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Mechanism of lowering cholesterol absorption by calcium as studied by in-vitro digestion model,
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Supplementary materials

The supplementary materials are organized in two general parts: in the first, we describe additional experimental procedures, whereas in the second, we present complementary experimental results.

The experimental procedures are listed as follows: Gas chromatography (section S1), procedure for fat and oil saponification and preparation of GC standards (sections S2 and S3), HPLC analysis of bile salts (section S4) and atomic absorption spectrometry (section S5).

Complementary experimental results are presented for: fatty acid composition of the studied fats and oils (Table S1), HPLC of bile salts (Figure S1), degree of fat lipolysis (Figure S2), concentration of FA and MG in the whole samples (Figure S3), cholesterol and FA composition of serum and permeate (Figure S4), phase distribution of FA and calcium (Figure S5), fraction of saturated FA in the permeate, as a function of calcium concentration, C_{Ca} (Figure S6), HPLC of bile salts at different calcium concentrations (Figure S7), comparison of the results in our study with the results of Simmonds et al. (1967) about the solubilization of cholesterol by unsaturated MG and unsaturated FA (Figure S8), correlation of the solubilized cholesterol with the solubilized total FA, unsaturated or saturated MG and FA (Figure S9).

S1. Gas chromatography (GC) procedure.

The GC analyses were performed on a TRACE GC apparatus (ThermoQuest, Italy) equipped with autosampler AS 2000. We used a capillary column Quadrex, USA, with the following specification: 5 % phenyl methylpolysiloxane, 10 m length, I.D. 0.53 mm, 0.1 μm film thickness. Cold on-column injection was used, at a secondary cooling time of 0.3 min. The injection volume was 1 μL. The oven was programmed as follows: start at 120 ºC, hold 2 min, ramp 1 to 325 ºC at 10 ºC/min, ramp 2 to 345 ºC at 5 ºC/min, hold 5 min. The flame-ionization detector (FID) temperature was set to 350 ºC. The carrier gas was helium, set at a constant pressure flow mode (60 kPa). The detector gases were hydrogen and air, with nitrogen as make-up gas. The secondary cooling gas was nitrogen with a purity of 99.99 %. All other gases were of 99.999 % purity.

Before injection, the samples were derivatized by mixing with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 1 h at 60 ºC.

The concentration of FA, MG, TG and cholesterol was calculated from the internal standard hexadecanol (cetanol), using correction factors of 1.16 and 1.90 for the FA and the TG, respectively. The correction factors were obtained from calibration curves with standard substances.

S2. Procedure for fat and oil saponification and preparation of GC standards.

To determine the fatty acid composition of the used fats and oils, they were saponified by the following procedure, adapted from IUPAC (Paquot, C. (1981). Standart methods for the analysis of oils, fats and derivatives (IUPAC), In *Pure & appl. Chem* (6th ed.)): First, we prepared 3.33 M alcoholic (80 % ethanol) solution of potassium hydroxide (KOH). Then, the fats and oils which are solids at room temperature (lard, cocoa butter, cow butter, palm oil) were melted at 50 ºC, 0.22 g of each were weighted in a separate vessel and 3 mL of the alc. KOH solution were added. The vessel was tightly closed and left for 4 hours in a heating oven set at 45 ± 5 ºC. The same procedure, but at room temperature, was applied to sunflower oil as it is in a liquid state. Every hour the mixture in the vessel was homogenized by hand shaking. Afterwards, the obtained clear solution was left to evaporate in a vacuum dryer overnight. The next day, 24.5 mL of water were added to the vessel and the pH was lowered to 2 by addition of 5.5 mL 2 M HCl. The sample was then treated with chloroform, and the extracted FFA were used in the preparation of the chromatography standards for the respective fats/oils.

S3. Procedure for preparation of GC standards.

To prepare standards for GC, mixtures of TG, MG, FFA and cholesterol were prepared to correspond to 50 % and 90 % fat/oil hydrolysis (defined as going from TG to MG). A separate couple of standards were prepared for each kind of oil or fat, by using the respective non-hydrolyzed fat/oil for the TG and the saponified extract for the FFA. Unsaturated MG (Danisco) was used in all these standards. The concentration of cholesterol (C_{chol}) in the 50 % hydrolysis standard was 0.6 mM, while C_{chol} in the 90 % hydrolysis standard was 0.3 mM.

