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Bioaccessibility of pomegranate seed oil using INFOGEST *in vitro* digestion model☆

Sarah Vellemans [a,*], Antoine Ide [a], Marion Duchatelet [a], Rachel Dubois [a], Antoine Demol [a], Eric Mignolet [a], Yvan Larondelle [a], Nathalie Delzenne [b], Cathy Debier [a]

[a] Louvain Institute of Biomolecular Science and Technology, UCLouvain, Croix du Sud 4-5, 1348 Louvain-la-Neuve, Belgium
[b] Metabolism and Nutrition Research Group, Louvain Drug Research Institute, UCLouvain, Av. E. Mounier 73, 1200 Brussels, Belgium

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ABSTRACT

Pomegranate seed oil has been widely studied for its health-promoting properties. However, the bioavailability of its conjugated linolenic acids, an essential factor for assessing their impact on health, remains poorly understood. This study used the harmonized *in vitro* protocol INFOGEST to investigate the bioaccessibility of pomegranate seed oil in comparison to olive oil. Bioaccessibility, key aspect of bioavailability, refers to the proportion of a nutrient released in the gastrointestinal tract and available for intestinal absorption. It was determined by measuring the ratio of fatty acids present in the absorbable fraction (monoglycerides and free fatty acids) to total fatty acids quantified by gas chromatography following solid-phase extraction. Results showed that the overall bioaccessibility of pomegranate seed oil was equivalent to that of olive oil. Notably, punicic acid (in pomegranate seed oil) and oleic acid (in olive oil) – both 18-carbon fatty acids present in equivalent quantities in the two oils, showed high and equivalent bioaccessibility. The presence of proteins (whey and soy protein isolates) did not affect the bioaccessibility of the major fatty acids under experimental conditions. The recovery index was high for all fatty acids, indicating minor degradation during *in vitro* digestion, and was not affected by the presence of oxygen. In conclusion, punicic acid is efficiently released by digestive lipases, suggesting good bioaccessibility of pomegranate seed oil. This finding paves the way for its potential application in oral therapeutic use.

1. Introduction

Numerous studies have demonstrated the positive impact of certain lipids on health, and some of them have been used for their therapeutic properties for centuries, demonstrating their potential to improve health and manage diseases (Fontes et al., 2017; Soppert et al., 2020). Among lipids, polyunsaturated fatty acids (PUFAs) are known for their bioactive properties. Some PUFAs have double bonds not separated by methylene group, known as conjugated fatty acids. Fatty acids with 18 carbons and two conjugated double bonds are part of conjugated linoleic acids (CLAs) while those that have three double bonds, of which at least two are conjugated are part of conjugated linolenic acids (CLnAs) (Dhar Dubey et al., 2019). Among the conjugated fatty acids, CLAs have been the most widely studied for their health-promoting effects. Over the last two decades, however, interest towards CLnAs has risen, partly because of their availability, as they are present in high amounts in some seed oils, such as punicic acid (PunA, C18:3 c9t11c13) present up to 70 % in pomegranate seed oil (PSO) or α-eleostearic acid (C18:3 c9t11t13) present up to 70 % in tung oil (*Vernicia fordii*), 50 % in bitter gourd oil (*Momordica charantia),* and 50 % in *Ricinodendron heudelotii* kernel oil (Fontes et al., 2017; Nikiema et al., 2019). Traditionally used for thousands of years as medicinal fruit, the pomegranate (*Punica granatum*) is grown in Mediterranean regions, Middle East and Asia with Iran, India, China and Pakistan being the main producers (Ambigaipalan et al., 2016; Yisimayili & Chao, 2022). In 2017, the world annual production was estimated at 5.9 million tons (Paul & Radhakrishnan, 2020). Pomegranate seeds, a by-product of the juice industry, hold significant value as a resource in the cosmetic, pharmaceutical and nutrition sectors (Teixeira da Silva et al., 2013; J. Wang et al., 2024). Moreover, among commercially available oils rich in CLnAs, PSO contains the highest level of CLnAs without any presumed toxic effects unlike tung oil (Edwards, 1964).

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* Corresponding author.
E-mail address: sarah.vellemans@uclouvain.be (S. Vellemans).

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In vitro and *in vivo* studies reported that CLnAs have health-promoting effects such as anti-cancer, anti-inflammatory, anti-obesity and anti-atherogenic properties (Dhar Dubey et al., 2019). Notably, PunA showed a strong anti-cancer property *in vitro* by inducing ferroptosis, a cell death due to the accumulation of lipid peroxides in cell membranes (Vermonden et al., 2021). Indeed, it has recently been shown that CLnAs are peroxidized by a different mechanism than conventional PUFAs, promoting the formation of elevated amounts of reactive aldehydes. Their high sensitivity to oxidation contributes to the induction of ferroptosis to a greater extent than non-conjugated PUFAs (Do et al., 2023). A particularly interesting aspect of CLnAs is their promising bioactivity at significantly lower doses compared to CLAs, which facilitates reaching therapeutic levels (Du et al., 2024).

