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Interaction between dietary bioactive peptides of short length and bile salts in submicellar or micellar state

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Abstract: Bile salts act as steroidal detergents in the gut, which could also interact with peptides and improve their bioavailability, but according to an unclear mechanism. The occurrence of direct interaction between milk bioactive peptides, Ile-Asn-Tyr-Trp, Leu-Asp-Gln-Trp, and Leu-Gln-Lys-Trp, and different bile salts in submicellar or micellar state was investigated by intrinsic fluorescence measurement and dynamic light scattering, above the critical micellar concentration, the latter being determined by isothermal titration calorimetry. The peptides form aggregates, spontaneously. In the presence of bile salts, some released peptide monomers were bound at the micellar surface. The lack of hydrogen bond involving the C12-OH group of the steroid skeleton and the acidic function of some bile salts might promote the interaction with the peptides, as well as the lack of the C12-OH group rather than that of the C7-OH group. At submicellar concentrations, sodium taurochenodeoxycholate and taurodeoxycholate readily interacted with the most hydrophobic peptide Ile-Asn-Tyr-Trp.



Vandœuvre-lès-Nancy, March 17, 2016

Revised submission FOODCHEM-D-15-05365

Dear Editor,

Thank you for having revised our article entitled:

“Interaction between dietary bioactive peptides of short length and bile salts in submicellar or micellar state”

By Justine Guerin, Alexandre Kriznik, Nick Ramalanjaona, Yves Le Roux, Jean-Michel Girardet

We have corrected the manuscript according to the requests of the two reviewers (see the attached file ‘Answers to reviewers’). Figure S1 has been corrected according to the request of reviewer 2. Please, could you reproduce Figure 3 in color on the Web and in black-and-white in print ?

Thanking you in advance,
Yours faithfully,

Dr. Jean-Michel GIRARDET

Answer to Reviewers

Reviewer #1

This manuscript described a study on the interaction between dietary bioactive peptides of short length and bile salts in submicellar or micellar state. Although there is some scientific significance in this manuscript, there are still some issues worth considering.

- 1. The major problem that needs attention is the lack of experiments on the molecular mechanism of some bile salts promoting the interaction with the peptides.**
- 2. After the interaction between bioactive peptides and bile salts, if the conformation of these peptides also changed, how to change?**
- 3. In fact, what I want to know is the authentic significance of this study.**
- 4. please rewrite the highlights.**

Firstly, we want to thank the reviewer for having revised our manuscript. It is important to note that this study mainly aims to describe and finally to highlight direct non-covalent interactions between short peptides of high physiological interest and pure bile salts. For this purpose, fluorescence spectroscopy and dynamic light scattering (DLS) were both combined to show that: i) pure peptides spontaneously aggregate in aqueous buffers, ii) peptide aggregates can be disrupted by some bile salts when added in the medium, iii) in some cases, the peptides seem to likely adsorb onto the surface of some bile salt micelles without disturbing their micellar structure. Obviously, some key features could be inferred from the results. However, determining the interaction mechanism of those peptide aggregates with bile salt micelles at the molecular level would deserve to be addressed to more detailed works because of the high complexity of the systems. As a starting point, NMR could be envisaged as a promising technology in identifying the molecular/functional determinants of the interaction between bile salts with those peptide aggregates. But, the NMR signature of the peptide could potentially be hidden by strong signals from the added bile salts. Alternatively, infrared spectroscopy could also help to provide information about conformational changes of the peptides, especially when they interact with bile salts. However, as shown in our study, the peptides formed aggregates in aqueous buffers, so that any significant changes in the infrared spectra could also involve disruption of the peptide aggregates by bile salts. More alternative strategies or approaches should thus be considered in order to go further towards molecular mechanisms.

Finally, for a clearer presentation of our study, the highlights were rewritten as required by the reviewer.

Reviewer #2

The paper is well written and results support the claims. Bile salts are one of the most studied among several compounds used to enhance nasal absorption. Kramer et al., 1994 have described how the attachment of a peptide to the C3 position of the bile acid increases uptake of the peptide. Given their importance, the development of accurate

and sensitive methods of instrumental analysis has been the subject of intensive research.

We thank the reviewer for the correction of the manuscript and for his/her good advices.

Page 3, lines 62-64: Indeed, the study of Kramer et al. (1994) seems to us very interesting and is quoted in the "Introduction" section of the revised version as following: "On the other hand, Kramer et al. (1994) have described how the covalent attachment of peptide to bile salt increases uptake of the peptide by ileal brush-border membrane vesicles via the specific intestinal absorption pathway for bile acids."

Page 21, lines 536-538: Reference Kramer et al. (1994) is added in the "References" section.

Minor comments:

- Table 3:

Particle sizes, all the 3 peptides (INYW, LDQW, LQKW) are following the similar trend in all bile salts except LDQW in NaTDC. Can the authors explain the discrepancy?

Indeed, it was a wrong value of particle size and we apologize to the reviewer. We have repeated this measurement in the same conditions as before and corrected the value (particle size of 3.8 nm instead of 5.1 nm) on Table 3 and the curve obtained with a mixture of LDQW and NaTDC on Figure S1 (supplementary data).

- DLS measurements:

1. Each sample should be measured twice, once with the attenuator position automatically optimized for determination of size distributions and a second time with maximum open attenuator position to ensure comparability of total scattering intensities.

Experiments are performed with samples of low concentration which do not scatter light much. Thus, in this case the attenuator has been set on a position allowing more light to pass through the sample. Moreover, each measurement is based on 12-16 scans by the Nanosizer device, with an automated attenuator position that allows to obtain better accuracy and reproducibility, which, with all the respect addressed to the reviewer, should not be modified as recommended by Malvern (manufacturer).

2. Did the influence of all buffer components on viscosity and Refractive index are accounted for or not?

Concerning the refractive index, we applied on it a corrective value including the contribution of the buffer as advised by the manufacturer (values pre-implemented into the software).

- ITC measurements:

A critical step in the analysis of an ITC thermogram is the distinction between the net reaction heat and the instrumental baseline power trace.

1. Did you carry out Automated baseline adjustment and peak integration (both pre- and post-injection baselines)?

We carry out an automated pre-baseline adjustment. For the post-baseline, we subtracted a blank run constituted of a buffer/buffer titration, but it was an insignificant process due to the huge heat signal measured for each bile salt (around 20 $\mu\text{cal/s}$ at the maximum of the injection peak). The sentence page 7 lines 164-165 is completed by "...and a blank titration (buffer into buffer) was subtracted".

2. Did you inject 1.4 ml sample or the 1.428 ml is the size of the cell?

Page 7, lines 159-161: The sentence is corrected as following: "During the titration, each concentrated bile salt solution was sequentially injected into a 1.428-ml reaction cell..." (cell volume: 1.428 ml).

3. What is reference power set on the instrument? What is the setting of the filter period?

We set a reference power of 2 $\mu\text{cal/s}$ adapted to the huge endothermic signals and the filter period was set to 2 s allowing the software to register the entire titration process (120 min). The sentence "Reference power was set at 2 $\mu\text{cal/s}$ adapted to the huge endothermic signals and the filter period was set to 2 s." is added in the text, page 7, lines 163-164.

Highlights

1/ Fluorescence spectroscopy and DLS were used to highlight peptide-bile salt interaction

2/ Antioxidant peptides from milk: LDQW, LQKW and INYW were studied as peptide models

3/ Self-aggregated peptides were dissociated by bile salt micelles

4/ Released peptides were likely bound to the surface of bile salt micelles

5/ INYW could especially interact with bile salts at submicellar concentrations

29

30 **ABSTRACT**

31

32 Bile salts act as steroidal detergents in the gut, which could also interact with peptides and improve
33 their bioavailability, but according to an unclear mechanism. The occurrence of direct interaction
34 between milk bioactive peptides, Ile-Asn-Tyr-Trp, Leu-Asp-Gln-Trp, and Leu-Gln-Lys-Trp, and
35 different bile salts in submicellar or micellar state was investigated by intrinsic fluorescence
36 measurement and dynamic light scattering, above the critical micellar concentration, the latter being
37 determined by isothermal titration calorimetry. The peptides form aggregates, spontaneously. In the
38 presence of bile salts, some released peptide monomers were bound at the micellar surface. The
39 lack of hydrogen bond involving the C12-OH group of the steroid skeleton and the acidic function
40 of some bile salts might promote the interaction with the peptides, as well as the lack of the C12-
41 OH group rather than that of the C7-OH group. At submicellar concentrations, sodium
42 taurochenodeoxycholate and taurodeoxycholate readily interacted with the most hydrophobic
43 peptide Ile-Asn-Tyr-Trp.

