Food & Function

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2016, **7**, 1932

Investigations of *in vitro* bioaccessibility from interesterified stearic and oleic acid-rich blends

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Received 20th October 2015,
Accepted 22nd February 2016

DOI: 10.1039/c5fo01272d

www.rsc.org/foodfunction

Interesterification was previously found to impact stearic acid absorption in a randomized cross-over study, when human volunteers consumed a 70 : 30 wt% high-oleic sunflower and canola stearin blend (NIE) compared to the same blend which had undergone either chemical (CIE) or enzymatic (EIE) interesterification. In this research, *in vitro* lipid digestion, bioaccessibility, and changes in undigested lipid composition and melting behavior of these same test fats were investigated using the dynamic, multi-compartmental TIM-1 digestion model and compared with the previous human study. Overall, TIM-1 bioaccessibility was higher with interesterification ($p < 0.05$). Oleic acid bioaccessibility was higher than stearic acid bioaccessibility for NIE, and *vice versa* for the interesterified blends ($p < 0.05$). Stearic acid was more concentrated in the undigested triacylglycerols (TAG) from NIE, corresponding to a relatively higher melting temperature of the undigested lipids. The results confirm the impact of TAG composition, fatty acid position and/or physical properties on lipid digestion. TIM-1 bioaccessibility was linearly correlated ($R^2 = 0.8640$) with postprandial serum TAG concentration in the human study. Therefore, the *in vitro* digestion model offered predictive insights related to the impacts of lipid interesterificaton on absorption.

Introduction

Interesterification re-arranges fatty acids (FA) within and between triacylglycerol (TAG) molecules using either a chemical or enzymatic catalyst.[1] As a result, it can be used to achieve lipids with desired melting ranges and physical properties for various food applications.[2] Interesterified (IE) lipids are of particular interest because of the ability to use saturated FA-based hardstocks to achieve solid consistency and plasticity without the need for *trans* FA from partially hydrogenated sources. Interesterification can also be used to produce lipids with desired nutritional functionalities, such as for infant formulas or enteral and parenteral products.[3] According to human infant and animal studies, changes in FA distribution, especially long chain saturated FA, can impact lipid absorption based on the selectivity of pancreatic TAG lipase for positions *sn*-1 and 3.[4,5] This activity leaves intact 2-monoacylglycerols,[6] with the constituent long chain saturated FA having a higher tendency for absorption.[4,7] For example, in infants and animal studies, stearic and palmitic acids were found to be better absorbed when located at the *sn*-2 position.[4] In contrast, adult human studies have indicated efficient saturated FA absorption, regardless of FA positional distribution.[5,8] Positional distribution has also been related to delayed chylomicron clearance of saturated FA, a scenario that may lead to postprandial lipemia and increased cardiovascular disease risk.[4] Therefore, further knowledge about the metabolic fate of structured lipids, based on saturated FA, is required.

We previously investigated differences in serum TAG and FA response in an acute human study,[9] where participants consumed stearic-rich IE fats either as a blend (NIE) or after the blend was chemically (CIE) or enzymatically (EIE) interesterified. There was no significant effect of interesterification on postprandial serum TAG response in the non-obese individuals (BMI <30 and/or waist circumference <102 cm, $n = 10$), although differences in postprandial serum FA composition indicated increases in oleic and stearic acid concentrations with CIE and EIE *versus* NIE. These differences in FA composition were also observed in the obese participant group (BMI >30 and/or waist circumference >102 cm, $n = 11$), along with significantly higher values of TAG AUC for CIE *versus* NIE. Only serum oleic acid increased following consumption of the NIE blend by both groups. It was speculated that the differences in physical properties, resulting from interesterification,

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contributed to the differences in postprandial FA composition between NIE and IE fats. Indeed, the importance of physical properties on lipid digestibility and absorption has long been suggested,[10] but remains equivocal, despite recent interest.[7,11–13] To advance knowledge about the health effects of dietary lipids, a better understanding of the interplay between FA positional distribution, TAG physical properties, digestibility, and absorption is necessary.

In vitro digestion studies have become popular for investigating dynamics occurring in the gastrointestinal tract and for investigating the potential health implications of food composition and structure.[11,14,15] Although not without limitations, the approach avoids some of the ethical, safety, and logistical issues associated with collecting samples from human participants.[16–19] Among the most advanced *in vitro* systems is the dynamic, multi compartmental, TIM-1 (TNO, Zeist, The Netherlands). The TIM-1 consists of stomach, duodenal, jejunal, and ileal compartments, all maintained at 37 °C, and can be coupled with the TIM-2 unit which simulates dynamics in the large intestine.[20] It is computer controlled to maintain predetermined volumes and concentrations of digestive fluids and pH in each of the glass compartments which consist of inner flexible silicone jackets which are contracted and relaxed using external pressure changes in order to mimic peristaltic movements.[21,22] To simulate intestinal absorption, the jejunal and ileal digestates are passed over semi-permeable 50 nm pore size membranes (Spectrum Milikros modules M80S-300-01P) through which the so-called bioaccessible molecules pass.[21,22] The ileal efflux therefore contains unabsorbed meal components which, *in vivo*, would be passed to the colon.[21] The ability to collect samples for analysis by a variety of techniques is an advantage of utilizing *in vitro* models like the TIM-1 to gain a mechanistic understanding of digestive events. The TIM-1 system was developed based on *in vivo* data[23,24] and good agreement has been reported with the results of human studies.[23,25]

