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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

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26 **Abstract**

27 β -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 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 -casein interaction has been successfully evaluated ($4.1 \pm 0.7 \times 10^{-}$
33 4 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 β -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 β -casein ($K_A = 23.5 \pm 1.9 \times 10^4$ M $^{-1}$) than vitamin
38 D3 ($K_A = 5.8 \pm 1.1 \times 10^4$ 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 -casein 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,
67 Maresca, Taieb, & Dumay, 2013). Among the four different caseins, α_{s1} -, α_{s2} -, β - and κ -
68 caseins, a particular interest is paid to β -casein. 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 -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 β -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 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

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 (2.99×10^{-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 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)-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 β -
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 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 μ M β -
161 casein stock solution was prepared in HBS-EP buffer (pH 7.4). **The β -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 μ g/mL in 10 mM acetate buffer at pH 3.6 and was
167 injected at 10 μ l/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 μ 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 (ϵ) 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 μ 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 β -casein coated flow cells, in the injection of the
194 running buffer at a flow rate of 30 μ 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 β -casein flow cells. Chloramphenicol, a negative control, was also injected
198 at 500 and 1000 μ 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 β -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 β -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:

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

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 β -casein concentration ($[\beta\text{-casein}]$) is obtained as the
216 difference between R_{\max} , maximum response due to analyte binding directly
217 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:

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

220 where $K_D = k_d/k_a$.

221 The effectiveness of the fitting is evaluated by the 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 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 β -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 β -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 β -caseins on CM5 sensor chip. Firstly, the β -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 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 -casein 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 -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 β 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 β -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 β -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 (2.99×10^{-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 β -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 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
315 analyses is $4.1 \pm 0.7 \times 10^{-4}$ M for curcumin in DMSO. The calculated χ^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 β -casein and hydrophobic low molecular weight molecules
324 such as curcumin. To further explore the binding of β -casein to curcumin and vitamin
325 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
332 fluorescence emission of β -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 β -casein concentration is below its CMC in order to
335 prevent its self-association. Fluorescence emission spectra of β -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 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 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 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 -casein tryptophan residue
351 microenvironment, as consequence of curcumin binding which strongly suggests the
352 hydrophobic character of curcumin and β -casein 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- β -
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 -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 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
362 aqueous solutions, at experimental pH (~ 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 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](#)):

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

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 β -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 μM 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 β -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 ± 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- β -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 β -casein (Mohammadi, Bordbar, Divsalar, Mohammadi, &**
395 **Saboury, 2009). Lower K_A values in the order of 1.10^3 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 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 ($3.8\text{-}7.7 \times 10^6$
408 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 β -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 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 -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 β -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 -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 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 β -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 β -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
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- β -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 β -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 β -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 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

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484

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633

634

635 **Figure captions**

636 **Figure 1:** Binding sensorgrams for curcumin interaction with immobilized β -casein on
637 CM5 biosensor at 25°C (A and B) and steady-state affinity analysis of curcumin
638 binding to β -casein fitted to a 1:1 interaction model (C and D). Two β -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 μ M. The experiment was repeated three times.

643

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

647

648 **Figure 3:** β -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 μ M β -casein. Curcumin was prepared at 3 mM in absolute
651 ethanol then added to β -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 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.

657

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 $\ln[\text{curcumin}]$ (A)
660 and $\ln[\text{Vitamin D3}]$ (B) for the binding parameter evaluation with β -casein at 25°C.
661