44

45

46 *Keywords:* bile salts, CMC, DLS, ITC, milk whey proteins, peptides, tryptophan intrinsic fluores-
47 cence

48

49 1. Introduction

50

51 Biological activities of peptides have been widely studied during the two last decades. Thus,
52 several milk-derived bioactive peptides are nowadays commercially available in functional foods
53 but also in ingredients (Mills, Ross, Hill, Fitzgerald, & Stanton, 2011). To prove their effectiveness
54 *in vivo*, there is a growing interest in the study of the bioavailability of such dietary bioactive
55 peptides in the gastrointestinal tract. In this context, bile salts, as steroidal detergents, may help
56 bioactive peptides in reaching their targets in their active form during the postprandial phase in the
57 gut. Indeed, they increase epithelial paracellular permeability by modulating tight junctions and
58 therefore, facilitate the transport of drugs and other nutrients through the intestinal barrier
59 (Mukaizawa et al., 2009). Cakir-Kiefer, Miclo, Balandras, Dary, Soligot, and Le Roux (2011) have
60 shown, for instance, that the bile salts protect the α -casozepine, an α_{s1} -casein decapeptide exhibiting
61 an anxiolytic activity, against hydrolysis by intestinal peptidases and also improve the transport of
62 α -casozepine across Caco-2 cell monolayer. On the other hand, Kramer et al. (1994) have described
63 how the covalent attachment of peptide to bile salt increases uptake of the peptide by ileal brush-
64 border membrane vesicles via the specific intestinal absorption pathway for bile acids.

65 Bile salts are components derived from the cholesterol molecule which display an
66 amphipathic complex structure: The convex side of their cyclopentanoperhydrophenanthrene
67 nucleus (steroid skeleton) is hydrophobic, with the presence of hydrogen and methyl groups,
68 whereas the concave side is hydrophilic due to the presence of hydroxyl groups. Bile salts also
69 possess a lateral chain that may be conjugated with taurine or glycine (Bloch & Watkins, 1978;
70 Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). Bile salts can be categorized
71 according to the number and position of hydroxyl groups but also to the presence or not of
72 conjugation. Due to the spatial geometry of the steroid skeleton, the bile salts can form micelles
73 with a low aggregation number. The morphology of these micelles is complex and still under study
74 (e.g. Posa & Sebenji, 2014). Below the critical micellar concentration (CMC), the bile salts are in

75 monomeric form, whereas in conditions neighboring CMC, small aggregates begin to form primary
76 micelles (Madenci & Egelhaaf, 2010). Bile salts are secreted in the liver and transported *via* the bile
77 into the intestine, where they improve digestion, for instance, by emulsifying products from
78 enzymatic breakdown of dietary fats by forming mixed micelles with the poorly-soluble nutrients
79 which will be further transported through the intestinal barrier (Verde & Frenkel, 2010). They also
80 play a major role in the enzymatic lipolysis by interacting with the colipase, an amphiphile
81 polypeptide of 10 kDa that anchors the pancreatic lipase at the surface of lipid droplets in the
82 intestine. The hydrophilic side of the colipase interacts with the lipase, whereas its hydrophobic side
83 is a common binding site for lipid droplets and bile salts (Kerfelec et al., 2008).

84 The enterohepatic circulation of bile salts contributes to the regulation of cholesterol
85 homeostasis. Many studies have reported the hypocholesterolemic effects of proteins. Peptides
86 released by proteolysis and exhibiting high bile salt binding capacity could inhibit the reabsorption
87 of bile salts in the ileum leading to decrease the blood cholesterol level. For instance, a β -
88 lactoglobulin-derived pentapeptide (Ile⁷¹-Ile-Ala-Glu-Lys⁷⁵) has been identified to have a
89 hypocholesterolemic effect according to an unclear mechanism but likely involving bile salt binding
90 properties (Nagaoka et al., 2001). More recently, some authors have reported the ability of peptide
91 hydrolysates to inhibit bile salt re-absorption in the ileum, and therefore to lower cholesterol levels
92 in the bloodstream (Perez-Galvez, Garcia-Moreno, Morales-Medina, Guadix, & Guadix, 2015).
93 This would also be directly linked to their capacity to bind bile salts. But the interactions at
94 molecular scale between bile salt and small peptides are still unclear.

95 Noteworthy, bile salts are also able to bind compounds such as drugs, proteins, or high-
96 molecular mass polypeptides, for which the molecular interactions have been studied in details. For
97 instance, interaction of phenothiazines, anxiolytic drugs, with bile salts such as sodium cholate
98 (NaC) and sodium deoxycholate (NaDC) has been recently investigated by measurement of
99 different physico-chemical parameters including CMC, surface tension, UV absorption, and
100 intrinsic fluorescence emission. The results showed that these drugs can interact with these bile salts

101 to form mixed micelles, either by electrostatic interactions involving the $(\text{CH}_3)_2\text{NH}^+$ group of the
102 drug molecule and the carboxylate function of the lateral chain of the bile salts, or by hydrophobic
103 interactions between the phenothiazine ring and the hydrophobic convex side of the steroid skeleton
104 (Mahajan & Mahajan, 2012). In another study, some authors have shown that oil-in-water
105 emulsions stabilized with either β -lactoglobulin or lactoferrin interact with a complex mixture of
106 bile salts and that the interaction capability mostly depends on the isoelectric point (pI) of the
107 proteins (Sarkar, Horne, & Singh, 2010; Singh & Sarkar, 2011). Another study using circular
108 dichroism has unveiled interaction between bile salts (NaC and NaDC), either in submicellar or
109 micellar forms, and polypeptides such as poly[Leu-Leu-Lys] and poly[Leu-Leu-Asp] displaying
110 molecular masses ranging from 94 to 140 kDa (D'Alagni, D'Archivio, & Giglio, 1993). According
111 to the authors, both NaC and NaDC may interact with the polypeptides either by hydrophobic
112 interaction involving their hydrophobic side and the Leu residues or by forming hydrogen bonds
113 between the hydroxyl groups located at C3 and C12 of the steroid skeleton and the amino function
114 of the Lys residues.

115 Many short peptide sequences, that are encrypted in dietary proteins and liberated by
116 bacterial or intestinal epithelial cell proteases, are known to play a beneficial role in human health.
117 They display a variety of activities such as antimicrobial, antioxidative, antithrombotic,
118 antihypertensive, anti-inflammatory, and immunomodulatory activities mainly investigated *in vitro*
119 (Mills et al., 2011). Nevertheless, only few clinical studies have demonstrated biological effects of
120 such dietary peptides (e.g. the antihypertensive lactotriptides Ile-Pro-Pro and Val-Pro-Pro;
121 Boelsma & Kloek, 2009). To reach their target and exert their biological activity *in vivo*, the
122 peptides of interest have to be (i) resistant toward hydrolysis by peptidases of the brush border, (ii)
123 absorbed through the gastrointestinal barrier, and (iii) carried in the bloodstream in an active form
124 and optionally across the blood-brain barrier.

125 The aim of the present study is to investigate non-covalent interactions between bioactive
126 peptides at effective concentrations and pure bile salts at neutral pH (close to pH within both

127 jejunum and ileum) as a function of the bile salt concentration, then to try to define whether such
128 interactions are rather polar or hydrophobic. The tetrapeptides Ile-Asn-Tyr-Trp (INYW) and Leu-
129 Asp-Gln-Trp (LDQW) derived from α -lactalbumin and Leu-Gln-Lys-Trp (LQKW) from β -
130 lactoglobulin after thermolysin hydrolysis were chosen as bioactive peptide models having free
131 radical scavenging activity *in vitro* (Sadat, Cakir-Kiefer, N'Negue, Gaillard, Girardet, & Miclo,
132 2011; Contreras, Hernandez-Ledesma, Amigo, Martin-Alvarez, & Recio, 2011). The peptide
133 LQKW also displays an antihypertensive activity in spontaneously hypertensive rats (Hernandez-
134 Ledesma, Miguel, Amigo, Aleixandre, & Recio, 2007). Several pure bile salts were selected
135 according to their physicochemical properties: The primary bile salts (produced in the liver) NaC,
136 sodium chenodeoxycholate (NaCDC), sodium glycocholate (NaGC), sodium taurocholate (NaTC),
137 and sodium taurochenodeoxycholate (NaTCDC), and the secondary bile salts (generated by
138 intestinal bacteria) NaDC and sodium taurodeoxycholate (NaTDC). The occurrence of electrostatic
139 or hydrophobic interactions was studied by measurements of intrinsic fluorescence emission due to
140 excitation of Trp residues, below and above the CMC of bile salts, and by dynamic light scattering
141 (DLS) above the CMC. Beforehand, isothermal titration calorimetry (ITC) was performed to
142 determine the CMCs of the bile salts under the experimental conditions used.