To fully harness the health benefits of CLnAs, their digestion in the gastrointestinal tract must be thoroughly studied to assess the feasibility of oral administration. Despite their efficacy at lower doses than CLAs and their abundance in oils, inefficient digestion could limit their potential. Currently, no reliable studies have evaluated PSO digestion using a validated *in vitro* protocol, highlighting the need for further research. Investigating their rate of intraluminal digestion will help determining whether oral administration is an effective delivery method. A key factor in this process is bioaccessibility - the proportion a component released from the food matrix, accessible to enzymes and available for absorption. Bioaccessibility is part of a broader concept, bioavailability, which includes additional steps such as absorption and transport to target tissues (Grundy et al., 2024; Rodrigues et al., 2022). Dietary lipids are mainly in the form of triglycerides (TG) requiring a complex digestion process including the successive combination of the emulsion formation, luminal hydrolysis by gastric and intestinal lipases, micelle formation, absorption across the epithelium and re-assembly into TG in the enterocytes. As digestive lipases are stereospecific for the outer chains (sn-1 and sn-3) of TG, their action leads to the release of one 2-monoglyceride (MG) and two free fatty acids (FFA), the two forms absorbable by enterocytes (McClements et al., 2008). The gold standard for studying food digestion is the use of *in vivo* studies in animals or humans, but these experiments are expensive, technically difficult and sometimes ethically questionable (Dupont et al., 2019; Pascoviche & Lesmes, 2021). To overcome this issue, *in vitro* methods have been developed to simulate the physiological digestion processes. To address the difficulty of comparing the results of different protocols existing in the literature, the COST INFOGEST network established a standardized *in vitro* digestion protocol (Brodkorb et al., 2019), recognized for its simplicity, accuracy and reproducibility. While static models cannot fully replicate human digestion, they remain valuable tools for assessing compound bioaccessibility in food matrices, demonstrating strong correlation with *in vivo* data (Bohn et al., 2018). In addition, INFOGEST protocol allows controlled investigations of the intraluminal phase of digestion, enabling a wide range of conditions to be tested. This study investigates whether CLnA structure influences hydrolysis by digestive enzymes, which could impact their potential for nutritional therapy. For this purpose, we used the standardized INFOGEST *in vitro* digestion protocol and the different lipid classes were separated through solid-phase extraction (SPE) after Bligh and Dyer extraction and injected onto gas chromatography equipped with flame ionization detector (GC-FID). The bioaccessibility was evaluated as the proportion of FFA and MG, compared to the total of lipids quantified. PSO was compared to olive oil (OO), selected as a reference due to its well-characterized digestion and high content of oleic acid (OA, C18:1 c9), an 18-carbon monounsaturated fatty acid present in similiar proportions to PunA in PSO. Given that lipids are rarely found isolated in the diet, but are generally present in the form of complex matrices whose components interact with each other, the influence of the presence of proteins on the bioaccessibility of oils was assessed by adding whey or soy protein isolates (WPI, SPI) to the system. Proteins, due to their high surface-active characteristics, can either enhance lipid digestion by stabilizing emulsions or hinder it by restricting enzyme access to lipid droplets (Iddir et al., 2020).

2. Materials and methods

2.1. Materials

PSO was purchased from Ol'Vita (Macinowice, Poland) and OO from Carl Roth (Karlsruhe, Germany). For *in vitro* digestion, rabbit gastric extract (RGE-15) was purchased from Lipolytech (Marseille, France), bovine bile, porcine pancreatin, and electrolytes (KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, $CaCl_2(H_2O)_2$), were purchased from Sigma-Aldrich (St. Louis, MO, USA). WPI and SPI were purchased from Purasana (Gullegem, Belgium). For lipid analysis, standards were purchased from Larodan (Solna, Sweden) and solvents from VWR Chemicals (Radnor, PA, USA) unless otherwise specified and were of GC-grade purity. For phytosterol analysis, plant sterol mixture was purchased from Cayman Chemicals (Ann Arbor, MI, USA) while betulin and β-sitosterol from Sigma-Aldrich. Ultra-pure water was used (Milli-Q, Merck Millipore, Burlington, MA, USA).

2.2. Sample preparation

Each sample that underwent the INFOGEST protocol contained 50 mg of oil mixed with Milli-Q water to reach a total of 1 g, with a water blank run in parallel. To investigate the impact of proteins on oil bioaccessibility, three oil-to-protein ratios (2:1, 1:1, 1:1.5) were tested, with protein levels below, equal to or above those of the oil, since previous studies showed that the impact of proteins was concentration-dependent (Iddir et al., 2020). These ratios remained within the solubility limits of pure proteins in water and were based on (i) previous studies investigating the impact of proteins on fat-soluble compounds (carotenoids) bioaccessibility (Iddir et al., 2020) and (ii) nutritionally relevant concentrations in a balanced meal. The amount of WPI and SPI needed to reach these ratios corrected by the respective protein content (80 % for WPI, 92.6 % for SPI) was solubilized in water prior to incorporation into the samples. The oils were enriched in phytosterols by incorporating the required quantities of β-sitosterol, followed by solubilizing through heating at 70 °C for 15 min and sonication for 5 min. Proper solubilization was verified by GC-FID.

