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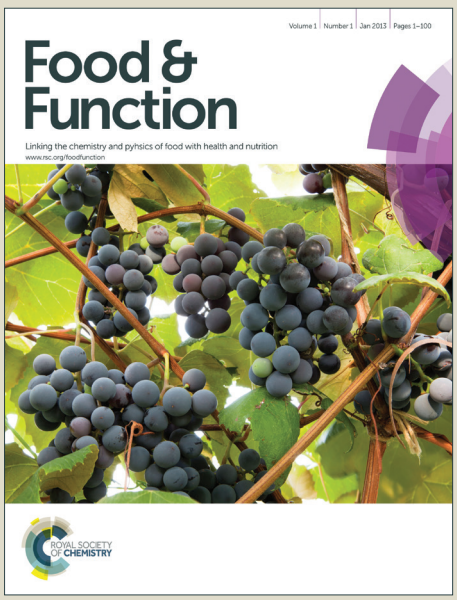

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Mechanisms of cholesterol and saturated fatty acid lowering by *Quillaja saponaria* extract, studied by *in vitro* digestion model

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ABSTRACT

Quillaja saponin extracts are known to reduce plasma cholesterol levels in humans. Here we study the mechanism of this effect with Quillaja Dry saponin extract (QD). *In vitro* model of triglyceride lipolysis is used to quantify the effect of QD on the solubilization of cholesterol and of the lipolysis products (fatty acids and monoglycerides) in the dietary mixed micelles (DMM). We found that QD extract decreases significantly both the cholesterol (from 80 % to 20 %) and saturated fatty acids (SFA, from 70 % to 10 %) solubilised in DMM. Series of dedicated experiments prove that QD may act by two mechanisms: (1) direct precipitation of cholesterol and (2) displacement of cholesterol from the DMM. Both mechanisms lead to increased cholesterol precipitation and, thus, render cholesterol bio-inaccessible. We prove also that the saponin molecules are not the active component of QD, because highly purified Quillaja extract with very similar saponin composition does not exhibit cholesterol-lowering or SFA-lowering effect. The effect of QD extract on cholesterol solubilisation is most probably caused by the high-molecular weight polyphenol molecules, present in this extract. The reduced SFA solubilisation is caused by Ca^{2+} ions of relatively high concentration (1.25 wt %), also present in QD extract, which precipitate the fatty acids into calcium soaps.

1. Introduction.

The *Quillaja saponaria* (soapbark) tree is native to central Chile and contains significant amounts of saponins in its bark. The saponins are a large family of biosurfactants, which contain a hydrophobic scaffold, called "aglycone", and a hydrophilic part, composed of one, two or three oligosaccharide chains. Saponins are divided into two main classes, according to the chemical structure of the aglycone: triterpenoid and steroid.[1] The Quillaja saponins are triterpenoid and have several sugar units, distributed in two oligosaccharide chains.[2]

Saponins possess a wide range of biological activities, one of them being the ability to inhibit cholesterol absorption and to decrease serum and liver cholesterol.[3-6] The latter effect has important implications for human health, as it could help the battle against hypercholesterolemia and the cardio-vascular problems, associated with it.[7]

The main sources of cholesterol in the body are its synthesis in the liver and its absorption in the digestive tract. More than 70 % of the cholesterol enters the digestive tract through the bile secretion and the rest originates from the food.[8] The synthesis of cholesterol in the liver is stimulated by saturated fatty acids (FA).[9] Therefore, an additional opportunity to lower serum cholesterol is to reduce the absorption of saturated FA in the gut.

The cholesterol-lowering activity of Quillaja saponins has been demonstrated both in animal studies[10] and in clinical trials with humans[11]. It was proposed that the saponin action must be inside the intestine, because the saponins are not absorbed in the gut.[12] Some saponins (soya, soapwort) inhibit intestinal bile salt (BS) reabsorption via formation of large saponin+BS aggregates, which enhances BS synthesis from cholesterol in the liver and, thus, leads to cholesterol depletion.[10,13,14] However, Quillaja saponins were found to decrease only slightly the BS reabsorption[10,13,14] which indicates a different cholesterol-lowering mechanism.

Oakenfull et al. observed that the intake of Quillaja saponins increases the amount of faecal cholesterol[10] and, therefore, they hypothesized that Quillaja saponins form insoluble complexes cholesterol+saponin.[10,15] Such a possibility is indirectly supported by the fact that other saponins, in particular digitonin[16] and alfalfa saponins[17-20] indeed form insoluble complexes with cholesterol. However, there is still no direct experimental evidence for the actual mechanism of action of Quillaja saponins.

A convenient approach to study the cholesterol-lowering mechanism of Quillaja saponins is provided by the *in vitro* digestion models. In the last decade, such models are increasingly being applied to assess the bioaccessibility of lipophilic substances, which are important for human health: drugs, carotenoids, anthocyanins, cholesterol, fatty acids or metal ions (iron, lead, etc.).[21-30] Compared to *in vivo* studies, the main advantages of using *in vitro* models are the better control of the experimental parameters which allows mechanistic understanding and theoretical analysis, the lack of ethical issues about the use of animals, lower cost and better reproducibility. The main disadvantage is that the extrapolation of the *in*

vitro results to the real *in vivo* situation is rarely straightforward and should be made very carefully.

In our previous study we described a relatively simple *in vitro* model for triglyceride (TG) lipolysis which mimics very closely the chemical composition of the fluids in the human stomach and small intestine.[29] To test its performance, we studied the effects of Ca^{2+} on the solubilization of cholesterol and lipolysis products in the dietary mixed micelles (DMM). We found that the solubilized cholesterol increases linearly with the concentration of solubilized free fatty acids (free FA) which evidenced for a co-solubilization of these two components in the bile micelles. This result means that the solubilization of cholesterol during *in vitro* digestion must be studied in combination with the solubilization of fatty acids, as these two components are interrelated.

To the best of our knowledge, the *in vitro* digestion models have not been used to study the effect of Quillaja saponins on cholesterol or free FA bioaccessibility. Therefore, the current study has the following major aims: (1) to investigate *in vitro* the effect of Quillaja extracts on the solubilization of cholesterol and free FA in the DMM, (2) to give a mechanistic explanation for the observed effects, and (3) to check whether the saponin molecules are the actual active components for the observed effects in the studied extracts.

We studied a crude, non-purified Quillaja extract (Quillaja Dry, QD), containing saponins, polyphenols, polysaccharides, calcium etc.[2] To clarify which are the active components in this non-purified extract, we studied also an ultra-pure Quillaja extract, containing 90 % saponins, and a polyphenol fraction, isolated from the original non-purified QD extract (polyphenols are also known to reduce serum cholesterol[31]).

2. Materials and methods.

The overall set of experiments included several complementary methods and systems. We studied the digestion of oil-in-water emulsions, where the triglyceride (TG) source was sunflower oil, lard or cocoa butter. The degree of TG lipolysis and the concentrations of the essential components (cholesterol, fatty acids, monoglycerides, etc.) were determined by gas chromatography (GC). The concentrations of the solubilised cholesterol and fatty acids were determined also in the aqueous phase, obtained after filtration (containing aggregates with size < 200 nm) or after centrifugation (aggregate size < 1 μm). The concentrations of the bile salts, polyphenols and saponins in the aqueous phase were determined by high pressure liquid chromatography (HPLC). Atomic absorption spectrometry (AAS) was used to determine the amount of Ca^{2+} ions in the non-purified Quillaja extract and in the polyphenol isolate. To distinguish between the effects of the non-purified Quillaja extract and the effects of Ca^{2+} (present in this extract) we performed experiments with additional Ca^{2+} ions in the reaction mixture. The size and shape of the molecular aggregates in the aqueous phase was studied by transmission electron cryo-microscopy (cryo-TEM).