143

144 **2. Materials and methods**

145

146 *2.1. Peptides and bile salts*

147

148 Peptides LDQW (560.26 Da), LQKW (573.33 Da), and INYW (594.28 Da) were
149 synthesized by Genosphere Biotechnologies (Paris, France). Purity of each peptide was further
150 improved (final purity upper than 97%) by reversed-phase high-performance liquid chromatography
151 as described in previous study (Sadat et al., 2011). Pure bile salt (NaC, NaCDC, NaDC, NaGC,
152 NaTC, NaTCDC, NaTDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

153

154 2.2. Isothermal titration calorimetry (ITC)

155

156 The different bile salts (at 50 mM) were solubilized in 20 mM
157 trishydroxymethylaminomethane/HCl (Tris/HCl) buffer, pH 7.0, containing 150 mM NaCl (Tris-
158 buffered saline or TBS). All the ITC experiments were carried out at 310 K in TBS using a VP-ITC
159 microcalorimeter (MicroCal, Northampton, MA, USA). During the titration, each concentrated bile
160 salt solution was sequentially injected into a 1.428-ml reaction cell, initially containing the degassed
161 buffer, to achieve the expected final concentrations. Each injection lasted 20 s, and intervals of 180
162 s between successive injections were applied. A rotating 290-ml micro-syringe permitted a constant
163 stirring at a speed of 300 rpm. Reference power was set at 2 μ cal/s adapted to the huge endothermic
164 signals and the filter period was set to 2 s. Experiments were carried out in duplicate to ensure the
165 repeatability of the method and a blank titration (buffer into buffer) was subtracted. The
166 experimental data were analyzed using the Origin 6.2 software provided by MicroCal.

167

168 2.3. Spectrofluorimetry

169

170 The analyses were carried out with a Xenius spectrofluorimeter (Safas, Monaco). Each
171 peptide was solubilized in TBS buffer at 3.4 μ M for LQKW and LDQW, and 6.6 μ M for INYW
172 (determined by UV absorbance measurement at 280 nm) and each bile salt (100 mM in TBS buffer,
173 determined by weighing) was mixed with the peptide solutions as volume/volume, in a quartz
174 cuvette with an optical path of 1 cm. The final concentrations were then of 1.7 μ M for LQKW and
175 LDQW and 3.3 μ M for INYW in order to reach *ca.* 50% of relative value of emission intensity, or
176 EMI, at the emission wavelength $\lambda = 358$ nm. The bile salts were used (i) at different concentrations
177 below and close to their CMCs previously determined and (ii) at a final concentration of 50 mM,
178 widely upper than their respective CMCs. For total fluorescence measurements, the selected step

179 was 2 nm and the bandwidth was 10 nm. The excitation wavelength was 295 nm to selectively
180 excite Trp residue of the peptides and the emission spectrum was recorded between 310 and 550 nm.
181 The photomultiplier voltage was adjusted between 675 and 800 V in order to obtain a signal close to
182 50% EMI. The measurements were carried out in duplicate, at 37 °C. The emission spectrum of
183 each bile salt (at adequate final concentration) formed a background noise and was systematically
184 subtracted from the emission spectrum of the corresponding peptide/bile salt mixture.

185

186 *2.4. Dynamic Light Scattering (DLS)*

187

188 The particle size determination of bile salt micelles and potentially, peptide aggregates was
189 performed by granulometry on a Zetasizer Nano-S model (Malvern Instruments, Malvern, UK).
190 Each pure peptide was analyzed by DLS at final concentrations of 3.3 µM for INYW and 1.7 µM
191 for LQKW and LDQW, either in TBS buffer or in ultra-pure water alone or in the presence of 0.1%
192 trifluoroacetic acid. Each pure bile salt was also analyzed by DLS at final concentration of 50 mM
193 in TBS buffer. Each peptide/bile salt mixture was prepared in TBS buffer, by taking the same final
194 concentrations as above. Prior to the DLS investigations, all samples were centrifuged at 21,130 g
195 for 30 min at 37 °C to eliminate dust or undesired particles having hydrodynamic diameter higher
196 than 1000 nm that could interfere the measure. The supernatant of each sample was gently
197 transferred into a quartz cuvette of 12 µl and the particle size measurements were performed in
198 triplicate at 37 °C, with a light diffusion at 173°. The data were collected in automatic mode and
199 analyzed using the associated software DTS version 4.2 (Malvern Instruments).

200

201 **3. Results and discussion**

202

203 *3.1 Experimental conditions*

204

205 The Trp-containing synthetic peptides used in this study corresponded to thermolytic
206 peptides of whey proteins (α -lactalbumin and β -lactoglobulin) that display interesting bioactive
207 activities: free radical scavenging activity for LDQW, LQKW and INYW but also antihypertensive
208 activity for LQKW (Contreras et al., 2011; Sadat et al., 2011). Since they possibly interact with bile
209 salts thanks to non-covalent interactions, those peptides are therefore interesting models to
210 investigate the influence of bile salts on the efficiency of peptide absorption through the
211 gastrointestinal barrier. Furthermore, the presence of tryptophan residues within their primary
212 sequence allowed them to be used as fluorescent tool to monitor their interaction with the bile salts
213 but also their effect on the CMCs by fluorescence spectroscopy. The peptide concentrations (1.7 μ M
214 for LQKW and LDQW, and 3.3 μ M for INYW) were in a same order of magnitude than the
215 reported effective concentrations of other peptides tested *in vivo*, such as the antibacterial
216 lactoferricin detected in the upper small intestine (Kuwata et al., 2001) and the 93-123 β -
217 caseinopeptide involved in gut protection (Plaisancié et al., 2013).

218 In the present experimental conditions, pH, the nature of the buffering agent, the ionic
219 strength, and the concentration of bile salts were carefully chosen. Physiologically, the micellar
220 solubilization of lipids by bile salts occurs efficiently when the bile salts have an adequate
221 concentration. The latter is reached only in the jejunum in which the pH was *ca.* 7.0. Besides, at
222 neutral pH, the three peptides have different states of charge and were therefore suitable models for
223 the present study: LDQW is negatively charged (pI = 3.80), INYW is in a zwitterionic state and
224 displays therefore a zero net charge (pI = 5.52), and LQKW is positively charged (pI = 8.75). The
225 Tris molecule was used as buffering agent, since it is not charged at pH 7.0 (pKa = 8.06 at 25 °C
226 and *ca.* 7.70 at 37 °C) and thus does not interact with the peptides. In the experiments performed in
227 the presence of bile salts in micellar form, the concentration of the bile salts was fixed at 50 mM, a
228 concentration widely greater than their respective CMCs in order to generate secondary aggregates
229 built from primary micelles (see below). All the experiments were performed in the presence of 150
230 mM NaCl, as the Na⁺ and Cl⁻ ions (as well as other electrolytes such as K⁺, Ca²⁺, and HCO³⁻ but in

231 a lesser extent) are secreted in the bile extract and contribute to the ionic strength in the intestinal
232 tractus.

233

234 *3.2 Determination of bile salt CMCs*

235

236 Bile salts undergo a rapid dynamic association-dissociation equilibrium to form self-
237 aggregates or micelles when their concentration reaches and exceeds CMC. Usually, CMC values of
238 bile salts are mostly determined either in pure water or in the presence of 150 mM NaCl, at 25 °C
239 (Fini & Roda, 1987), but the temperature as well as the ionic strength exert an influence on the
240 micelle formation: an increase of their respective values promotes a decrease of the bile salt micelle
241 size by minimizing the electrostatic repulsions (Maestre, Guardado, & Moya, 2014). Because self-
242 aggregation continues to proceed with increasing concentration above CMC, the detection of the
243 lowest concentration at which the first aggregates form, strongly depends upon the physical-
244 chemical conditions particularly (Carey, 1985). It was thus crucial to determine the CMC of each
245 bile salt under our experimental conditions precisely, i.e. in the presence of 150 mM NaCl, at pH
246 7.0, and at 37 °C prior to study the possible interactions between the peptides and the bile salts in
247 submicellar concentrations and in the vicinity of their CMC.

