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 etal. (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 µcal/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 µcal/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 µcal/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

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

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Running title: Interaction peptides-bile salts

Abbreviations: CMC, critical micellar concentration; DLS, dynamic light scattering; EMI, emission fluorescence intensity; ITC, isothermal titration calorimetry; NaC, sodium cholate; NaCDC, sodium chenodeoxycholate;NaDC, sodium deoxycholate; NaGC, sodium glycocholate; NaTC, sodium taurocholate; NaTCDC, sodium taurochenodeoxycholate; NaTDC, sodium taurodeoxycholate, pI, isoelectric point; TBS, Tris-buffered saline; Tris, trishydroxymethylaminomethane.

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.

Keywords: bile salts, CMC, DLS, ITC, milk whey proteins, peptides, tryptophan intrinsic fluorescence

1. Introduction

Biological activities of peptides have been widely studied during the two last decades. Thus, several milk-derived bioactive peptides are nowadays commercially available in functional foods but also in ingredients (Mills, Ross, Hill, Fitzgerald, & Stanton, 2011). To prove their effectiveness *in vivo*, there is a growing interest in the study of the bioavailability of such dietary bioactive peptides in the gastrointestinal tract. In this context, bile salts, as steroidal detergents, may help bioactive peptides in reaching their targets in their active form during the postprandial phase in the gut. Indeed, they increase epithelial paracellular permeability by modulating tight junctions and therefore, facilitate the transport of drugs and other nutrients through the intestinal barrier (Mukaizawa et al., 2009). Cakir-Kiefer, Miclo, Balandras, Dary, Soligot, and Le Roux (2011) have shown, for instance, that the bile salts protect the α-casozepine, an α_{s1}-casein decapeptide exhibiting an anxiolytic activity, against hydrolysis by intestinal peptidases and also improve the transport of α-casozepine across Caco-2 cell monolayer. 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.

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

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

The enterohepatic circulation of bile salts contributes to the regulation of cholesterol homeostasis. Many studies have reported the hypocholesterolemic effects of proteins. Peptides released by proteolysis and exhibiting high bile salt binding capacity could inhibit the reabsorption of bile salts in the ileum leading to decrease the blood cholesterol level. For instance, a β-lactoglobulin-derived pentapeptide (Ile^{71}-Ile-Ala-Glu-Lys^{75}) has been identified to have a hypocholesterolemic effect according to an unclear mechanism but likely involving bile salt binding properties (Nagaoka et al., 2001). More recently, some authors have reported the ability of peptide hydrolysates to inhibit bile salt re-absorption in the ileum, and therefore to lower cholesterol levels in the bloodstream (Perez-Galvez, Garcia-Moreno, Morales-Medina, Guadix, & Guadix, 2015). This would also be directly linked to their capacity to bind bile salts. But the interactions at molecular scale between bile salt and small peptides are still unclear.

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

to form mixed micelles, either by electrostatic interactions involving the $(CH_3)_2NH^+$ group of the drug molecule and the carboxylate function of the lateral chain of the bile salts, or by hydrophobic interactions between the phenothiazine ring and the hydrophobic convex side of the steroid skeleton (Mahajan & Mahajan, 2012). In another study, some authors have shown that oil-in-water emulsions stabilized with either β-lactoglobulin or lactoferrin interact with a complex mixture of bile salts and that the interaction capability mostly depends on the isoelectric point (pI) of the proteins (Sarkar, Horne, & Singh, 2010; Singh & Sarkar, 2011). Another study using circular dichroism has unveiled interaction between bile salts (NaC and NaDC), either in submicellar or micellar forms, and polypeptides such as poly[Leu-Leu-Lys] and poly[Leu-Leu-Asp] displaying molecular masses ranging from 94 to 140 kDa (D'Alagni, D'Archivio, & Giglio, 1993). According to the authors, both NaC and NaDC may interact with the polypeptides either by hydrophobic interaction involving their hydrophobic side and the Leu residues or by forming hydrogen bonds between the hydroxyl groups located at C3 and C12 of the steroid skeleton and the amino function of the Lys residues.