The standards used for determination of the retention times in GC were: myristic acid (98%, Fluka, cat. no. 70082), palmitic acid (98%, Riedel de Haen, cat. no. 27734), stearic acid (97%, Acros, cat. no. 174490025), oleic acid (85%, Tokyo Kasei Kogyo, cat. no. 00011), 2-oleyl glycerol (95%, Sigma, cat. no. M2787) and dipalmitin (99%, Sigma, cat. no. D2135), cholesterol (95%, Sigma, cat. no. 26740), and triolein (99%, Fluka, cat. no. 92859).

S4. HPLC analysis of bile salts. The analysis was performed on a Shimadzu (Japan) apparatus, equipped with two high-pressure mixing binary gradient pumps (LC-20AD), DGU-14A four-line membrane degasser, SIL-10ADvp autosampler, CTO-10ASvp wide temperature range column oven, and SPD-10Avp UV-VIS detector. The separation was carried out on 25cm x 4.6 mm i.d., 5 μm particle size, Ascentis® C18 column (Supelco, USA) and a guard column Ascentis® C18 Supelguard™ Guard Cartridge (5 μm particle size, 2 cm x 4 mm i.d.). All samples were filtered through 200 nm filters (section 2.4) and kept at 37 ºC prior injection.

For the separation of bile salts we developed a new procedure, in which we used gradient elution with 1.5 vol. % formic acid in water (A) and 1.5 vol. % formic acid in acetonitrile (B). The total flow rate was kept at 1 ml/min for the whole time of the analysis (35 min). The system was run with the following gradient program: from 40 % B to 75 % B for 20 min, from 75 % B to 40 % B for the next 2 min and constant flow at 40 % B for 13 min. The sample injection volume was 20 μL. The UV-VIS detector was set at λ = 200 nm and the column temperature was 37 ºC.

The standards for determination of the retention times in HPLC were: 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 major bile salt peaks in the extract have been separated well by this procedure, see Figure S1A. However, the identification of these peaks was difficult, because only some of them had retention times close to our standards (Figure S1B). Thus, in our study we analyzed the changes in the areas of the main bile salt peaks at low (1 mM) and high calcium concentration (11 mM).

S5. Atomic absorption spectrometry (AAS) procedure. Calcium concentration in the aqueous phase was determined by AAS on an instrument Analyst 400 (PerkinElmer, USA) in an air/acetylene flame. The samples were prepared as follows: after filtration of the reaction mixture by the procedure described in Section 2.4, part of the sample was taken and diluted with solutions containing NaCl, KCl, NaN_3 and Na_2EDTA. We added Na_2EDTA to these solutions to prevent calcium precipitation, while NaN_3 was added as an antibacterial agent. The dilution was designed in such a way that all studied solutions had similar Ca^{2+} concentrations, falling around the middle of the calibration curves. The final concentrations of Na^+ and K^+ ions in the calibration solutions was equal to that in the studied solutions, because these elements interfere with calcium when measured by AAS (J. B. Willis (1960). The determination of metals in blood serum by atomic absorption spectroscopy: calcium, Spectrochim. Acta, 16(3),259–272.)

Table S1. Fatty acid composition of the studied fats and oils, as determined by GC (described in sections S1-S3). The quoted percentages are defined as weight of given fatty acid, divided by the weight of all visible fatty acids in GC chromatogram.

Fatty acids, wt. %	**SFO**	**Lard**	**Palm oil**	**Cow butter**	**Cocoa butter**
Lauric acid (C12:0)	0	0	0.9	2.6	0
Myristic acid (C14:0)	0	1.4	1	10.8	0
Palmitoleic acid (C16:1)	0	1.9	0	1.6	0
Palmitic acid (C16:0)	7.1	29.4	46.5	32	26.6
Oleic + linoleic acid (C18:1,2)	87.2	45.4	44.1	33.3	34.8
Stearic acid (C18:0)	5.7	21.9	7.5	19.7	38.6
Total Saturated FFA (SFFA)	12.8	52.7	55.9	65.1	65.2
Total Unsaturated FFA (UFFA)	87.2	47.3	44.1	34.9	34.8