248 Determination of the CMCs was carried out by ITC measurements according to the method
249 of Garidel, Hildebrand, Neubert, and Blume (2000). The second derivative of the titration isotherm
250 gives the CMC values of the bile salts with accuracy. The CMCs of NaTDC and NaTCDC were the
251 lowest among the bile salts investigated in the present study (CMC = 1.8 mM for NaTDC and
252 NaTCDC; Fig. 1). This was due to their particularly pronounced amphipathic nature, as NaTDC and
253 NaTCDC possess together the highest polar side chain (sulfonate group) and the lowest content of
254 polar hydroxyl groups (2 OH groups at C3 and C12 of the steroid skeleton for NaTDC and at C3
255 and C7 for NaTCDC; Table 1) leading to increase the hydrophobicity of the steroid skeleton.
256 Generally, CMCs determined for the seven kinds of bile salts in our experimental conditions

257 remained close to those reported by several authors (Table 1). In the present study, the aggregation
258 number (N_{agg}) was not taken into account as a relevant parameter, since it increases with the bile salt
259 concentration above the CMC to form bigger aggregates (Garidel et al., 2000).

260

261 3.3 Self-aggregation of peptides

262

263 It was worthy to note that all the three peptides were able to form aggregates in solution at
264 neutral pH, as shown by DLS performed with the pure peptides in TBS (main particle sizes of 178,
265 527, and 489 nm for LQKW, INYW, and LDQW, respectively; Table 2). However, the peptides in
266 their monomeric form (less than 600 Da) were not detectable, as the DLS can measure sizes
267 corresponding to a range of molecular masses between 10^3 and 2×10^7 Da. Therefore, the proportion
268 of molecules undergoing a spontaneous self-association remained unfortunately unknown.

269 The nature of the peptide self-association was mainly thought hydrophobic, probably
270 involving the C-terminal Trp lateral chain, as the peptides were able to self-associate either in pure
271 water or in the presence of 150 mM NaCl, but also under acidic conditions (0.1% trifluoroacetic
272 acid in water at pH *ca.* 2.5) as observed for INYW for instance. However, electrostatic interactions
273 such as salt bridge involving β -COO⁻ of the Asp residue and α -NH₃⁺ of Leu could not be excluded
274 in the self-association observed for LDQW. Indeed, this acidic peptide formed the biggest
275 aggregates at neutral pH and the smallest ones at pH 2.5 compared to that observed for the other
276 peptides in the same pH conditions. On the other hand, the most basic peptide LQKW displayed a
277 reverse behavior as aggregates increased with a decreasing pH or in the absence of ionic strength
278 which also suggested hydrophilic interactions.

279 Similarly, a well-known peptide aggregation phenomenon involving both electrostatic and
280 hydrophobic forces concerns insulin. Smirnova et al. (2015) have reported that the aggregation
281 process of insulin is pH-dependent, due to a compromise between electrostatic and hydrophobic
282 strengths. Indeed, the inhibition of the self-assembly of insulin by arginine added to the medium at

283 pH 7 is rather due to the formation of hydrophobic interactions between the guanidinium group of
284 Arg and aromatic residues of the native insulin molecule. At pH 8, the aromatic residues are
285 however located at the surface of the insulin molecule that is unfolded and prone to readily self-
286 assembly even in the presence of Arg.

287

288 *3.4 Interaction peptides/bile salts in submicellar state*

289

290 The pure peptides LDQW and LQKW displayed maximal fluorescence emission intensities
291 of 56 and 62% EMI, respectively, at $\lambda_{\max} = 358$ nm under our experimental conditions. The pure
292 peptide INYW emitted a maximal fluorescence of 46% EMI, although its concentration was twice
293 of those of LDQW and LQKW to reach *ca.* 50% EMI. In all cases, fluorescence quenching due to
294 the presence of aspartic acid and glutamine residues in LDQW, glutamine and lysine residues in
295 LQKW and asparagine and tyrosine residues in INYW could be reasonably expected according to
296 the study provided by Chen and Barkley (1998). However, an additional loss of fluorescence
297 intensity due to possible exciton coupling due to possible interactions between aromatic residues
298 could also be considered in the case of INYW, as demonstrated decades ago (Kasha, 1963; Kasha,
299 1991; Scholes & Ghiggino, 1994). This would be in a good agreement with the possible
300 hydrophobic interaction between the aromatic lateral chains of the peptide as envisaged above.
301 Nevertheless, since all experiments were performed with fixed amount of peptides, and since
302 intrinsic tryptophan fluorescence are well-known to be sensitive towards changes in the
303 environment polarity, those peptides have been chosen as fluorescent tool to report their own
304 interaction with the bile salts but also to monitor their own effect on micelles formation. To this end,
305 we systematically determined the fluorescence variation ΔF as the difference between the total
306 fluorescence emitted by the peptides in the presence of bile salts compared to that of the free
307 peptides at $\lambda_{\max} = 358$ nm (ΔF is expressed in % of EMI variation).

308 The two charged peptides, LQKW and LDQW, displayed very low fluorescence variations

309 against increasing concentrations of NaC and NaCDC bile salts (Figs. 2a and 2b). Although no
310 apparent CMCs were measured, we could not exclude that both peptides interact with the bile salts
311 since a significant increase of their respective ΔF values in the presence of micellar concentrations
312 of NaC or NaCDC was observed (Figs. 3b and 3c). Altogether, these results suggested that in both
313 cases, the peptides might delay micelles formation and therefore interact with NaC or NaCDC. At
314 the molecular scale, salt bridges involving negative charges carried by the lateral chains of the bile
315 salt monomers (COO^- or SO_3^-) and the positive charges of the peptides ($\alpha\text{-NH}_3^+$ or $\varepsilon\text{-NH}_3^+$) should
316 be considered in addition to the polar interactions within the aggregated peptides and beside
317 hydrophobic interactions. This suggested that, in submicellar conditions, possible changes in the
318 tryptophan environment between the aggregated forms and the bound forms of those peptides were
319 too weak to be simply monitored by fluorescence. This might thus explain the low changes of ΔF
320 observed for submicellar concentrations of NaC or NaCDC.

321 By contrast with LQKW and LDQW, experiments performed with the most hydrophobic
322 peptide, INYW, clearly showed that the latter peptide interacted with the bile salts below and in the
323 vicinity of their CMCs. Indeed, experiments performed in the presence of NaC and NaCDC, were
324 for instance characterized by an increase of ΔF values whereas a drop of ΔF could be observed in
325 the presence of NaTDC and NaTCDC. In all cases, it was thus possible to determine an apparent
326 CMC (CMC_{app}), *i.e.* a CMC determined in the presence of disturbing agent. CMC_{app} values
327 obtained with NaC and NaCDC were comparable to that obtained for free NaC and NaCDC. On the
328 other hand, CMC_{app} values of NaTDC and NaTCDC obtained in the presence of INYW were about
329 four fold higher than those measured in the absence of the peptide. Although satisfying, these
330 results also showed that the bile salts interacted differently with INYW according to their
331 conjugation state. Indeed, in experiments performed with NaC or NaCDC, the interaction with the
332 peptide was associated with an increase of the ΔF values. This would suggest that those
333 unconjugated bile salts (whatever the number of hydroxyl groups carried by the steroid skeleton)
334 would be able to disrupt peptide aggregation leading thus to the recovery of the peptide

335 fluorescence thanks to the loss of the exciton coupling assumed to be associated with the
336 hydrophobic self-association of the peptide. Nevertheless, the disruption of the peptide aggregates
337 seemed neither to disturb the micellar process nor the respective structures of the primary bile salt
338 micelles.

339 On the other hand, it is thought that INYW rather used hydrophobic interactions to bind
340 NaTDC or NaTCDC. Indeed, the loss of the fluorescence intensity observed could be explained by
341 a direct uptake of the peptide aggregates by the bile salts during the early micellar process leading
342 thus to a simple fluorescence quenching. Although this assumption required further investigations to
343 be asserted, hydrophobic interactions between the peptide and those conjugated bile salts would be
344 in an agreement with a disturbance of primary micelles formation, since NaTDC and NaTCDC are
345 the two most tensioactive bile salts and because these latter need a high number of monomers for
346 hydrophobic self-assembling ($N_{agg} = 18$ and 22 for NaTCDC and NaTDC, respectively; Table 1).