The purpose of this study was to investigate the *in vitro* lipid digestibility and FA bioaccessibility of interesterified (CIE and EIE) and NIE stearic and oleic acid-rich lipids using the TIM-1 *in vitro* digestion model and to compare the results with the observed differences in postprandial blood lipids from our previous human study.[9]

Methodology

Materials

Pancrex V powder (lipase activity = 25 000 units per g, protease activity = 1400 units per g, and amylase activity = 30 000 units per g) and *Rhizopus* lipase (150 000 units per mg F-AP-15) were obtained from Paines & Byrne Ltd (Surrey, UK) and Amano Enzyme Inc. (Nagoya, Japan), respectively. Fresh pig bile (Farm to Fork, Warren, NJ, USA) was collected, pooled and aliquoted into single portions and stored at −20 °C until use.[15] Trypsin from bovine pancreas (7500 *N*-α-benzoyl-L-arginine ethyl ester (BAEE) units per mg, T9201), pepsin from porcine gastric mucosa (2500 units per mg protein, P7012), α-amylase type II-A from *Bacillus* species (≥1500 units per mg protein A-6380) and all other chemicals utilized with the TIM-1 were purchased from Sigma Aldrich (St. Louis, MO, USA). High oleic sunflower oil (76% oleic acid) was purchased from Vegetol 80RBD, Acatris, Minneapolis, MN, USA, and fully hydrogenated canola stearin (88% stearic acid) was purchased from CanAmera, Oakville, ON, Canada. Sodium methoxide and *Candida antarctica* lipase (Novozym 435, immobilized on polyacrylic resin) were purchased from Sigma, St Louis, MO, USA and, Novozymes Biopharma US Inc., NC, USA, respectively. The non-esterified FA analysis kits (NEFA-HR2) were purchased from Wako Pure Chemical Industries (Wako Diagnostics, VA, USA).

The test fats

NIE, CIE, and EIE were prepared as previously described.[9] Briefly, NIE was a 70 : 30 wt% blend of high oleic sunflower oil and fully hydrogenated canola stearin. This NIE blend was then either chemically interesterified (CIE) using sodium methoxide (0.3 wt%) or enzymatically interesterified (EIE) using *Candida antarctica* lipase at 5 wt%.

LC/MS regiospecific analysis of the test fats

The regiospecificity of CIE and EIE TAG were analyzed by liquid chromatography/mass spectrometry (LC/MS). Analyses were done using a Shimadzu LC/MS system consisting of a Nexera UHPLC coupled to an 8030 triple quadrupole mass spectrometer. The mobile phase was pumped at a gradient of 1 mL min^{-1} through three Agilent ChromSpher 5 lipids columns (5 μm, 4.6 × 250 mm) connected in series and maintained at 30 °C. The mobile phase, where solvent A was heptane–2-propanol–acetonitrile (99.8 : 0.1 : 0.1) and solvent B was heptane–2-propanol–acetonitrile (99 : 1 : 1), was held for 15 min at 5% B, then ramped to 20% B over 55 min, followed by a change to 72% B over 50 min. The mobile phase was returned to the original composition at 135 min, and held there for 60 min before the next injection. The column eluate was directed unsplit into the mass spectrometer's APCI source. Positive ion mass spectra were recorded as Q3 scans from *m*/*z* 450 to 1050 at a scan rate of 1363 μ s^{-1}. Argon was used as the collision gas at 230 kPa. The APCI source temperature, heat block, and desolvation line were maintained at 325, 200, and 250 °C, respectively, and nitrogen was used as the nebulizing and drying gas, at flow rates of 3 and 5 L min^{-1}, respectively. Samples were 5 mg mL^{-1} in mobile phase solvent A and the injection volume was 0.4 μL.

Meal preparation

Meals consisting of NIE, CIE, and EIE for the TIM-1 *in vitro* digestions were prepared to resemble those consumed by the participants in our human study.[9] The non-obese participants had an average body weight of 86.2 ± 2.4 kg (BMI <30 kg m^{-2} and/or waist circumference <102 cm) and consumed three slices of toasted white bread (120 g, Wonder™, 285 calories, 3 g fat (0.6 g saturated), 1.5 g fibre, and 10.5 g protein), 500 g of water and 1 g test fat per kg body mass. Meal proportions

for the TIM-1 experiments were calculated accordingly, such that the 100 g meal consisted of 17.0, 12.2, and 70.8 g of toast, fat and water, respectively, and the TIM-1 parameters were programmed to be representative of the gastrointestinal environment of a healthy adult under fed state conditions. As per Robinson *et al.*[9] the day before a TIM-1 digestion, each fat was melted to erase the crystal memory and stored overnight at 4 °C. White bread (Wonder™, Weston Bakeries, Toronto, ON; ingredients: enriched white wheat flour, water, sugar/glucose–fructose, yeast, vegetable oil (soya bean and/or canola), salt, defatted soya flour, calcium propionate, stearoyl-2-lactylate, monoacylglycerols) was toasted, cooled, and the test fat spread as per the above ratio. Toast with fat was then combined with room temperature water in a Magic Bullet™ blender (Homeland Housewares®, Los Angeles, CA, USA) and pulsed 20 times to mimic the mastication process and enable loading into the TIM-1.