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

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

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

2. Materials and methods

2.1. Peptides and bile salts

Peptides LDQW (560.26 Da), LQKW (573.33 Da), and INYW (594.28 Da) were synthesized by Genosphere Biotechnologies (Paris, France). Purity of each peptide was further improved (final purity upper than 97%) by reversed-phase high-performance liquid chromatography as described in previous study (Sadat et al., 2011). Pure bile salt (NaC, NaCDC, NaDC, NaGC, 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 µcal/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 µM for LQKW and LDQW, and 6.6 µM 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 µM for LQKW and
175 LDQW and 3.3 µM for INYW in order to reach *ca*. 50% of relative value of emission intensity, or
176 EMI, at the emission wavelength λ = 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

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

2.4. Dynamic Light Scattering (DLS)

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

3. Results and discussion

3.1 Experimental conditions

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

In the present experimental conditions, pH, the nature of the buffering agent, the ionic strength, and the concentration of bile salts were carefully chosen. Physiologically, the micellar solubilization of lipids by bile salts occurs efficiently when the bile salts have an adequate concentration. The latter is reached only in the jejunum in which the pH was *ca.* 7.0. Besides, at neutral pH, the three peptides have different states of charge and were therefore suitable models for the present study: LDQW is negatively charged (pI = 3.80), INYW is in a zwitterionic state and displays therefore a zero net charge (pI = 5.52), and LQKW is positively charged (pI = 8.75). The Tris molecule was used as buffering agent, since it is not charged at pH 7.0 (pKa = 8.06 at 25 °C and *ca*. 7.70 at 37 °C) and thus does not interact with the peptides. In the experiments performed in the presence of bile salts in micellar form, the concentration of the bile salts was fixed at 50 mM, a concentration widely greater than their respective CMCs in order to generate secondary aggregates built from primary micelles (see below). All the experiments were performed in the presence of 150 mM NaCl, as the Na^+ and Cl^- ions (as well as other electrolytes such as K^+, Ca^{2+}, and HCO^{3-} but in

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

3.2 Determination of bile salt CMCs

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

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

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

3.3 Self-aggregation of peptides

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

The nature of the peptide self-association was mainly thought hydrophobic, probably involving the C-terminal Trp lateral chain, as the peptides were able to self-associate either in pure water or in the presence of 150 mM NaCl, but also under acidic conditions (0.1% trifluoroacetic acid in water at pH *ca*. 2.5) as observed for INYW for instance. However, electrostatic interactions such as salt bridge involving β-COO^- of the Asp residue and α-NH_3^+ of Leu could not be excluded in the self-association observed for LDQW. Indeed, this acidic peptide formed the biggest aggregates at neutral pH and the smallest ones at pH 2.5 compared to that observed for the other peptides in the same pH conditions. On the other hand, the most basic peptide LQKW displayed a reverse behavior as aggregates increased with a decreasing pH or in the absence of ionic strength which also suggested hydrophilic interactions.

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

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

3.4 Interaction peptides/bile salts in submicellar state

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

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 (α-NH_3^+ or ε-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

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

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

3.5 Interaction peptides/bile salt micelles

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

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

involving NaTDC and NaTCDC with those peptides led to a drop of ΔF values (Table 3 and Fig. 3). Discussing such results in terms of binding strength or about the nature of the interaction between the reactants is highly difficult since the fluorescence properties of the tryptophan are strongly dependent upon its direct environment. Furthermore, no significant wavelength shift of the maximum emission peak of the peptides was observed upon addition of bile salts, indicating that the interactions mainly affect the fluorescence quantum yield of the peptides. This allows us to only discuss about the nature of the peptide-micelles interaction according mainly to the exposure level of Trp towards the solvent (water) but also in some extent towards electron-deficient groups such as -COOH or $-NH_3^+$ since both factors are known to reduce tryptophan fluorescence quantum yield in some cases. Therefore, the jump of the ΔF values observed from interaction of the peptides with micelles made from NaCDC, NaC, but also those from NaTC, NaGC and NaDC, could be roughly attributed to a lesser exposure of the tryptophan residue towards the solvent in an agreement with an increase of their fluorescence intensities. It was not excluded in this context that a part of the peptides could be incorporated within the layers of the micelles as they could also be located on the surface of the micelles (avoiding neighboring polar electron-deficient groups) or in the inner side of the micellar structure assuming that the latter were less polar than the solvent. These aspects about the interaction between the peptides and those bile salts should be further explored through further investigations, but could explain the delay suggested as regards the micelles formation discussed in Fig. 2 (at least for LQKW and LDQW).