347

348 *3.5 Interaction peptides/bile salt micelles*

349

350 The total fluorescence emission variations (ΔF) of each peptide were determined at $\lambda_{max} =$
351 358 ± 1 nm (λ_{max} slightly varied depending on the polarity of the local environment) from the
352 emission spectra performed in the presence of bile salts at micellar concentrations. In all cases, the
353 results clearly showed that all peptides interacted with the micelles formed from the bile salts
354 previously used but also with those from NaDC, NaGC, NaTC, since significant changes of ΔF
355 values upon the addition of the bile salts were observed. The use of several kinds of bile salts
356 provided an interesting range of different structures useful to better define the importance of some
357 chemical groups in the interaction between the peptides and the bile salt micelles.

358 Experiments carried out in the presence of NaC, NaCDC, NaDC, NaGC, and NaTC were
359 generally associated with an increase of the maximal intensity of fluorescence emission (ΔF from 7
360 to 56%; Table 3) compared to that observed with the peptide alone. On the other hand, interaction

361 involving NaTDC and NaTCDC with those peptides led to a drop of ΔF values (Table 3 and Fig. 3).
362 Discussing such results in terms of binding strength or about the nature of the interaction between
363 the reactants is highly difficult since the fluorescence properties of the tryptophan are strongly
364 dependent upon its direct environment. Furthermore, no significant wavelength shift of the
365 maximum emission peak of the peptides was observed upon addition of bile salts, indicating that
366 the interactions mainly affect the fluorescence quantum yield of the peptides. This allows us to only
367 discuss about the nature of the peptide-micelles interaction according mainly to the exposure level
368 of Trp towards the solvent (water) but also in some extent towards electron-deficient groups such as
369 $-\text{COOH}$ or $-\text{NH}_3^+$ since both factors are known to reduce tryptophan fluorescence quantum yield in
370 some cases. Therefore, the jump of the ΔF values observed from interaction of the peptides with
371 micelles made from NaCDC, NaC, but also those from NaTC, NaGC and NaDC, could be roughly
372 attributed to a lesser exposure of the tryptophan residue towards the solvent in an agreement with an
373 increase of their fluorescence intensities. It was not excluded in this context that a part of the
374 peptides could be incorporated within the layers of the micelles as they could also be located on the
375 surface of the micelles (avoiding neighboring polar electron-deficient groups) or in the inner side of
376 the micellar structure assuming that the latter were less polar than the solvent. These aspects about
377 the interaction between the peptides and those bile salts should be further explored through further
378 investigations, but could explain the delay suggested as regards the micelles formation discussed in
379 Fig. 2 (at least for LQKW and LDQW).

380 By contrast, it was to note that the fluorescence of those three peptides appeared quenched
381 by the two most powerful amphiphiles NaTCDC and NaTDC. In addition, the quenching of Trp
382 intrinsic fluorescence was relatively important, as the ΔF values varied from -44 to -88% according
383 to the nature of the mixture. The bile salts are planar amphiphiles and the formation of primary
384 micelles is due to agglomeration of hydrophobic surfaces in a back-to-back manner (Carey, 1985).
385 As the bile salt concentration increases widely above the CMC, the primary micelles form larger
386 self-aggregates (secondary aggregates) by association of the hydrophilic surfaces *via* formation of

387 hydrogen bonds between hydroxyl groups. According to Kawamura et al. (1989), the growth of the
388 micelle occurs with alternating up and down orientation of the bile salt monomers, the hydrophobic
389 side of the steroid skeleton being shielded from water. Alternatively, Campanelli, Candeloro de
390 Santis, D'Archivio, and Giglio (1991) suggest that the association of the bile salt monomers
391 proceeds by electrostatic bonds leading to a helical association of the bile salts with their
392 hydrophobic sides exposed to water. Thus, in the context of NaTCDC and NaTDC micelles, the
393 peptides should not interact with the micellar core but rather with the micellar surface through
394 either electrostatic bonds or hydrophobic interaction in a geometry allowing Trp to be more exposed
395 towards the solvent. As discussed for LQKW and LDQW, the specific nature of the interaction
396 between INYW and NaTCDC or NaTDC should also be considered with care in further
397 investigations.

398 As speculative, all of those suggestions required to take into account the nature of the
399 hydrophilic groups of the bile salt used in a further analysis to better understand how the micelle
400 layers could affect the quantum yield of the tryptophan of peptides. Recently, it was reported that
401 the hydroxyl group carried by the carbon C12 of the steroid skeleton can form a hydrogen bond
402 with the carboxylic function of the lateral chain, whereas the formation of a hydrogen bond *via* the
403 C7-OH group is unlikely (Posa & Sebenji, 2014). Furthermore, it is well-established that the
404 microenvironment of the C12-OH group is more hydrophobic than that of the C7-OH group (Posa,
405 2012). The lack of hydroxyl group at C12 may thus enhance the hydrophobicity in a more important
406 manner than the absence of hydroxyl group at C7 explaining some specific binding behavior. For
407 instance, comparing the results obtained with NaCDC micelles to that observed for NaDC micelles
408 might be associated to general stronger binding of the peptides to NaCDC assuming that both kinds
409 of micelles had the same effect upon the peptide quantum yield because of an enhanced
410 hydrophobic interaction. As another instance, NaC, NaGC, and NaDC were able to form such
411 hydrogen bonds with the peptide, which would help as additional bond to the hypothetical
412 hydrophobic interactions promoting thus the increase of the peptide fluorescence. By contrast, the

413 absence of the C12-OH group in NaCDC and NaTCDC or the presence of a sulfonate function
414 instead of the carboxylate function in NaTC, NaTCDC, and NaTDC made these latter bile salts
415 unable to form such a hydrogen bond. In the case of the taurine-conjugated bile salts, this hydrogen
416 bond is either very weak or not available due to the particularly very acidic feature of the sulfonate
417 function (Ueyama, Takahashi, Onoda, Okamura, & Yamamoto, 2007). To compare, the pKa values
418 of the taurine-conjugated bile salts are 1.9 against 5.0 and 3.9 for the unconjugated and glycine-
419 conjugated bile salts, respectively (Roba et al., 1983; Table 1). And noticeably, those bile salts
420 showed the most important fluorescence variations, either positive (micelles of NaTC and NaCDC)
421 or negative (micelles of NaTCDC and NaTDC) according probably to the position of the tryptophan
422 residue during the interaction with the peptide. The absence of hydrogen bond between the steroid
423 skeleton (at C12) and the acidic function of the lateral chain was therefore an important factor to
424 consider in the ability of bile salts micelles to bind peptides.

425 The interactions between the peptides and the micellar bile salts also obviously depended on
426 the hydrophilic/hydrophobic nature of the peptides. For instance, the negatively charged peptide
427 LDQW appeared to interact more strongly with NaGC than the two other peptides ($\Delta F = +36\%$;
428 Table 3). An explanation might come from the repulsive negative charge of the Asp side chain kept
429 at a distance the carboxylate function of the lateral chain of NaGC from the steroid skeleton and
430 prevented the hydrogen bond with the C12-OH group by a competitive process. As another instance,
431 in the context of LQKW/NaCDC mixture, LQKW might readily interact *via* its $\epsilon\text{-NH}_3^+$ group with
432 the carboxylate function of the lateral chain available at the surface of the micelle of NaCDC, as no
433 hydrogen bond at C12 of the steroid skeleton was formed. On the other hand, formation of a salt
434 bridge between the side chain of Lys and the taurine-conjugated bile salt micelles could not be
435 envisaged, since the spectra obtained for the mixtures of the two other peptides with either
436 NaTCDC or NaTDC were almost similar to those of LQKW/NaTCDC and LQKW/NaTDC,
437 respectively.

