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**Molecular interactions governing the incorporation of cholecalciferol and retinyl-
palmitate in mixed taurocholate-lipid micelles**

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Abbreviations: NaTC (sodium taurocholate), RP (retinyl palmitate), D₃ (cholecalciferol), cmc
(critical micelle concentration), cac (critical aggregation concentration), LDP (lipid digestion
products), PC (phosphatidylcholine), Lyso-PC (L- α -lysophosphatidylcholine palmitoyl), POPC
(2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine).

26

27 **Abstract**

28 Cholecalciferol (D_3) and retinyl palmitate (RP) are the two main fat-soluble vitamins found in
29 foods from animal origin. It is assumed that they are solubilized in mixed micelles prior to their
30 uptake by intestinal cells, but only scarce data are available on the relative efficiency of this
31 process and the molecular interactions that govern it. The extent of solubilization of D_3 and RP
32 in micelles composed of lipids and sodium taurocholate (NaTC) was determined. Then, the
33 molecular interactions between components were analyzed by surface tension and surface
34 pressure measurements. The mixture of lipids and NaTC allowed formation of micelles with
35 higher molecular order, and at lower concentrations than pure NaTC molecules. D_3
36 solubilization in the aqueous phase rich in mixed micelles was several times higher than that of
37 RP. This was explained by interactions between NaTC or lipids and D_3 thermodynamically
38 more favorable than with RP, and by D_3 self-association.

39

40 **Keywords:** bioaccessibility; surface pressure; bile salt; compression isotherm; lipid monolayer;
41 vitamin A; vitamin D; phospholipid.

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43

44 **1. Introduction**

45

46 Retinyl esters and cholecalciferol (D_3) (Figure 1) are the two main fat-soluble vitamins
47 found in foods of animal origin. There is a renewed interest in deciphering their absorption
48 mechanisms because vitamin A and D deficiency is a public health concern in numerous
49 countries, and it is thus of relevance to identify factors limiting their absorption to tackle this
50 global issue. The fate of these vitamins in the human upper gastrointestinal tract during

digestion is assumed to follow that of dietary lipids (Borel *et al.* 2015). This includes emulsification, solubilization in mixed micelles, diffusion across the unstirred water layer and uptake by the enterocyte via passive diffusion or apical membrane proteins (Reboul *et al.* 2011). Briefly, following consumption of vitamin-rich food sources, the food matrix starts to undergo degradation in the acidic environment of the stomach, which contains several enzymes, leading to a partial release of these lipophilic molecules and to their transfer to the lipid phase of the meal. Upon reaching the duodenum, the food matrix is further degraded by pancreatic secretions, promoting additional release from the food matrix, and both vitamins then transfer from oil-in-water emulsions to mixed micelles (and possibly other structures, such as vesicles, although not demonstrated yet). As it is assumed that only free retinol can be taken up by enterocytes, retinyl esters are hydrolyzed by pancreatic enzymes, namely pancreatic lipase, pancreatic lipase-related protein 2 and cholesterol ester hydrolase (Desmarchelier *et al.* 2013). Bioaccessible vitamins are then taken up by enterocytes via simple passive diffusion or facilitated diffusion mediated by apical membrane proteins (Desmarchelier *et al.* 2017). The apical membrane protein(s) involved in retinol uptake by enterocytes is(are) yet to be identified but in the case of D₃, three proteins have been shown to facilitate its uptake: NPC1L1 (NPC1 like intracellular cholesterol transporter 1), SR-BI (scavenger receptor class B member 1) and CD36 (Cluster of differentiation 36) (Reboul & Borel 2011). Both vitamins then transfer across the enterocyte towards the basolateral side. The transfer of vitamin A is mediated, at least partly, by the cellular retinol-binding protein, type II (CRBP_{II}), while that of vitamin D is carried out by unknown mechanisms. Additionally, a fraction of retinol is re-esterified by several enzymes (Borel & Desmarchelier 2017). Vitamin A and D are then incorporated in chylomicrons in the Golgi apparatus before secretion in the lymph.

The solubilization of vitamins A and D in mixed micelles, also called micellarization or micellization, is considered as a key step for their bioavailability because it is assumed that the

non-negligible fraction of fat-soluble vitamin that is not micellarized is not absorbed (Desmarchelier *et al.* 2013). Mixed micelles are mainly made of a mixture of bile salts, phospholipids and lysophospholipids, cholesterol, fatty acids and monoglycerides (Hernell *et al.* 1990). These compounds may form various self-assembled structures, e.g., spherical, cylindrical or disk-shaped micelles (Walter *et al.* 1991, Leng *et al.* 2003) or vesicles, depending on their concentration, the bile salt/phospholipid ratio (Walter *et al.* 1991), the phospholipid concentration, but also the ionic strength, pH and temperature of the aqueous medium (Madenci & Egelhaaf 2010; Salentinig *et al.* 2010; Cheng *et al.* 2014). Fat-soluble micronutrients display large variations with regards to their solubility in mixed micelles (Sy *et al.* 2012; Gleize *et al.* 2016) and several factors are assumed to account for these differences (Desmarchelier & Borel 2017, for review).