TIM-1 *in vitro* digestions

Additional information about the TIM-1 methodology used, including a schematic of the system, can be found in Speranza *et al.*[15] A small intestinal electrolyte solution (SIES: 85.6 mM NaCl, 8.01 mM KCl, 2.25 mM $CaCl_2$) and a 7% pancreatin solution (from Pancrex V powder) were prepared. Prior to introducing the meal into the TIM-1, duodenal start residue (15 g SIES, 30 g fresh porcine bile, 2 mg trypsin solution, 15 g of 7% pancreatin solution), jejunal start residue (40 g SIES, 80 g fresh porcine bile, 40 g of 7% pancreatin solution), and ileal start residue (160 g SIES) were injected into their respective compartments and the system was heated to 37 °C. The initial amount of gastric juice was simulated by loading the gastric compartment with 5 g of gastric enzyme solution (600 U mL^{-1} pepsin and 40 U mL^{-1} *Rhizopus* lipase in gastric electrolyte solution: 4.8 g L^{-1} NaCl, 2.2 g L^{-1} KCl, 0.22 g L^{-1} $CaCl_2$).

100 g of the meal mixture, prepared as above, was weighed and combined with 95 g of gastric electrolyte solution (without the gastric enzymes). After adding 11 mg of amylase and 50 g of water to this meal mixture, the pH was adjusted to 5.5 (by drop-wise addition of 0.1 M HCl). After one minute, this pH-adjusted meal mixture was then immediately introduced to the TIM-1 gastric compartment, along with 50 g of water-rinse. The experiment was carried out using conditions representative of the fed state and the TIM-1 was operated as previously described.[15] A preprogrammed computer protocol controlled the secretion of 1 M HCl to the gastric compartment and regulated gastric emptying, intestinal transit times, and pH. Fluid secretions had the following flow rates; duodenal secretions: fresh porcine bile at 0.5 mL min^{-1}, 7% pancreatin solution at 0.25 mL min^{-1}, and SIES at 3.2 mL min^{-1}; jejunal secretion: SIES and 10% fresh procine bile at 3.2 mL min^{-1}; ileal secretion: SIES at 3.0 mL min^{-1}. The duodenal, jejunal, and ileal compartments were maintained at pH 6.5, 6.8, and 7.2, respectively, by controlled secretion of a 1 M sodium bicarbonate solution.

Sample collection

The jejunal and ileal dialysates (containing the bioaccessible components) and ileal efflux (reflecting the undigested and/or unabsorbed contents passing to colon) were collected at 30, 60, 90, 120, 180, 240, 300, and 360 min from the time of introducing the meal to the TIM-1. The collected dialysates were cooled on ice, weighed and stored at −20 °C for subsequent analysis. Collected ileal efflux samples for each time point were pooled and chilled on ice. Each test fat was run in duplicate. The dialysates were analyzed for total bioaccessible FFA and the bioaccessibility of each major FA, as described below. The ileal efflux was analyzed for FA composition of the separated TAG and FFA fractions and melting behaviour of the extracted lipids was investigated.

Total FFA measurements

The total FFA concentration was quantified for each bioaccessible (*i.e.* dialyzed) lipid fraction at each time point. Samples were extracted into acidic hexane and FFA quantified using a commercial NEFA enzymatic kit with spectrophotometric analysis at 550 nm (Spectramax plus, Molecular Devices Corporation, CA, USA).[26]

FA composition of bioaccessible lipids

The FA composition of the bioaccessible TIM-1 jejunal and ileal lipids (*i.e.* combined lipolysis and absorption) were determined by GC, as previously described, using C17 : 0 as an internal standard.[27] Briefly, total lipids were extracted by the Folch method[28] with transesterification using 14% boron trifluoride in methanol (Sigma Aldrich, St. Louis, MO, USA). Methyl esters were re-suspended in hexane and analyzed using an Agilent 7890A GC equipped with flame ionization detection (Agilent Technologies Inc., DE, USA). For comparison with the Robinson *et al.* human study,[9] relative concentrations of palmitic, stearic, oleic, and linoleic acids are reported.

FA composition of ileal efflux lipids

Lipids were extracted from thawed (at 4 °C) ileal efflux samples.[29] Extracted lipids were saponified and resuspended in 100 μL hexane and spotted on thin layer chromatography (TLC) plates[27] using a solvent mixture of 80 : 20 : 1 petroleum ether : ethyl ether : glacial acetic acid. After visualization under UV light and in comparison with standards, bands for TAG and FFA were identified, scraped, methylated and the lipids were analyzed after re-suspending in hexane. There were no mono- or diacylglycerol bands visible in the efflux samples (data not shown), suggesting these species were digested and/or bioaccessible. The background biliary contribution was assumed similar across all treatments by maintaining consistent conditions. Relative concentrations of palmitic, stearic, oleic, and linoleic acids were determined, as above.

Melting behavior by differential scanning calorimetry (DSC)

Lipids were extracted[29] from the ileal efflux samples and 5–10 mg were weighed and sealed in alodined aluminum DSC pans. Samples of the extracted lipids and NIE, EIE, and CIE

were melted at 80 °C for 30 min (in the sealed pans) and crystallized overnight at 4 °C. Melting and re-crystallization behaviors were analyzed using a Q2000 model (TA Instruments, Mississauga, ON, Canada) and peak temperatures determined using the system software (TA Instruments Universal Analysis 2000 software, TA Instruments, ON, Canada). The experimental protocol consisted of an initial temperature of 5 °C followed by heating at 5 °C min^{-1} to 85 °C, isothermal conditions for 3 min and cooling at 5 °C min^{-1} to 5 °C.