438 To investigate if the peptides were able to bind the surface of the bile salt micelles, the

439 hydrodynamic diameter of the micelles was determined by DLS experiments in the absence and in
440 the presence of peptides. The pure bile salts formed micelles displaying hydrodynamic diameter
441 values comprised between 2.2 and 4.2 nm (Table 2), in good agreement with those reported (for
442 instance, the hydrodynamic diameter for NaC micelles at 26.5 mM is 3.0 nm; Garidel, Hildebrand,
443 Knauf, & and Blume, 2007). Due to their hydrophobic nature, the four dihydroxylated bile salts
444 displayed the greatest micelle sizes. In the peptide/bile salt mixtures, the size of the bile salt
445 micelles were slightly increased at the exception of the NaDC micelles (average increase of *ca.* 13%;
446 Table 3), whereas the peptide aggregates were partly or fully disrupted (Fig. S1). By taking into
447 account the overall results from the fluorescence experiments and DLS measurements, it was
448 suggested that the peptides were mainly bound at the surface of the bile salt micelles. These
449 interactions were directly observable by the DLS method, although the monomeric peptides display
450 small molecular masses (less than 600 Da) and were in very low proportion comparatively to the
451 bile salts (peptide/bile salt ratio of approximately 1/5000). It is however reported that a difference of
452 only 0.5 nm of the hydrodynamic radius measured by DLS (i.e. an increase from 2.5 to 3.0 nm) is
453 sufficient to unveil a conformational change of a protein (e.g. calmodulin) interacting with a target
454 peptide (Papish, Tari, & Vogel, 2002). According to these authors, the DLS method offers results
455 closely comparable to those obtained by other methods such as small-angle X-ray or neutron
456 scattering. It was then thought that a low increase of the particle size might be attributed to the
457 formation of mixed peptide/bile salt micelles, for which the peptides mainly cover the surface.
458 Complementary to the results of the fluorescence measurements, the DLS results also supported the
459 hypothesis that the absence of hydrogen bond at C12 of the steroid skeleton might be a condition to
460 confer to bile salts good adsorption properties of peptides.

461

462 **4. Conclusion**

463

464 The present study was an approach of the molecular mechanism of interaction of milk

465 protein-derived antioxidant peptides with submicellar or micellar bile salts. The interaction closely
466 depended upon the hydrophilic-hydrophobic balance of the peptide. This work has also highlighted
467 that the lack of the hydrogen bond involving the C12-OH group of the steroid skeleton and the
468 acidic function of the lateral chain of some bile salts promoted the interaction between the bile salt
469 micelles and peptides, as well as the lack of the C12-OH group rather than that of the C7-OH group.

470 The most hydrophobic bile salts, i.e. NaTDC and NaTCDC, were able to interact at
471 submicellar concentrations with the most hydrophobic peptide studied, i.e. INYW. At concentration
472 higher than the CMC, NaTDC and NaTCDC micelles were revealed as possible efficient
473 dissociating agents toward peptide aggregates and were able to adsorb at the micelle surface all the
474 peptides studied, mainly through hydrophobic bonds.

475 In the intestine, it was then difficult to predict if peptides of complex hydrolysates provided
476 by the diet might interact with bile salts or not and, therefore, pass through the intestinal barrier by
477 the mean of paracellular transport or transmembrane transporters (Ganapathy & Miyauchi, 2005) in
478 order to fully exert their biological activity *in vivo*. Nevertheless, the hydrophobic peptides might be
479 the best protected against proteolytic digestion in the gut by adsorption onto the micellar surface of
480 the bile salts, especially those devoid of hydrogen bond at position C12 of the steroid skeleton. As
481 such, most of the peptides exhibiting antioxidant properties are concerned, as they are often
482 hydrophobic.

483

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485

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489

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599

Legends to Figures

Fig. 1. Titration isotherms of (a) sodium taurodeoxycholate (NaTDC) and (b) sodium taurochenodeoxycholate (NaTCDC) determined by isothermal titration calorimetry (ITC). The critical micellar concentration (CMC) corresponds to the x -intercept of the second derivative (in insets). C , bile salt concentration; H , enthalpy.

Fig. 2. Total fluorescence emission variation (ΔF at $\lambda_{\max} = 358$ nm) of INYW (3.3 μM ; squares), LQKW (1.7 μM ; triangles), and LDQW (1.7 μM ; circles) in the presence of bile salts at submicellar concentration and in the vicinity of the CMC. (a) Sodium cholate (NaC), (b) sodium chenodeoxycholate (NaCDC), (c) sodium taurodeoxycholate (NaTDC), and (d) sodium taurochenodeoxycholate (NaTCDC). Apparent critical micellar concentration (CMC_{app}) was defined as the intersection point between the tangent at the inflection point and the slant asymptote at high values of concentrations as shown by dashed lines on the graphs.

Fig. 3. Total fluorescence emission spectra (ΔF at $\lambda_{\max} = 358 \pm 2$ nm) of (a) INYW (3.3 μM), (b) LQKW (1.7 μM), and (c) LDQW (1.7 μM) in the presence of bile salts at 50 mM. NaC, sodium cholate, NaCDC, sodium chenodeoxycholate, NaDC, sodium deoxycholate, NaGC, sodium glycocholate, NaTC, sodium taurocholate, NaTCDC, sodium taurochenodeoxycholate, and NaTDC, sodium taurodeoxycholate.

Supplementary data:

Fig. 1S. Hydrodynamic diameters of peptides/bile salt micelles determined by dynamic light scattering (DLS). The different bile salts (50 mM) and the peptides (a) INYW (3.3 μM), (b) LQKW (1.7 μM), and (c) LDQW (1.7 μM) were mixed in TBS buffer. The particle size distribution of pure peptides is represented in blue, of pure bile salts in green, and of the peptide/bile salt mixture in red.

Table 1

Isothermal titration calorimetry (ITC) determination of the critical micellar concentration (CMC) of bile salts in the presence of 150 mM NaCl. This table also regroups different physicochemical parameters such as the position of hydroxyl functions on the steroid skeleton, the molecular mass (MM), the pKa, the reported CMC in pure water or in the presence of 150 mM NaCl, and the reported aggregation number (N_{agg}) in the presence of 150 mM NaCl.

Bile salt	Hydroxyl group position	MM (Da)	pKa ^a ($\pm 0,1$)	CMC (mM)		N_{agg} ^e (150 mM Na ⁺ , pH ca. 8 and 25 °C)
				Water ^b	150 mM Na ⁺ ^c	
Primary bile salt						
NaC	3 α 7 α 12 α	430.60	5.0	13	9.9 (11 ^b)	3 \pm 1
NaCDC	3 α 7 α	414.60	5.0	9	5.4 (4 ^b)	8 \pm 3
NaGC	3 α 7 α 12 α	487.60	3.9	12	8.5 (10 ^b)	6 \pm 2
NaTC	3 α 7 α 12 α	537.69	1.9	10	8.5 (6 ^b)	5 \pm 3
NaTCDC	3 α 7 α	521.69	1.9	2.5-3.0	1.8 (2.1 ^d)	18 \pm 2
Secondary bile salt						
NaDC	3 α 12 α	414.60	5.0	10	4.0 (3 ^b)	15 \pm 3
NaTDC	3 α 12 α	521.70	1.9	1-4	1.8 (2.0 ^e)	22 \pm 3

^aFrom Roda et al. (1983).

^bFrom Fini & Roda (1987).

^cDetermination of the CMC by ITC in the present study.

^dFrom Stevens et al. (1989).

^eFrom Carey (1985).

Table 2

Main particle sizes of peptides solubilized in different media and of bile salts in TBS buffer determined by dynamic light scattering (DLS). The standard deviation was $\pm 2\%$ ($n = 3$).

Peptide or bile salt	TBS		Ultra-pure water		0.1% trifluoroacetic acid in water	
	Particle size (nm)	Proportion (%)	Particle size (nm)	Proportion (%)	Particle size (nm)	Proportion (%)
INYW	527	73	535	80	392	100
LQKW	178	45	400	90	544	84
LDQW	489	63	490	100	382	89
NaC	2.5	68	-	-	-	-
NaCDC	3.4	60	-	-	-	-
NaDC	4.2	65	-	-	-	-
NaGC	2.3	84	-	-	-	-
NaTC	2.2	93	-	-	-	-
NaTCDC	3.4	97	-	-	-	-
NaTDC	3.5	98	-	-	-	-

Table 3

Variation of the total fluorescence emission (ΔF) of peptides in the presence of bile salt micelles and particle size distribution of each peptide/bile salt mixture. Different particles of a same sample are separated by a slash symbol.