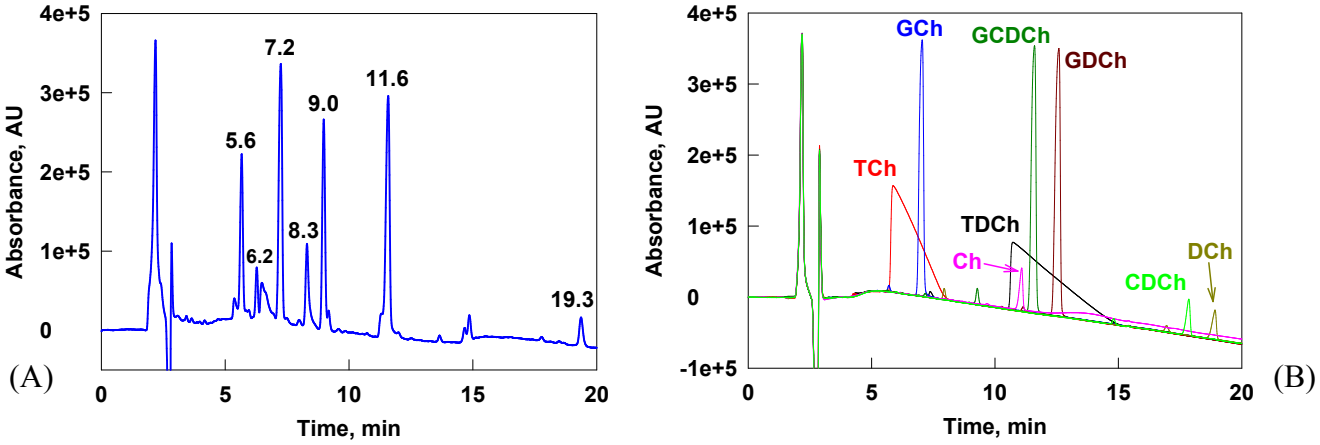


Figure S1. HPLC chromatogram of (A) bile salts present in permeate obtained by filtration of a lipolysis solution; the major bile salts elute at 5.6, 6.2, 7.2, 8.3, 9.0, 11.6 and 19.3 min, (B) single bile salt standards, plotted in the same scale, as function of the retention time: Taurocholic acid (TCh, 6.2 min), glycocholic acid (GCh, 7 min), taurodeoxycholic acid (TDCh, 10.7 min), cholic acid (Ch, 11 min), glycochenodeoxycholic acid (GCDCh, 11.5 min), glycodeoxycholic acid (GDCh, 12.6 min), chenodeoxycholic acid (CDCh, 17.8 min) and deoxycholic acid (DCh, 18.8 min).