Figure 1

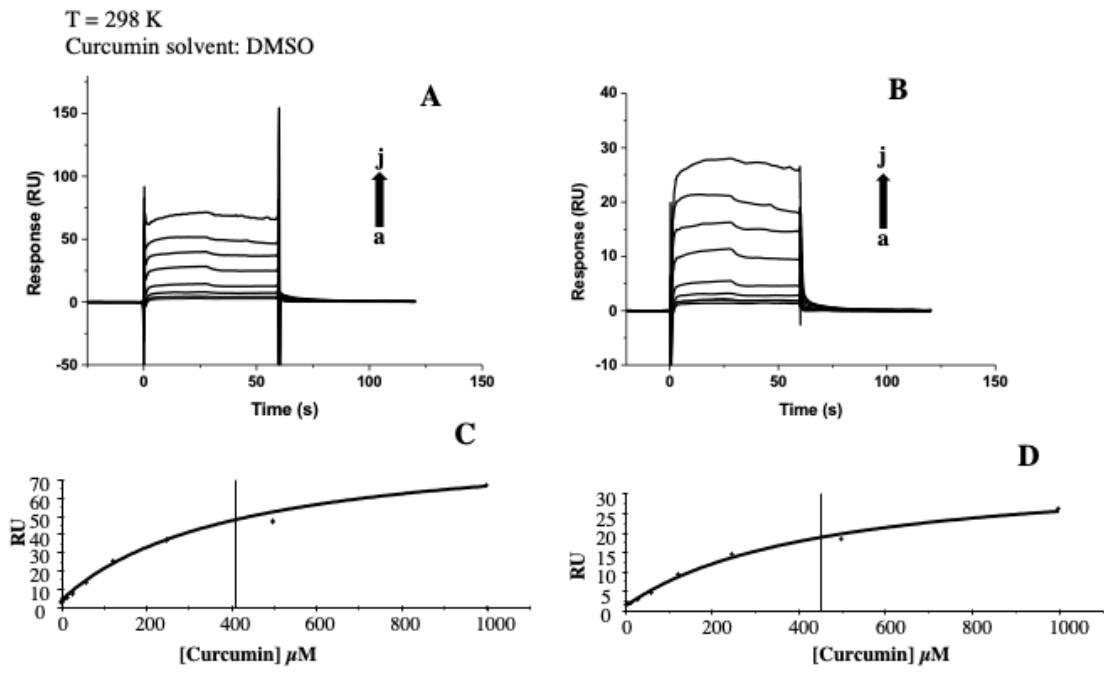


Figure 2

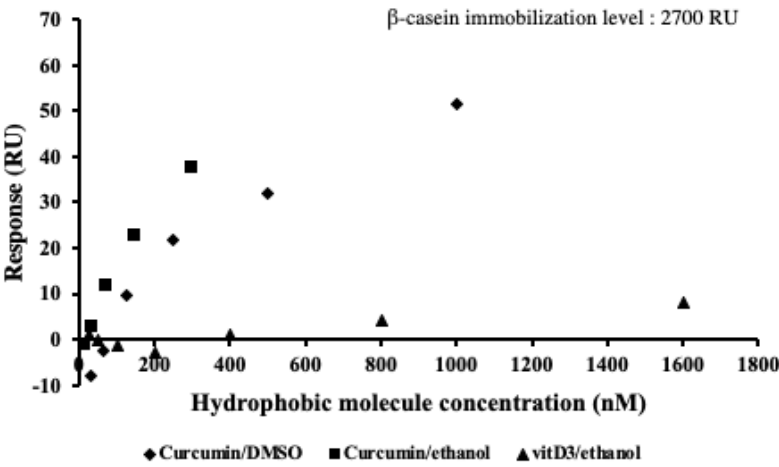


Figure 3

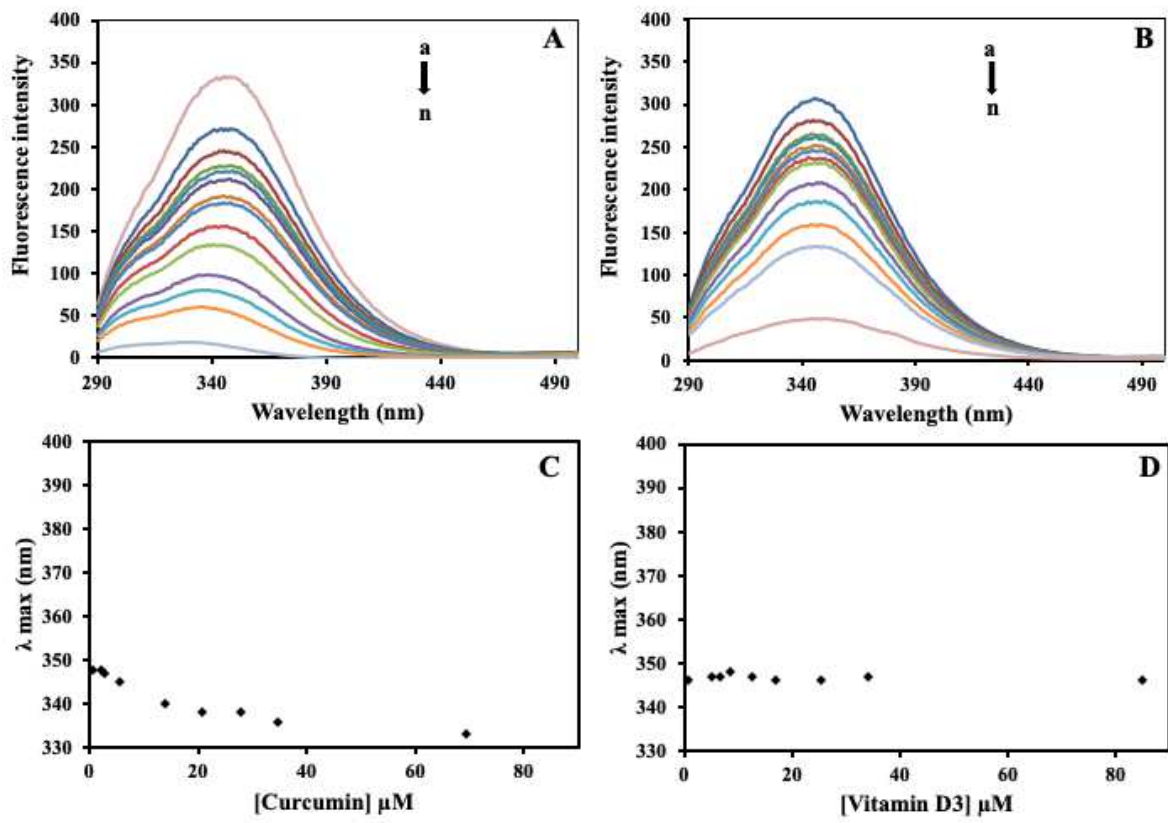
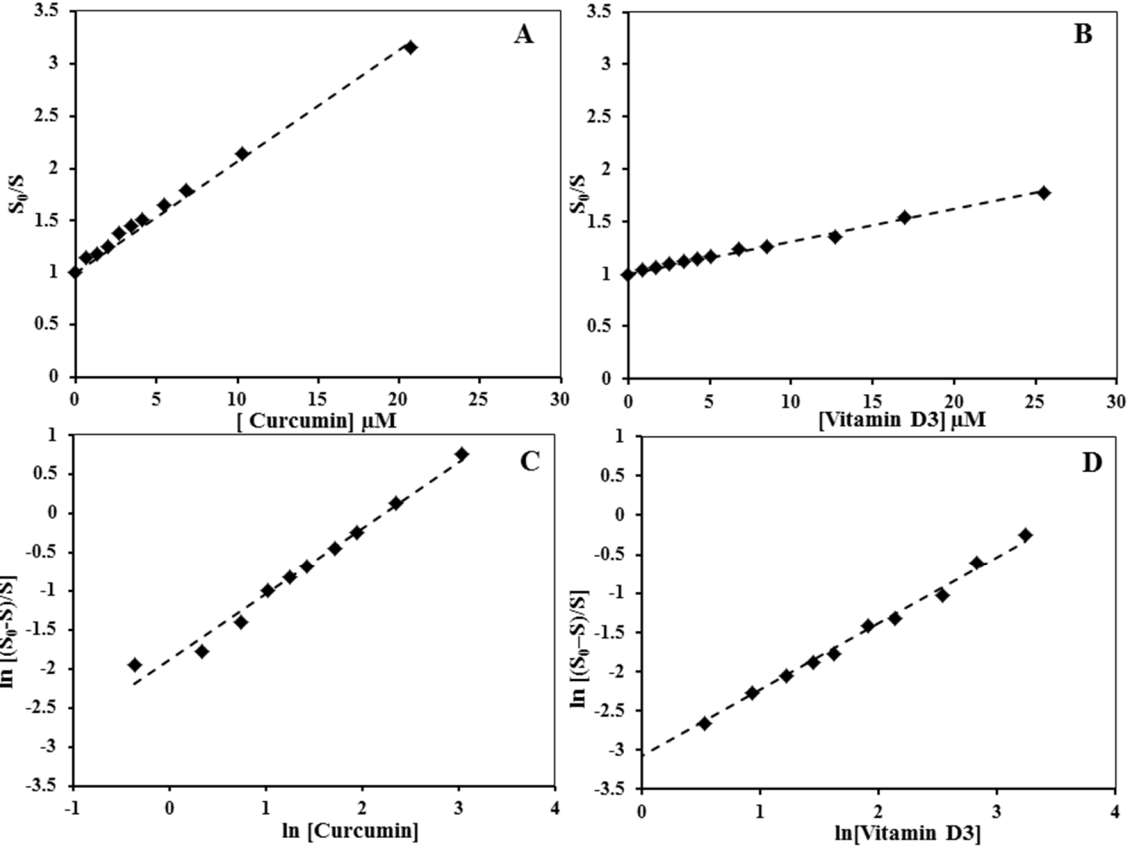


Figure 4



1 **Table 1:** Binding parameters of the interactions between curcumin or vitamin D3 and β -
 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 ± 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 ($p < 0.05$)

6