Statistical analysis

Two-way analysis of variance (ANOVA) was used to compare lipolysis and FA composition between samples and Tukey's test was performed for multiple comparisons testing using GraphPad Prism (v 6.0e, GraphPad Software, San Diego, CA). Normality of the data was confirmed before each analysis using D'Agostino and Pearson Omnibus normality testing. Pearson correlation analysis was used to compare the TIM-1 *in vitro* bioaccessiblity (%) with serum TAG concentration (mmol L^{-1}) obtained from the non-obese group in the human study.[9] Data is reported as mean ± SEM and $p < 0.05$ was considered statistically significant.

Results and discussion

TIM-1 digestion: lipid bioaccessibility

The FA and TAG compositions of NIE, CIE, and EIE are shown in Table 1. Importantly, they are consistent with the composition reported in the human study[9] and show that the FA composition of the three test fats was similar, although they varied considerably in terms of TAG species.

Table 1 Fatty acid and triacylglycerol compositions of NIE, CIE, and EIE

	NIE	CIE	EIE
Fatty acid species (wt%)			
16:0	5.1 ± 0.1	5.0 ± 0.0	5.1 ± 0.0
18:0	27.9 ± 0.0	30.0 ± 0.0	29.1 ± 0.0
18:1n-9	58.9 ± 0.1	56.4 ± 0.1	57.3 ± 0.1
18:2n-6	4.6 ± 0.0	4.7 ± 0.0	4.7 ± 0.0
Total	96.5 ± 0.0	96.2 ± 0.2	96.2 ± 0.0
	NIE	CIE	EIE
Triacylglycerol species[a] (wt%)			
OOS	4.2 ± 0.1	34.8 ± 2.2[b]	34.1 ± 0.7[b]
OOO	36.7 ± 0.9	25.3 ± 3.3	26.4 ± 0.3
SOS	nd	14.0 ± 0.8[b]	11.2 ± 0.5[b]
OOL	21.4 ± 0.4	6.8 ± 3.2	7.5 ± 0.2
PLO	2.3 ± 0.0	3.9 ± 0.3	4.1 ± 0.3
OPO	3.7 ± 0.1	2.3 ± 0.0	3.1 ± 0.7
PPP	2.4 ± 0.2	2.7 ± 0.2	2.4 ± 0.3
POS	nd	2.8 ± 0.3	1.7 ± 0.5
PPS	nd	1.3 ± 0.1	1.1 ± 0.4
SSP	2.2 ± 0.1	0.5 ± 0.4	0.2 ± 0.1
SSS	23.8 ± 0.4	0.8 ± 0.3	0.6 ± 0.2
Total	96.4 ± 3.8	95.0 ± 3.4	92.2 ± 3.4

[a] P: palmitic acid, S: stearic acid, O: oleic acid, L: linoleic acid. nd: not detected. [b] The following TAG species were resolved by LC/MS analysis. For CIE, OOS/OSO = 29.0/10.9%; SOS/SSO = 4.3/11.1% (wt%). For EIE, OOS/OSO = 29.4/9.9% and SOS/SSO = 4.3/10.5% (wt%).

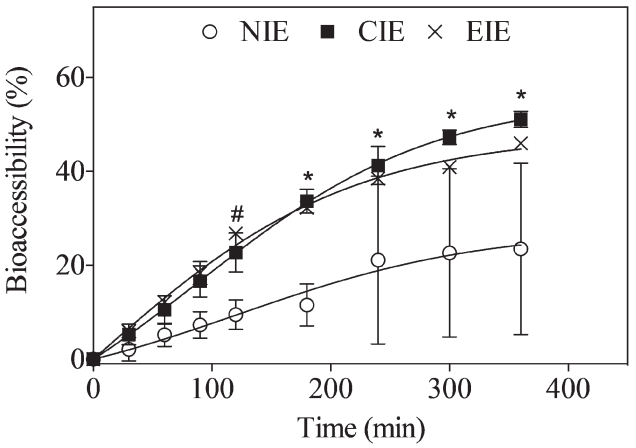

Fig. 1 Bioaccessibility (%) of NIE, CIE, and EIE during 6 h *in vitro* TIM-1 digestion ($n = 2$). Data reported as mean ± SEM. # Significant treatment differences ($p < 0.05$) observed between NIE and EIE. * Significant treatment differences ($p < 0.05$) observed between NIE and both IE blends. Overall ANOVA $p = 0.013$ for time × test fat interaction.

Fig. 1 shows the TIM-1 lipid bioaccessibility obtained for NIE, CIE, and EIE based on the determination of FA concentrations of the jejunal and ileal dialysate samples throughout the 6 h digestion protocol. There was a trend towards higher bioaccessibility for the IE *versus* NIE fats. Statistically significant differences were observed between NIE and EIE (but not CIE) starting at 120 min. Thereafter, NIE had significantly lower lipid bioaccessiblity than the IE fats ($p < 0.05$). Ultimately, the bioaccessibility achieved at 6 h was also higher for the IE blends ($p < 0.05$) compared with the NIE sample, but the two esterified blends did not differ ($p > 0.05$). Lipids rich in SSS are notoriously waxy and difficult to work with. The NIE sample was visibly less evenly distributed throughout the TIM-1 compartments than the IE samples. Notably, at 240 minutes and beyond, the variability in the NIE, TIM-1 bioaccessiblity data was also much higher than for the IE samples (Fig. 1). This may correlate with the high melting temperature of SSS (*i.e.* 73.1 °C[30]) present in NIE and the fact that TIM-1 has a gastric emptying half life of 70 min.[23] Therefore, the physical state of NIE visibly impacted TIM-1 digestion dynamics.