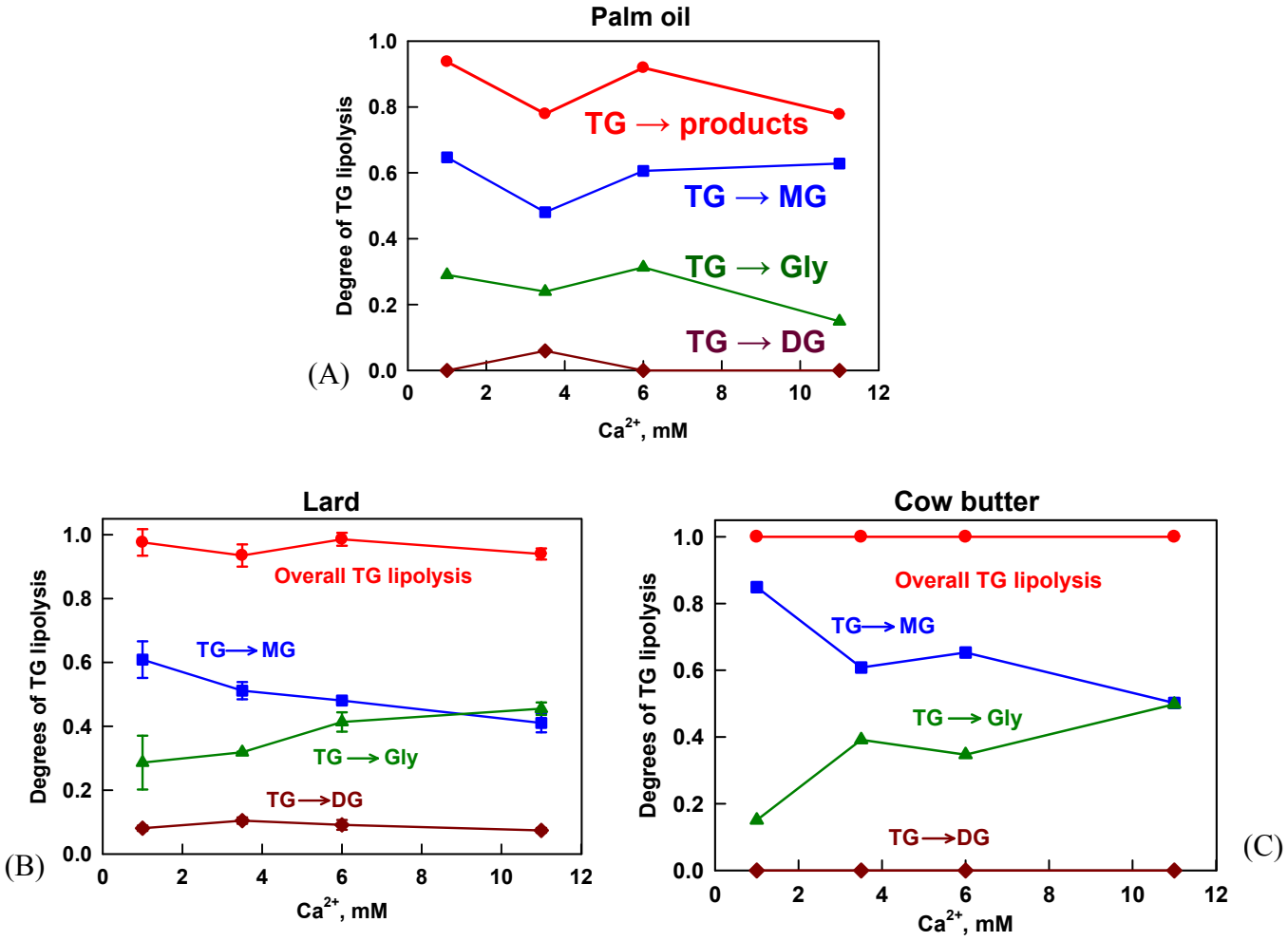


Figure S2. Degrees of lipolysis for (A) palm oil, (B) lard and (C) cow butter: overall degree of TG lipolysis, α (red dots), degree of lipolysis to diglycerides, β (brown diamonds), degree of lipolysis to monoglycerides, γ (blue squares) and degree of lipolysis to glycerol, δ (green triangles). The respective definitions are given in the text. The results are averaged from (at least) two separate experiments.