The mixed micelle lipid composition has been shown to significantly affect vitamin absorption. For example, the substitution of lysophospholipids by phospholipids diminished the lymphatic absorption of vitamin E in rats (Koo *et al.* 2001). In rat perfused intestine, the addition of fatty acids of varying chain length and saturation degree, i.e. butyric, octanoic, oleic and linoleic acid, resulted in a decrease in the rate of D₃ absorption (Hollander *et al.* 1978). The effect was more pronounced in the ileal part of the small intestine following the addition of oleic and linoleic acid. It was suggested that unlike short- and medium-chain fatty acids, which are not incorporated into micelles, long-chain fatty acids hinder vitamin D absorption by causing enlargement of micelle size, thereby slowing their diffusion towards the enterocyte. Moreover, the possibility that D₃ could form self-aggregates in water (Meredith *et al.* 1984), although not clearly demonstrated, has led to question the need of mixed micelles for its solubilization in the aqueous environment of the intestinal tract lumen (Rautureau and Rambaud 1981; Maislos and Shany 1987).

This study was designed to compare the relative solubility of D₃ and RP in the aqueous phase rich in mixed micelles that exists in the upper intestinal lumen during digestion, and to dissect, by surface tension and surface pressure measurements, the molecular interactions existing between these vitamins and the mixed micelle components that explain the different solubility of D₃ and RP in mixed micelles.

2. Materials and methods

2.1. Chemicals

2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (POPC) (phosphatidylcholine, ≥99%; Mw 760.08 g/mol), 1-palmitoyl-sn-glycero-3-phosphocholine (Lyso-PC) (lysophosphatidylcholine, ≥99%; Mw 495.63 g/mol), free cholesterol (≥99%; Mw 386.65 g/mol), oleic acid (reagent grade, ≥99%; Mw 282.46 g/mol), 1-monooleoyl-*rac*-glycerol (monoolein, C18:1,-*cis*-9, Mw 356.54 g/mol), taurocholic acid sodium salt hydrate (NaTC) (≥95%; Mw 537.68 g/mol), cholecalciferol (>98%; Mw 384.64 g/mol; melting point 84.5°C; solubility in water: 10⁻⁴-10⁻⁵ mg/mL; logP 7.5) and retinyl palmitate (>93.5%; Mw 524.86 g/mol; melting point 28.5°C; logP 13.6) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Chloroform and methanol (99% pure) were analytical grade reagents from Merck (Germany). Ethanol (99.9%), n-hexane, chloroform, acetonitrile, dichloromethane and methanol were HPLC grade reagents from Carlo Erba Reagent (Peypin, France). Ultrapure water was produced by a Milli-Q[®] Direct 8 Water Purification System (Millipore, Molsheim, France). Prior to all surface tension, and surface pressure experiments, all glassware was soaked for an hour in a freshly prepared hot TFD4 (Franklab, Guyancourt, France) detergent solution (15% v/v), and then thoroughly rinsed with ultrapure water. Physico-chemical properties of D₃ and RP were retrieved from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>).

2.2. Micelle formation

The micellar mixture contained 0.3 mM monoolein, 0.5 mM oleic acid, 0.04 mM POPC, 0.1 mM cholesterol, 0.16 mM Lyso-PC, and 5 mM NaTC (Reboul *et al.* 2005). Total component concentration was thus 6.1 mM, with NaTC amounting to 82 mol%. Two vitamins were studied: crystalline D₃ and RP.

Mixed micelles were formed according to the protocol described by Desmarchelier *et al.* (2013). Lipid digestion products (LDP) (monoolein, oleic acid, POPC, cholesterol and Lyso-PC, total concentration 1.1 mM) dissolved in chloroform/methanol (2:1, v/v), and D₃ or RP dissolved in ethanol were transferred to a glass tube and the solvent mixture was carefully evaporated under nitrogen. The dried residue was dispersed in Tris buffer (Tris-HCl 1mM, CaCl₂ 5mM, NaCl 100 mM, pH 6.0) containing 5 mM taurocholate, and incubated at 37 °C for 30 min. The solution was then vigorously mixed by sonication at 25 W (Branson 250W sonifier; Danbury, CT, U.S.A.) for 2 min, and incubated at 37 °C for 1 hour. To determine the amount of vitamin solubilized in structures allowing their subsequent absorption by enterocytes (bioaccessible fraction), *i.e.* micelles and possibly small lipid vesicles, whose size is smaller than that of mucus pores (Cone 2009), the solutions were filtered through cellulose ester membranes (0.22 µm) (Millipore), according to Tyssandier *et al.* 2003. The resulting optically clear solution was stored at -20 °C until vitamin extraction and HPLC analysis. D₃ and RP concentrations were measured by HPLC before and after filtration. For surface tension measurements and cryoTEM experiments, the mixed micelle systems were not filtered.