2.1. Materials. Two types of Quillaja extracts were kindly donated by Desert King Co. in Chile: Quillaja Dry 100 NP and SuperSap. The first product is obtained by spray-drying of non-purified aqueous extract of Quillaja tree bark and contains 26 wt % saponins (determined via HPLC by the producer), 1.25 wt % Ca^{2+} (determined by AAS in the current study), polyphenols, phenolic acids and polysaccharides.[2] The colour of this powder is brown, because of the polyphenols present in the extract. The second extract, SuperSap, is highly purified, contains 90 % saponins, and appears as white powder. The average molecular weight of Quillaja saponins is around 1650 g/mol.[32,33] The latter value is used to calculate the molar concentration of saponin in the performed experiments.

The following fats and oils were purchased from a local grocery store and were used without purification: lard (Boni Holding Ltd.), cocoa butter (Chemax Pharma Ltd.), and sunflower oil (SFO, product of Kaliakra Ltd.). We determined by GC their fatty acid compositions, which vary significantly: from 18 % saturated FA in SFO, to 41 and 85 % in lard and cocoa butter, respectively (see Table S1 in the Supplementary materials section).

Pancreatin from porcine pancreas, 4 × USP specifications, was obtained from Sigma-Aldrich (Cat N P1750). It contains pancreatic lipase and colipase at a molar ratio of 1:1[34,35] and a range of other enzymes, such as amylase, trypsin, ribonuclease and protease. The lot number of the used pancreatin sample was 029K1095, with a lipase activity of at least 8 USP units. One unit corresponds to the amount of pancreatin that liberates 1 μEq of acid per minute at pH = 9, T = 37 °C, using an olive oil emulsion as a substrate.

As bile salts source we used porcine bile extract, obtained from Sigma-Aldrich (Cat. No. B-8631) which contains 50 wt % bile salts, 6 wt % phosphatidylcholine and less than 0.06 wt % Ca^{2+}.[36] GC analysis showed that it contains also 1.2 ± 0.1 wt % cholesterol and 6.7 wt % fatty acids.

Pepsin from porcine gastric mucosa (Fluka, cat. No. 77160) with lot number 1238420 was used to mimic the "stomach" stage in the *in vitro* model. The activity of this pepsin is 643 U/mg. One unit here corresponds to the amount of enzyme, which increases the absorbance at 280 nm by 0.001 per minute at pH = 2.0 and 37 °C, when using hemoglobin as substrate.

The standards used for determination of the retention times in GC and HPLC were: myristic acid (98 %, Fluka), palmitic acid (98 %, Riedel de Haen), stearic acid (97 %, Acros), oleic acid (85 %, Tokyo Kasei Kogyo), 1-oleyl glycerol (99 %, Sigma), dipalmitin (99 %, Sigma), cholesterol (99 %, Fluka), triolein (99 %, Fluka). As bile salts standards we used cholic acid, deoxycholic acid, chenodeoxycholic acid, glycocholic acid, sodium taurocholate, sodium glycochenodeoxycholate, sodium glycodeoxycholate and sodium taurodeoxycholate, all obtained from Sigma, with purity ≥ 95 %.

All aqueous solutions were prepared using deionized water from water-purification system Elix 3 (Millipore, USA). For preparation of electrolyte solutions we used NaCl

(product of Merck), KCl (product of Merck), $CaCl_2$ (product of Fluka) and $NaHCO_3$ (product of Teokom), all of purity higher than 99 %.

Cholesterol was purchased from Sigma (> 95 %, cat. No. 26740).

2.2. Emulsion preparation. The fat-in-water emulsions of cocoa butter and lard were prepared in the following way: first, the respective fat was melted at T = 50 °C, then 30 mL fat were added to 20 mL emulsifier solution, which was also thermostated at 50 °C. Then, emulsification was performed with a rotor-stator homogenizer Ultra Turrax T25 (Janke & Kunkel GmbH & Co, IKA-Labortechnik), operating at 13 500 rpm for 5 min at T = 50 °C. The emulsifier solution contained 1 wt % of the surfactant Tween 80 (product of Sigma), 10 mM NaCl and 0.1 g/L NaN_3 (as a preservative). The obtained stock emulsions were immediately used in the lipolysis experiments: the required amount of the stock emulsion was taken by a pipette and diluted in the electrolyte-enzyme solutions, as explained in section 2.3. The emulsions of sunflower oil were prepared following the same procedure, without heating.

The drop size distribution in these emulsions was determined by video-enhanced optical microscopy.[37,38] For each sample, the diameters of at least 1000 drops were measured. The accuracy of the optical measurements was estimated to be ± 0.3 μm.[37] The mean drop size in the studied emulsions was characterized by the volume-surface diameter, d_{32}, calculated from the relation:

$$d_{32} = \sum N_i d_i^3 \Big/ \sum N_i d_i^2 \qquad (1)$$

where N_i is the number of drops with diameter d_i. Our emulsions had d_{32} = 13 ± 2 μm for cocoa butter, d_{32} = 7 ± 2 μm for lard and d_{32} = 20 ± 3 μm for sunflower oil.

2.3. In vitro digestion model. The used in-vitro model is developed by Vinarov et al.[29] Briefly, it consists of two stages, which simulate the digestion in the stomach and in the small intestine. In the "stomach" stage, the pH is acidic (pH = 1.3) and the protease pepsin is present. In the experiments aimed to study the effect of calcium concentration, at this stage we added the required volume of 1M $CaCl_2$ solution using a micropipette, to achieve the desired final calcium concentration. In the following "intestinal" stage, we introduced sodium bicarbonate to increase pH to around 6.2 and then added the bile extract and pancreatin (containing pancreatic lipase, proteases and other digestive enzymes). The pH in the "intestinal" stage of the experiments increased gradually from 6.2 to 7.5 for 4 h, mimicking the pH-profile observed *in vivo*.[29]

The Quillaja extract was dissolved in the saline solution (59 mM NaCl + 35 mM KCl + 3.5 mM $CaCl_2$), one day before the experiment. The pepsin, bile salts and pancreatin solutions were prepared directly at 37 °C, just before their use in the actual lipolysis experiments.

The experimental protocol of solution mixing and the concentrations of the main components in the final reaction mixture are shown in Figure 1. Note that the reaction mixture contains also phospholipids, cholesterol and fatty acids, which originate from the bile extract (their concentration depends on the particular batch of the bile source).

After a total reaction time of 4.5 h, we added the drug Orlistat (Xenical®, Roche) to inhibit completely the pancreatic lipase. Afterwards, the oil soluble components in the sample were extracted with chloroform or the sample was filtered/centrifuged to obtain clear aqueous phase for further analysis of the lipids solubilized in the dietary mixed micelles (DMM).

2.4. Separation of the aqueous phase for analysis of its lipid content. To analyse the lipid solubilization at the end of the *in vitro* digestion experiment, we separated the DMM from the much bigger oil droplets and solid precipitates by filtration or centrifugation.

(A) Filtration. The reaction mixture was first filtered through a filter paper with pore size of 2-3 μm and 84 g/m^2 weight (BOECO, Germany). The filtration was carried out in a glass funnel and the filtrate was collected in a glass flask. Afterwards the obtained permeate was further filtered through a 200 nm nylon syringe filter (Minisart NY25, Sartorius, Germany). All operations were performed at 37 ºC. The obtained permeate was clear and was then subjected to chloroform extraction, as described in section 2.5.

(B) Centrifugation. The reaction mixture was centrifuged for 1 h at 3620 g (4500 rpm) in SIGMA 3-16PK centrifuge, at 37 ºC. Afterwards, the aqueous serum was withdrawn by syringe and the solubilised lipophilic substances were extracted with chloroform.