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

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

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

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

The interactions between the peptides and the micellar bile salts also obviously depended on the hydrophilic/hydrophobic nature of the peptides. For instance, the negatively charged peptide LDQW appeared to interact more strongly with NaGC than the two other peptides (ΔF = +36%; Table 3). An explanation might come from the repulsive negative charge of the Asp side chain kept at a distance the carboxylate function of the lateral chain of NaGC from the steroid skeleton and prevented the hydrogen bond with the C12-OH group by a competitive process. As another instance, in the context of LQKW/NaCDC mixture, LQKW might readily interact *via* its ε-NH_3^+ group with the carboxylate function of the lateral chain available at the surface of the micelle of NaCDC, as no hydrogen bond at C12 of the steroid skeleton was formed. On the other hand, formation of a salt bridge between the side chain of Lys and the taurine-conjugated bile salt micelles could not be envisaged, since the spectra obtained for the mixtures of the two other peptides with either NaTCDC or NaTDC were almost similar to those of LQKW/NaTCDC and LQKW/NaTDC, respectively.

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

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

4. Conclusion

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

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

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

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

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References

Bloch, C. A., & Watkins, J. B. (1978). Determination of conjugated bile acids in human bile and duodenal fluid by reverse-phase high-performance liquid chromatography. *Journal of Lipid Research, 19*, 510−513.

Boelsma, E., & Kloek, J. (2009). Lactotripeptides and antihypertensive effects: a critical review. *British Journal of Nutrition, 101*, 776−786.

Cakir-Kiefer, C., Miclo, L., Balandras, F., Dary, A., Soligot, C., & Le Roux, Y. (2011). Transport across Caco-2 cell monolayer and sensitivity to hydrolysis of two anxiolytic peptides from α_{s1}-casein, α-casozepine, and α_{s1}-casein-f91–97: effect of bile salts. *Journal of Agricultural and Food Chemistry, 59*, 11956−11965.

Campanelli, A.R., Candeloro de Santis, S., D'Archivio, A. A., & Giglio, E. (1991). Cristal structures of bile salts: Sodium taurocholate. *Journal of Inclusion Phenomena and Molecular Recognition in Chemistry, 11*, 247−256.

Carey, M. C. (1985). Physical-chemical properties of bile acids and their salts. In H. Danielsson & J. Sjövall (Eds.), *Sterols and Bile Acids* (pp. 345−397). Amsterdam: Elsevier Science (The Netherlands).

Chen, Y., & Barkley, M. D. (1998). Toward understanding tryptophan fluorescence in proteins. Biochemistry, 37, 9976−9982.

Contreras, M. d. M., Hernandez-Ledesma, B., Amigo, L., Martin-Alvarez, P. J., & Recio, I. (2011). Production of antioxidant hydrolyzates from a whey protein concentrate with thermolysin: Optimization by response surface methodology. *LWT - Food Science and Technology, 44*, 9−15.

D'Alagni, M., D'Archivio, A. A., & Giglio, E. (1993). On the interaction of polypeptides with bile salts or bilirubin-IXα. *Biopolymers, 33*, 1553−1565.

Fini, A., & Roda, A. (1987). Chemical properties of bile acids. IV. Acidity constants of glycine-conjugated bile acids. *Journal of Lipid Research, 28*, 755−759.

517 Ganapathy, V., & Miyauchi, S. (2005). Transport systems for opioid peptides in mammalian tissues.
518 *The AAPS Journal, 7*, E852−E856.
519 Garidel, P., Hildebrand, A., Neubert, R., & Blume, A. (2000). Thermodynamic characterization of
520 bile salt aggregation as a function of temperature and ionic strength using isothermal
521 titration calorimetry. *Langmuir, 16*, 5267−5275.
522 Garidel, P., Hildebrand, A., Knauf, K., & Blume, A. (2007). Membranolytic activity of bile salts:
523 Influence of biological membrane properties and composition. *Molecules, 12*, 2292−2326.
524 Hernandez-Ledesma, B., Miguel, M., Amigo, L., Aleixandre, M. A., & Recio, I. (2007). Effect of
525 simulated gastrointestinal digestion on the antihypertensive properties of synthetic β-
526 lactoglobulin peptide sequences. *Journal of Dairy Research, 74*, 336−339.
527 Kasha, M. (1963). Energy transfer mechanisms and the molecular exciton model for molecular
528 aggregates. *Radiation Research, 20*, 55−71.
529 Kasha, M. (1991). Energy transfer, charge transfer and proton transfer in molecular composite
530 systems. *Basic Life Science, 58*, 231−251.
531 Kawamura, H., Murata, Y., Yamagushi, T., Igimi, H., Tanaka, M., Sugihara, G., et al. (1989). Spin-
532 label studies of bile salt micelles. *Journal of Physical Chemistry, 93*, 3321−3326.
533 Kerfelec, B., Allouche, M., Colin, D., Van Eyck, M.H., Brasseur, R., & Thomas, A. (2008).
534 Computational study of colipase interaction with lipid droplets and bile salt micelles.
535 *Proteins: Structure, Function and Genetics, 73*, 828−838.
536 Kramer, W., Wess, G., Neckermann, G., Schubert, G., Fink, J., Girbig, F., et al. (1994). Intestinal
537 Absorption of Peptides by Coupling to Bile Acids. *Journal of Biological Chemistry, 269*,
538 10621−10627.
539 Kuwata, H., Yamauchi, K., Teraguchi, S., Ushida, Y., Shimokawa, Y., Toida, T., et al. (2001).
540 Functional fragments of ingested lactoferrin are resistant to proteolytic degradation in the
541 gastrointestinal tract of adult rats. *Journal of Nutrition, 131*, 2121−2127.
542 Madenci, D., & Egelhaaf, S. U. (2010). Self-assembly in aqueous bile salt solutions. *Current*