2.3. Self-micellarization of D₃

Molecular assemblies of D₃ were prepared in Tris buffer using the same protocol as for mixed micelles. D₃ was dissolved into the solvent mixture and after evaporation, the dry film was hydrated for 30 min at 37°C with taurocholate-free buffer. The suspension was then

sonicated. All D₃ concentrations reported in the surface tension measurements were obtained from independent micellarization experiments - not from the dilution of one concentrated D₃ solution.

2.4. Surface tension measurements

Mixed micelle solutions were prepared as described above, at concentrations ranging from 5.5 nM to 55 mM, with the same proportion of components as previously mentioned. The surface tension of LDP mixtures hydrated with a taurocholate-free buffer, and that of pure taurocholate solutions were also measured at various concentrations. The solutions were poured into glass cuvettes. The aqueous surface was cleaned by suction, and the solutions were left at rest under saturated vapor pressure for 24 hours before measurements. For penetration studies, glass cuvettes with a side arm were used, allowing injection of NaTC beneath a spread LDP or vitamin monolayer. Surface tension measurements were performed by the Wilhelmy plate method, using a thermostated automatic digital tensiometer (K10 Krüss, Germany). The surface tension γ was recorded continuously as a function of time until equilibrium was reached. All experiments were performed at $25 \pm 1^\circ\text{C}$ under saturated vapor pressure to maintain a constant level of liquid. The reported values are mean of three measurements. The experimental uncertainty was estimated to be 0.2 mN/m. Surface pressure (π) values were deduced from the relationship $\pi = \gamma_0 - \gamma$, with γ_0 the surface tension of the subphase and γ the surface tension in the presence of a film.

2.5. Surface pressure measurements

Surface pressure-area π -A isotherms of the LDP and LDP-vitamin mixtures were obtained using a thermostated Langmuir film trough (775.75 cm², Biolin Scientific, Finland) enclosed into a Plexiglas box (Essaid *et al.* 2016). Solutions of lipids in a chloroform/methanol

(9:1, v/v) mixture were spread onto a clean buffer subphase. Monolayers were left at rest for 20 minutes to allow complete evaporation of the solvents. They were then compressed at low speed ($6.5 \text{ \AA}^2 \cdot \text{molecule}^{-1} \cdot \text{min}^{-1}$) to minimize the occurrence of metastable phases. The experimental uncertainty was estimated to be 0.1 mN/m. All experiments were run at $25 \pm 1^\circ\text{C}$. Mean isotherms were deduced from at least three compression isotherms. The surface compressional moduli K of monolayers were calculated using Eq. 1:

$$K = -A \left(\frac{d\pi}{dA} \right)_T \quad (\text{Eq. 1})$$

Excess free energies of mixing were calculated according to Eq. 2:

$$\Delta G^{EXC} = \int_0^\pi (X_L A_L + X_{VIT} A_{VIT}) d\pi \quad (\text{Eq. 2})$$

with X_L and A_L the molar fraction and molecular area of lipid molecules, and X_{VIT} and A_{VIT} the molar fraction and molecular area of vitamin molecules, respectively (Ambike *et al.* 2011).

2.6. Cryo-TEM analysis

A drop (5 μL) of LDP-NaTC micellar solution (15 mM), LDP-NaTC- D_3 (3:1 molar ratio) or pure D_3 “micellar suspension” (5 mM, theoretical concentration) was deposited onto a perforated carbon-coated, copper grid (TedPella, Inc); the excess of liquid was blotted with a filter paper. The grid was immediately plunged into a liquid ethane bath cooled with liquid nitrogen (180°C) and then mounted on a cryo holder (Da Cunha *et al.* 2016). Transmission electron measurements (TEM) measurements were performed just after grid preparation using a JEOL 2200FS (JEOL USA, Inc., Peabody, MA, U.S.A.) working under an acceleration voltage of 200 kV (Institut Curie). Electron micrographs were recorded by a CCD camera (Gatan, Evry, France).

2.7. Vitamin analysis

2.7.1. Vitamin extraction.

D₃ and RP were extracted from 500 µL aqueous samples using the following method (Desmarchelier *et al.* 2013): retinyl acetate was used as an internal standard and was added to the samples in 500 µL ethanol. The mixture was extracted twice with two volumes of hexane. The hexane phases obtained after centrifugation ($1200 \times g$, 10 min, 10°C) were evaporated to dryness under nitrogen, and the dried extract was dissolved in 200 µL of acetonitrile/dichloromethane/methanol (70:20:10, v/v/v). A volume of 150 µL was used for HPLC analysis. Extraction efficiency was between 75 and 100%. Sample whose extraction efficiency was below 75% were re-extracted or taken out from the analysis.

