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Selective quantification of free fatty acids reveals matrix-dependent differences in lipid bioaccessibility

Nicholas S. Rivera [a,1], Thomas Montebugnoli [a,1], Alessandra Bordoni [a,b,*]

[a] Department of Agricultural and Food Sciences, DISTAL, Alma Mater Studiorum, University of Bologna, Piazza Goidanich 60, 47521 Cesena (FC), Italy
[b] Interdepartmental Centre of Agrifood Industry Research, Alma Mater Studiorum, University of Bologna, via Quinto Bucci 336, 47521 Cesena (FC), Italy

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ABSTRACT

Current *in vitro* approaches to lipid bioaccessibility typically quantify total fatty acid methyl esters (FAMEs) in the bioaccessible (micellar) fraction after digestion, assuming complete hydrolysis of esterified lipids. However, this operational definition does not discriminate between released and hydrolyzed lipids and may systematically overestimate the fraction immediately available for absorption. This study proposes an analytical approach that operationally differentiates between lipid release and free fatty acid formation. Following INFOGEST digestion, free fatty acids (FFAs) were isolated from the bioaccessible fraction by solid-phase extraction and quantified by gas chromatography. Four ready-to-eat foods differing in matrix structure (walnuts, canned mackerel, cream cheese, biscuits) were used as test matrices. Bioaccessibility, operationally defined as FFAs relative to total fatty acids in undigested food, ranged from 67.6% to 9.9%. In three of four matrices, a substantial proportion of solubilized lipids were not recovered as FFAs, indicating that conventional FAME-based quantification would likely overestimate bioaccessibility. The fatty acid profile of the bioaccessible fraction differed from the original composition, suggesting matrix-dependent differences in fatty acid release patterns. These findings highlight limitations of the prevailing analytical practice for lipid bioaccessibility assessment and provide a more compositionally rigorous framework, with implications for digestion studies, and food composition data interpretation.

1. Introduction

Dietary lipids, commonly called fats, are the major source of energy per unit of weight in the diet (38 kJ/g - 9 kcal/g). They are macronutrients that, in addition to providing energy, serve many other functions in the body; they are components of cell membranes, precursors of bioactive compounds such as prostaglandins, prostacyclins, and thromboxanes, and transporters of fat-soluble nutrients. The main components of dietary fats are triglycerides (TAGs). Mono- (MAGs), diacylglycerols (DAGs), and phospholipids (PLs) are present in smaller amounts. Animal fats also contain other lipid species such as free cholesterol and cholesterol esters (CE) (EFSA Panel on Dietetic Products, Nutrition, and Allergies NDA, 2010).

The first step in fat processing in the gastrointestinal tract is the release from the food matrix. Then, to be made available for hydrolysis, fats must be emulsified. In the stomach, emulsification occurs through mechanical action, and in adults, only a small portion of dietary fats are ultimately hydrolyzed. Following gastric digestion, in the duodenum, bile produced by the liver acts as a surfactant to produce a fine emulsion that is more readily accessible for hydrolysis by pancreatic enzymes. The hydrolyzed lipid-soluble components (free fatty acids -FFAs-, 2-MAGs, lyso-PLs, free cholesterol) integrate with bile salts in mixed micelles to diffuse between intestinal microvilli and interact with transporters located on the luminal surface of enterocytes (Walther et al., 2019).

Abbreviations: CE, cholesterol esters; DAGs, diglycerides; DHA, docosahexaenoic acid; DRI, dietary reference intake; FAs, fatty acids; FAMEs, fatty acid methyl-esters; FFAs, free fatty acids; FID, flame ionization detector; GC, gas-chromatography; IS, internal standard; MAGs, monoglycerides; MUFA, monounsaturated fatty acids; ND, not digested; PLs, phospholipids; PUFAs, polyunsaturated fatty acids; RTE, ready to eat; BF, bioaccessible fraction; SFAs, saturated fatty acids; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SPE, solid-phase extraction; SSF, simulated salivary fluid; TAGs, triglycerides.

* Corresponding author at: Department of Agricultural and Food Sciences, DISTAL, Alma Mater Studiorum, University of Bologna, Piazza Goidanich 60, 47521 Cesena (FC), Italy.
E-mail addresses: nicholas.rivera@unibo.it (N.S. Rivera), thomas.montebugnoli2@unibo.it (T. Montebugnoli), alessandra.bordoni@unibo.it (A. Bordoni).
[1] equally contributed to this work

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TAGs are molecules composed of a glycerol backbone to which three fatty acids (FAs) are attached, and for nutritional adequacy and health outcomes, the type of esterified fatty acids present in food TAGs is crucial. The importance of the qualitative content of FAs in foods is increasingly evident and confirmed by numerous studies. Saturated fatty acids (SFAs) have been linked to an increased risk of cardiovascular disease when consumed in large quantities, and while there is some controversy about the need to reduce intake of these FAs for cardiovascular prevention (Yamada et al., 2025), EFSA recommendations indicate that SFAs intake should be as low as possible within the context of a nutritionally adequate diet (EFSA Panel on Dietetic Products, Nutrition, and Allergies NDA, 2010). Conversely, a diet including foods rich in polyunsaturated fatty acids (PUFAs), particularly long-chain omega-3s, is believed to have a positive impact on health (Li et al., 2025; Sadeghi et al., 2025; Yan et al., 2024). In their systematic review, Schoeneck & Iggman (2021) demonstrated with strong evidence that foods rich in unsaturated fatty acids and low in saturated and trans fatty acids cause at least moderate reductions (i.e., 0.20–0.40 mmol/L) in serum LDL cholesterol (Schoeneck & Iggman, 2021).