Opinion in Colloid & Interface Science, 15, 109−115.

Maestre, A., Guardado, P., & Moya, M. L. (2014). Thermodynamic Study of Bile Salts Micellization. *Journal of Chemical and Engineering Data, 59*, 433−438.

Mahajan, S., & Mahajan, R. K. (2012). Interactions of phenothiazine drugs with bile salts: Micellization and binding studies. *Journal of Colloid and Interface Science, 387*, 194−204.

Maldonado-Valderrama, J., Wilde, P., Macierzanka, A., & Mackie, A. (2011). The role of bile salts in digestion. *Advances in Colloid and Interface Science, 165*, 36−46.

Mills, S., Ross, R. P., Hill, C., Fitzgerald, G. F., & Stanton, C. (2011). Milk intelligence: Mining milk for bioactive substances associated with human health. *International Dairy Journal, 21*, 377−401.

Mukaizawa, F., Taniguchi, K., Miyake, M., Ogawara, K., Odomi, M., Higaki, K., et al. (2009). Novel oral absorption system containing polyamines and bile salts enhances drug transport via both transcellular and paracellular pathways across Caco-2 cell monolayers. *International Journal of Pharmacology, 367*, 103−108.

Nagaoka, S., Futamura, Y., Miwa, K., Awano, T., Yamauchi, K., Kanamaru, Y., et al. (2001). Identification of novel hypocholesterolemic peptides derived from bovine milk β-lactoglobulin. *Biochemical and Biophysical Research Communications, 281*, 11−17.

Papish, A. L., Tari, L. W., & Vogel, H. J. (2002). Dynamic light scattering study of calmodulin-target peptide complexes. *Biophysical Journal, 83*, 1455−1464.

Perez-Galvez, R., García-Moreno, P. J., Morales-Medina, R., Guadix, A., & Guadix, E. M. (2015). Bile acid binding capacity of fish protein hydrolysates from discard species of the West Mediterranean Sea. *Food and Function, 6*, 1261−1267.

Plaisancié, P, Claustre, J, Estienne, M, Henry, G, Boutrou, R, Paquet, A, et al. (2013). A novel bioactive peptide from yoghurts modulates expression of the gel-forming MUC2 mucin as well as population of goblet cells and Paneth cells along the small intestine. *Journal of Nutritional Biochemistry, 24*, 213−221.