Peptide and bile salt	ΔF (%)	Particle sizes (nm)	Proportions (%)
INYW			
NaC	+ 19	2.9/302	90.2/7.2
NaCDC	+ 53	3.6/281	57.5/34.7
NaDC	+ 7	4.1/385	53.0/43.3
NaGC	- 5	2.9	100
NaTC	+ 29	2.5/369	66.4/32.1
NaTCDC	- 45	3.7/809	91.5/2.6
NaTDC	- 88	3.7/306	85.2/14.8
LDQW			
NaC	+ 24	2.6/728	90.8/3.7
NaCDC	+ 56	3.7/262	56.6/37.0
NaDC	+ 8	4.2/308	77.0/23.0
NaGC	+36	2.4/760	66.5/30.2
NaTC	+ 19	2.4/620	73.5/20.8
NaTCDC	- 54	3.9	98.8
NaTDC	- 85	3.8/366	84.7/10.0
LQKW			
NaC	+ 37	2.7/302	90.2/7.2
NaCDC	s.s.	3.6/281	57.5/34.7
NaDC	+ 15	4.1/395	53.0/43.3
NaGC	+ 15	2.9	100
NaTC	+ 37	2.5/369	66.4/32.1
NaTCDC	- 44	3.7/809	91.5/2.6
NaTDC	- 85	3.7/306	85.2/14.8

s.s., saturated signal. In bold character, main particles. The standard deviation of the particle size was $\pm 2\%$ (n= 3).

Figure 1

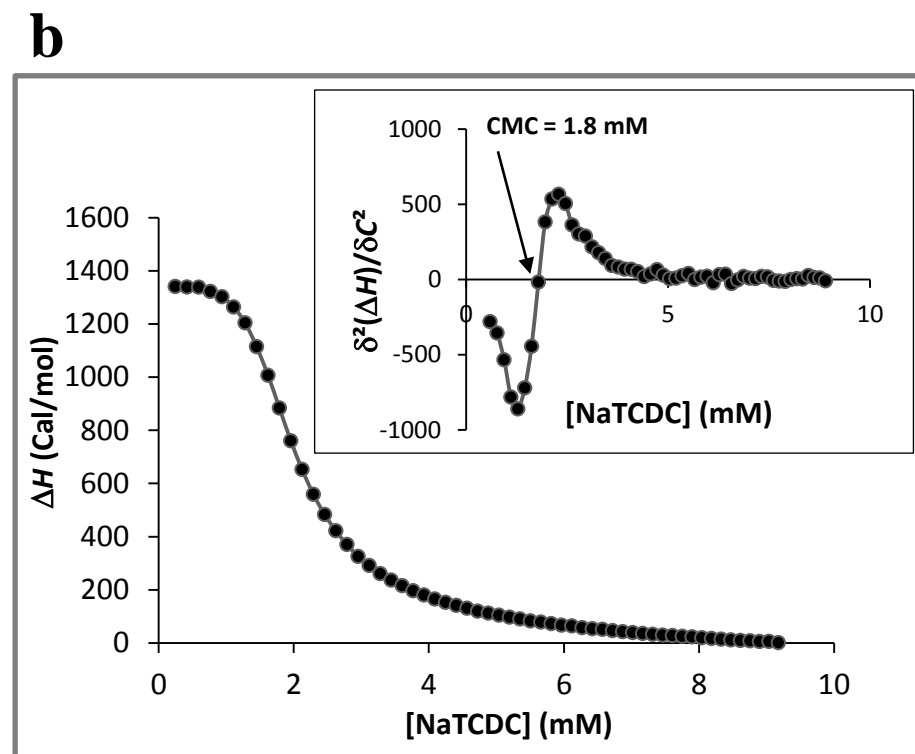
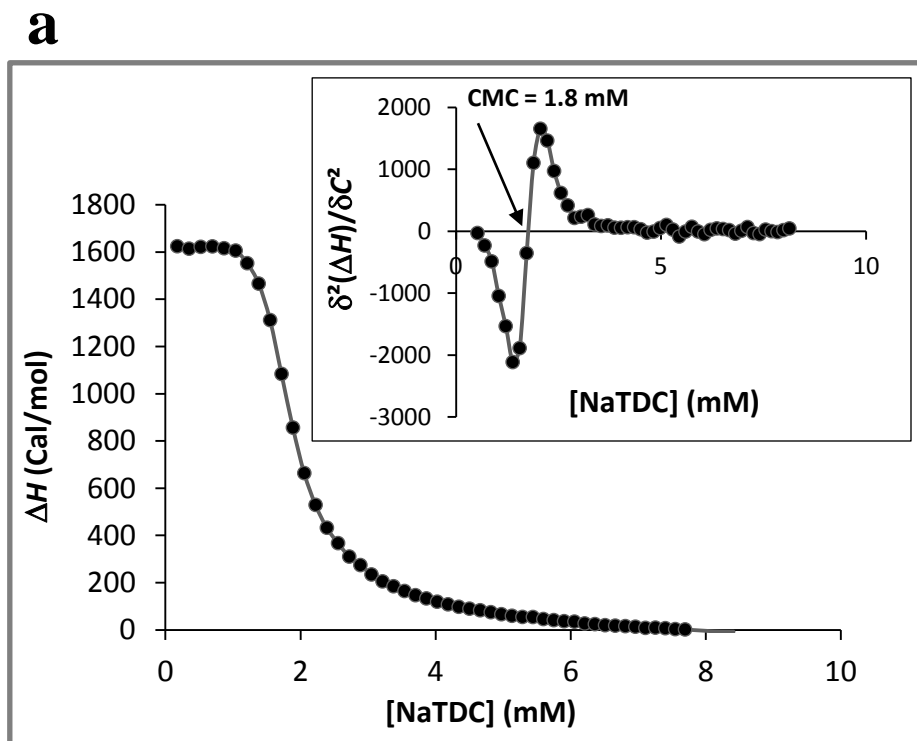