2.7.2. Vitamin HPLC analysis.

D₃, and RP and retinyl acetate were separated using a 250 x 4.6-mm RP C18, 5-µm Zorbax Eclipse XDB column (Agilent Technologies, Les Ulis, France) and a guard column. The mobile phase was a mixture of acetonitrile/dichloromethane/methanol (70:20:10, v/v/v). Flow rate was 1.8 mL/min and the column was kept at a constant temperature (35 °C). The HPLC system comprised a Dionex separation module (P680 HPLC pump and ASI-100 automated sample injector, Dionex, Aix-en-Provence, France). D₃ was detected at 265 nm while retinyl esters were detected at 325 nm and were identified by retention time compared with pure (>95%) standards. Quantification was performed using Chromeleon software (version 6.50, SP4 Build 1000) comparing the peak area with standard reference curves. All solvents used were HPLC grade.

2.8. Statistical analysis

Results are expressed as means $\pm$ standard deviation. Statistical analyses were performed using Statview software version 5.0 (SAS Institute, Cary, NC, U.S.A.). Means were compared by the non-parametric Kruskal-Wallis test, followed by Mann-Whitney U test as a post hoc test for pairwise comparisons, when the mean difference using the Kruskal-Wallis test was found to be significant ($P < 0.05$). For all tests, the bilateral alpha risk was $\alpha = 0.05$.

3. Results

3.1. Solubilization of D₃ and RP in aqueous solutions rich in mixed micelles

D₃ and RP at various concentrations were mixed with micelle components (LDP-NaTC). D₃ and RP concentrations were measured by HPLC before and after filtration of aggregates with a diameter smaller than 0.22 μm (Figure 2). D₃ and RP solubilization in the solution that contained mixed micelle solution followed different curves: D₃ solubilization was linear ($R^2 = 0.98$, regression slope = 0.71) and significantly higher than that of RP, which reached a plateau with a maximum concentration around 125 μM .

The morphology of the LDP-NaTC and LDP-NaTC-D₃ samples before filtration was analyzed by cryoTEM. In Figure 3, micelles are too small to be distinguished from ice. At high LDP-NaTC concentration (15 mM) small and large unilamellar vesicles (*a*), nano-fibers (*b*) and aggregates (*c*) are observed (Figure 3A). Both nano-fibers and aggregates seem to emerge from the vesicles. In the presence of D₃ at low micelle and D₃ concentration (5 mM LDP-NaTC + 1.7 mM D₃) (Figures 3B and 3C), the morphology of the nano-assemblies is greatly modified. Vesicles are smaller and deformed, with irregular and more angular shapes (*a'*). There are also more abundant. A difference in contrast in the bilayers is observed, which would account for leaflets with asymmetric composition. Some of them coalesce into larger structures, extending along the walls of the grid (*d*). Fragments and sheets are also observed (figure 3B). They exhibit

irregular contour and unidentified membrane organization. The bilayer structure is not clearly observable. New organized assemblies appear, such as disk-like nano-assemblies (e) and emulsion-like droplets (f). At higher concentration (15 mM LDP-NaTC + 5 mM D₃ in figure 3D), the emulsion-like droplets and vesicles with unidentified membrane structure (g) are enlarged. They coexist with small deformed vesicles.

3.2. Compression properties of LDP components, the LDP mixture and the vitamins

To better understand the mechanism of D₃ and RP interaction with LDP-NaTC micelles, we focused on the interfacial behavior of the various components of the system. We first determined the interfacial behavior of the LDP components and their mixture in proportions similar to those in the micellar solution, by surface pressure measurements. The π -A isotherms are plotted in Figure 4A. Based on the calculated compressibility modulus values, the lipid monolayers can be classified into poorly organized ($K < 100$ mN/m, for lyso-PC, monoolein, and oleic acid), liquid condensed ($100 < K < 250$ mN/m, for POPC and the LDP mixture) and highly rigid monolayers ($K > 250$ mN/m, for cholesterol) (Davies & Rideal 1963).

The interfacial behavior of the two studied vitamins is illustrated in Figure 4B. D₃ shows a similar compression profile to that of the LDP mixture, with comparable surface area and surface pressure at collapse ($A_c = 35 \text{ \AA}^2$, $\pi_c = 38$ mN/m) but a much higher rigidity, as inferred from the comparison of their maximal K values (187.4 mN/m and 115.4 mN/m for D₃ and LDP, respectively). RP exhibits much larger surface areas and lower surface pressures than D₃. The collapse of its monolayer is not clearly identified from the isotherms, and is estimated to occur at $\pi_c = 16.2$ mN/m ($A_c = 56.0 \text{ \AA}^2$), as deduced from the slope change in the π -A plot.