569 Posa, M. (2012). Hydrophobicity and self-association of bile acids with a special emphasis on oxo
570 derivatives of 5-β-cholanic acid. *Current Organic Chemistry, 16*, 1876−1904.
571 Posa, M., & Sebenji, A. (2014). determination of number-average aggregation numbers of bile salts
572 micelles with a special amphasis on their oxo derivatives - The effect of the steroid skeleton.
573 *Biochimica et Biophysica Acta, 1840*, 1072−1082.
574 Roda, A., Hofmann, A. F., & Mysels, K. J. (1983). The influence of bile salt structure on self-
575 association in aqueous solutions. *Journal of Biological Chemistry, 258*, 6362−6370.
576 Sadat, L., Cakir-Kiefer, C., N'Negue, M.-A., Gaillard, J.-L., Girardet, J.-M., & Miclo, L. (2011).
577 Isolation and identification of antioxidative peptides from bovine α-lactalbumin.
578 *International Dairy Journal, 21*, 214−221.
579 Sarkar, A., Horne, D. S., & Singh, H. (2010). Interactions of milk protein-stabilized oil-in-water
580 emulsions with bile salts in a simulated upper intestinal model. *Food Hydrocolloids, 24*,
581 142−151.
582 Scholes, D. S., & Ghiggino, K. P. (1994). Electronic interactions and interchromophore excitation
583 transfer. *Journal of Physical Chemistry, 98*, 4580−4590.
584 Singh, H., & Sarkar, A. (2011). Behaviour of protein-stabilised emulsions under various
585 physiological conditions. *Advances in Colloid and Interface Science, 165*, 47−57.
586 Smirnova, E., Safenkova, I., Stein-Margolina, V., Shubin, V., Polshakov, V., & Gurvits, B. (2015).
587 pH-responsive modulation of insulin aggregation and structural transformation of the
588 aggregates. *Biochimie*, *109*, 49−59.
589 Stevens, R. D., Lack, L., Collins, R. H., Meyers, W. C., Jr., & Killenberg, P. G. (1989). Effects of
590 monosulfate esters of taurochenodeoxycholate on bile flow and biliary lipids in hamsters.
591 *Journal of Lipid Research, 30*, 673−679.
592 Ueyama, N., Takahashi, K., Onoda, A., Okamura, T., & Yamamoto, H. (2007). Inorganic-
593 organiccalcium carbonate composite of synthetic polymer ligands with an intramolecular
594 NH···O hydrogen bond. In K. Naka (Ed.), *Biomineralization II: Mineralization Using*

595 *Synthetic Polymers and Templates* (pp. 155−194). Springer-Verlag Berlin Heidelberg
596 (Germany).
597 Verde, A. V., & Frenkel, D. (2010). Simulation study of micelle formation by bile salts. *Soft Matter,*
598 *6*, 3815−3825.
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 λ_{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 λ_{max} = 358 ± 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] (± 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	3α7α12α	430.60	5.0	13	9.9 (11[b])	3 ± 1
NaCDC	3α7α	414.60	5.0	9	5.4 (4[b])	8 ± 3
NaGC	3α7α12α	487.60	3.9	12	8.5 (10[b])	6 ± 2
NaTC	3α7α12α	537.69	1.9	10	8.5 (6[b])	5 ± 3
NaTCDC	3α7α	521.69	1.9	2.5-3.0	1.8 (2.1[d])	18 ± 2
Secondary bile salt						
NaDC	3α12α	414.60	5.0	10	4.0 (3[b])	15 ± 3
NaTDC	3α12α	521.70	1.9	1-4	1.8 (2.0[e])	22 ± 3

[a]From Roda et al. (1983).

[b]From Fini & Roda (1987).

[c]Determination of the CMC by ITC in the present study.

[d]From Stevens et al. (1989).

[e]From 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 ±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 ±2% (n= 3).

