Electronic Supplementary Material (ESI) for Food & Function.
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Supplementary information

SI1 Negative control of *In vitro* digestion assays

To ensure that all drops in pH, leading to NaOH titration, were caused by release of FFAs through enzymatic reactions, negative control assays were performed, in which no enzyme was added. The negative controls were performed as single determination for each assay type. Any background titration observed during the negative control assays was later subtracted from the enzyme assays. As a negative control for pepsin and pancreatin, the pepsin and pancreatin supernatants were substituted with the equivalent volume pure gastric or intestinal medium, respectively. The rHGL extract was substituted with an equivalent volume of the solvent in which the rHGL concentrate was dissolved (20 mM sodium-acetate, 150 mM NaCl, and 10% isopropanol).

SI2 Droplet size distributions of undigested NAN1 and SPCemul

The droplet size of NAN1 showed a bimodal distribution with approximately 76% of the lipid found in small droplets with a diameter of ≈ 0.2 µm, and 24% found in larger droplets with a diameter of ≈ 15 µm, resulting in an average D[4,3] particle size of 4.6 ± 1.2 µm. On the day of production (day 1), SPCemul showed a unimodal distribution with an average droplet diameter of 0.83 ± 0.00 µm. At day 3, the droplet size distribution had settled into a bimodal distribution with approximately 27% of the lipid found in small droplets with a diameter of ≈ 1 µm and 73% found in large droplets with a diameter of ≈ 5.8 µm, resulting in an average D[4,3] droplet size of 5.5 ± 1.9 µm. A small portion of the lipid also appeared to have coalesced into larger lipid droplets with a size of approximately 50 µm. As no noteworthy change in the droplet size distribution was observed from day 3 to 5 after production, which was when the *in vitro* digestions were carried out, only the average of the SPCemul (day 3-5) measurements is shown in **Figure 2C**.

SI3 Back titration

The *in vitro* gastro-intestinal lipid digestion of NAN1 and SPCemul was determined titrimetrically through continuous titration of FFAs released during digestion. Only deprotonated FFAs lead to titration with NaOH. Any FFAs protonated during the gastric and intestinal digestion, became deprotonated and hence titratable during the BT. The distribution between the FFAs titrated during gastric digestion, intestinal digestion and the BT of *in vitro* digestion of NAN1 and SPCemul is provided in **Figure SI1A** and **B**. Approximately 43% of the FFAs released during digestion of NAN1 (**Figure S1A**) and 60% of SPCemul (**Figure SI1B**) were deprotonated at pH $\leq$ 6.5 and hence titrated during the gastro-intestinal digestion, while the remaining FFAs became deprotonated during the BT. One explanation, for the difference in the degree of deprotonation, could be that the FFAs released during digestion of NAN1 has higher pK_a values than those released from SPCemul, indicating that the FFA composition was different in NAN1 and SPCemul. Since the FFA composition in the un-digested NAN1 and SPCemul was not determined in this study, this could not be confirmed. Despite the difference in the buffer capacity of NAN1 and SPCemul (**Figure 2B**), the buffer capacity was not believed to be the cause of the difference in deprotonation, as blank assays were used to adjust for background titration caused by differences in buffer capacity.

The relationship between the FFAs titrated during gastro-intestinal digestion and during the BT is provided in **Figure SI1C** and shows a good linear regression for both NAN1 (r^2=0.999) and SPCemul (r^2=0.993), which shows that the continuous titration of FFAs during the individual digestion steps in this study can be used to represent both FFAs in the protonated and deprotonated state.