Lipid digestion is generally considered to be high (~95%) under healthy physiological conditions.[31] That said, the issue of stearic acid digestibility and absorption is a matter of long and considerable debate.[10] It was previously reported that the impact of stearic acid on plasma lipids, red blood cells and platelet FA composition occurs at a slower rate compared to short chain saturated FA.[32] In contrast, a higher bioaccessibility coefficient was observed for stearic acid compared to oleic acid in a previous TIM-1 study involving conjugated linoleic acid-rich milk and milk emulsions.[25] SSS digestibility, specifically, is often regarded as being low,[12] although estimates differ and the evidence for this is largely based on animal

models. In one rat study, stearic acid digestibility from unblended SSS was reportedly 0.15 g per g stearic acid.[10] In another rat study, lipid absorption of SSS, OOO and safflower oil was 73, 98 and 97%, respectively,[33] providing evidence of lower absorption of the highly saturated FA. The issue of stearic acid digestibility and absorption is additionally complicated by the fact that it can depend on dose as well as method of sample preparation,[10] and be influenced by factors such as high calcium levels in feed which may contribute to soap formation. For example, as measured in lymph output in rats, SSS digestibility was improved by blending with OOO.[5] This may occur through disruption of the crystalline networks[10] and potential formation of eutectics.

Lipid bioaccessibility was related to TAG species present and melting behaviour. It is hypothesized that SSS crystallinity, at body temperature, limits lipase accessibility in part, because bile salt emulsification of solid fats is limited.[10] Fig. 2a shows the presence of a single melting peak for NIE at 62.0 ± 0.0 °C and a range of melting events for the IE fats. In CIE, melting events were observed at 15.0 ± 1.4, 32.0 ± 0.0, and 43.0 ± 1.4 °C. In EIE, melting events were observed at 2.0 ± 2.8 and 26.0 ± 2.8 °C. According to Table 1, although all fats contained approximately 30% stearic acid, the IE blends contained a much more diverse group of TAG species than NIE, with the predominant TAG species (~40%) being OOS and OSO. These TAG have melting temperatures of 24.0 and 24.2 °C [30] *i.e.* below 37 °C. Furthermore, NIE had a solid fat content of 18.6% at 37 °C while the IE samples contained only 5.4–5.6% solid fat at this temperature.[9] Therefore, the presence of ~25% SSS contributed to a much higher solids content for NIE *versus* the IE blends and these differences in physical properties were associated with differences in *in vitro* digestion handling and observed lower lipid bioaccessibility from NIE. It has been speculated that physical properties of saturated FA-rich blends, including some IE samples, may contribute to attenuated postprandial lipemia.[5,34] For example, Berry and Sanders[7] speculated that shea butter, a native fat with high proportion of stearic acid in the *sn*-1/3 positions resulted in a lower lipemic response compared to high oleic sunflower oil partly because of associated differences in physical properties.[7] In our previous human study,[9] no significant differences were observed between values of incremental TAG area under the curve (57.0 ± 44.4, 83.3 ± 83.8, and 116.6 ± 80.9 mmol L^{-1} × 6 h for NIE, CIE, and EIE, respectively; $p > 0.05$) for non-obese individuals.[9] Of note, in the obese study participants, the TAG AUC values were significantly higher for the CIE (242.4 ± 145.2 mmol L^{-1} × 6 h) *versus* NIE (105.8 ± 116.8 mmol L^{-1} × 6 h), but not for NIE *versus* EIE (193.2 ± 130.1 mmol L^{-1} × 6 h) or CIE *versus* EIE.[9] The overall tendency was for lower lipemia after consumption of NIE *versus* the IE fats. It is possible the small sample size and duration of the human study (6 h) may have precluded the ability to detect significant differences among the test fat treatments. Notably, there are reports that plasma TAG concentrations can remain elevated beyond 6 h postprandial, particularly in the case of higher melting fats.[35,36] The difference among the obese and non-obese groups could relate to differences in post-absorptive lipid handling, given that insulin resistance can lead to elevated postprandial TAG related to competition for hepatic receptors between chylomicrons and very low density lipoproteins that impede the uptake of chylomicron remnants.[37] Regardless, the *in vitro* experiments support that the physical properties of the NIE and IE blends impact lipemia, suggesting a role for the physical properties in impacting digestive processing and lipemia. Wang *et al.*[13] recently attempted to clarify the relative contributions of TAG structure and solid fat content on lipid metabolism in mice fed IE lipids rich in palmitic and oleic acids for six weeks. Solid fat content, at the levels examined (*i.e.* 0–21.9% at 37 °C), did not impact lipid metabolism, although TAG positional distribution had an impact on fasting non-esterified FA and glucose concentrations.