Figure 2

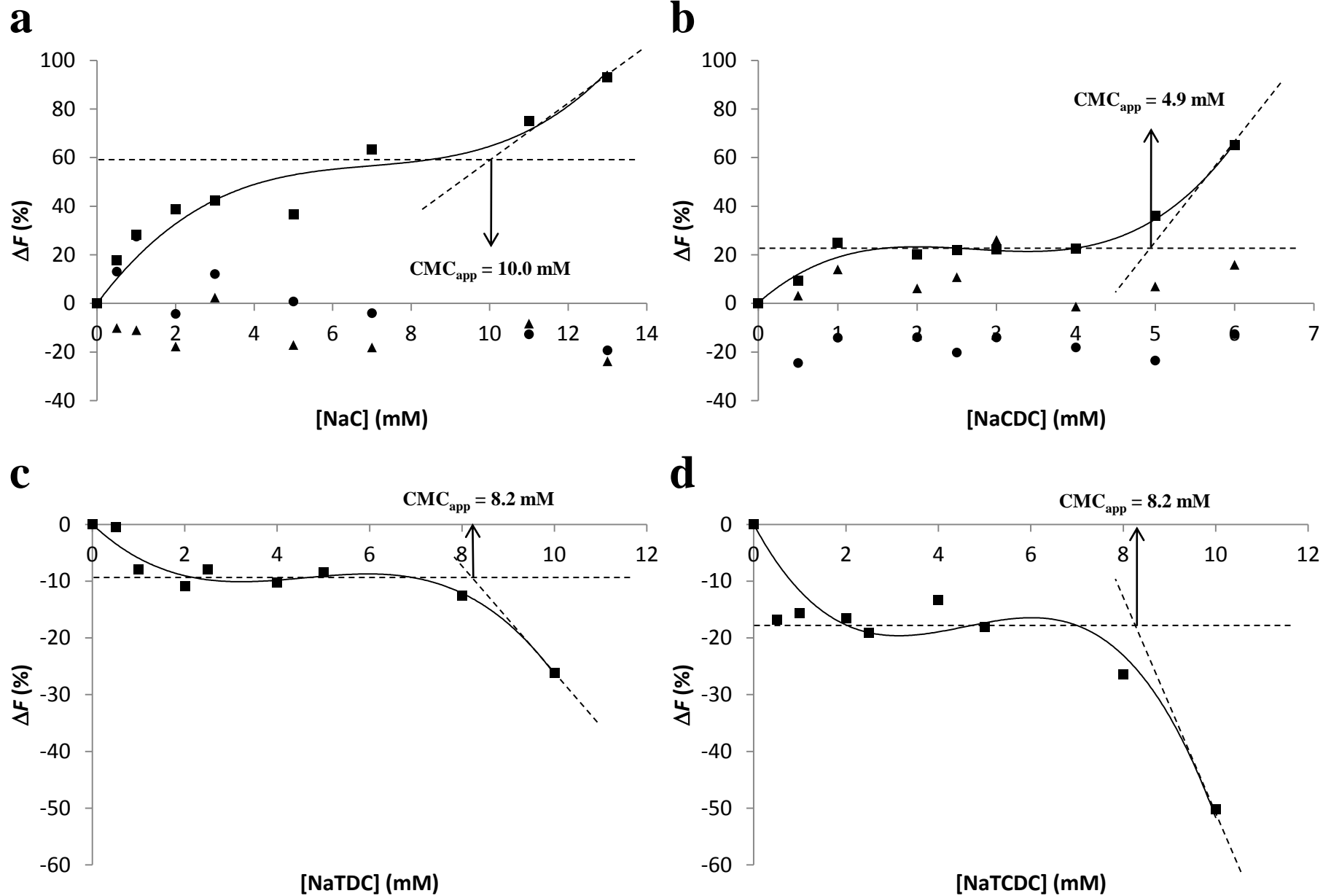


Figure 3 in color

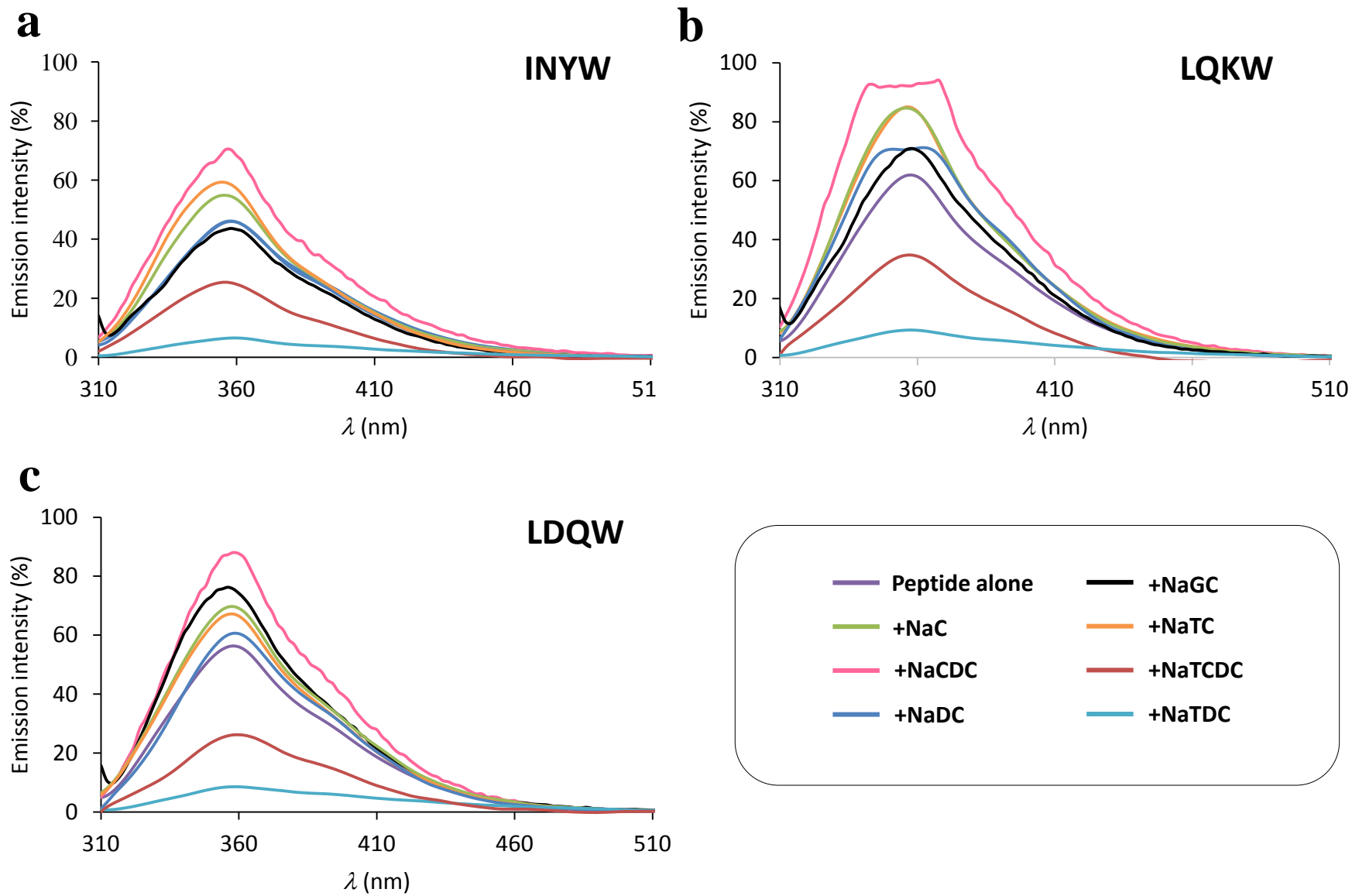


Figure 3 in black & white

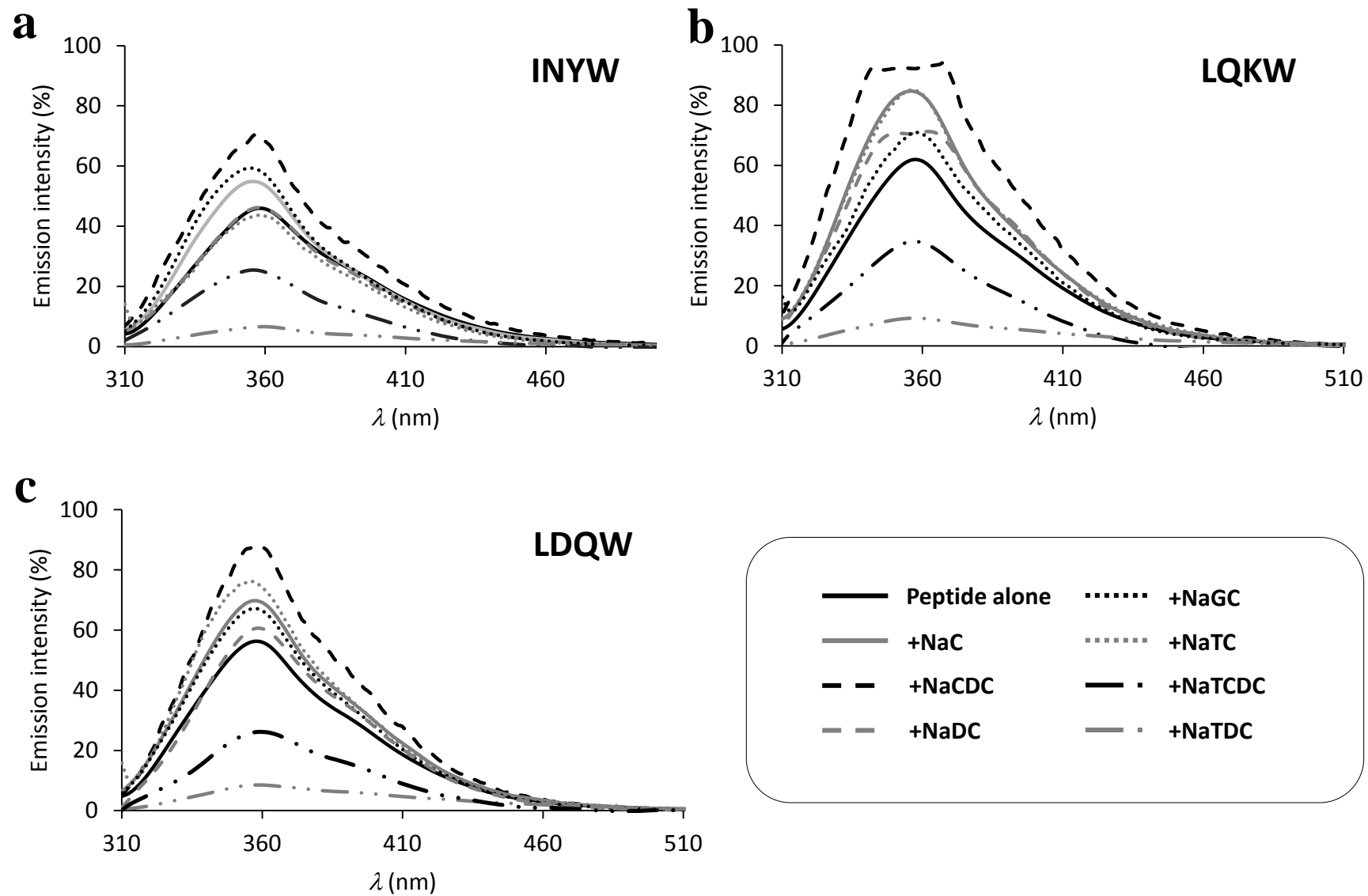


Figure S1

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