löfås, S., & Lindquist, G. (1991). Immobilization of proteins to a
 carboxymethyldextran-modified gold surface for biospecific interaction analysis in
 surface plasmon resonance sensors. *Analytical Biochemistry*, 198(2), 268–277.
- Khumsupan, P., Ramirez, R., Khumsupan, D., & Narayanaswami, V. (2011).
 Apolipoprotein E LDL receptor-binding domain-containing high-density
 lipoprotein: a nanovehicle to transport curcumin, an antioxidant and anti-amyloid
 bioflavonoid. *Biochemica et Biophysica Acta*, *1808*(1), 352–359.
- Lakowicz, J. R. (2006). Principles of fluorescence spectroscopy. Principles of *Fluorescence Spectroscopy Principles of Fluorescence Spectroscopy*, Springer,
 New York, USA, 3rd edn.
- Letchford, K., Liggins, R., & Burt, H. (2008). Solubilization of hydrophobic drugs by
 methoxy poly(ethylene glycol)-block-polycaprolactone diblock copolymer
 micelles: theoretical and experimental data and correlations. *Journal of Pharmaceutical Sciences*, 97(3), 1179–1190.
- Majhi, A., Rahman, G., Panchal, S., & Das, J. (2010). Binding of curcumin and its long
 chain derivatives to the activator binding domain of novel protein kinase C. *Bioorg*

- 548 *Med Chem*, 18(4), 1591–1598.
- 549 Marchesseau, S., Mani, J. C., Martineau, P., Roquet, F., Cuq, J. L., & Pugnière, M.
- 550 (2002). Casein interactions studied by the surface plasmon resonance technique.
 551 *Journal of Dairy Science*, 85(11), 2711–21.
- Mcgaughey, G. B., Gagne, M., & Rappe, A. K. (1998). π-stacking interactions alive and
 well in proteins. *The Journal of Biological Chemistry*, 273(25), 15458–15463.
- Mehranfar, F., Bordbar, A. K., Fani, N., & Keyhanfar, M. (2013). Binding analysis for
 interaction of diacetylcurcumin with β-casein nanoparticles by using fluorescence
 spectroscopy and molecular docking calculations. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *115*, 629–635.
- Mehranfar, F., Bordbar, A. K., Keyhanfar, M., & Behbahani, M. (2013).
 Spectrofluoremetric and molecular docking study on the interaction of
 bisdemethoxycurcumin with bovine β-casein nanoparticles. *Journal of Luminescence*, 143, 687–692.
- Moeiniafshari, A. A., Zarrabi, A., & Bordbar, A. (2015). Exploring the interaction of
 naringenin with bovine β-casein nanoparticles using spectroscopy. *Food Hydrocolloids*, 51, 1–6.
- Mohammadi, F., Bordbar, A.K., Divsalar, A., Mohammadi, K., & Saboury, A.A.
 (2009). Analysis of binding interaction of curcumin and diacetylcurcumin with
 human and bovine serum albumin using fluorescence and circular dichroism
 spectroscopy. *Protein Journal*, 28, 189-196.
- 569 Myszka, D.G., Jonsen, M.D., & Graves, B.J. (1998). Equilibrium analysis of high
 570 affinity interactions using Biacore. *Analytical Biochemistry*, 265, 326-330.
- 571 Nadi, M.M., Kooshk, M.R.A., Mansouri, K., Ghadami, S.A.G., Amani, M., Ghobadi,
- 572 S., & Khodarahmi, R. (2015). Comparative spectroscopic studies on curcumin

- stabilization by association to bovine serum albumin and casein: A perspective on
 drug-delivery application. *International Journal of Food Properties*, 18, 638-659.
- 575 O'Connell, J. E., Grinberg, V. Y., & De Kruif, C. G. (2003). Association behavior of β576 casein. *Journal of Colloid and Interface Science*, 258(1), 33–39.
- 577 Okuro, P. K., Furtado, G. F., Sato, A. C. ., & Cunha, R. L. (2015). Structures design for
 578 protection and vehiculation of bioactives. *Current Opinion in Food Science*, *5*, 67–
 579 75.
- Park, Y. J., Kim, H. K., Lim, D. W., & Lee, E. K. (2015). Effects of pH and protein
 conformation on in-solution complexation between bovine α-lactalbumin and oleic
 acid : Binding trend analysis by using SPR and ITC. *Process Biochemistry*, *50*(9),
 1379–1387.
- Plesa, M., Kim, J., Paquette, S. G., Gagnon, H., Ng-Thow-Hing, C., Gibbs, B. F.,
 Coulton, J. W. (2011). Interaction between MMACHC and MMADHC, two
 human proteins participating in intracellular vitamin B12 metabolism. *Molecular Genetics and Metabolism*, 102(2), 139–148.
- Ranadheera, C. S., Liyanaarachchi, W. S., Chandrapala, J., Dissanayake, M., &
 Vasiljevic, T. (2016). Utilizing unique properties of caseins and the casein micelle
 for delivery of sensitive food ingredients and bioactives. *Trends in Food Science*
- 591 *and Technology*, 57, 178–187.
- Sahu, A., Kasoju, N., & Bora, U. (2008). Fluorescence study of the curcumin-casein
 micelle complexation and its application as a drug nanocarrier to cancer cells. *Biomacromolecules*, 9(15), 2905–2912.
- Schuck, P., & Zhao, H. (2010). The role of mass transport limitation and surface
 heterogeneity in the biophysical characterization of macromolecular binding
 processes by SPR biosensing. *Methods in Molecular Biolgy*, 627, 15–54.