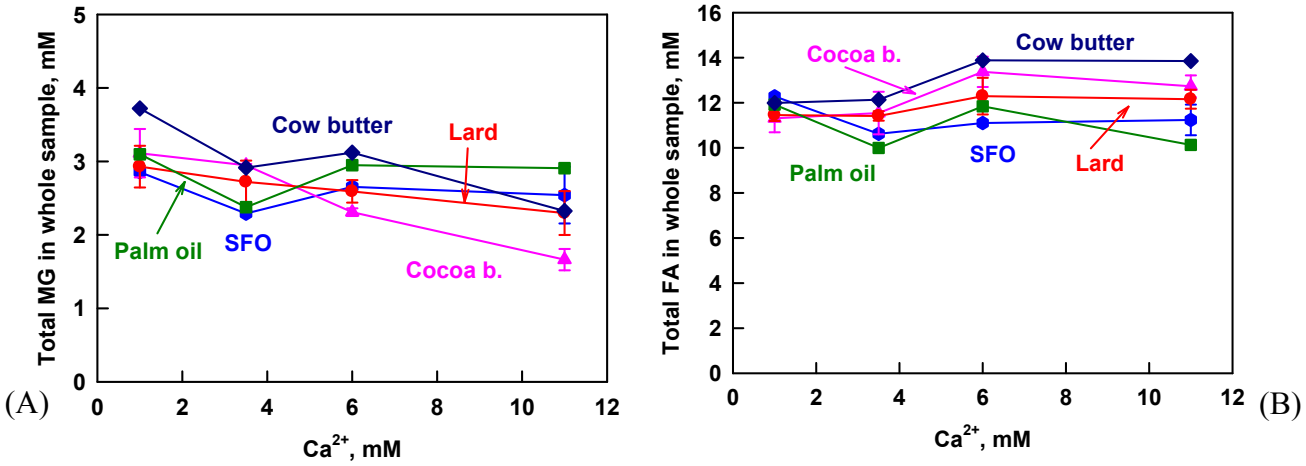


Figure S3. Concentration of (A) total monoglycerides and (B) total fatty acids in the whole sample, after lipolysis of oil-in-water emulsions of Cocoa butter (pink triangles), Palm oil (green squares), Lard (red circles), Cow butter (dark blue diamonds) and Sunflower oil (blue hexagons). The results are averaged from (at least) two separate experiments.

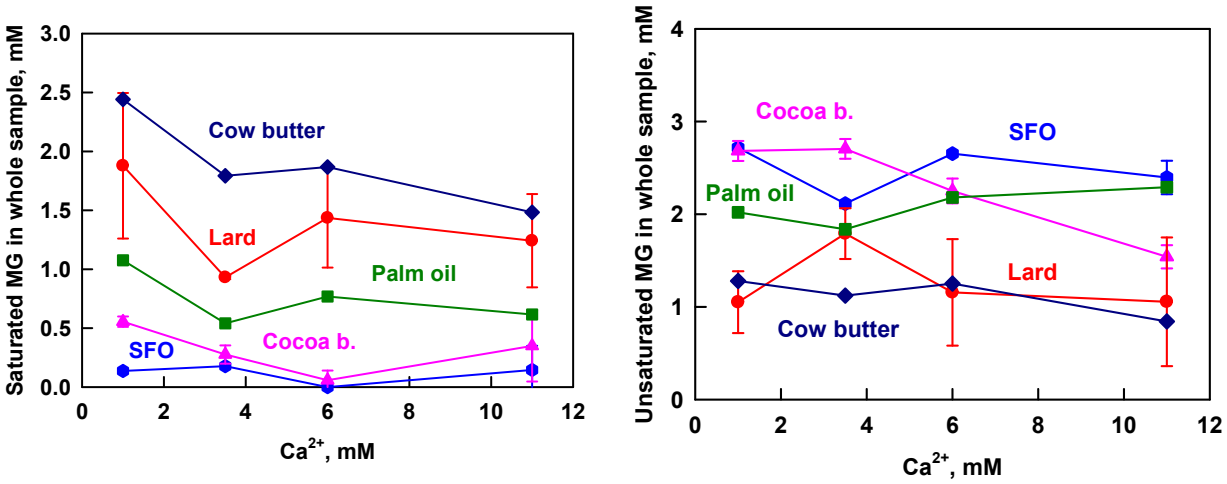


Figure S4. Concentration of (A) saturated monoglycerides and (B) unsaturated monoglycerides in the whole sample, after lipolysis of oil-in-water emulsions of Cocoa butter (pink triangles), Palm oil (green squares), Lard (red circles), Cow butter (dark blue diamonds) and Sunflower oil (blue hexagons). The results are averaged from (at least) two separate experiments.