2.5. Extraction of the lipophilic substances by chloroform. After stopping the lipolysis reaction with Orlistat granules, the reaction mixture was allowed to cool down to room temperature and its pH was decreased to pH = 2 by adding HCl (to decrease the solubility of the fatty acids in the aqueous phase). Next, 6 mL chloroform was added and the sample was sonicated for 15 min. After every 5 min of sonication, the sample was vigorously agitated by hand shaking. The obtained complex dispersion was centrifuged for 30 min at 3620 g (4500 rpm) which led to separation of clear aqueous and chloroform phases, indicating that the lipophilic substances were transferred into the non-polar phase. The obtained chloroform phase was further analyzed by GC and the recovery of the cholesterol, fatty acids (FA), mono- and di-glycerides (MG and DG), and tri-glycerides (TG) was found to be ≥ 90 %.

The same procedure was applied for analysis of the aqueous phases, separated by filtration or centrifugation, as described in section 2.4.

2.6. Degree of TG hydrolysis. The digestion of edible oils or fats by the pancreatic lipase occurs via consecutive reactions, where the TG is hydrolyzed to DG and MG.[39] To quantify the extent of TG lipolysis in the end of the experiment, we use the total degree of TG lipolysis, α, defined as:

$$\alpha = \frac{C_{TG}^{INI} - C_{TG}}{C_{TG}^{INI}} \quad (3)$$

Here C_{TG}^{INI} is the initial molar concentration of TG (which is known in advance), while C_{TG} is the molar concentration of the remaining, non-hydrolyzed TG, as determined by GC. The value of α accounts for the relative amount of TG that has been transformed into MG or DG.

Specific details about the experimental protocols are provided in the Supplementary material sections S1-S7 and the related Figures S1-S5, available online.

3. Experimental results.

All results presented in section 3 are performed with the non-purified extract Quillaja Dry (QD). Note that the latter contains 1.25 wt % Ca^{2+}. Therefore, by varying QD concentration we change both the saponin and calcium concentrations in the reaction mixture.

The ultra-pure Quillaja saponins extract (SuperSap) and the QD polyphenol isolate were of limited quantity and, therefore, they were used only in several series of control experiments, described later in section 4.

First we describe the effect of QD on the degree of *in vitro* digestion of SFO, cocoa butter and lard, at different Ca^{2+} concentrations, section 3.1. The obtained results are used afterwards in the description of the effect of QD on the solubilization of cholesterol (section 3.2) and fatty acids and monoglycerides (section 3.3). Finally, in section 3.4 we present and discuss cryo-TEM micrographs of the molecular aggregates present in the aqueous phase.

3.1. Effect of Quillaja Dry (QD) on the degree of TG lipolysis, at different Ca^{2+} concentrations.

We studied the lipolysis of three edible fats with very different fatty chain compositions: sunflower oil (18 % saturated FA), lard (41 % saturated FA) and cocoa butter (85 % saturated FA). Thus we could study systematically the effect of QD on the lipolysis of saturated and unsaturated fats.

The performed experiments show that we have complete TG lipolysis, $\alpha \approx 1$, for all studied fats, at all concentrations of QD studied (from 0 to 3 mM saponin), see Figure S6A in the Supplementary materials. Therefore, we can conclude that the presence of QD and the fatty acid profile of the TG do not affect the degree of its *in vitro* lipolysis at long reaction times. This result is in agreement with our previous results,[30] showing that the fat type has no effect on the degree of TG lipolysis in the absence of QD, at long reaction times.

On the other hand, by varying the concentration of QD extract (C_{QD}) we change also the concentration of calcium, C_{Ca} – the addition of 1 mM saponin in the QD extract is accompanied with the addition of 2 mM of Ca^{2+} ions, present in this extract. To check for possible interfering effects of Ca^{2+} ions, we varied C_{Ca} at fixed QD concentration, by adding

different amounts of 1 M Ca^{2+} solution in the reaction mixture (as explained in section 2.3). The final C_{Ca} in the mixture was varied between 1 and 11 mM in the absence of QD, and between 3 and 13 mM in the presence of 1 mM QD. The obtained results showed that TG lipolysis is almost complete, $\alpha \geq 0.9$, at all studied C_{Ca}, regardless of the presence of QD (Figure S6B in the Supplementary materials). Therefore, calcium has no significant effect on the degree of *in vitro* TG lipolysis, at long reaction times, in agreement with our previous studies.[29,30]

In all performed experiments, the TG was hydrolysed mainly to MG, whereas very little or no DG was found at the end of the lipolysis experiment (data by GC analysis, not shown). Consequently, the main components in the reaction mixture, at the end of the lipolysis experiment are FA, MG and cholesterol (plus bile salts, phospholipids and enzymes added with the bile and enzyme sources). The total concentrations of FA and MG in the sample at the end of the experiment are approximately the same for all oils and fats: ≈ 12 mM FA and ≈ 3.5 mM MG, whereas the saturation of these components is very different, depending on the specific oil or fat used in the *in vitro* experiment.

Summarizing, the TG lipolysis is almost complete for all studied fats ($\alpha \geq 0.9$), under all conditions studied. The degree of lipolysis does not depend on QD presence (saponins varied between 0 and 3 mM), Ca^{2+} concentration (1 to 13 mM) or fat saturation (between 18 and 85 %).

3.2. Effect of Quillaja Dry on the solubilization of cholesterol

We studied the solubilization of cholesterol in both small aggregates (d < 200 nm) and large aggregates (d between 200 and 1000 nm) using two methods for aqueous phase separation: filtration and centrifugation (Section 2.4). The aqueous phase obtained after filtration is henceforth referred to as “the permeate”, whereas that obtained by centrifugation is called “the serum”. In the following subsections, we present the effect of QD on the cholesterol solubilization in the permeate (subsection A) and in the serum (subsection B).

(A) Effect of Quillaja Dry on the solubilized cholesterol in the permeate.

We studied the effect of QD on the cholesterol solubilization in the permeate using SFO, lard and cocoa butter emulsions. The cholesterol (CH) solubilization is presented in per cent, calculated from the ratio [CH in the permeate] / [CH in the whole sample].

The dependence of the fraction of solubilised cholesterol in the permeate on C_{QD} for SFO, lard and cocoa butter is compared in Figure 2A. One sees that the cholesterol solubilization decreases significantly with the increase of C_{QD} for all studied fats. This effect is most pronounced for cocoa butter, where the solubilized cholesterol decreases from ≈ 80 % (in the absence of QD) down to ≈ 15 % (at the highest QD concentration).

With respect to the effect of the fatty acid type on the cholesterol solubilization in presence of QD, one sees no significant difference between SFO, lard and cocoa butter. Considering that cocoa butter contains mainly saturated FA (≈ 85 %), whereas SFO is mostly

composed of unsaturated FA (≈ 82 %), we conclude that the degree of fatty acid saturation has no significant effect on the solubilization of cholesterol in the presence of QD.

In our previous studies, we found that the increase in Ca^{2+} concentration can lower the concentration of solubilized cholesterol, by decreasing the solubilization of fatty acids and monoglycerides which, in their turn, affect the cholesterol solubilization.[29,30] To determine whether the calcium present in QD affects the cholesterol solubilisation, we varied the total concentration of Ca^{2+} ions, C_{Ca}, between 1 and 11 mM in the absence of QD, and between 3 and 13 mM in the presence of 1 mM QD. Cocoa butter and lard emulsions were used in this series of experiments.