Gas chromatographic FA analysis was performed on the bioaccessible lipids obtained from the jejunal and ileal dialysates. These results are presented in Table 2. The analysis was focused on palmitic, stearic, oleic, and linoleic acids, as these were the major FA present in the test fats and were analyzed in the human study.[9] However, the discussion focuses on stearic and oleic acids, given the *in vitro* biliary contributions of

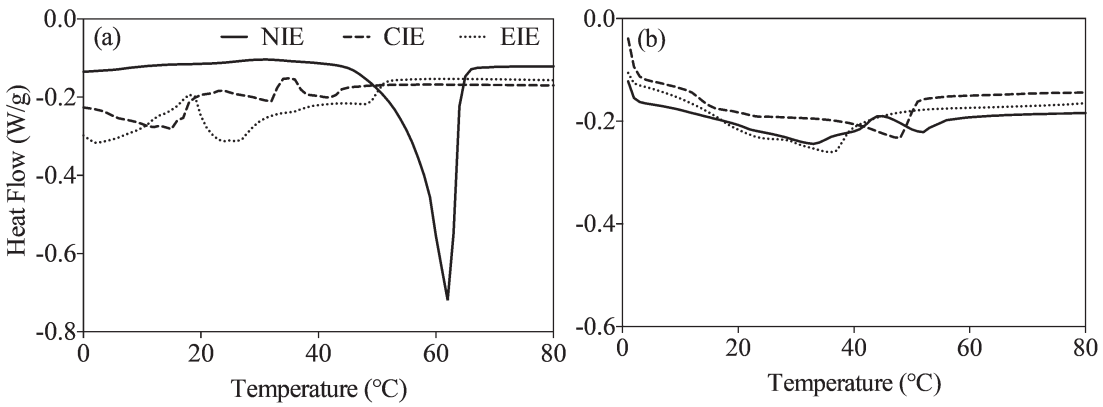

Fig. 2 DSC thermograms of (a) NIE, CIE, and EIE fats and (b) of extracted lipids from the NIE, CIE, and EIE ileal efflux from the TIM-1 digestion simulator (n = 3).

Table 2 Fatty acid (palmitic, stearic, oleic, and linoleic acids) concentration (cumulative, wt%) of bioaccessible lipids following 6 h TIM-1 digestion of NIE, CIE, and EIE[a]

	NIE	CIE	EIE
Palmitic acid (16:0)	29.5 ± 0.2[a]	29.1 ± 0.6[a]	32.9 ± 0.6[b]
Stearic acid (18:0)	18.0 ± 0.4[a]	20.5 ± 0.1[b]	20.0 ± 0.1[b]
Oleic acid (18:1n-9)	22.9 ± 0.7[a]	18.4 ± 0.6[b]	17.0 ± 0.8[b]
Linoleic acid (18:2n-6)	29.6 ± 0.3[a]	32.0 ± 0.1[b]	30.1 ± 0.2[a]
	100.0	100.0	100.0

[a] Mean ± SEM. [a,b]Values within each row with different superscript letters differ significantly ($p < 0.05$).

palmitic and linoleic acids which are the main saturated FA and unsaturated FA, respectively, in bile phospholipids.[38] With CIE and EIE, the bioaccessibility of stearic acid was significantly higher than for oleic acid. In contrast, the bioaccessible lipids from NIE were significantly higher in oleic acid ($p < 0.05$). These trends may be related to differences in the stereospecific positioning of stearic acid between the test fats. Regiospecific TAG analysis of EIE and CIE by LC/MS indicated ratios in the range of 2 : 1 for OOS : OSO and 1 : 2.5 for SOS : SSO (wt%, data not shown). Therefore, combined with the data in Table 1, it is estimated that 25–27% of the IE sample TAG had stearic acid located at *sn*-2. Although there was similarly around 25–29% stearic acid located at *sn*-2 in NIE, approximately 25.5% of this was in the form of SSS and SSP. Therefore, there was limited digestibility and release of stearic acid from these molecules which have relatively high melting temperatures (*i.e.* 73.1 and 65.2, respectively °C [30]). It is commonly accepted that FA located at the *sn*-2 position remain preferentially esterified during digestion, enter the bile salt mixed micelles as part of 2-monoacylglycerols, and are absorbed by the enterocytes.[31] Animal and human infant studies[4,5] have indicated that stearic acid located at *sn*-1/3 can have lower absorption because of the possible formation of insoluble FA soaps with calcium (particularly with a calcium-rich diet[39]) and release of insoluble acylglycerols after they are hydrolyzed by pancreatic TAG lipase.[10] As reviewed by Livesey,[10] in a rat study, stearic acid hydrolysis was higher for OSO and OOS (99.2 and 96.4%, respectively) than from SOS and OSS (70.5 and 60.1%, respectively) due to the release of poorly digestible acylglycerol intermediates. It was concluded that, under physiologically relevant concentrations of calcium and magnesium ions, this factor may contribute more to stearic acid digestibility than the formation of insoluble soaps.[10] The results of the present study support that stearic acid positional distribution in IE lipids favors higher overall lipid bioaccessibility, and specifically stearic acid bioaccessibility, compared to NIE.

In our previous human study,[9] postprandial increases in stearic and oleic acids were observed following consumption of EIE and CIE, but only serum oleic acid concentration increased with NIE. The trends observed for stearic acid bioaccessibility with the TIM-1 were similar, although oleic acid bioaccessibility was higher for NIE than EIE and CIE ($p < 0.05$). Oleic acid in NIE was predominantly in the form of OOO, followed by OOL. In contrast, mixed TAG species of mono-, di-, and tri-olein were present in the IE blends (Table 1). Previously, the rate of TAG removal from TAG-rich lipoproteins in healthy normolipidemic participants was reported to be faster for OOO, followed by OOL and OOS.[40] Therefore, the observed higher bioaccessibility of oleic acid from NIE can be attributed to its positional distribution.