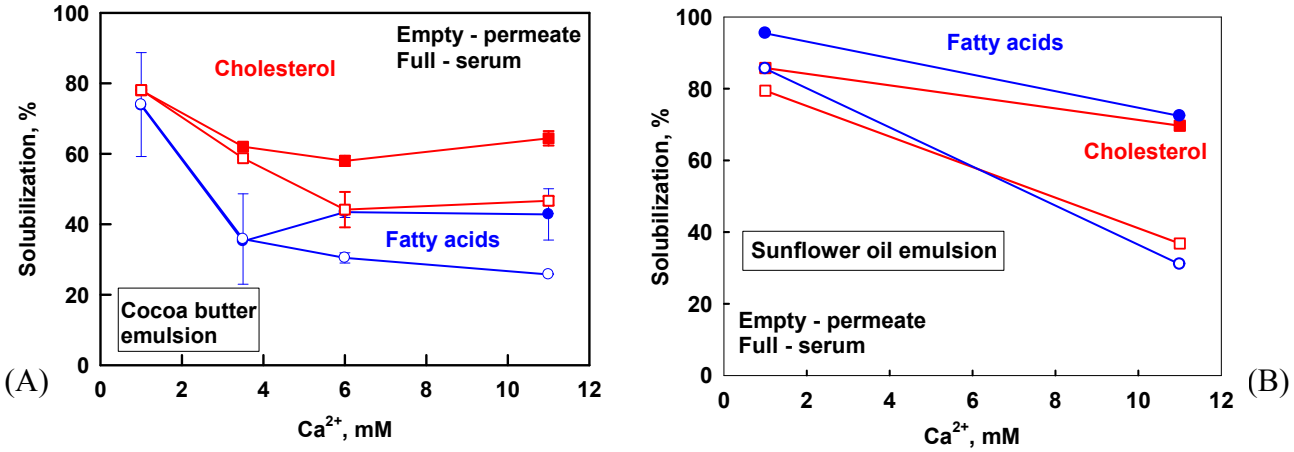


Figure S5. Percentage of solubilized cholesterol (red squares) and total FA (blue circles) in the serum (full symbols) or in the permeate (empty symbols), as a function of calcium concentration for in vitro digestion of (A) cocoa butter emulsion and (B) sunflower oil emulsion.

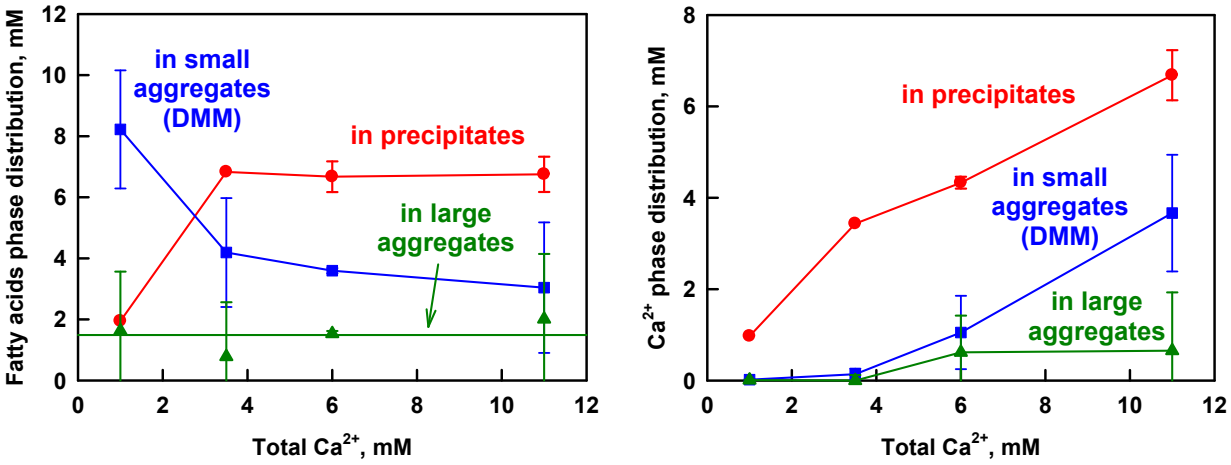


Figure S6. Distribution of (A) fatty acids and (B) calcium ions between small aggregates (blue squares), large aggregates (green triangles) and precipitates (red circles), as a function of the total calcium concentration. The concentration of components in the small aggregates is determined directly from analysis of the permeate, whereas for the large aggregates it is calculated from the difference [serum-permeate]. The experimental data is an average of at least two separate experiments. The solid lines are guide to the eye.