2.3. In vitro digestion with the INFOGEST method

In vitro digestion was performed according to the INFOGEST 2.0 protocol (Brodkorb et al., 2019). Briefly, samples were exposed to three successive digestives phases: oral, gastric, and intestinal, which consist of incubating the sample in a 15 mL tube at 37 °C under agitation on a rotating wheel at 40 rpm (Tube Revolver Rotator, Thermo Fisher Scientific, Waltham, MA, USA) with simulated digestive fluids containing electrolytes and enzymes as detailed in the INFOGEST 2.0 protocol. All enzyme activities were tested prior to *in vitro* digestion, as recommended by the INFOGEST protocol, and the needed amount of each enzyme was added to reach the specified activity levels. The oral phase consisted of a 1:1 (wt/wt) dilution of the food with simulated salivary fluid and a 2-min incubation. The use of salivary α-amylase was omitted in our study due to the nature of our samples. The oral bolus was then diluted 1:1 (v/v) with simulated gastric fluid and gastric enzymes (pepsin and lipase from RGE) to reach 60 U of lipase/mL of digesta and incubated at pH 3 for 2 h. Finally, gastric chyme was diluted 1:1 (v/v) with simulated intestinal fluid, bovine bile salts (10 mmol of bile salts/L of digesta) and porcine pancreatin (100 U of trypsin/mL of digesta) and incubated for 2 h. Tubes were flushed with argon before the gastric and intestinal phases in order to eliminate the air in the headspace. At the end of digestion, samples were homogenized with Ultra-Turrax for 10 s at 15,000 rpm before sampling and stored at −80 °C until analysis.

2.4. Bioaccessibility assessment through Bligh & Dyer extraction, solid phase extraction and GC-FID

Lipid extraction was performed with methanol/chloroform/water (2:1:0.8; v/v/v) according to the Bligh and Dyer method (Bligh & Dyer, 1959) and lipid classes separation was performed with solid-phase extraction (SPE). In brief, after extraction, samples were dried under a stream of nitrogen at 30 °C, resuspended into chloroform and loaded on SPE columns (Bond Elut-NH2, Agilent Technologies, Santa Clara, CA, USA). Neutral lipids, FFA and phospholipids (PL) were eluted with chloroform/2-propanol (2:1; v/v), diethyl ether/acetic acid (98:2; v/v) and methanol respectively. Neutral lipids were dried under a steam of nitrogen at 30 °C, resuspended in hexane and loaded on a new SPE column. Phytosterols esters (PE), TG, diglycerides (DG) and MG were eluted with hexane, hexane/dichloromethane/diethyl ether (99:10:1; v/v/v), hexane/ethyl acetate (85:15; v/v) and chloroform/methanol (2:1; v/v) respectively (Kaluzny et al., 1985). An internal standard composed of 1-monopentadecanoin, triheptadecanoin, 10(*Z*)-diheptadecenoin, nonadecanoic acid, 1,2-dibehenoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL, USA), cholesteryl tricosanoate was added in each sample at the initial step to evaluate the extraction yield of each lipid class, quantify any loss and ensure proper separation of lipid classes. Samples were dried under a stream of nitrogen at 30 °C and methylated under alkaline conditions (KOH 0.1 mol/L in methanol at 70 °C for 1 h) and then under acidic conditions (HCl 1.2 mol/L in methanol at 70 °C for 12 min). Methylation conditions were optimized to ensure efficient derivatization across all lipid classes while preventing oxidative degradation, as confirmed by peroxide value measurements (data not shown). Resulting fatty acids methylesters (FAMEs) were extracted with hexane. Before injection, methyl-undecanoate was added in each sample as injection standard. Fames were separated by GC (Trace 1310, Thermo Fisher Scientific) equipped with a RT-2560 column (biscyanopropylpolysiloxane, 100 m × 0.25 mm × 0.2 μm; Restek) flowed with H_2 as carrier gas. The GC temperature program was as follows: initial temperature of 80 °C, increase at 25 °C/min up to 175 °C, hold for 25 min; increase at 10 °C/min up to 200 °C; hold for 20 min; increase at 10 °C/min up to 220 °C; hold for 5 min; increase at 10 °C/min up to 235 °C, hold for 15 min; decrease at 20 °C/min to initial temperature of 80 °C. FAMEs were detected using a FID kept at a constant temperature of 255 °C and flowed by air (350 mL/min), H_2 (35 mL/min) and N_2 (40 mL/min). An external standard composed of 45 pure FAMEs was used for peak identification and quantification. Chromatograms were processed using ChromQuest 5.0 software (Thermo Fisher Scientific, Waltham, MA, USA). Data were normalized with the internal and injection standards and blanks were subtracted. Concentrations of fatty acids in the different lipid classes after INFOGEST *in vitro* digestion were expressed as mmol/L of digesta (*i.e.* digested samples).

2.5. Oil composition analysis

The fatty acid profile and the lipid class composition of the oils were analyzed using the same method as described above. In brief, Bligh & Dyer extraction, SPE and two-step methylation were performed on 20 mg of oil and injected onto GC-FID. The fatty acid profile is expressed as percentage of the total of fatty acids. To quantify phytosterols, oil was first saponified in alkaline conditions (KOH 2 mol/L in methanol at 50 °C for 2 h) then the unsaponifiable fraction was extracted with hexane and derivatization was carried out to form trimethylsilyl (TMS) ether derivatives. Phytosterols were separated by GC-FID equipped with a Rxi-5Sil column (30 m × 0.25 mm × 0.25 μm, Restek) flowed with N_2 as carrier gas. The GC temperature program was as follows: the initial temperature of 70 °C, increase at 30 °C/min up to 290 °C, hold for 22 min; increase at 30 °C/min up to 320 °C; hold for 5 min. Identification and quantification of phytosterols was determined with a plant sterol mixture as external standard and betulin was used as internal standard.

Table 1
Concentrations of fatty acids in the different lipid classes after INFOGEST *in vitro* digestion of olive oil (OO) and pomegranate seed oil (PSO). Results are expressed as mean ± standard deviation (n = 3). Free fatty acids (FFA), monoglycerides (MG), diglycerides (DG), triglycerides (TG), phospholipids (PL), phytosterol esters (PE). Significance was established by two-way ANOVA with Bonferroni multiple comparisons and is indicated by capital letters (A) for comparisons between different lipid classes within the same oil and by lowercase letters (a) for comparisons between oils within the same lipid class.