The obtained results show that the addition of QD in the reaction mixture significantly lowers the solubilized cholesterol, at all studied calcium concentrations, C_{Ca} (Figure 2B). Calcium decreases cholesterol solubilization in the absence of QD, however, it has minor effect in the presence of 1 mM QD. Therefore, we can conclude that the strong cholesterol-lowering effect of QD is due mainly to the active component of the extract (e.g. saponins or polyphenols), whereas the contribution of the Ca^{2+} ions, present in this extract, is much smaller.

(B) Effect of Quillaja Dry on solubilized cholesterol in the serum.

There are two ways to explain the reduced solubilization of cholesterol by the QD extract: (1) QD induces cholesterol precipitation or (2) QD forms large colloidal aggregates with cholesterol, which do not precipitate, but do not pass through the pores of 200 nm filter. It was shown earlier that such large aggregates can form during *in vitro* lipid digestion, in the absence of Quillaja extracts.[29] To distinguish between these two possibilities, we studied the concentration of cholesterol in the serum and in the sediment, formed after centrifugation.

The serum was obtained after centrifugation at intermediate centrifugal force (3620 g), so that the solid precipitates sedimented at the bottom of the centrifugal vial, whereas the large colloidal aggregates (up to 1 μm) remained dispersed in the solution.[29]

The cholesterol content in the permeate (after filtration), the serum and the sediment, after lipolysis of a cocoa butter emulsion, is presented in Figure 3. One sees that the cholesterol solubilized in the serum is only slightly higher than that solubilized in the permeate. This comparison shows that the cholesterol is solubilized mainly in the small aggregates, with size below 200 nm.

At high concentration of QD, where the solubilized cholesterol is decreased to 20 %, the other 80 % of cholesterol were found in the sediment (the triangle in Figure 3), thus satisfying the mass balance requirement.

Thus we conclude that: (1) The increase of QD concentration leads to cholesterol precipitation, (2) As a result, the concentration of cholesterol, solubilised in the DMM, is significantly reduced, (3) This effect can be only partially explained by the presence of Ca^{2+} in the QD extract.

3.3. Effect of Quillaja Dry on the solubilization of fatty acids and monoglycerides.

(A) Effect of QD on solubilized fatty acids.

We studied also the solubilization of fatty acids and monoglycerides in the DMM. As the non-purified Quillaja extract contains high amounts of Ca^{2+} (1.25 wt %), one can expect these calcium ions to reduce FA solubilisation by causing a precipitation of calcium-FA soaps.[30] To distinguish between the effect of Ca^{2+} and the possible effects of the other components in the QD extract, we performed three types of experiments: (1) we varied the concentration of the QD extract, which changes both C_{QD} and C_{Ca}; (2) we varied Ca^{2+} concentration at constant C_{QD} =1 mM, and (3) we varied C_{Ca} in the absence of QD. These experiments were performed with cocoa butter emulsions and with SFO emulsions.

The effect of QD concentration on the solubilized FA in the permeate, studied with cocoa butter emulsions, is presented in Figure 4A. One sees that the increase of C_{QD} from 0 to 3 mM, significantly decreases the solubilized saturated FA: from 6.5 to 1 mM. On the other hand, the solubilized unsaturated FA are not affected by QD and remain around 2 mM. The latter result reflects the preferential precipitation of saturated FA (SFA), as compared to unsaturated FA[30], as well as the lower concentration of unsaturated FA in the cocoa butter.

To understand whether this decrease of FA solubilization is due to the calcium present in the QD extract, we plotted the above results for SFA as a function of C_{Ca} (1 mM QD provides 2 mM Ca^{2+}), together with the results from the experiments where we varied C_{Ca} in the absence and in the presence of 1mM QD (Figure 4B). One sees that the curves of these three different sets of experiments overlap: therefore, the reduction of SFA solubilization by QD extract is due exclusively to the Ca^{2+} ions present in this extract.

Similar trend is observed for the unsaturated FA after lipolysis of lard and sunflower oil emulsion: their solubilization decreases with the increase of QD concentration, which is due to the high calcium content of the QD extract (see Figure S7 in the Supplementary materials).

To confirm further that the fatty acids are precipitated by calcium, we analysed the concentration of SFA in the serum after centrifugation, after lipolysis of cocoa butter emulsion. The obtained results (Figure S8 in Supplementary materials) showed that the non-solubilized SFA are, indeed, precipitated and that the compositions of the serum (after centrifugation) and of the permeate (after filtration) are the same.

Finally, experiments performed in the presence of 8 mM of the calcium-binding agent EDTA and 1.5 mM QD (total C_{Ca} = 4 mM) showed a significant recovery of the solubilisation of saturated FA in the DMM: 4.5 mM, compared to 2.4 mM in absence of EDTA. In contrast, cholesterol solubilization remained very low even in the presence of EDTA: 28 % compared to 78 % in absence of QD and C_{Ca} = 1 mM. This is direct evidence that (1) the effect of QD on FA solubilisation is due exclusively to the calcium ions present in QD and (2) the significant decrease of cholesterol solubilization by QD is only partially due to its high calcium content.

(B) Effect of QD on solubilized monoglycerides.

The effect of QD concentration on the solubilized MG in the permeate was studied with cocoa butter and sunflower oil emulsions. QD had not any effect on MG solubilization for both fats (Figure S9A in Supplementary materials). The concentration of MG solubilized in the serum and in the permeate was found to be the same (Figure S9B in Supplementary materials), similarly to the results for FA and cholesterol. These results indicate that the MG are solubilized mainly in the aggregates smaller than 200 nm.

Summarizing the results from this section: (1) QD decreases the solubilization of saturated and unsaturated FA, (2) This effect is due exclusively to the calcium ions present in this extract, which causes precipitation of insoluble calcium-FA soaps, (3) QD has no effect on MG solubilisation, and (4) both FA and MG are solubilized mainly in the small aggregates with diameter < 200 nm.

3.4. Cryo-TEM observations of the molecular aggregates in the aqueous phase.

The fact that the aqueous phases, obtained via centrifugation and filtration, have the same composition of cholesterol, fatty acids and monoglycerides, could be explained if there is no significant difference between the aggregates present in the serum and in the permeate.

To check this assumption, we analysed by cryo-TEM the serum and the permeate from experiments with 1.5 mM QD, obtained after lipolysis of SFO emulsion, Figure 5. Indeed, similar colloidal structures are observed in both micrographs: small micelles, with dimensions around 6 nm and long, worm-like micelles, with a cross-section of ≈ 14 nm and length of 200-500 nm. The regular grainy appearance of the worm-like micelles suggests that they may be composed of sticky primary micelles, arranged in a staggered or spiral configuration. The size of the small micelles corresponds to that reported in literature for bile salt micelles.[40-42] On the other hand, similar worm-like aggregates are reported for mixed solutions of bile salts and saponins by other authors.[14]

Thus, we can conclude that in presence of QD extract, the cholesterol and lipolysis products are solubilized in small micelles of diameter ≈ 6 nm and in worm-like micelles of larger cross-section. The bigger aggregates with size between 0.2 and 1 μm, observed under similar conditions in the absence of QD[29] are not formed in the presence of QD.

4. Mechanism and active components in QD extract.

4.1. Mechanism of lowering of cholesterol solubilization by Quillaja Dry.

In a previous study we showed that the increase of solubilized FA or MG leads to a corresponding increase in the solubilized cholesterol.[30] As QD can decrease the concentration of solubilized FA (as shown for all studied fats in section 3.3), due to the presence of Ca^{2+} ions, we must investigate the role of this effect for the cholesterol solubilization.