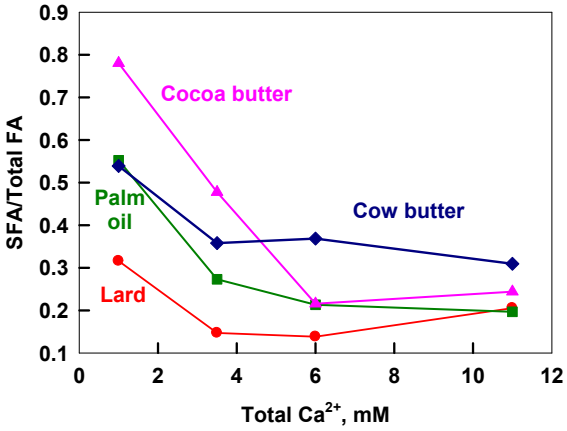


Figure S7. Fraction of SFA in the mixture (SFA+UFA), as determined in permeate, as a function of calcium concentration, for cocoa butter (pink triangles), palm oil (green squares), cow butter (blue diamonds) and lard (red circles).

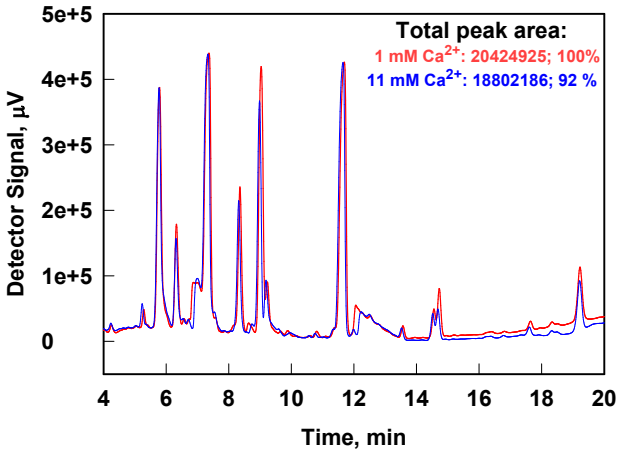
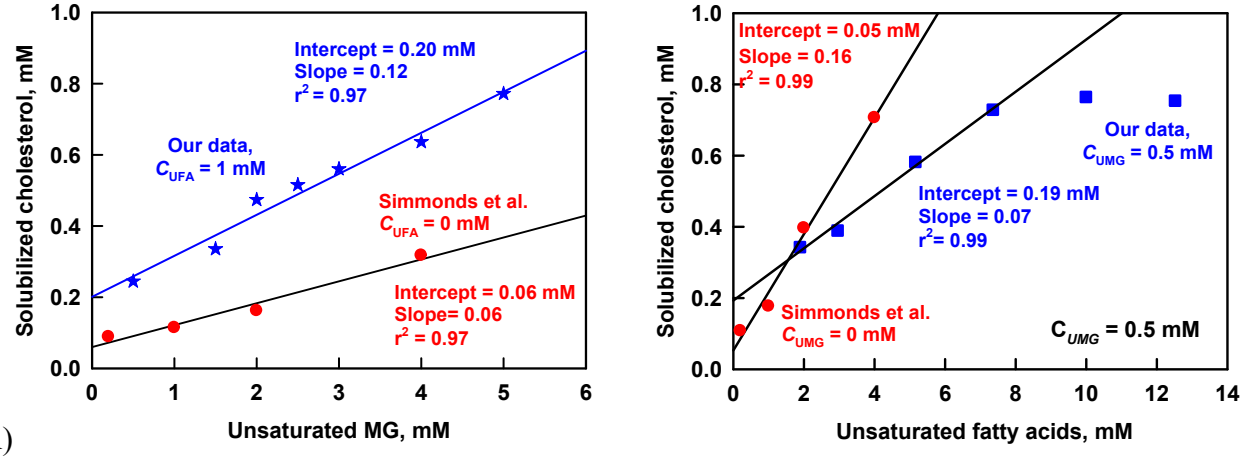


Figure S8. HPLC chromatograms of the bile salts in the permeate in presence of 1 mM (red line) or 11 mM Ca^{2+} (blue line), after lipolysis of cocoa butter emulsions.



(A) (B)

Figure S9. Solubilization of cholesterol by (A) unsaturated MG and (B) unsaturated FA. Results from Simmonds et al. (1967) (red dots) are compared to those of the present study (blue squares).

