Supplemental Document

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Evaluation of the changes in human milk lipid composition and conformational state with Raman spectroscopy during a breastfeed: supplement

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Adequate assignment of vibrational bands is crucial for the analysis of the human milk Raman spectra. The macronutrients of human milk are fat, carbohydrates and proteins [1, 2]. The concentrations of these nutrients in human milk are ~36 g/L for fat, ~72 g/L for lactose, ~10 g/L for oligosaccharides and ~9 g/L proteins [1]. As fat and lactose are present with the highest concentrations, Raman spectroscopic measurements on pure lipids and pure lactose were performed. This allowed for adequate assignment of vibrational bands in the acquired human milk Raman spectra of this study and for adequate quantification of lipid composition in human milk. The same confocal Raman spectroscope as discussed in the primary manuscript was used for measuring the pure samples. The goal of this supplement is to present the results of the Raman spectroscopic measurements on the most abundant components of human milk.

Materials and Methods. Only the most abundant lipids in human milk were evaluated. 95 to 98% of the lipids in human milk are triacylglycerol (TAG) molecules [3]. These molecules contain three fatty acids [3]. The most abundant fatty acids in human milk in order of abundance are oleic acid, palmitic acid, linoleic acid, myristic acid and stearic acid. Therefore, the pure lipids glyceryl trioleate (18:1, TOA), glyceryl tripalmitate (16:0, TPA), glyceryl trilinoleate (18:2, TLA), glyceryl trimyristate (14:0, TMA) and glyceryl tristearate (18:0, TSA) were measured. All pure lipids (Sigma Aldrich, US) were ≥99% pure and in powder form. Each lipid was individually dissolved in chloroform with a concentration of 0.40 g/mL. Three droplets of 20 µL of the lipid-chloroform solution was pipetted on a RVS substrate (microRIM) with an interval of 1 minute. The chloroform was evaporated during at least one hour before the measurements were performed. This resulted in a thin layer of pure lipid on the substrate. A spacer of 80 µm was attached to a borosilicate glass cover slip and placed on top of the sample before measurements. No sealing was applied. Per pure lipid sample, 900 spectra were measured in one layer by scanning a raster pattern of 30 x 30 points over an area of 56 x 56 µm. Anti-trapping mode was used and the integration time for each point measurement was 150 ms. The average intensity of these 900 spectra was used for the assignment of vibrational bands and quantification of lipid composition and conformational state.

Lactose (ThermoFisher Scientific, country) was diluted in PBS to a concentration of 100 mg/mL. A 50 µL droplet of lactose solution was pipetted on top of the RVS substrate. A spacer of 160 µm was attached to a borosilicate glass cover slip and placed on top of the sample. No sealing was applied. 1600 spectra were measured in one layer in total by scanning a raster pattern of 40 x 40 points over an area of 56 x 56 µm. Anti-trapping mode was used and the integration time of each point measurement was 250 ms. To correct for the dominant Raman contribution from water, the pure PBS spectrum was fitted to each individual spectrum, using a least squares algorithm, and the fitted spectrum was subtracted to result in a water-corrected Raman spectrum. The average intensity of these corrected 1600 spectra was used for assignment of vibrational bands from lactose in human milk.

Results pure lipids. Normalised Raman spectra of all measured lipids are shown in figure S1. The lipids TMA, TPA and TSA are lipids with saturated fatty acids, but the fatty acid length is different. The main difference between the Raman spectra of these three lipids is the intensity of the bands corresponding to the hydrocarbon chains (bands located at 1297, 1440, 2850, 2890 and 2940 cm^{-1} [4]) with respect to the intensity of the band originating from the glycerol backbone (band located at 1745 cm^{-1} [4]). The bands corresponding to the hydrocarbon chains have a higher intensity in case the number of carbon atoms of the fatty acids are higher. Another difference between these saturated fatty acids is the location of the band located around 1100 cm^{-1}. This location is 1094, 1102 and 1106 cm^{-1} for respectively the lipids TMA, TPA and TSA. Thus, the length of fatty acids can be investigated using Raman spectroscopy.

The lipids TSA, TOA and TLA are lipids with fatty acids with the same number of carbon atoms, but with a different degree of unsaturation. The double bond of the unsaturated fatty acids results in the bands located at 1260, 1655 and 3010 cm^{-1} as can be seen in figure S1. These bands correspond to C=C-H scissoring, C=C stretching and C=C-H stretching respectively [4]. The intensity of these bands increase linearly with the degree of unsaturation of the lipids as similarly found by Weng et al. [5].