With this aim in view, we plot the fraction of solubilized cholesterol, as a function of the concentration of solubilized FA + MG in the permeate, after lipolysis of cocoa butter,

SFO and lard emulsions, for experiments performed at different concentrations of QD extract and Ca^{2+} ions (Figure 6). One sees a linear correlation between the solubilized cholesterol and solubilised FA + MG, which evidences for co-solubilization of these components, in agreement with our previous studies.[29,30] The QD extract reduces the intercept of this dependence, which evidences that this extract decreases specifically the cholesterol solubilization, regardless of the presence of FA or MG.

We conclude from the above results that QD extract reduces the cholesterol solubilisation by two complementary effects. First, the calcium ions present in this extract are able to precipitate the fatty acids, thus decreasing the cholesterol solubilisation. Second, QD extract has a specific ability to reduce the cholesterol solubilisation which is super-imposed over the first effect. The combination of these two mechanisms is making the QD extract so effective in decreasing the cholesterol solubilization in our *in vitro* experiments.

Let us discuss now the possible mechanisms of the specific effect of QD on the cholesterol solubilization. In section 3.2 we found that QD induces cholesterol precipitation. This precipitation effect could be explained by three different mechanisms, schematically illustrated in Figure 7: (1) QD may precipitate first the bile salts, thus decreasing the solubilization capacity of the aqueous phase, with subsequent precipitation of the "residual" cholesterol, (2) The active components in QD may directly form an insoluble complex with the cholesterol, due to an extra-high affinity between the reacting substances, and (3) The active component in QD may solubilise in the DMM, thus displacing and forcing the cholesterol to precipitate. Note that all these mechanisms would result in cholesterol precipitation and, therefore, we need more elaborated approaches to distinguish between these options. This effort is worthy, because the different mechanisms rely on different specific interactions between the key components in the reaction mixture.

To test whether mechanism 1 is operative, we analysed the concentration of bile salts in the permeate by HPLC, in presence and in absence of 1 mM QD, after lipolysis of cocoa butter emulsion (the analytical procedure is described in the Supplementary materials). Note that we observed a significant decrease in the solubilized cholesterol (from ≈ 80 % to 33 %, see Figure 2) at this QD concentration. However, the concentration of the total bile salts in the permeate decreased only by around 12 %, see Figure 8. This small reduction of the BS concentration is unlikely to explain the 2.5-fold decrease in the solubilised cholesterol. Another observation which contradicts mechanism 1 comes from animal studies by other authors,[10,14] where the ingestion of Quillaja saponins led to very small increase of the faecal bile acids.

Thus, mechanisms 2 and/or 3 remain as possible explanations for the effect of QD on cholesterol solubilization. To distinguish between mechanisms 2 and 3, we performed experiments at two different bile salt concentrations, while maintaining fixed the concentrations of QD and cholesterol. If cholesterol is precipitated directly, due to strong interaction with QD components, its solubilization would not be affected by the increase of bile salt concentration – such a result would support mechanism 2. In contrast, if the

concentration of solubilized cholesterol increases upon increase of the bile salts, this would mean that the increased concentration of DMM enhances cholesterol solubilization, thus supporting mechanism 3 (QD components displace the cholesterol from the DMM).

We performed such experiments with cocoa butter emulsions and the obtained results showed an increase of the cholesterol solubilization (from 0.169 to 0.235 mM) with the increase of bile salt concentration (from 15 to 30 mM), at fixed 1 mM QD and 0.8 mM cholesterol in the whole sample. This corresponds to an increase of cholesterol solubilization from 21 to 29 %, which is smaller than the expected 2-times increase, if mechanism 3 is fully operative. For comparison, in our previous work with *Sapindus trifoliatus* saponins we showed that cholesterol solubilization increased by 1.8 times upon doubling the bile salt concentration, which was interpreted as a clear indication that mechanism 3 is dominant.[43]

To further clarify the effect of QD extract, we performed additional experiments where apart from doubling the concentration BS, we increased proportionally the concentrations of total calcium, emulsion and enzymes. In this way, we increased the concentration of BS from 15 to 30 mM, while maintaining the same ratios of [Ca^{2+}/BS], [reaction products/BS] and [Ca^{2+}/reaction products]. Again, the cholesterol solubilization increased from 21 to 29 % (viz. by 1.4 times, instead of 2 times), in agreement with the results described in the previous paragraph. Thus, the cholesterol-lowering effect of QD cannot be explained simply by mechanism 2 (with expected small effect of BS concentration) or 3 (with expected doubled increase in solubilisation).

Therefore, the obtained results do not allow us to conclude whether mechanism 2 or mechanism 3 is prevailing for QD extracts. Most probably, both mechanisms 2 and 3 are operative and play some role in the decrease of cholesterol solubilization by QD (Figure 7).

4.2. Determination of the type of active components in QD.

As explained in section 2.1, QD is a crude extract, containing 26 wt % saponins, 1.2 wt % Ca^{2+} and a wide variety of other chemical compounds (polyphenols, phenolic acids, polysaccharides, etc.) of unknown concentrations.[2]

To determine whether the observed decrease of cholesterol solubilization is driven by the saponins present in this crude extract, we performed additional experiments with: (1) highly purified Quillaja saponin extract, which contains > 90 % saponins and is used in pharmaceutical applications (with commercial name SuperSap) and (2) polyphenols, isolated by us from the QD extract.

The results for the percentage of solubilized cholesterol, at a SuperSap concentration of 1 and 3 mM saponins, are presented in Figure 9 for cocoa butter and lard emulsions. One sees that SuperSap does not affect the cholesterol solubilization, in contrast to QD extract which decreases the solubilized cholesterol from 80 % to 15 % for both fats.

To check whether the saponin profile of the QD extract and SuperSap is the same, we performed additional analysis by HPLC. The obtained chromatograms showed that the main saponin peaks in the two extracts are very similar (see Figure S10 in Supplementary

materials). Therefore, we must conclude that the saponins are not the active component in the studied QD extract.

To check for the role of the other major class of molecules present in QD, namely the polyphenols (PP), we isolated the PP by dialysis and studied their effect on the cholesterol solubilization. The procedures for PP isolation and their characterization by UV-VIS spectrophotometry, HPLC and atomic absorption spectrometry (AAS) are described in the Supplementary materials. The total PP content in the obtained QD dialysate was determined spectrophotometrically[44] and a comparison with the PP remaining inside the dialysis chamber showed that ≈ 80 % of the PP were successfully separated from the saponins. The other 20 % of polyphenols with larger molecular mass remained in the chamber with the saponins, without permeating through the dialysis membrane. Further characterization of the isolated PP fraction by HPLC and AAS evidenced that (1) only the largest PP molecules were not separated, (2) the PP isolate did not contain any saponins, and (3) the concentration of Ca^{2+} in the PP isolate was ≈ 1 mM.

The concentration of PP in the separated dialysate was found to correspond to the total PP present in a solution of 1 mM QD. Therefore, the effect of the separated PP on cholesterol solubilization was compared with that of 1 mM QD. Since it is possible that the lowering of cholesterol is due to a combination of PP + saponins, we studied also the effect of PP isolate + 1 mM pure saponins from SuperSap. To study the effect of the components of the QD extract that remained on the inner side of the dialysis membrane (high molecular weight polyphenols and saponins), we diluted this solution to a concentration corresponding to 1 mM QD saponins.

The results from this comparison are presented in Figure 10. One sees that the inner dialysis fraction of QD reduces cholesterol to the same extent as the 1 mM QD extract, whereas the effect of separated PP is the same as the effect of 3 mM Ca^{2+} (cholesterol solubilization is reduced by ≈ 25 %). The addition of saponins from SuperSap to the separated PP did not affect the cholesterol solubilization. Thus, the effect of the PP isolate, which contains smaller polyphenol molecules, is due to the calcium present therein. On the other hand, the effect of the larger polyphenols that remain inside the dialysis chamber cannot be explained with calcium.