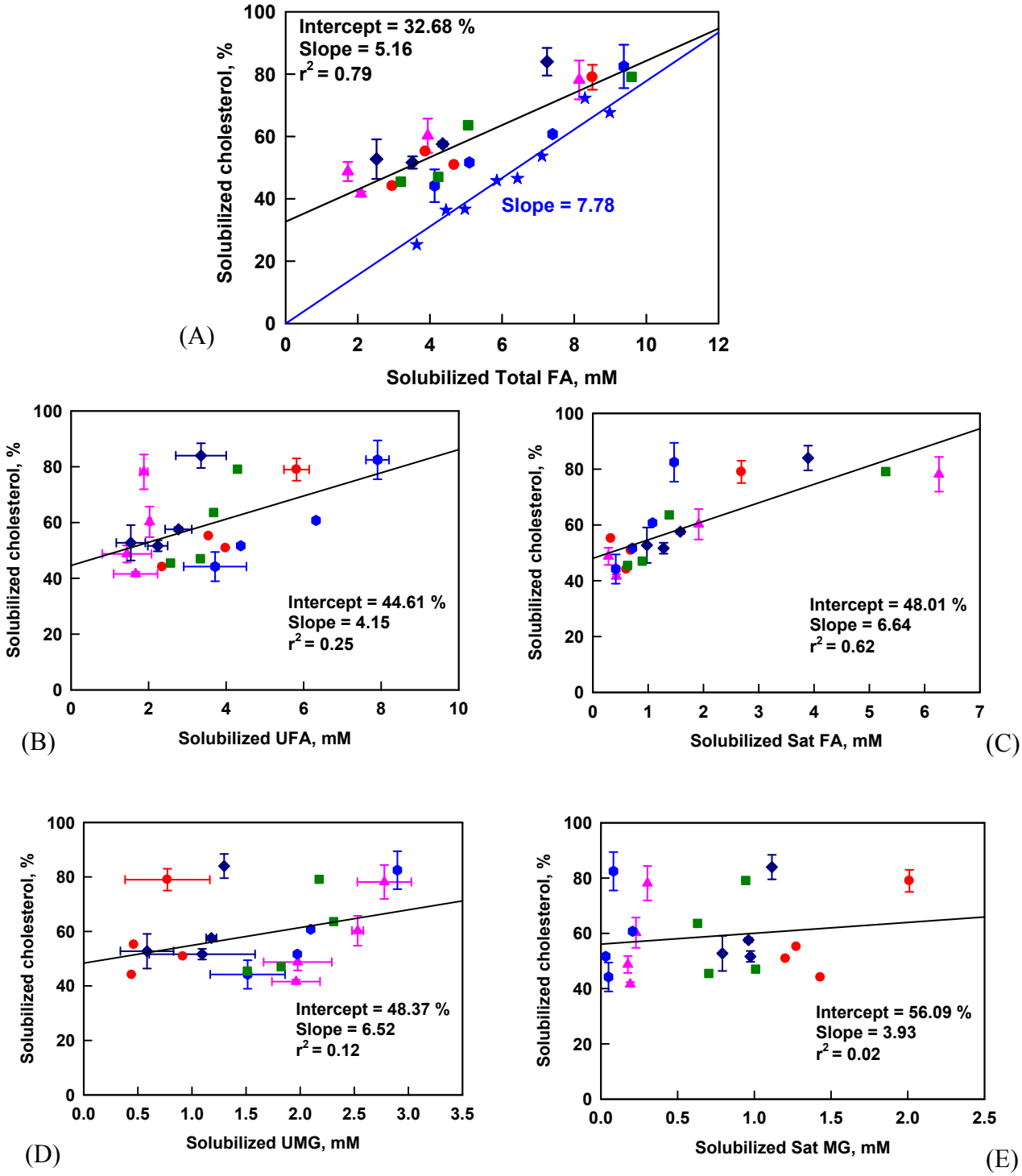


Figure S10. Solubilization of cholesterol, as a function of (A) solubilized total FA, (B) and (C) solubilized unsaturated and saturated FA, (D) and (E) solubilized unsaturated and saturated MG after lipolysis of Cocoa butter (pink triangles), Palm oil (green squares), Cow butter (dark blue diamonds), Lard (red circles) and Sunflower oil (blue hexagons) emulsions, Sunflower oil at pH=6.8 from Vinarov et al. (2012) (blue stars), at different concentrations of Ca^{2+} ions. The results are average from (at least) two separate experiments.