Lastly, it should be noted that the lipids TMA, TPA and TSA were crystalline at room temperature and the lipids TOA and TLA were liquid at room temperature. The lipid phase can also be measured using Raman spectroscopy

and results in three main observable differences. The first difference between spectra of lipids in the crystalline and liquid phase is the width of the bands originating from C-C stretching, twisting and bending, C-H stretching or C=O stretching vibrations. These bands of lipids in the crystalline phase are sharp bands, whereas these bands of lipids in the liquid phase are broad and overlap as can be observed for the bands at 1060, 1100, 1130, 1260, 1300, 1440, 1460, 1745, 2850 and 2940 cm^{-1} in figure S1. Secondly, the intensity of the C-C stretching vibrational bands (located at 1060, 1100 and 1130 cm^{-1} [4, 6]) are lipid phase related. The fatty acids of lipids in the crystalline phase are in the energetically favourable trans conformation resulting in a high intensity of the bands at 1060 and 1130 cm^{-1}, while the fatty acids of lipids in the liquid phase are in the gauche conformation, resulting in a high intensity of the band at 1100 cm^{-1}. Finally, the intensity of the band originating from C-H symmetric stretching vibration (located at 2850 cm^{-1} [4, 6, 7]) is almost lipid phase independent while the band originating from C-H antisymmetric stretching vibration (located at 2850 cm^{-1} [4, 6, 7]) is related to intermolecular packing and therefore lipid phase related. The band located at 2890 cm^{-1} is high in case of close interchain packing of the fatty acids (i.e. crystalline phase) and the intensity of this band is low (not distinguishable from the other bands) in case of high intrachain conformational disorder (i.e. liquid phase).

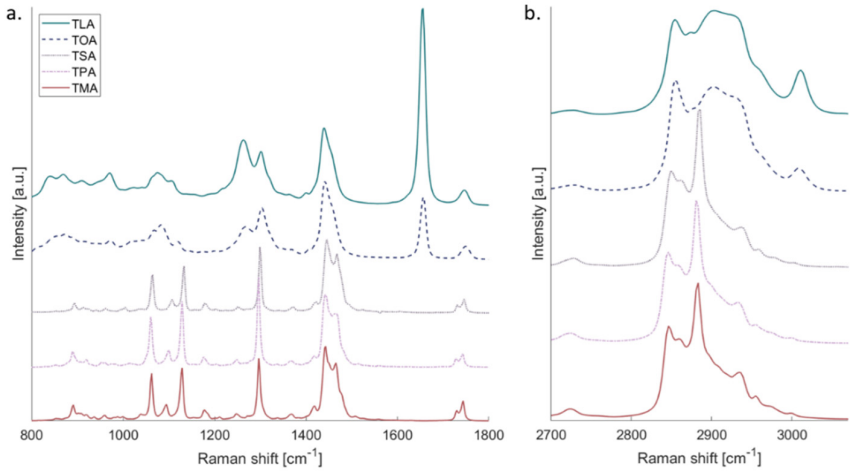

Figure S1: Raman spectra at room temperature of the pure lipids glyceryl trimyristate (14:0, TMA), glyceryl tripalmitate (16:0, TPA), glyceryl tristearate (18:0, TSA), glyceryl trioleate (18:1, TOA) and glyceryl trilinoleate (18:2, TLA) (a) in the fingerprint region and (b) in the high Raman shift region.

Results pure lactose. The average corrected lactose spectrum is presented in figure S2. The highest intensity bands in the lactose spectrum are located at 880, 1120, 1465 and 2900 cm^{-1}. These bands originate from respectively C-O-C deformation, C-O-C stretching, C-C deformations and C-H stretching [8]. The spectrum of lactose shows broad bands compared to the lipid spectra. Furthermore, some of these bands overlap with the bands in lipids spectra, namely located at 1060, 1100, 1130, 1260, 1460 and 2940 cm^{-1}).

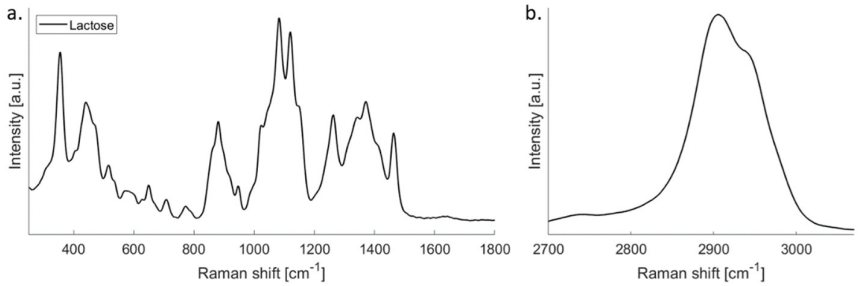

Figure S2: Raman spectra at room temperature of 100 mg pure lactose dissolved in 1 mL PBS corrected for water (a) in the fingerprint region and (b) in the high Raman shift region.

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