3.3. Self-assembling properties of D₃ in an aqueous solution

Since D₃ showed an interfacial behavior similar to that of the lipid mixture, and since it could be solubilized at very high concentrations in an aqueous phase rich in mixed micelles (as shown in Figure 2), its self-assembling properties were more specifically investigated. Dried D₃ films were hydrated with the sodium taurocholate free-buffer. Surface tension measurements at various D₃ concentrations revealed that the vitamin could adsorb at the air/solution interface, and significantly lower the surface tension of the buffer to $\gamma_{\text{cmc}} = 30.6 \text{ mN/m}$. A critical micellar concentration ($\text{cmc} = 0.45 \text{ }\mu\text{M}$) could be deduced from the γ -log C relationships and HPLC assays. Concentrated samples D₃ samples were analyzed by cryo-TEM (Figure 3E and 3F). Different D₃ self-assemblies were observed, including circular nano-assemblies (*h*) coexisting with nano-fibers (*i*), and large aggregates (*j*) with unidentified structure. The analysis in depth of the circular nano-assemblies allowed to conclude that they were disk-like nano-assemblies, rather than nanoparticles.

3.4. Interaction of LDP with NaTC

To better understand how the two studied vitamins interacted with the mixed micelles, we compared the interfacial behaviors of the pure NaTC solutions, LDP mixtures hydrated by NaTC-free buffer, and LDP mixtures hydrated by the NaTC buffered solutions (full mixed micelle composition). The LDP mixture composition was maintained constant, while its concentration in the aqueous medium was increased. The concentration of NaTC in the aqueous phase was also calculated so that the relative proportion of the various components (LDP and NaTC) remained unchanged in all experiments. From the results plotted in Figure 5A, the critical micellar concentration (cmc) of the LDP-NaTC mixture was 0.122 mM ($\gamma_{\text{cmc}} = 29.0 \text{ mN/m}$), a concentration 50.8 times lower than the concentration used for vitamin solubilization. The cmc values for the LDP mixture and the pure NaTC solutions were 0.025 mM ($\gamma_{\text{cmc}} = 24.0 \text{ mN/m}$), and 1.5 mM ($\gamma_{\text{cmc}} = 45.3 \text{ mN/m}$), respectively.

Experiments modeling the insertion of NaTC into the LDP film during rehydration by the buffer suggested that only few NaTC molecules could penetrate in the condensed LDP film (initial surface pressure: $\pi_i = 28$ mN/m) and that the LDP-NaTC mixed film was not stable, as shown by the decrease in surface pressure over time (Figure 5B).

3.5. Interaction of D_3 and RP with NaTC

The surface tension of the mixed NaTC-LDP micelle solutions was only barely affected by the addition of 0.1 or 1 mM D_3 or RP: the surface tension values increased by no more than 2.8 mN/m. Conversely, both vitamins strongly affected the interfacial behavior of the NaTC micellar solution, as inferred from the significant surface tension lowering observed (- 7.0 and - 8.1 mN/m for RP and D_3 , respectively).

3.6. Interaction of D_3 and RP with lipid digestion products

The interaction between the vitamins and LDP molecules following their insertion into LDP micelles was modeled by compression of LDP/ D_3 and LDP/RP mixtures at a 7:3 molar ratio. This ratio was chosen arbitrarily, to model a system in which LDP was in excess. The π -A isotherms are presented in Figures 6A and 6B. They show that both vitamins modified the isotherm profile of the lipid mixture, however, not in the same way. In the LDP/ D_3 mixture, the surface pressure and molecular area at collapse were controlled by LDP. For LDP/RP, despite the high content in LDP, the interfacial behavior was clearly controlled by RP. From the isotherms in Figures 6A and 6B, compressibility moduli and excess free energies of mixing were calculated and compared (Figures 6C, and 6D). D_3 increased the rigidity of LDP monolayers, whereas RP disorganized them. The negative ΔG^{EXC} values calculated for the LDP- D_3 monolayers at all surface pressures account for the good mixing properties of D_3 and the

lipids in all molecular packing conditions. Conversely for RP, the positive and increasing ΔG^{EXC} values with the surface pressure demonstrate that its interaction with the lipids was unfavorable.