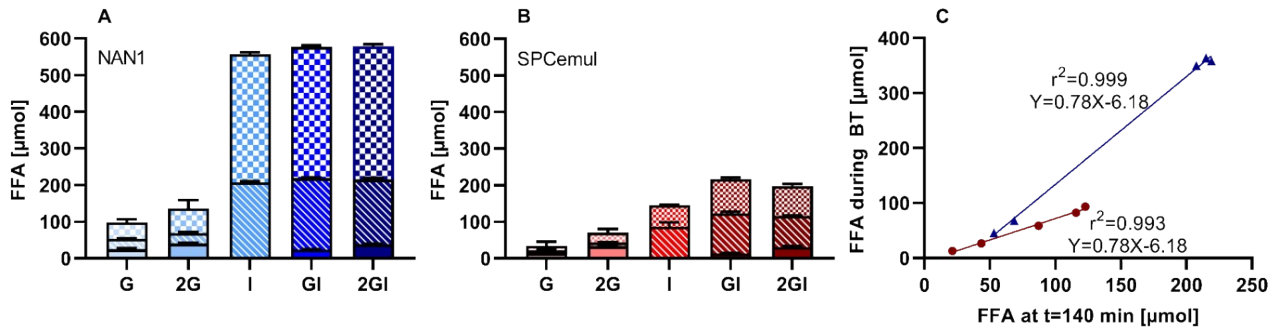

Figure SI1 – Summary of the *in vitro* pediatric gastro-intestinal digestion of NAN1 and SPCemul. A) FFA titrated during the gastric step (0-50 min): solid, during the intestinal step (50-140 min): striped, and during BT to pH 9: checkered of NAN1, mean ± SD (n=3). B) FFA titrated during the gastric step (0-50 min): solid, during the intestinal step (50-140 min): striped, and during BT to pH 9: checkered of SPCemul, mean ± SD (n=3). C) Linear regression of all FFAs titrated during the gastric and intestinal step (t=0-140 min) against FFA titrated during BT to pH 9 for NAN1: ▲ and SPCemul: ● (mean shown).

SI4A HPLC-ELSD method development and limit of quantification

The ELSD response is primarily dependent on the mass of the analyte and independent of the degree of saturation and the length of acyl chains. For instance, the ELSD response is quite similar for the two free fatty acids; oleic acid and palmitic acid, as reported by Hubert *et al.* (2017).[1] Thus, ELSD allows quantification of lipid classes based on a single lipid species.[2]

The elution times for TAGs, DAGs, MAGs and FFAs were based on the elution times of representative standard sets of the individual lipid species (**Figure SI2A**).

MAGs and FFAs could not be sufficiently separated, for which reason MAGs and FFAs in the digestion samples were integrated and quantified together. This tendency was also observed in a study by Carvalho *et al.* (2012) where a similar approach was used.[3] The calibration curves used for quantification of the lipid species in the digestion sample, shown in **Figure SI2B**, revealed the usual non-linear detector response vs. concentration provided by the ELSD. The data was linearized using a double logarithmic plot before linear regression (log-log scale; y=ax+b) weighted by $1/(\log \text{area})^2$ resulting in good fits with r^2 values between 0.972 and 0.995.

The limit of detection (LOD) was determined based on visual evaluation of calibration samples of increasingly higher concentrations (injection volumes of 15 µL). The concentration of a calibration sample with a compound peak between 2-3 times higher than the background noise level, peak-to-peak, was set as the LOD. This approach gives a good and realistic value for the LOD as is also supported in the literature.[4] According to Carr and Wahlich, the limit of quantification (LOQ) can be estimated to 3 times the LOD value.[5] The corresponding LOD and LOQ can be seen in **Table SI1**.