Figure 1

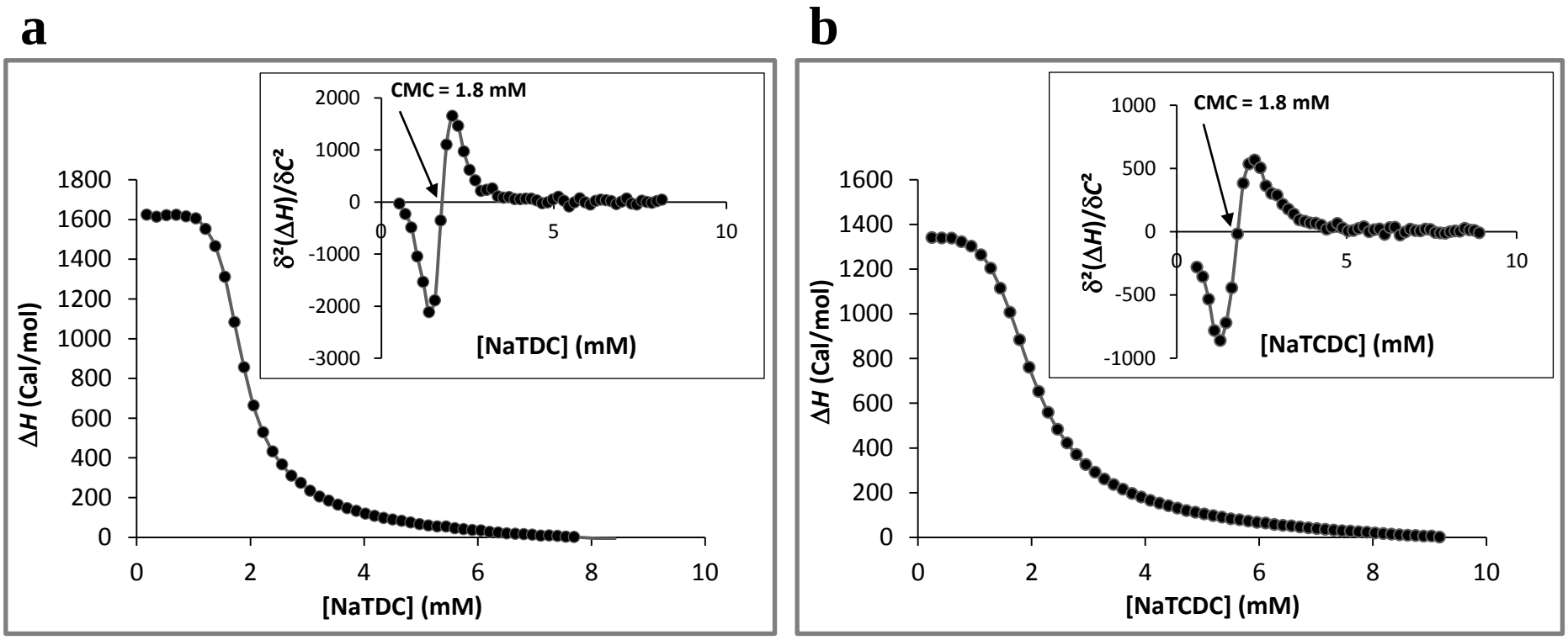

Figure 2

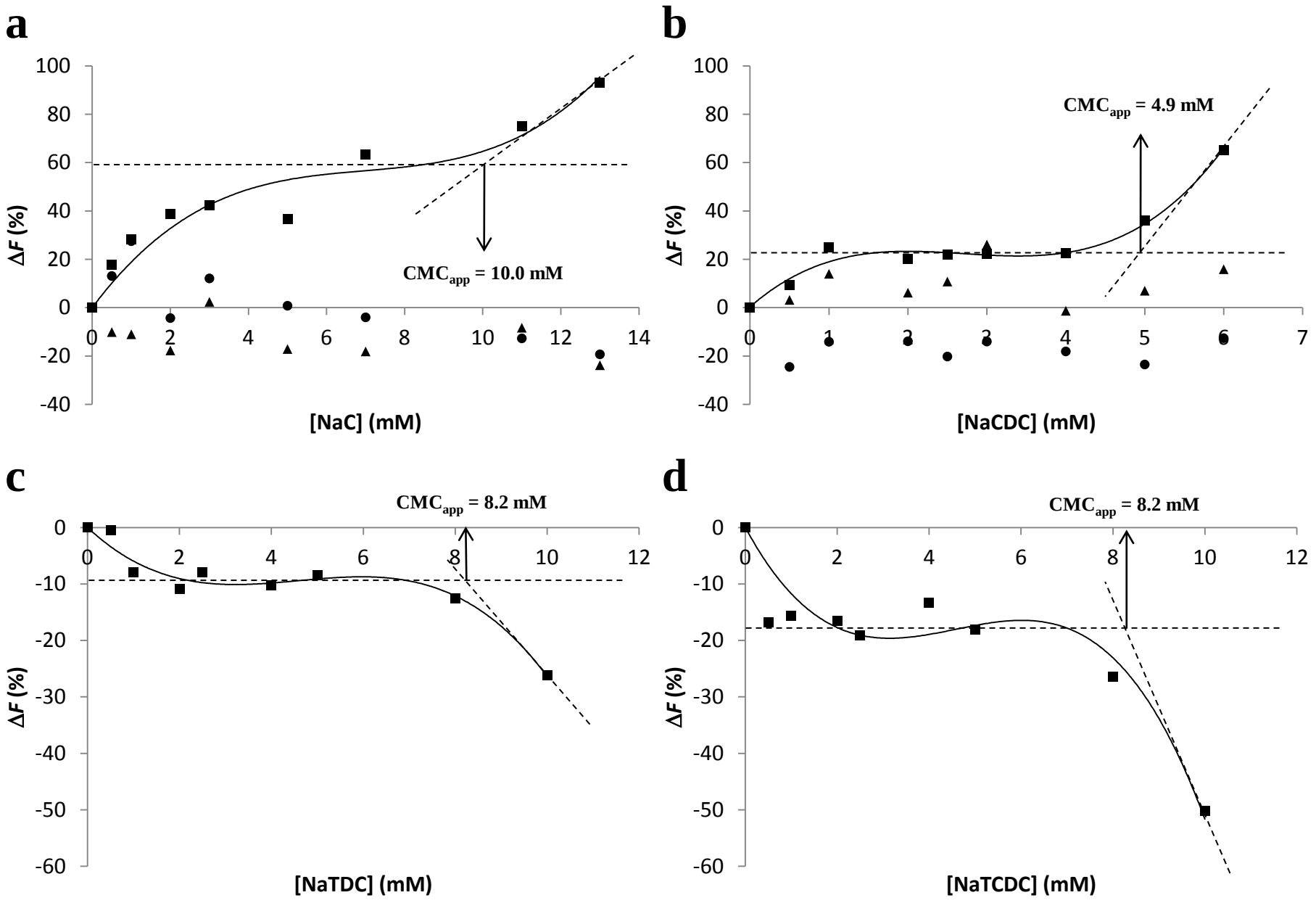
a
100
80
60
40
20
0
-20
-40
ΔF (%)
0
2
4
6
8
10
12
14
CMC$_{app}$ = 10.0 mM
[NaC] (mM)
b
CMC$_{app}$ = 4.9 mM
[NaCDC] (mM)
c
CMC$_{app}$ = 8.2 mM
-10
-30
-50
-60
[NaTDC] (mM)
d
CMC$_{app}$ = 8.2 mM
[NaTCDC] (mM)