TIM-1 digestion: unabsorbed lipids in the ileal efflux

Samples of the TIM-1 ileal efflux were analyzed to characterize components of the digestate, which are expected to reach the colon (Fig. 3). Lipids in the ileal efflux reflect components that have been digested, but not absorbed, as well as some undigested components. Therefore, in Fig. 3, the TAG constitute undigested lipids, while the FFA reflect those FA, which were hydrolyzed but not bioaccessible. The undigested TAG of the three test fats consisted predominantly of stearic acid followed by palmitic acid. The undigested NIE TAG were also significantly higher in stearic acid than the IE blends (*i.e.* 86.5 ± 0.3, 61.1 ± 2.8, and 44.3 ± 1.7% for NIE, CIE, and EIE, respectively,

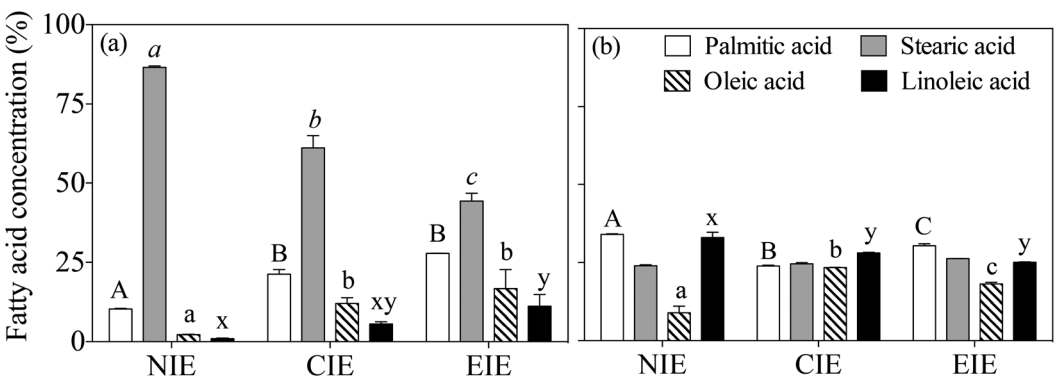

Fig. 3 Palmitic, stearic, oleic, and linoleic acid composition (wt%) of triacylglycerol (a) and free fatty acid (b) fractions of lipids remaining in the 6 h pooled TIM-1 efflux from NIE, CIE and EIE (mean ± SEM, $n = 2$). Different letters indicate significant differences within each FA between test fats in each graph ($p < 0.05$).

$p < 0.05$). Also, the undigested NIE TAG contained significantly less oleic acid compared to the IE blends, further confirming the relatively higher bioaccessibility of oleic acid from NIE. The amount of stearic acid in the NIE FFA fraction was 24 ± 0.4%. Collectively, this indicates that the predominant form of stearic acid excretion was in the form of TAG, suggesting a high level of undigested lipids. When lipid excretion is high (*i.e.* more than 7 g daily based on a 100 g fat diet), the condition known as steatorrhea occurs. Lipid malabsorption can result in high levels of undigested TAG in the intestines.[41] That said, we postulate that fecal excretion of undigested saturate-rich lipid species may go undetected based on their solid consistency. This is consistent with a rat feeding study which reported 6.6 ± 0.3, 16.8 ± 1.5, and 79.1 ± 1.0% fat present in the animals' feces, with no observed signs of gastrointestinal distress when rats were fed feed with liquid TAG, solid TAG or a solid alkane emulsion, respectively.[42]

Most stearic acid present in the CIE and EIE ileal efflux samples was present as TAG *versus* FFA, indicating a propensity for lower lipolysis of the TAG species present. The constituent SOS, SSO, SPO, and PSO in the IE blends have melting temperatures above physiological temperature (*i.e.* 37 °C) and their solid state may limit pancreatic access.[4] In the CIE and EIE efflux, FFA consisted of 24–26% stearic acid, which may not have been absorbed because it is known to form calcium soaps.[3] Due to their higher melting temperature, TAG species such as SOS and SSO may undergo less digestion due to their solid state at 37 °C compared to molecules such as OSO and OOS that will be in the liquid state. Stearic acid positional distribution can also come into play for these lower melting TAG, in that stearic acid at *sn*-2 (as in OSO) can be better absorbed from the *sn*-2 monoacylglycerol, which forms during digestion compared to stearic acid at *sn*-1 or 3 that is released by pancreatic lipase, but then forms a calcium soap. Previous reports also suggest that fats mainly composed of long chain FA are associated with slower rates of gastric emptying in humans, compared to short or medium chain FA[43] enabling slower, and more efficient, fat digestion. A limitation of *in vitro* digestion models is the inability to replicate such digestion dynamics.

Fig. 2b shows that lipids extracted from the ileal efflux following digestion of NIE, EIE or CIE have several melting events. Two peak melting events were observed in NIE at 33 ± 0.0 and 53 ± 1.4 °C. Single, distinct melting peaks were observed for CIE and EIE at 48 ± 1.4 and 36 ± 0.0 °C, respectively. Therefore, lipid fractions from the NIE and CIE efflux were solid at body temperature. In contrast, the EIE lipids from the efflux were in the liquid state at 37 °C. This may relate to the fact that, despite similar TAG profiles, EIE contained more partial glycerides (*i.e.* 6.6 *versus* 2.0% DAG in EIE *versus* CI, respectively, and 0% in NIE).[9] Chemical interesterification is a random process and, since *Candida antarctica* lipase (Novozym 435, Novozymes Biopharma US Inc., NC, USA) is also non-specific, both processes are expected to result in TAG species of similar composition. It would be interesting to investigate the EIE ileal efflux fat further to understand the reasons for the low bioaccessibiliy of TAG with relatively lower melting temperatures. However, the very low fat content (<0.2%) in the efflux makes it challenging to extract sufficient lipids for further analysis. There also is an unavoidable contribution from the biliary lipids that impact the melting properties of the ileal efflux fat, although this applies similarly to all three fats tested.