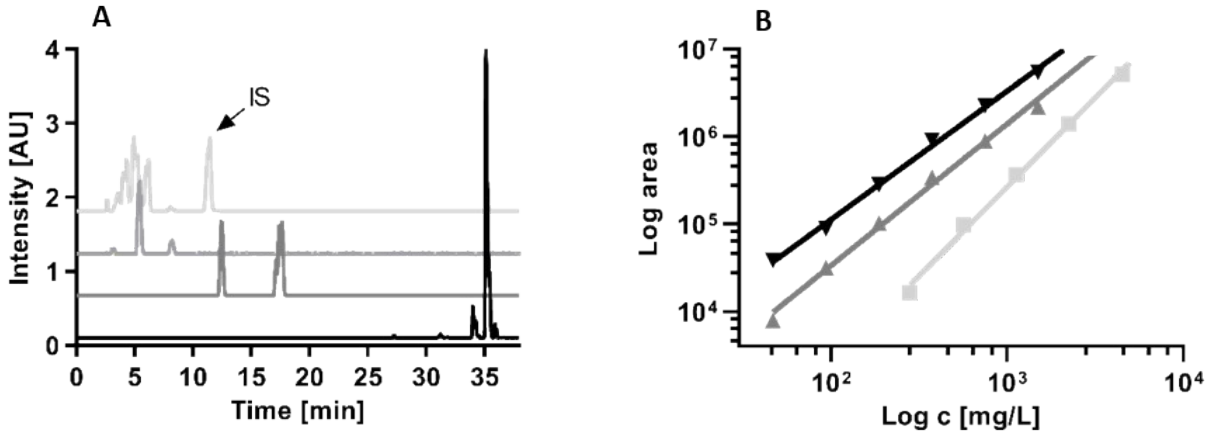

Figure SI2 – HPLC-ELSD method development: Detecting and quantifying neutral lipids. A) Grouping of the lipid classes based on representative standard sets of TAG, DAG, MAG and FFA mixtures. The groups were FFA: (0-8.1 min), MAG: (0-8.1 min), DAG: (10.4-25.4 min), TAG: (26.7-37.1 min). Arachidic acid (C20:0) was added to the FFA group to show the retention time of the internal standard (IS). B) Calibration curves used for chromatographic quantification of lipid classes. Palmitic acid (C16:0): was used for FFA/MAG (R^2=0.978), dipalmitin (C16:0): was used for DAG (R^2=0.972), and glyceryl tripalmitate (C16:0): was used for TAG (R^2=0.985).

Table SI1 – HPLC-ELSD method development: Limit of detection (LOD) and limit of quantification (LOQ) for each lipid class by HPLC-ELSD. TAG: glyceryl tripalmitate (16:0), DAG: dipalmitin (16:0) and FFA: palmitic acid (C16:0).

Compound	LOD [mg/L]	LOQ [mg/L]
FFA	40.0 mg/L (0.60 µg on column)	120
DAG	15.0 mg/L (0.23 µg on column)	45.0
TAG	3.00 mg/L (0.045 µg on column)	9.00

SI4B HPLC-ELSD quantification of *in vitro* digestion

The digestibility of the NAN1 and SPCemul by rHGL and pancreatin was assessed by indirect quantification by titration and by direct quantification by HPLC-ELSD, intended as control. Unless otherwise stated, all results presented in this section are based on the HPLC-ELSD analyses and are presented as mean ± SD.

At the end of the gastric digestion (t=50 min), digestion with 3.75 rHGL resulted in a release of 1630 ± 241 mg/L FFA/MAG for NAN1 and 361 ± 110 mg/L FFA/MAG for SPCemul, (average of G and GI) (**Figure 4A** and **D**). At the end of the gastro-intestinal digestion (t=140 min), digestion of NAN1 with no rHGL and 3.75 TBU/mL rHGL followed by 26.5 TBU/mL pancreatin resulted in 14,666 ± 1913 mg/L FFAs/MAGs (I) and 14,972 ± 1118 mg/L FFAs/MAGs for (GI) (**Figure 4A**). Digestion of SPCemul resulted in 5543 ± 498 mg/L FFAs/MAGs (I) and 9527 ± 2514 mg/L FFAs/MAGs (GI) (**Figure 4B**).

As was observed with titration, this difference between the mean values for I and GI at t=140 min were larger for SPCemul than for NAN1. However, due to variation in sampling homogeneity for SPCemul, the difference was not statistically significant (p=0.0993). The general observed trend; however, still supports the titration data **(Figure 4B)**.

The ratio of the gastric contribution to the gastro-intestinal digestion (FFAs released at t=50 min relative to t=140 min) for NAN1 (GI) was the same when quantified using titration (11 ± 0.4%) and HPLC-ELSD (11 ± 2%). For SPCemul (GI) the ratio was higher when the digestion was quantified using titration (10.5 ± 0.7%) compared with HPLC-ELSD (4 ± 0.8%), as only low amounts of FFAs/MAGs were detected by HPLC-ELSD at 50 min, see **Figure 4A** and **B**. The underlying cause was not believed to be due to an analytical error, but could be caused by creaming in SPCemul that would hamper strict homogeneous sampling of SPCemul compared with NAN1, which appeared to be a stable, as no creaming was observed.

To conclude, direct quantification of *in vitro* gastro-intestinal digestion of NAN1 and SPCemul was found to generally support the observations from the indirect quantification by titration.

References

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