4. Discussion

The objective of this study was to compare the solubility of RP and D₃ in aqueous solutions containing mixed micelles, and to decipher the molecular interactions that explain their different extent of solubilization. Our first experiment revealed that the two vitamins exhibit very different solubilities in an aqueous medium rich in mixed micelles. Furthermore, the solubility of D₃ was so high that we did not observe any limit, even when D₃ was introduced at a concentration > 1mM in the aqueous medium. To our knowledge, this is the first time that such a difference is reported. Cryo-TEM pictures showed that D₃ dramatically altered the organization of the various components of the mixed micelles. The spherical vesicles were deformed with angular shapes. The nano-fibers initiating from the vesicles were no longer observed. Large irregular in shape vesicle and sheets, disk-like nano-assemblies and emulsion-like droplets appeared in LDP-NaTC-D₃ mixtures, only. The existence of so many different assemblies would account for a different interaction of D₃ with the various components of mixed micelles, and for a reorganization of the components. D₃ could insert in the bilayer of vesicles and deform them, but also form emulsion-like droplets with fatty acids and monoglyceride. It is noteworthy that these emulsion-like droplets were not observed in pure D₃ samples, nor mixed micelles. Since previous studies have shown that both bile salts and some mixed micelle lipids, e.g. fatty acids and phospholipids, can modulate the solubility of fat-soluble vitamins in these vehicles (Yang & McClements 2013), we decided to study the

interactions of these two vitamins with either bile salts or micelle lipids to assess the specific role of each component on vitamin solubility in mixed micelles.

The characteristics of pure POPC, Lyso-PC, monoolein, and cholesterol isotherms were in agreement with values published in the literature (Pezron *et al.* 1991; Flasinsky *et al.* 2014; Huynh *et al.* 2014). For oleic acid, the surface pressure at collapse was higher ($\pi_c = 37$ mN/m) and the corresponding molecular area ($A_c = 26$ Å²) smaller than those previously published (Tomoaia-Cotisel *et al.* 1987), likely due to the pH of the buffer solution (pH 6) and the presence of calcium.

The interfacial properties of D₃ were close to those deduced from the isotherm published by Meredith *et al.* (1984) for a D₃ monolayer spread from a benzene solution onto a pure water subphase. The molecular areas at collapse are almost identical in the two studies (about 36 Å²), but the surface pressures differ (30 mN/m in Meredith and coworkers' study, and 38 mN/m in ours). Compressibility modulus values show that D₃ molecules form monolayers with higher molecular order than the LDP mixture, which suggests that they might easily insert into LDP domains.

As could be expected from its chemical structure, RP exhibited a completely different interfacial behavior compared to D₃ and the LDP, even to lyso-PC which formed the most expanded monolayers of the series, and displayed the lowest collapse surface pressure. The anomalous isotherm profile of lyso-PC has been attributed to monolayer instability and progressive solubilization of molecules into the aqueous phase (Heffner *et al.* 2013). The molecular areas and surface pressures for RP have been compared to those measured by Asai and Watanabe (2000) for RP monolayers spread from benzene solutions at 25°C onto a water subphase. Their values are much lower than ours, accounting for even more poorly organized monolayers. The low collapse surface pressure could correspond to molecules partially lying onto the aqueous surface, possibly forming multilayers above 16 mN/m as inferred from the

continuous increase in surface pressure above the change in slope of the isotherm. The maximal compressibility modulus confirms the poor monolayer order. The significant differences in RP surface pressure and surface area compared to the LDP mixture might compromise its insertion and stability into LDP domains.

The dogma in nutrition is that fat-soluble vitamins need to be solubilized in bile salt micelles to be transported to the enterocyte and then absorbed. It is also well known that although NaTC primary micelles can be formed at 2-3 mM with a small aggregation number, concentrations as high as 10-12 mM are usually necessary for efficient lipid solubilization in the intestine (Baskin & Frost 2008). Due to their chemical structure bile salts have a facial arrangement of polar and non-polar domains (Madenci & Egelhaaf 2010). Their self-assembling (dimers, multimers, micelles) is a complex process involving hydrophobic interaction and cooperative hydrogen bonding, highly dependent on the medium conditions, and that is not completely elucidated. The cmc value for sodium taurocholate in the studied buffer was particularly low compared to some of those reported in the literature for NaTC in water or sodium chloride solutions (3-12 mM) (Kratohvil *et al.* 1983; Meyerhoffer & McGown 1990; Madenci & Egelhaaf 2010). At concentrations as high as 10-12 mM, NaTC molecules form elongated cylindrical “secondary” micelles (Madenci & Egelhaaf 2010; Bottari *et al.* 1999). The cryoTEM analysis did not allow to distinguish micelles from the ice. In our solubilization experiment, the concentration of NaTC did not exceed 5 mM. Nevertheless, the micelles proved to be very efficient with regards to vitamin solubilization.