	Lipid classes	Olive oil (OO)	Pomegranate seed oil (PSO)
Concentrations of fatty acids in the different lipid classes (mmol/L of digesta)	FFA	$10.6 \pm 1.3^{A,a}$	$8.3 \pm 1.1^{A,b}$
	MG	$3.8 \pm 1.2^{B,a}$	$2.5 \pm 0.2^{BC,a}$
	DG	$0.9 \pm 1.2^{C,a}$	$3.2 \pm 2.0^{B,b}$
	TG	$1.3 \pm 0.6^{BC,a}$	$1.2 \pm 0.7^{BC,a}$
	PE	$0.8 \pm 0.4^{C,a}$	$0.4 \pm 0.1^{C,a}$

2.6. Bioaccessibility index and recovery index

Bioaccessibility refers to the proportion of the component released from the food matrix during digestion that is available for absorption. In the case of oils, the products of digestion of the main component (*i.e.* TG) available for absorption are FFA and MG (McClements et al., 2008). Bioaccessibility was calculated as the ratio between the concentrations of fatty acids in FFA and MG lipid classes and the concentrations of fatty acids present in all lipid classes measured by GC-FID following SPE (Eq. (1)).

$$Bioaccessibility\ index\ (BI)(\%) = \frac{[FFA]_i + [MG]_i}{[FFA]_i + [MG]_i + [DG]_i + [TG]_i + [PE]_i} x\,100 \quad (1)$$

With i for each individual fatty acid (mmol/L of digesta).

The recovery index (RI) of a fatty acid was calculated by comparing its amount after *in vitro* digestion with its amount in the initial oil sample (Eq. (2)). This factor is used to assess losses likely to occur during *in vitro* digestion.

$$Recovery\ index\ (RI)(\%) = \frac{Fatty\ acid\ content\ of\ digesta}{Fatty\ acid\ content\ of\ initial\ sample\ x\ dilution\ factor}\ x\,100 \quad (2)$$

2.7. Statistical analysis

All experiments were performed in triplicate, and results are expressed as mean ± standard deviation. Statistical analyses were performed with JMP Pro 17 (SAS institute inc., Cary, NC, USA) and GraphPad 10 (GraphPad Software, San Diego, CA, USA). Depending on the experimental design, comparisons were performed using Student's *t*-

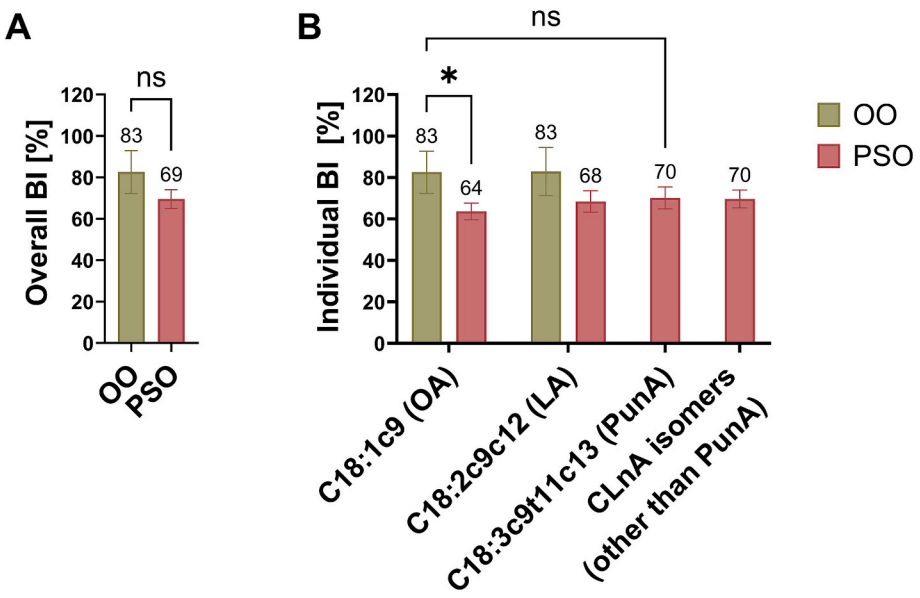

Fig. 1. A. Overall bioaccessibility index (BI) for olive oil (OO) and pomegranate seed oil (PSO), all fatty acids combined. B. BI for individual fatty acids of OO and PSO, *i.e.* oleic acid (OA), linoleic acid (LA), punicic acid (PunA) and conjugated linolenic acid (CLnA) isomers. Results are expressed as mean ± standard deviation ($n = 3$). Significance was determined by Student's *t*-tests with Bonferroni correction.

tests or linear models, followed by *post hoc* analyses (Bonferroni-corrected t-tests or Tukey's tests) when appropriate. The level of statistical significance was set at $p \leq 0.05$ for all analyses.

3. Results and discussion

3.1. Oils composition

The major fatty acids in PSO were PunA (61.9 %), the other CLnA isomers (22.4 %), linoleic acid (LA, C18:2 c9c12, 5.0 %), OA (4.4 %), palmitic acid (PA, C16:0, 2.7 %) and stearic acid (SA, C18:0, 1.9 %) while OO contained mainly OA (75.2 %) followed by PA (11.0 %), LA (6.5 %) and SA (3.6 %). Since the most abundant fatty acids in each oil (*i.e.* PunA in PSO and OA in OO) contain 18 carbons and are present in similar proportions, and given that OO is well known and frequently used in *in vitro* digestion studies, OO was selected as a control edible oil.