The above results indicate that the cholesterol-lowering properties of QD extract are most likely due to the large polyphenol molecules, present in this extract. Additional experiments with well separated polyphenol fractions would be very helpful to prove unambiguously this conclusion.

5. Conclusions.

We investigated the effect of non-purified Quillaja saponaria extract (Quillaja Dry, QD) on the solubilization of cholesterol and fatty acids, by quantifying their solubilization in an *in vitro* digestion model. The main conclusions can be summarized as follows:

(1) QD extract decreases significantly the solubilization of cholesterol in the small dietary mixed micelles (spherical or worm-like) which are able to pass the mucus layer of the small intestine.

(2) QD extract induces cholesterol precipitation by two possible mechanisms: (1) Direct precipitation of cholesterol by the active component present in the QD extract, and (2) Displacement of the cholesterol molecules from the DMM by the active component in QD (competitive solubilisation of this active component and cholesterol, Figure 7). Further experiments are needed to clarify which of these two mechanisms is prevailing.

(3) The active components for cholesterol-lowering in QD extract are not the saponins, but the large molecular-weight polyphenols, present in this extract.

(4) The solubilization of saturated fatty acids in the DMM decreases significantly in the presence of QD (from 70 to 10 %). This effect is explained by the presence of calcium ions of high concentration in the QD extract, which form insoluble calcium soaps in the presence of fatty acids.

This work shows the capability of our digestion model[29] to assess the bioaccessibility of lipophilic substances, which need solubilisation in the DMM to be absorbed in the gut. We demonstrated also that, when combined with appropriate complementary methods, such *in vitro* studies can provide valuable information about the detailed colloidal mechanisms and the key components governing the observed effects. Of particular interest is the clarification of the specific intermolecular interactions which control the studied phenomena.

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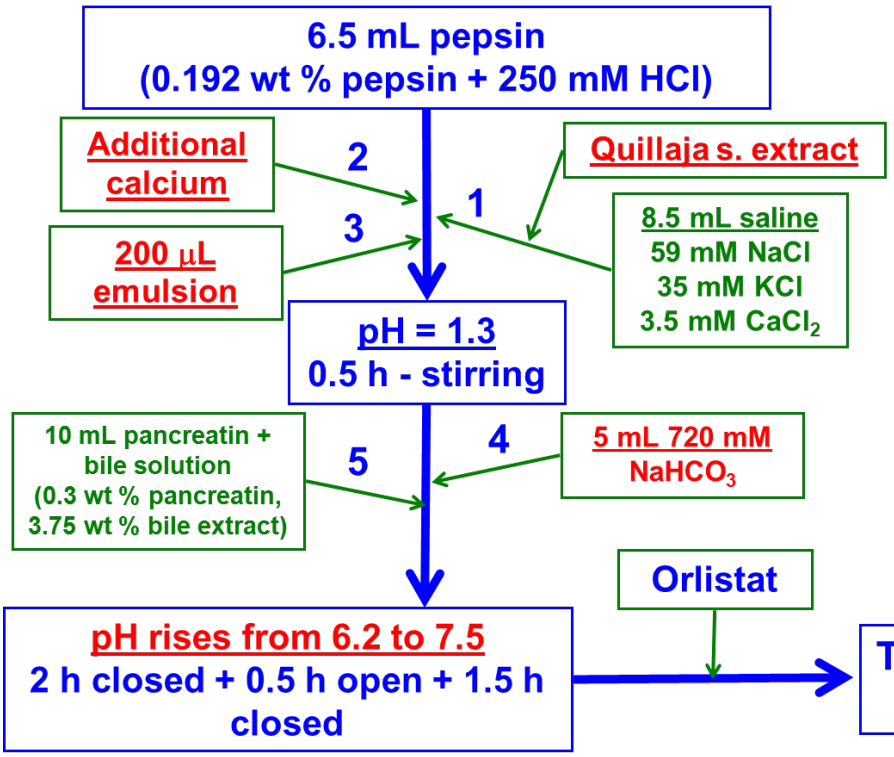


Component	Concentration, mM
Fat or Oil	4.2
Bile salts	15
Fatty acids (from bile)	3
Cholesterol (from bile)	0.4
Lecithin (from bile)	1.0
HCO_3^-	66
Na^+	137
Cl^-	83
K^+	10
Ca^{2+}	1

Figure 1. Schematic presentation of the protocol for mixing the solutions and performing the lipolysis reaction in the *in vitro* digestion model. The inserted table shows the molar concentrations of the main components in the complete reaction mixture in the "intestinal" stage of the model, after mixing all solutions.

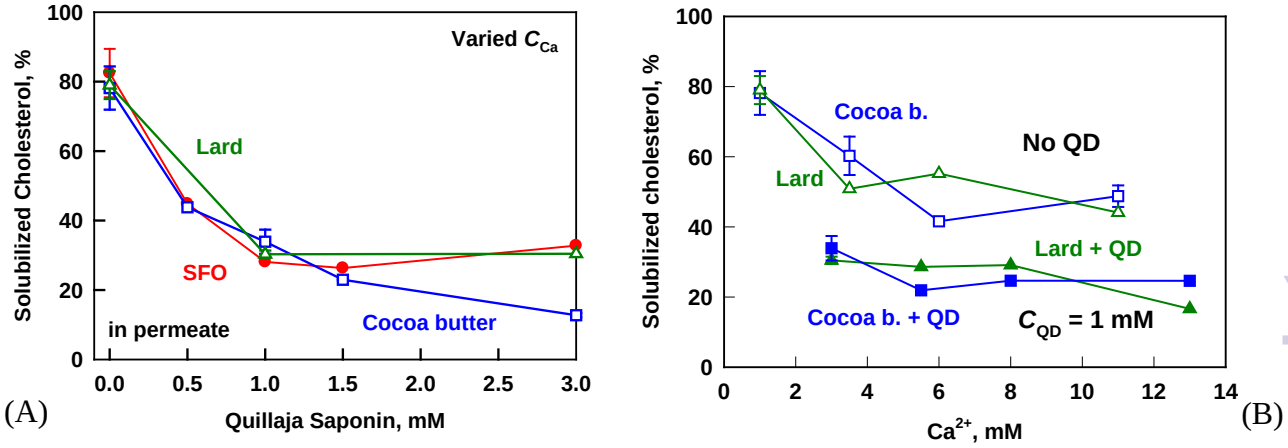


Figure 2. Fraction of solubilized cholesterol in the permeate, as a function of: (A) Quillaja saponin concentration, obtained after lipolysis of sunflower oil (red circles), cocoa butter (blue squares) or lard (green triangles) emulsions; (B) Ca^{2+} concentration for emulsions of lard (green triangles) or cocoa butter (blue squares), in presence (full symbols) or in absence (empty symbols) of QD extract. The concentration of cholesterol in the whole sample is 0.4 mM in all experiments. The presence of Ca^{2+} ions in QD is accounted for in (B). The experimental data are averaged from (at least) two separate experiments.