When bile salts and lipids are simultaneously present in the same environment, they form mixed micelles (Hernell *et al.* 1990). Bile salts solubilize phospholipid vesicles and transform into cylindrical micelles (Cheng *et al.* 2014). Walter *et al.* (1991) suggested that sodium cholate cylindrical micelles evolved from the edge of lecithin bilayer sheets. Most published studies were performed at high phospholipid/bile salt ratio. In our system, the

concentration of the phospholipids was very low compared to that of NaTC. We observed however the presence of vesicles, and nano-fiber structures emerging from them. In their cryoTEM analysis, Fatouros *et al.* (2009) compared bile salt/phospholipid mixtures to bile salt/phospholipid/fatty acid/monoglyceride ones at concentrations closer to ours. They observed only micelles in bile salt/phospholipid mixtures. However, in the presence of oleic acid and monoolein, vesicles and bilayer sheets were formed. This would account for a reorganization of the lipids and bile salts in the presence of the fatty acid and the monoglyceride. We therefore decided to study the interactions between bile salts and LDP. The results obtained show that the surface tension, the effective surface tension lowering concentration, and cmc values were very much influenced by LDP. The almost parallel slopes of Gibbs adsorption isotherms for pure NaTC and mixed NaTC-LDP suggest that LDP molecules inserted into NaTC domains, rather than the opposite. This was confirmed by penetration studies, which showed that NaTC (0.1 mM) could hardly penetrate in a compact LDP film. So, during lipid hydration, LDP molecules could insert into NaTC domains. The presence of LDP molecules improved NaTC micellarization.

After having determined the interfacial properties of each micelle component and measured the interactions between NaTC and LDP, we assessed the ability of D₃ and RP to solubilize in either NaTC or NaTC-LDP micelles. Surface tension values clearly show that both vitamins could insert in between NaTC molecules adsorbed at the interface, and affected the surface tension in the same way. The interfacial behavior of the molecules being representative of their behavior in the bulk, it is reasonable to think that both D₃ and RP can be solubilized into pure NaTC micelles. For the mixed NaTC-LDP micelles, the change in surface tension was too limited to allow conclusions, but the solubilization experiments clearly indicated that neither vitamin was solubilized to the same extent.

Solubilization experiments and the analysis of vitamin-NaTC interaction cannot explain why the LDP-NaTC mixed micelles solubilize D₃ better than RP. Therefore, we studied the interfacial behavior of the LDP mixture in the presence of each vitamin, to determine the extent of their interaction with the lipids. The results obtained showed that D₃ penetrated in LDP domains and remained in the lipid monolayer throughout compression. At large molecular areas, the π -A isotherm profile of the mixture followed that of the LDP isotherm with a slight condensation due to the presence of D₃ molecules. Above 10 mN/m, an enlargement of the molecular area at collapse and a change in the slope of the mixed monolayer was observed. However, the surface pressure at collapse was not modified, and the shape of the isotherm accounted for the insertion of D₃ molecules into LDP domains. This was confirmed by the surface compressional moduli. D₃ interacted with lipid molecules in such manner that it increased monolayer rigidity ($K_{\max} = 134.8$ mN/m), without changing the general organization of the LDP monolayer. The LDP-D₃ mixed monolayer thus appeared more structured than the LDP one. D₃ behavior resembles that of cholesterol in phospholipid monolayers, however without the condensing effect of the sterol (Ambike *et al.* 2011). The higher rigidity of LDP monolayer in the presence of D₃ could be related to the cryo-TEM pictures showing the deformed, more angular vesicles formed with LDP-NaTC-D₃. The angular shape would account for vesicles with rigid bilayers (Kuntsche *et al.* 2011).

For RP, the shape of the isotherms show evidence that lipid molecules penetrated in RP domains, rather than the opposite. Indeed, the π -A isotherm profile of the LDP-RP monolayer is similar to that of RP alone. The insertion of lipid molecules into RP domains is also attested by the increase in the collapse surface pressure from 16 to 22 mN/m. Partial collapse is confirmed by the decrease in the compressibility modulus above 22 mN/m. Thus, RP led to a destructuration of the LDP mixed monolayer and when the surface density of the monolayer increased, the vitamin was partially squeezed out from the interface. The calculated ΔG^{EXC}

values for both systems suggest that insertion of D₃ into LDP domains was controlled by favorable (attractive) interactions, whereas mixing of RP with LDP was limited due to unfavorable (repulsive) interactions, even at low surface pressures. According to Asai and Watanabe (2000), RP can be partially solubilized in the bilayer of phospholipids (up to 3 mol%), and the excess is separated from the phospholipids, and dispersed as emulsion droplets stabilized by a phospholipid monolayer.

On the whole, the information obtained regarding the interactions of the two vitamins with NaTC and LDP explain why D₃ is more soluble than RP in an aqueous medium rich in mixed micelles. Both vitamins can insert into pure NaTC domains, but only D₃ can also insert into the LDP domains in LDP-enriched NaTC micelles.

Furthermore, the results obtained suggest that this is not the only explanation. Indeed, since it has been suggested that D₃ could form cylindrical micelle-like aggregates (Meredith *et al.* 1984), we hypothesize that the very high solubility of D₃ in the aqueous medium rich in mixed micelles was partly due to the solubilization of a fraction of D₃ as self-aggregates. Indeed, we observed that D₃ at concentrations higher than 0.45 μ M, could self-assemble into various structures including nano-fibers. To our knowledge, no such structures, especially nanofibers, have been reported for D₃ so far. Rod diameter was smaller than 10 nm, much smaller than for the rods formed by lithocholic acid, for example (Terech *et al.* 2002). They were similar to those observed in highly concentrated LDP-NaTC mixtures, which seemed formed *via* desorganization of lipid vesicles. Disk-like and aggregates with unidentified structure, also observed in concentrated D₃ samples, could be related to these nano-fibers.