TG represented the main lipid class in oils, accounting for 87.3 % in PSO and 92.8 % in OO. The other lipid classes were present in much lower amounts: DG were found at 8.0 % in PSO and 3.7 % in OO, FFA at 3.5 % in PSO and 1.2 % in OO, and PE at 0.1 % in PSO and 2.1 % in OO. MG and PL were present in trace amounts (<0.2 % or not detected).

3.2. The bioaccessibility of PunA in PSO is high and equivalent to OA in OO

Concentrations of fatty acids in the different lipid classes after *in vitro* digestion are presented in Table 1. The overall bioaccessibility index (BI) was determined by the ratio of the total fatty acids present in absorbable fractions, FFA and MG, to the total fatty acids present in all lipid fractions and is presented in Fig. 1A while the BI for each individual fatty acid is shown in Fig. 1B. Only 18-carbon fatty acids representing at least 5 % of total fatty acids in the oil were considered. The main lipid class present in both oils after *in vitro* digestion was FFA. The following lipid classes were DG, MG, TG, and PE in PSO and MG, TG, DG and PE in OO, in descending order. There were fewer FFA in PSO than in OO, but more fatty acids in the form of DG (Table 1). There has been a shift in the main lipid class, from TG to FFA, compared to the initial composition of the oils. Following *in vitro* digestion, 82.6 ± 10.3 % of OO lipids were transformed into absorbable fraction namely FFA and MG, reflecting overall BI (Fig. 1A). This result is in accordance with previous studies showing a high bioaccessibility of OO, reaching more than 70 % (Alberdi-Cedeño et al., 2020). Regarding PSO, the overall BI reached 69.5 ± 4.5 %, which despite a slight downward trend, did not significantly differ from that of OO. When examining individual fatty acids, OA bioaccessibility was lower in PSO (63.6 ± 4.0 %) compared to OO (82.5 ± 10.2 %) (Fig. 1B). Regarding the major fatty acids in both oils, the bioaccessibility of PunA in PSO (70.1 ± 5.3 %) did not differ from that of OA in OO. The other CLnA isomers, for their part, reached a BI of 69.6 ± 4.3 %. Taken together, these results indicate that PSO is as bioaccessible as OO, particularly for fatty acids present in equivalent proportions in the oils, PunA in PSO compared to OA in OO, despite a lower bioaccessibility for OA between oils.

The proportion of fatty acids in oils could be an important factor for bioaccessibility, particularly considering the competition of digestive lipases for TG. This is one of the reasons why we selected OO as a control since its major fatty acid is present in similar proportion than PunA in PSO. Additionally, the position on the TG backbone is critical, as digestive lipases specifically cleave at sn-1 and sn-3 positions. It has been shown that two oils with the same fatty acid composition but different TG structures result in different bioaccessibility due to the location specificity of pancreatic lipase (Y. Wang et al., 2023). The type of fatty acid on the glycerol backbone is also expected to impact bioaccessibility since the hydrolysis of short- and medium-chain fatty acids is faster than that of long-chain fatty acids (McClements et al., 2008). Here, both fatty acids share the same carbon chain length, 18 carbons, but different degrees of unsaturation. The number, position and configuration of the double bonds do not appear to have had any impact on the bioaccessibility of PunA compared to OA. Conjugated fatty acids therefore appear to be digested like non-conjugated monounsaturated fatty acids of the same carbon chain length. Taken together, the high bioaccessibility of PSO in this study aligns with findings from Bañares et al. (2023), who investigated the bioaccessibility and the anti-inflammatory effect of PSO and OO. However, it is difficult to make a direct comparison due to major methodological differences. First, they did not follow the INFOGEST protocol, but a distinct approach with different incubation times, enzymes selection, pH and electrolyte concentrations. Specifically, their gastric digestion lasted one hour, with porcine pepsin as the only digestive enzyme present and NaCl/HCl as electrolytes whereas INFOGEST recommends 2 h incubation with RGE containing gastric pepsin and gastric lipase, and a broader list of electrolytes (KCl, KH_2PO_4,

Table 2
Phytosterol composition of olive oil (OO) and pomegranate seed oil (PSO). Data are presented means ± standard deviations (n = 3). Not detected (ND). Significance between oils was determined by Student's t-tests with Bonferroni correction.

	Phytosterols	Olive oil (OO)	Pomegranate seed oil (PSO)
Phytosterol content (mg/kg)	Campesterol	115.2 ± 14.4[a]	630.2 ± 18.6[b]
	Stigmasterol	ND	340.7 ± 11.0
	β-sitosterol	1731.2 ± 56.3[a]	5646.0 ± 157.4[b]
	Total	1846.3 ± 70.6[a]	6616.8 ± 8[b]

$NaHCO_3$, NaCl $MgCl_2$, $(NH_4)_2CO_3$, HCl and $CaCl_2$). Similarly, their intestinal digestion followed a one-hour protocol with phosphatidylcholine, bile salts, cholesterol, porcine pancreatin and phospholipase A2, whereas INFOGEST recommends a 30-min incubation with bovine salts, followed by a two-hour incubation with porcine pancreatin in presence of electrolytes (KCl, KH_2PO_4, $NaHCO_3$, NaCl $MgCl_2$, $(NH_4)_2CO_3$, HCl and $CaCl_2$). Secondly, INFOGEST requires the use of enzymatic assays to accurately determine the enzyme quantity required, ensuring precise and reproducible digestion conditions. This approach contrasts with Bañares et al. (2023), who relied solely on weight-based enzyme addition, potentially affecting digestion reproducibility. Finally, the major difference lies in the subsequent analyses to evaluate bioaccessibility. They performed centrifugation after digestion to separate an oil phase, considered as undigested lipids, a middle micellar aqueous phase and a