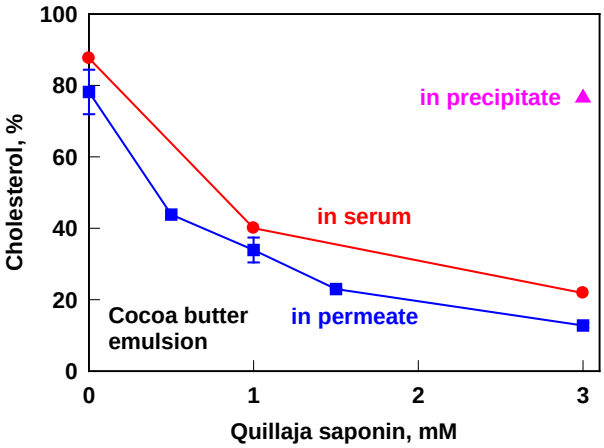


Figure 3. Fraction of cholesterol in the permeate after filtration through 200 nm filter (blue squares), in the serum after centrifugation (red circles) and in the centrifuged precipitate (pink triangle), as a function of QD concentration, obtained after lipolysis of cocoa butter emulsions. The cholesterol in the whole sample (before filtration or centrifugation) is 0.4 mM. The experimental data for the permeate are averaged from (at least) two separate experiments.

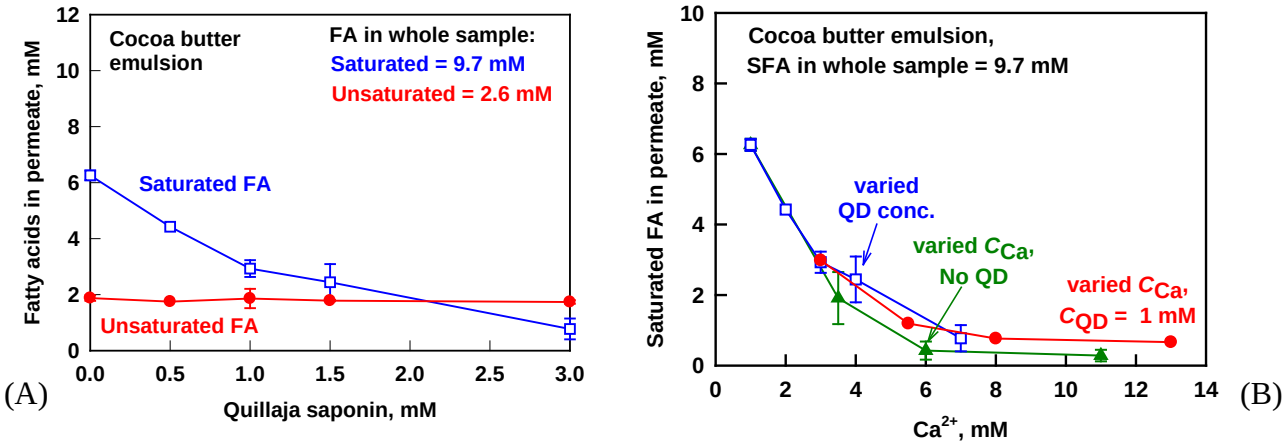
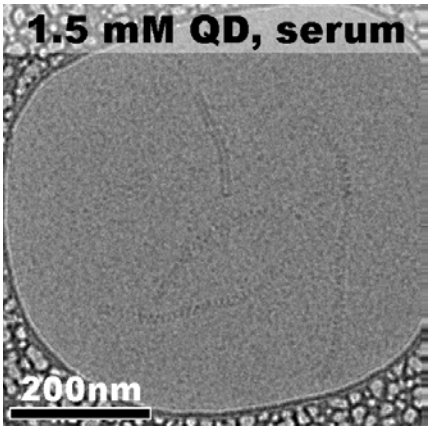
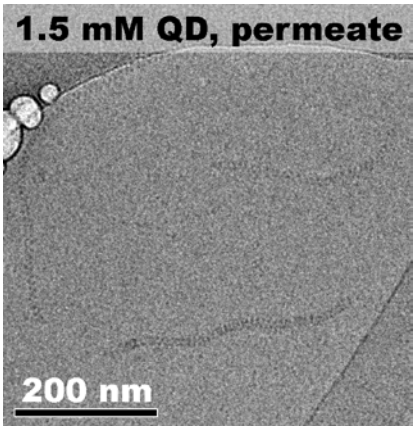


Figure 4. (A) Concentration of the solubilized saturated (blue squares) and unsaturated (red circles) free FA, as a function of QD concentration. The concentration of free FA in the whole sample (before filtration) is 9.7 mM for the saturated and 2.6 mM for the unsaturated FA. (B) Concentration of the solubilized saturated free FA, as a function of C_{Ca} for experiments performed at different QD concentrations (blue squares), upon C_{Ca} variation in the absence of QD (green triangles) or upon C_{Ca} variation in presence of 1 mM QD (red circles). 1 mM of saponins in QD corresponds to addition of 2 mM Ca^{2+} ions from the QD extract. All data are obtained after lipolysis of cocoa butter emulsions and are averaged from (at least) two separate experiments.

(A) 



(B)

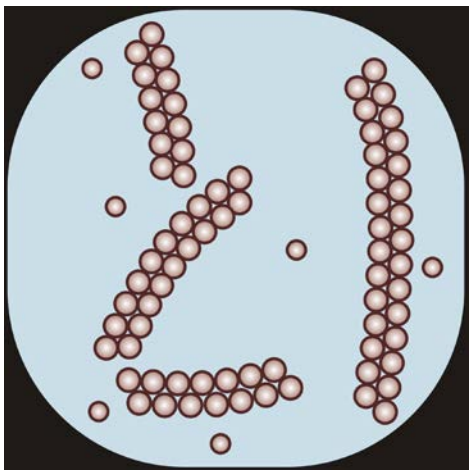

(C)

Figure 5. Cryo-TEM micrographs of the aqueous phase after lipolysis of sunflower oil-in-water emulsions, separated by centrifugation (A) or filtration (B), in presence of 1.5 mM QD. In both pictures, one sees small dietary micelles (which appear like dark dots) and elongated, worm-like micelles. The lipolysis experiments are performed as described in section 2.3, except for the duration of the "intestinal" phase which was cut from 4 to 1 h. The pH profile was varied as in the other lipolysis experiments, from 6.2 to 7.5. (C) Schematic presentation of the complex structure of the worm-like micelles.

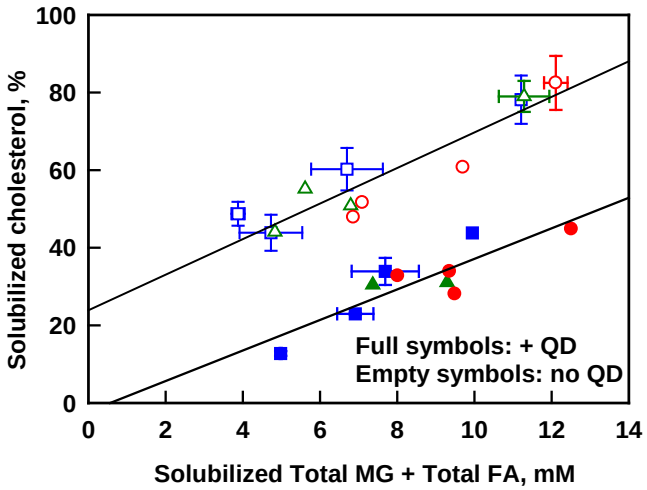


Figure 6. Solubilized cholesterol in the permeate (after filtration), as a function of the concentration of MG + FA in the permeate, for samples in presence of QD (full symbols) and for samples in absence of QD (empty symbols), obtained after lipolysis of cocoa butter (blue squares), lard (green trianlges) and SFO (red circles) emulsions. The concentration of cholesterol in the whole sample is 0.4 mM.