Correlations between TIM-1 and human study results

When a correlation analysis was performed between the TIM-1 lipid bioaccessibilty data and the healthy non-obese participants' serum postprandial TAG concentration at each corresponding time point, a significant positive correlation (R^2 = 0.8640; $p < 0.05$) was observed (Fig. 4). TIM-1 bioaccessibility was significantly different between IE and NIE starting at 180 min of *in vitro* digestion, and for both IE fats thereafter. Similarly, *in vivo* serum TAG concentrations trended to being higher for the IE fats than for NIE during the 6 h period ($p > 0.05$). These observations suggest that IE fats may be absorbed more rapidly and warrant further investigation. There was no correlation found between serum TAG AUC values, *i.e.* an indicator of cumulative absorption, in the healthy participants and the TIM-1 total lipid bioaccessibility at 360 min (R^2 = 0.7588; p = 0.3269). This relates to the fact that differences were observed between the fats in the TIM-1 study, but not in the human study in terms of serum TAG AUC values.[9] Of note, there was a trend towards higher blood lipids for CIE and EIE *versus* NIE in the healthy study participants. These differences are consistent with TIM-1 results showing lower digestibility and bioaccessibility of the NIE *versus* IE lipids. The efficiency of human lipid metabolism, significant intra-individual variability and/or low participant numbers might explain this apparent discrepancy. Support for this can also be found in the data from the obese human study participants. Because the TIM-1 conditions utilized reflect those of healthy individuals,[22] comparisons with that group of partici-

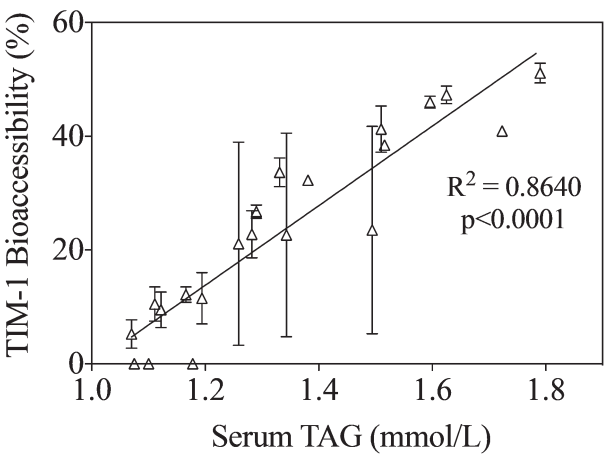

Fig. 4 Correlation analyses between TIM-1 6 h bioaccessibility (%, error bars indicate the SEM, n = 2) and human study 6 h postprandial serum TAG concentration of non-obese subjects (mmol L^{-1}, n = 10) fed the same test fats.

pants were prioritized. However, it is interesting that, in the obese participants, serum TAG concentrations over time and TAG AUC were higher for CIE *versus* NIE.[9] The TIM-1 results would predict this lower impact on postprandial lipemia following consumption of NIE *versus* the IE blends. However, in contrast to data from healthy participants, a significant correlation was not observed between the serum TAG concentrations and TIM-1 lipid bioaccessibility over time for the obese participants (R^2 = 0.1723; p = 0.0613). Also, as with the healthy participants, serum TAG AUC following consumption of the lipids was not correlated with TIM-1 total lipid bioaccessibility at 360 min (R^2 = 0.3591; p = 0.5909). Lastly, both the human and TIM-1 results reflect differences in terms of FA absorption between the IE and NIE fats, *i.e.* stearic acid absorption and bioaccessibility were higher with consumption of the CIE and EIE compared with NIE.

Conclusions

The objective of this work was to investigate the impact of TAG structure and physical properties of NIE and CIE and EIE blends with similar FA composition on *in vitro* digestive processing and in comparison with previously observed postprandial lipemic effects. The results confirm previous evidence that TAG molecular structure and melting point impact lipid digestion. Moreover, they help to validate the use of the TIM-1 in modeling and predicting human digestion, as the TIM-1 bioaccessibility data showed good linear correlation with human serum TAG concentration. The lower concentrations of SSS in the IE *versus* NIE blends and the corresponding lower melting temperatures and solid fat contents at 37 °C were associated with higher *in vitro* TIM-1 bioaccessibility and lower variability. Therefore, physical properties and composition of the fat blends impacted digestion. The presence of higher proportions of undigested stearic acid in the ileal efflux of NIE indicates the lower SSS digestibility and the limited TAG digestibility of stearic acid-containing molecules was shown by the presence of 44–61% (relative percentage) stearic acid in the IE undigested TAG. Results from the TIM-1 concerning the effects of interesterification mirrored the trend towards higher lipid absorption from the IE blends *versus* NIE. The TIM-1 experiments indicated potential differences in lipid absorption between the IE and NIE, supporting that sophisticated *in vitro* models can aid in the study of mechanisms impacting the digestion of structured lipids.

Acknowledgements

The authors would like to acknowledge Dr John Carney, Solazyme Inc. for the LC/MS TAG analysis and Lois Xinjie Lin for assistance with the TIM-1. We also acknowledge the financial support of Canada's Natural Sciences and Engineering Research Council.

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