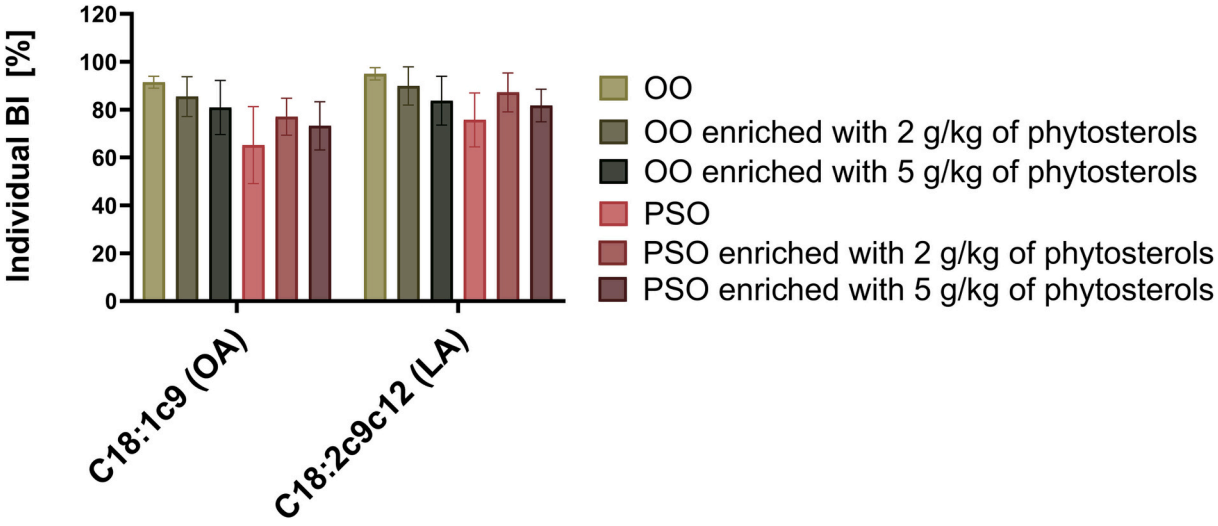

Fig. 2. Bioaccessibility Index (BI) for individual fatty acids of olive oil (OO) and pomegranate seed oil (PSO), *i.e.* oleic acid (OA), linoleic acid (LA), punicic acid (PunA), after enrichment with phytosterols. Results are expressed as mean ± standard deviation (n = 3). Significance between conditions for each fatty acid of each oil was determined by Tukey's tests. No significant difference was found between conditions.

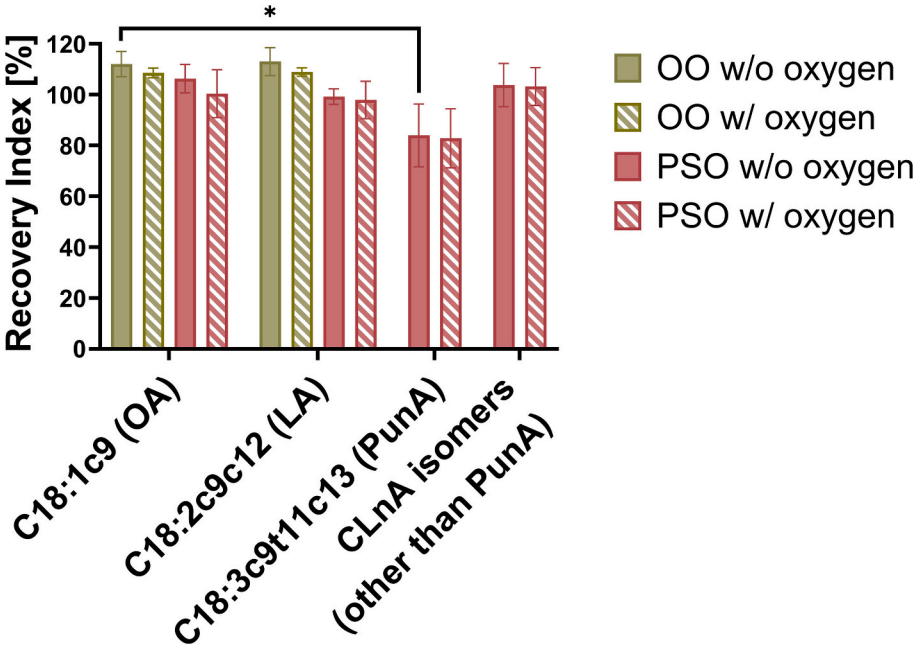

Fig. 3. Recovery index of fatty acids present in olive oil (OO) and pomegranate seed oil (PSO) after *in vitro* digestion in presence or absence of oxygen. Results are expressed as means ± standard deviation (n = 3). Significance was determined by Student's t-tests with Bonferroni correction. No significant difference was found between oxygen conditions within each fatty acid.