Figure 3 in color

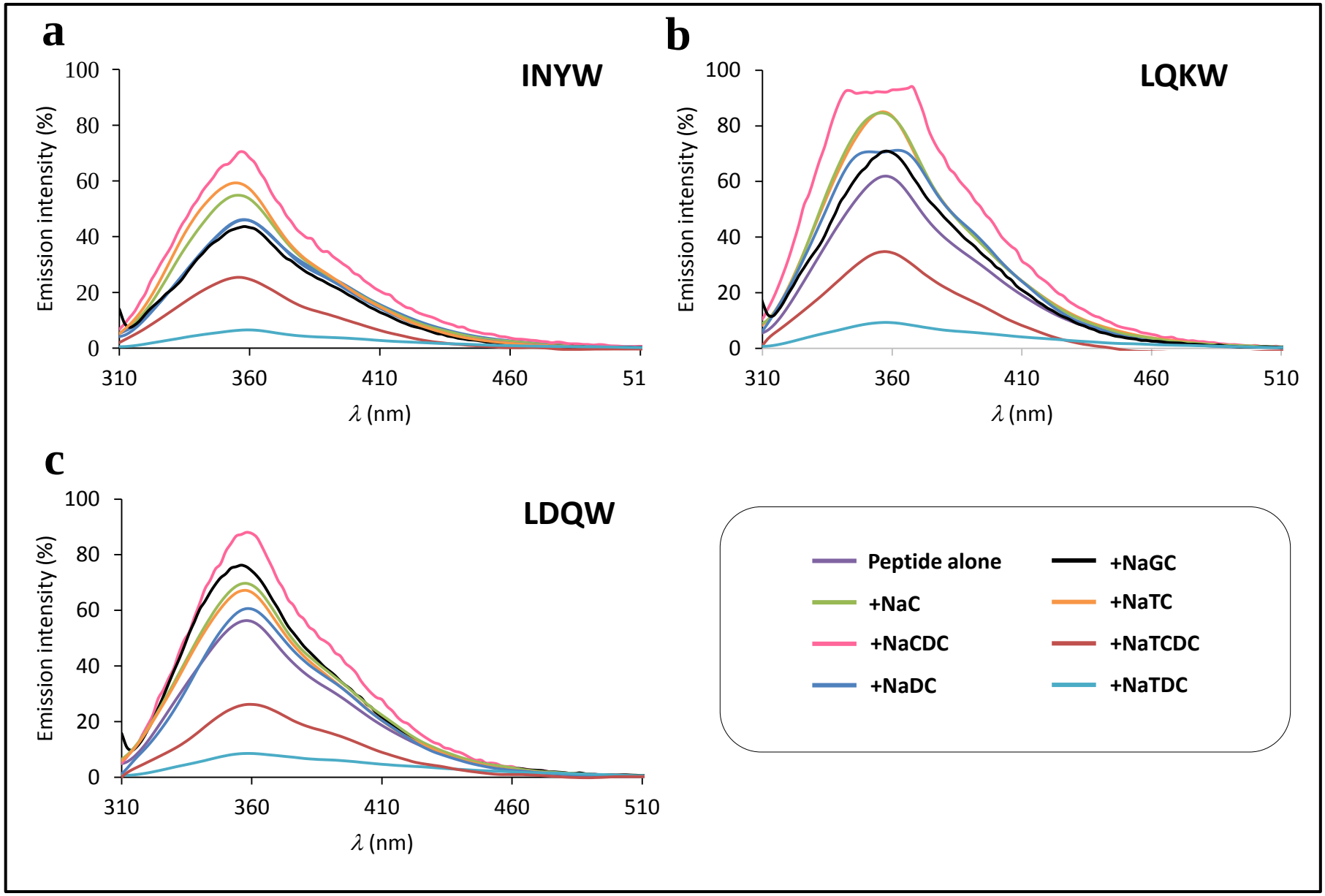

Figure 3 in black & white