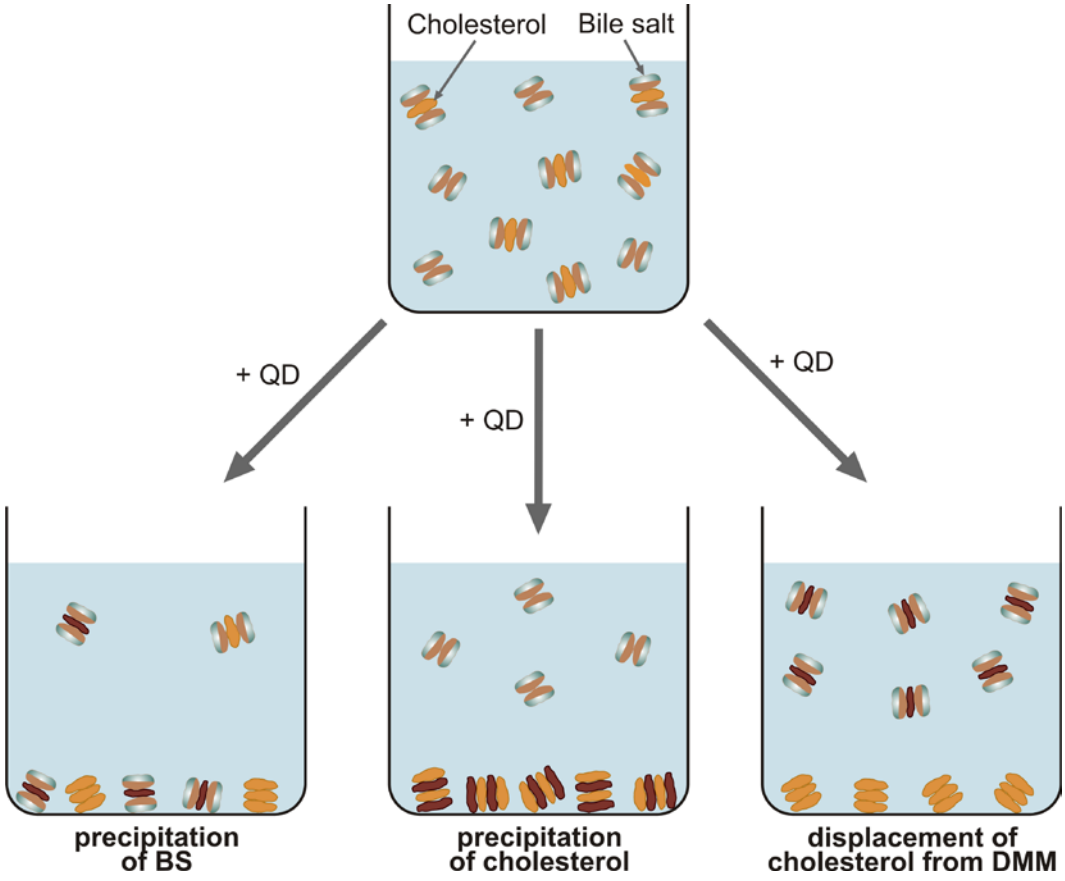


Figure 7. Schematic presentation of the three main mechanisms of interaction between the key components which might lead to cholesterol precipitation. From left to right: (1) The active components in QD extract precipitate the bile salts. As a result, the aqueous phase has lower solubilisation capacity and the excess of cholesterol precipitates. (2) The active components in QD have very strong affinity to cholesterol which leads to formation of mixed precipitates. (3) The active components in QD have strong affinity to the dietary mixed micelles and are able to displace the cholesterol from these micelles. The displaced cholesterol is forced to precipitate, due to its very low solubility in water.

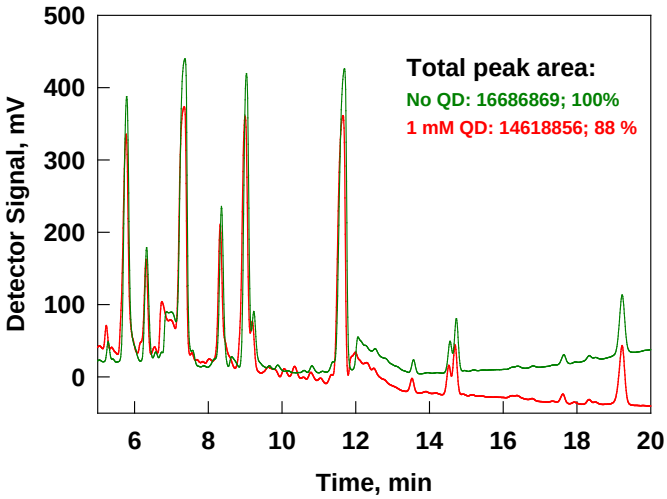


Figure 8. HPLC chromatogram of the bile salts present in the permeate in the absence (green curve) or in the presence (red curve) of 1 mM QD. The total decrease of the peak area in the chromatogram, as a result of QD addition in the reaction mixture, is 12 % only.

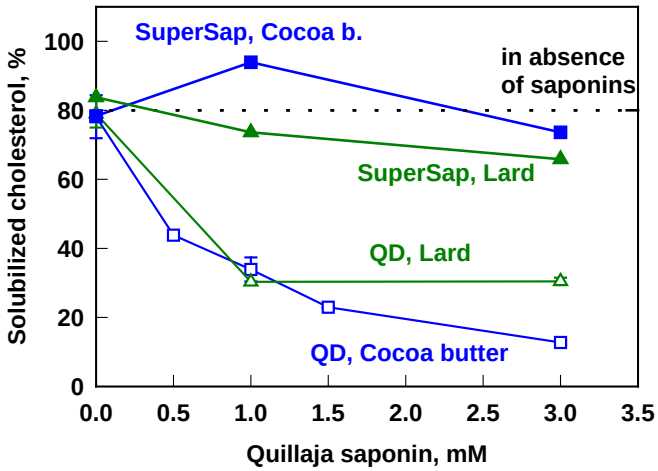


Figure 9. Fraction of solubilized cholesterol in the permeate, as a function of Quillaja saponin concentration, obtained after lipolysis of cocoa butter emulsions (blue squares) or lard emulsions (green triangles). The Quillaja saponins originate either from non-purified QD extract (empty symbols) or from purified SuperSap extract (full symbols). The concentration of cholesterol in the whole sample is 0.4 mM. The experimental data for QD are averaged from (at least) two separate experiments.

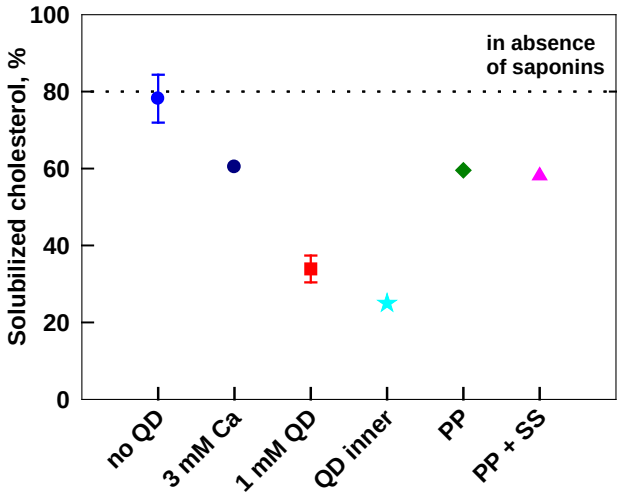


Figure 10. Percentage of solubilized cholesterol in the permeate: in absence of QD (blue circle), in presence of 2 mM calcium (dark blue circle), in presence of 1 mM QD (red square), in presence of the inner dialysis fraction of 1 mM QD (cyan star), in presence of the polyphenol (PP) fraction of 1 mM QD (without the saponins, green diamond), or in presence of the PP fraction of 1 mM QD + 1 mM SuperSap (PP + SS, pink triangle). The experiments are performed with cocoa butter emulsions. The concentration of cholesterol in the whole sample is 0.4. The experimental data for QD are averaged from (at least) two separate experiments.