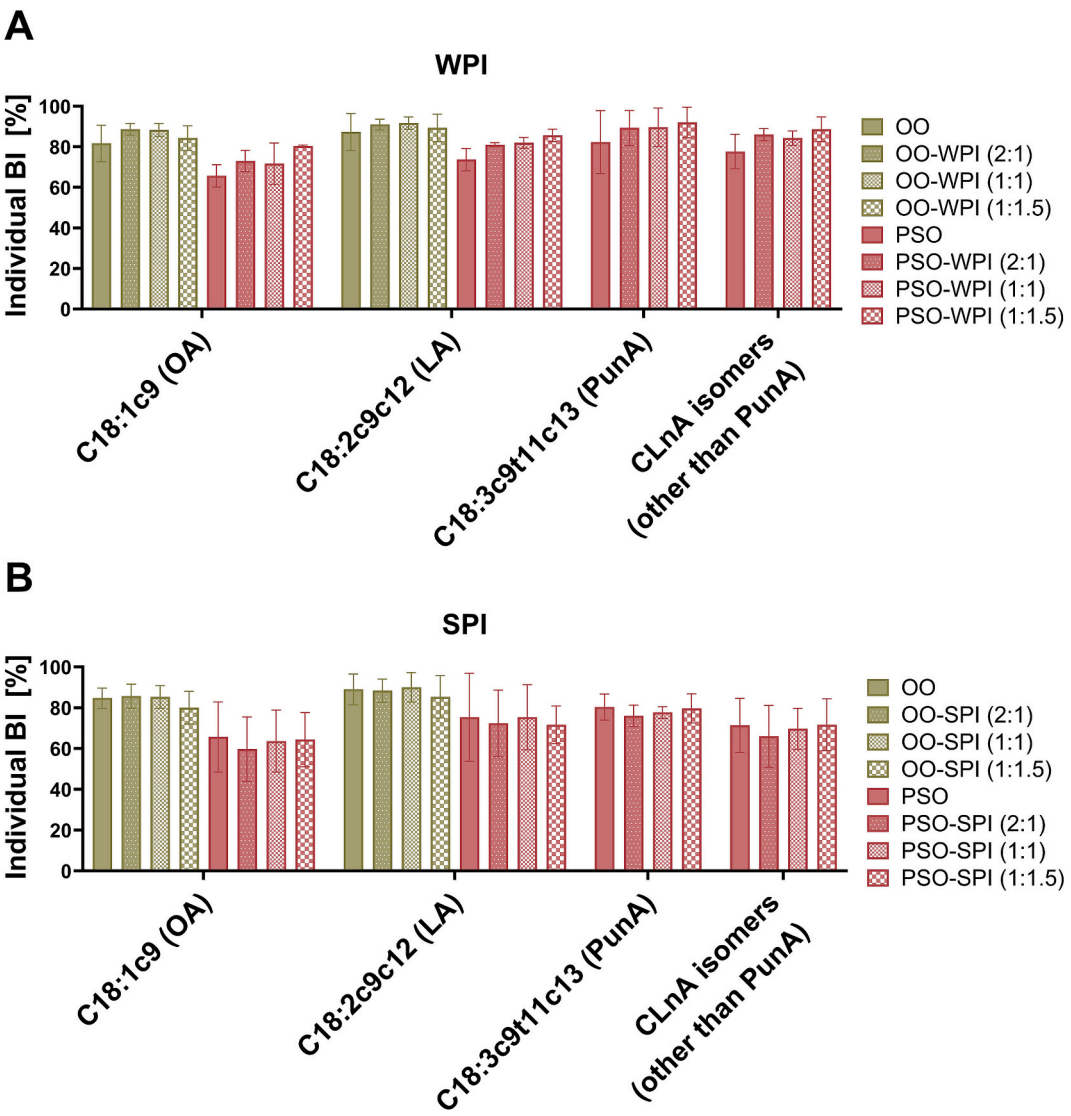

Fig. 4. Impact of whey and soy protein isolates (WPI, SPI) at increasing concentrations on the bioaccessibility index (BI) for individual fatty acids of olive oil (OO) and pomegranate seed oil (PSO), *i.e.* oleic acid (OA), linoleic acid (LA), punicic acid (PunA) and conjugated linolenic acid (CLnA) isomers. Results are expressed as mean ± standard deviation (n = 3). Significance was determined by Tukey's test. No significant difference was found between conditions with each fatty acid.

precipitate phase. The fatty acid profile was not analyzed, FFA being quantified by titration and other lipids classes by liquid chromatography coupled with a diode array and evaporative light scattering detectors. According to their method, bioaccessibility was considered good if the oil phase represented less than 20 % of the total digestion product and if the total percentage of MG was higher than 20 % (Bañares et al., 2023). In our study, we performed a SPE to analyze the fatty acid profile in each lipid class, assessing the bioaccessibility of each fatty acid by the proportion present as FFA and MG relative to the total fatty acids present in all lipid classes.