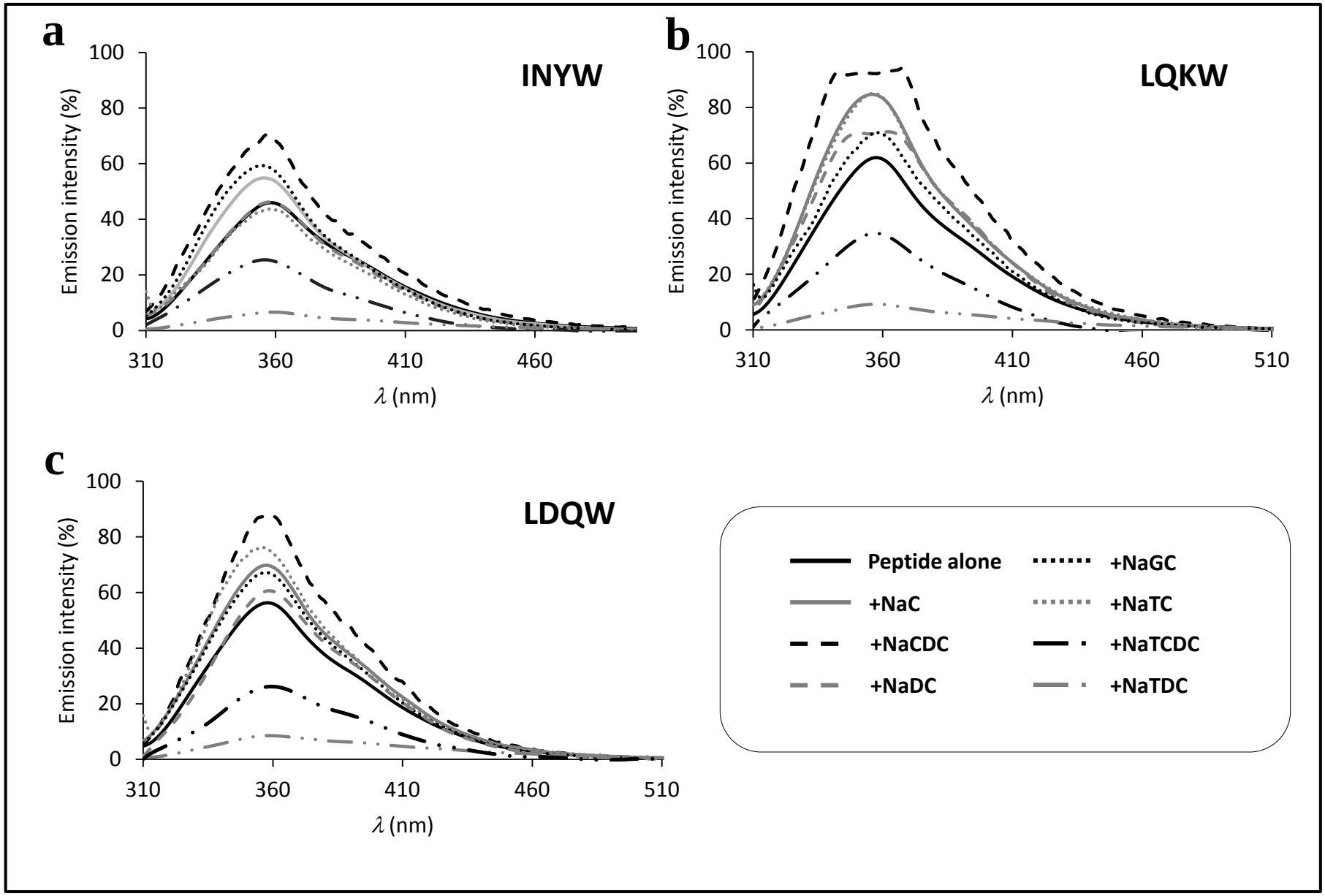

Figure S1

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