- Semo, E., Kesselman, E., Danino, D., & Livney, Y. D. (2007). Casein micelle as a
 natural nano-capsular vehicle for nutraceuticals. *Food Hydrocolloids*, 21(5–6),
 936–942.
- 601 Shapira, A., Davidson, I., Avni, N., Assaraf, Y. G., & Livney, Y. D. (2012). β-Casein
- 602 nanoparticle-based oral drug delivery system for potential treatment of gastric
- 603 carcinoma: Stability, target-activated release and cytotoxicity. *European Journal of*

604 *Pharmaceutics and Biopharmaceutics*, 80(2), 298–305.

- 605 Swaisgood, H. E. (2003). Chemistry of the caseins. In P. F. Fox & P. L. H. McSweeney
 606 (Eds.), *Advanced Dairy Chemistry* (Vol. 1, pp. 139–202). New York.
- 607 Thompson, A. K., Singh, H., & Dalgleish, D. G. (2010). Use of surface plasmon
- 608 resonance (SPR) to study the dissociation and polysaccharide binding of casein
- micelles and caseins. *Journal of Agricultural and Food Chemistry*, 58(22), 11962–
 11968.
- 611 Vachali, P., Li, B., Nelson, K., & Bernstein, P.S. (2012). Surface plasmon resonance
- 612 (SPR) studies on the interactions of carotenoids and their binding proteins.
 613 *Archives of Biochemistry and Biophysics*, *519*, 32-37.
- Vieira da Silva, B., Barreira, J. C. M., & Oliveira, M. B. P. P. (2016). Natural
 phytochemicals and probiotics as bioactive ingredients for functional foods:
 Extraction, biochemistry and protected-delivery technologies. *Trends in Food Science and Technology*, *50*, 144–158.
- 618 Vieth, R., Kimball, S., Hu, A., & Walfish, P. G. (2004). Randomized comparison of the
- 619 effects of the vitamin D3 adequate intake versus 100 mcg (4000 IU) per day on
- 620 biochemical responses and the wellbeing of patients. *Nutrition Journal*, *3*, 8.
- 621 Walstra, P., Wouters, J. T., & Geurts, T. J. (2006). Dairy Science and Technology.
- 622 Second edition (Taylor & F, Vol. 4).

- 623 Watrelot, A. A., Tien Tran, D., Buffeteau, T., Deffieux, D., Le Bourvellec, C., Quideau,
- S., & Renard, C. M. G. C. (2016). Applied surface science immobilization of
 flavan-3-ols onto sensor chips to study their interactions with proteins and pectins
 by SPR. *Applied Surface Science*, *371*, 512–518.
- 627 Williams, M. A. K., & Daviter, T. (2013). Protein-Ligand Interactions: Methods and
- 628 *Applications*. (M. A. K. Williams & T. Daviter, Eds.) (2nd ed.). Humana Press.
- Kiao, C. Q., Jiang, F. L., Zhou, B., Li, R., & Liu, Y. (2011). Interaction between a
 cationic porphyrin and bovine serum albumin studied by surface plasmon
 resonance, fluorescence spectroscopy and cyclic voltammetry. *Photochemical &*
- 632 *Photobiological Sciences*, *10*(7), 1110–7.
- 633

635 Figure captions

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

643

Figure 2: Steady-state affinity analysis of β-casein binding to curcumin and vitamin D3
 respectively, prepared in different solvents for stock solutions then diluted in HBS-EP+.

646 β -case in immobilization level was 2 700 RU.

647

648 **Figure 3:** β-case in intrinsic fluorescence emission spectra (T=25°C, λ_{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 μM β-casein. Curcumin was prepared at 3 mM in absolute

ethanol then added to β -case in 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 μ M. Vitamin D3 was prepared at 3 mM in absolute ethanol then added to β -

- 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 μ M. The experiment
- 656 was repeated three times.

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





Figure 2











Table 1: Binding parameters of the interactions between curcumin or vitamin D3 and β casein at 25°C obtained by SPR and fluorescence quenching.

Method	SPR	Fluorescence quenching		
	K _A	K _A	n	K _{sv}
	$(\times 10^3 \mathrm{M}^{-1})$	$(\times 10^4 \mathrm{M}^{-1})$		(× 10 ⁴ M ⁻¹)
Curcumin	2.45 ± 1.42	23.5 ± 1.9^{a}	0.74 ± 0.05^{a}	13.5 ± 2.0
Vitamin D3	-	5.8 ± 1.1^{b}	0.82 ± 0.04^{a}	3.8 ± 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)