3.3. Higher phytosterol content in PSO does not explain the lower bioaccessibility of OA

The phytosterol content of the oil could influence the overall bioaccessibility of PSO, as it has been reported that phytosterols might reduce the rate of lipid digestion by forming a barrier around the lipid droplets due to their hydrophobic nature, impeding access by digestive lipases. Previous work has shown that increasing phytosterol concentrations proportionally decrease the lipid digestibility of β-sitosterol-enriched sunflower oil (Wilde et al., 2019). However, phytosterol levels in oils reported in the literature are highly variable, making comparisons very difficult as they depend on many factors such as harvest time, genotypes, growing areas, climatic conditions, or different oil extraction methods (Iriti et al., 2023). Therefore, the quantification of phytosterols was carried out in both oils (Table 2). The main phytosterol present in both oils was β-sitosterol, followed by campesterol while stigmasterol was only detected in PSO. The total phytosterol content was higher in PSO than in OO, supporting the hypothesis that phytosterols could reduce the bioaccessibility of the fatty acids present in the oil. The fact that the percentage of PE was higher in OO than in PSO (*cf.* 3.1.) while total phytosterol quantification showed much higher levels of phytosterols in PSO indicates that the proportion of esterified phytosterols is minor. To investigate whether the phytosterol content of PSO could contribute to the lower bioaccessibility of certain fatty acids, we conducted an additional experiment. OO was enriched with β-sitosterol to match the phytosterol levels naturally present in PSO. In parallel, PSO was similarly enriched with the same amount of β-sitosterol as in OO. As shown in Fig. 2, phytosterol enrichment had no significant impact on the bioaccessibility of OA or LA in either oil. These results suggest that the elevated phytosterol content in PSO is unlikely to be the primary factor driving for the observed trend of reduced bioaccessibility.

3.4. Fatty acid recovery indexes are high, indicating minor loss during in vitro digestion

The recovery index (RI) was calculated by comparing the amount of a fatty acid in the digesta with its amount in the initial oil sample, to check whether degradation had occurred during *in vitro* digestion. Our results revealed that more than 80 % of all fatty acids were recovered after digestion (Fig. 3). A lower RI was however observed for PunA compared to OA in OO, which could be explained by its greater sensitivity to oxidation linked to conjugated double bonds. To evaluate the role of oxygen, we replicated the *in vitro* digestion experiment without argon flushing prior to gastric and intestinal phases. The presence of oxygen had no impact on the RI of any fatty acids in the two oils, indicating that even though PunA losses might be due to oxidation, they were not increased in absence of argon flush. However, there is no clear consensus on oxygen removal during the INFOGEST digestion protocol, and most standard digestion protocols do not even address this issue. Several studies have highlighted the crucial importance of studying oxidation processes during digestion, as the dissolved oxygen levels reduce the bioaccessibility of polyphenols for example (Eran Nagar et al., 2023). More importantly, the presence of oxygen in the upper digestive tract can promote oxidation and thereby the formation of toxic compounds with adverse health effects (Alberdi-Cedeño et al., 2020; Martin-Rubio et al., 2019).

3.5. WPI and SPI have no impact on fatty acid bioaccessibility

Dietary lipids are not only found as oils, but are often part of complex food matrices such as oil-in-water emulsions, water-in-oil emulsions or as lipid inclusions in carbohydrate or protein matrices (Michalski et al., 2017). Proteins influence lipid digestion in contrasting ways: they act as surfactants which stabilize emulsions, but they can also hinder enzymatic access to lipid droplets (Iddir et al., 2020). To investigate protein effects in our model, we assessed the impact of WPI and SPI on the bioaccessibility of OO and PSO. Our results indicate that neither increasing concentrations of WPI or SPI did not enhance the bioaccessibility of major fatty acids in both oils (Fig. 4). Studies on the impact of proteins on the bioaccessibility of lipids or fat-soluble food components are limited and findings vary depending on protein type and concentration. For instance, SPI has been shown to enhance FFA release from oil emulsions, whereas WPI decreases it (Iddir et al., 2020; Malaki Nik et al., 2011). Discrepancies between our study and previous research likely arise from variations in protein concentration and sample preparation. Iddir et al. (2020) demonstrated that lipid-to-protein ratios play a critical role in determining the influence of proteins on bioaccessibility. Notably, depending on the ratio, the positive or negative effects of proteins could diminish or even disappear, underscoring the complexity of protein-lipid interactions in digestion. Further research on colloid and interfacial science is required to elucidate how proteins and, more generally, food matrix composition influence CLnA bioaccessibility and digestion dynamics.

4. Conclusion

Over the past 20 years, there has been a growing interest towards the health-promoting properties of CLnAs. To explore their potential for nutritional therapy, the present work examined the bioaccessibility of PSO in comparison to OO with the harmonized *in vitro* digestion protocol INFOGEST. By assessing the influence of oxygen exposure, protein presence and phytosterols through a fatty acid-specific lens - an approach, to our knowledge, not previously undertaken - we provide new insights into CLnA digestion. Our results show that CLnAs are efficiently digested *in vitro* with PSO exhibiting bioaccessibility levels comparable to OO. However, a lower bioaccessibility of OA was noticed in PSO, independent of its phytosterol content. High recovery indexes for all fatty acids suggest minimal loss during *in vitro* digestion. Our findings support the concept that PunA-containing TG are well hydrolyzed by digestive lipases, making oral administration a suitable option. The bioaccessibility of the major fatty acids was not affected by the presence of proteins (WPI and SPI) in our experimental conditions. This study provides the first comparative assessment of CLnA-rich oil digestion using a validated *in vitro* digestion model, providing valuable insights into their bioaccessibility. However, it does not fully capture the complexity of lipid digestion and absorption. Further research should explore digestion of CLnA-rich oils beyond bioaccessibility, including absorption, biotransformation by enterocytes, and release into the systemic circulation.

CRediT authorship contribution statement

Sarah Vellemans: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Antoine Ide:** Writing – review & editing, Methodology. **Marion Duchatelet:** Investigation. **Rachel Dubois:** Investigation. **Antoine Demol:** Investigation. **Eric Mignolet:** Methodology. **Yvan Larondelle:** Writing – review & editing, Funding acquisition, Conceptualization. **Nathalie Delzenne:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Cathy Debier:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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