In our solubilization experiments, which were performed at much higher D₃ concentrations, both insertion of D₃ molecules into NaTC and LDP domains, and D₃ self-

assembling could occur, depending on the kinetics of insertion of D₃ into the NaTC-DLP mixed micelles.

5. Conclusion

The solubilization of a hydrophobic compound in bile salt-lipid micelles is dependent upon its chemical structure and its ability to interact with the mixed micelles components. Most hydrophobic compounds are expected to insert into the bile salt-lipid micelles. The extent of the solubilizing effect is, however, much more difficult to predict. As shown by others before us, mixed micelles components form a heterogeneous system with various molecular assemblies differing in shape and composition. The conditions of the medium (pH, ionic strength and temperature) affect the formation of these molecular assemblies, although we did not study this effect on our system. Our results showed that D₃ displayed a higher solubility in mixed micelle solutions than RP. This difference was attributed to the different abilities of the two vitamins to insert in between micelle components, but it was also explained by the propensity of D₃, contrarily to RP, to self-associate into structures that are readily soluble in the aqueous phase. It is difficult to predict the propensity of a compound to self-association. We propose here a methodology that was efficient to distinguish between two solubilizing behaviors, and could be easily used to predict the solubilization efficiency of other hydrophobic compounds. Whether the D₃ self-assemblies are available for absorption by the intestinal cells needs further studies.

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Conflicts of interest: DP, ED and VLD are employed by Adisseo. Adisseo markets formulated vitamins for animal nutrition.

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Figure Captions

Figure 1: Chemical structures for D₃ and RP.

Figure 2: Solubilization of D₃ and RP in aqueous solutions rich in mixed micelles: (●), cholecalciferol; (□), retinyl palmitate. For D₃ $R^2 = 0.98$, and regression slope = 0.71.

Figure 3: Cryo-TEM morphology of (A) 15 mM mixed LDP-NaTC micelles, (B) and (C) 5 mM mixed LDP-NaTC micelles + 1.7 mM D₃, (D) 15 mM mixed LDP-NaTC micelles + 5 mM D₃, (E) and (F) pure D₃ assemblies: (a) spherical vesicles, (a') small deformed vesicles, (b) nano-fibers, (c) aggregates, (d) large deformed vesicle fragments (sheets), (e) disk-like nano-assembly, (f) emulsion-like droplet, (g) large deformed vesicle, (h) disk-like nano-assemblies, (i) nano-fibers, (h) aggregates. All samples were prepared by the dry film hydration method, without filtration. Scale bars: 100 nm.

Figure 4: Mean compression isotherms for (A) the pure micelles components and the LDP mixture, and (B) D₃ (solid line) and RP (dashed line) spread at the air/buffer interface from a chloroform-methanol (9:1) solution (25°C). POPC (●), LysoPC (○), monoolein (△), cholesterol (▲), oleic acid (□), LDP (dashed line).

Figure 5: (A) Adsorption isotherms for LDP hydrated in NaTC-free buffer (○), LDP hydrated in NaTC-containing buffer solution (■), and pure NaTC (◆) solutions at 25°C. (B) Surface pressure increment upon injection of NaTC beneath a condensed LDP monolayer ($\pi_i = 28$ mN/m). The final NaTC concentration in the subphase was 0.1 mM. The initial increase in surface pressure following NaTC addition to the subphase was an injection effect.

669 **Figure 6:** π -A isotherms (A,B), compressibility moduli (C) and excess free energies (D) for
670 LDP-vitamin monolayers (25°C). A: LDP mixture (dashed line), pure D₃ (dashed-dot line) and
671 LDP-D₃ (7:3) mixture (solid line); B: LDP mixture (dashed line), RP (dashed-dot line) and
672 LDP-RP (7:3) mixture (solid line). C: LDP mixture (dashed line), LDP-D₃ (7:3) mixture (solid
673 line), and LDP-RP (7:3) mixture (dashed-dot-dot line). D: LDP-D₃ (7:3) (square) and LDP-RP
674 (7:3) (triangle) mixtures.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Highlights :

- Cholecalciferol D_3 exhibits dramatic solubilization in simulated intestinal medium
- Both lipids and bile salt in mixed micelles contribute to D_3 solubilization
- D_3 self-association is an additional factor impacting the solubilization process
- Retinyl palmitate (RP) is poorly solubilized in the mixed micelle solutions
- High excess free energy of mixing of RP with the lipids explains its poor inclusion