Evidence of the importance of the quantity and quality of FAs consumed through food raises a nontrivial question: does chemical composition alone reliably reflect the fraction of lipids that becomes available for absorption during digestion? Indeed, to be absorbed in the intestine and thus exert their effects in the body, TAGs must become bioaccessible during digestion, that is, be released from the food matrix and hydrolysed in a suitable form (Fernández-García et al., 2009). An assessment of the bioaccessibility of fats could better reflect the role of the food containing them in the diet. Therefore, assessing bioaccessibility is gaining momentum, and *in vitro* protocols are considered an ideal tool for investigating bioaccessibility, at least as a first step in nutritional studies and for guiding the formulation of new foods (Lesmes, 2023). Although *in vitro* digestion models are widely used to estimate lipid bioaccessibility, there is no consensus on its operational definition (Bohn et al., 2018). In many studies, lipid bioaccessibility is inferred from the total fatty acids recovered after digestion in the bioaccessible fraction (BF), commonly obtained as the aqueous/micellar phase after centrifugation. However, this fraction may contain different lipid species, including free fatty acids (FFAs), MAGs, and residual esterified lipids associated with mixed bile-salt micelles or dispersed colloidal structures. Therefore, total FAME-based quantification does not distinguish between fatty acids present as FFAs and those still in esterified forms, potentially leading to overestimation of the fraction immediately available for absorption.

From this perspective, since the bioaccessibility of food components in isolation differs from the bioaccessibility of the same components when they are part of foods (Miller et al., 2023), evaluating the matrix effect, that is, the impact of the food matrix, has become increasingly important. The bioaccessibility of nutrients/food components in general - and of lipids specifically – within a given matrix is not fixed, as it can be modulated by structural and compositional factors (Martínez-Sánchez et al., 2024). Food structure can influence the physical organization of nutrients, thereby affecting digestion dynamics and the extent to which lipids are released and transformed during digestion.

Therefore, the present study was designed to (i) apply a selective analytical approach to quantify FFAs in the BF after *in vitro* digestion; (ii) compare this approach with conventional total FAME-based estimations of lipid bioaccessibility; (iii) explore how different food matrices influence the proportion of FFAs in the BF. To this purpose, four different commercial foods were digested *in vitro* using the INFOGEST protocol. The four foods chosen had in common that they were ready to eat (RTE) and that they were often evaluated nutritionally based on their quantitative and qualitative fat content, either positively (high in PUFA) or negatively (high in SFA). However, they differed in their structural and compositional characteristics, including lipid organization and matrix complexity.

To evaluate bioaccessible lipids, the digested food obtained at the end of INFOGEST digestion was centrifuged and lipids were extracted from the BF (Francisco et al., 2020). As the characteristics of the food matrix modulate the effectiveness of extraction methods in the recovery of total lipids, in preliminary experiments lipids were extracted using both the Folch et al. (1957) and the Bligh & Dyer (1959) methods. Total lipids in the BF were quantified by weight and by gas-chromatography, after methyl-esterification and using an internal standard (IS). The evaluation of total lipids and fatty acid methyl-esters (FAMEs) concentration and profile was also performed in the corresponding not digested (ND) samples. Finally, FFAs were separated from the total lipids extracted from the BF of the digested food using solid-phase extraction (SPE), and quantified by gas-chromatography.

The experimental workflow is schematized in Fig. 1.

2. Material and methods

2.1. Materials and chemicals

Experiments were performed on four different commercial foods (biscuits, shelled walnuts, canned mackerel in brine, and cream cheese) purchased from local supermarkets. For each food, three different production batches were considered.

Digestive enzymes were purchased from Sigma-Aldrich (Burlington, MA, USA): α-amylase from human saliva (Type IX-A, measured activity 48 U/mg), pepsin from porcine gastric mucosa (measured activity 1358 U/mg), and pancreatin from porcine pancreas (measured trypsin activity 6.6 U/mg). Porcine bile extract, fatty acid methyl esters standard mix and the internal standard (pentadecanoic acid C15, 10 mg/ml) were also purchased from Sigma-Aldrich (Burlington, MA, USA). All reagents and solvents were purchased from the same company except where specified otherwise.

2.2. In vitro digestion

The foods were in vitro digested according to the standardized static INFOGEST protocol (Minekus et al., 2014), using oral, gastric and intestinal phases with α-amylase, pepsin, pancreatin and bile salts. The stock electrolyte solutions making up simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were formulated following the protocol instructions. The amount of each enzyme preparation added was calculated according to its activity in order to achieve the enzymatic activities specified by the INFOGEST protocol. *In vitro* digestions of the different foods were performed in triplicates using 5 g of food for each digestion, as well as a blank weighing 5 g of distilled water and adding the same enzymes and bile added in the digestion of the food.

Prior to *in vitro* digestion, all food samples were prepared to approximate oral processing conditions. Soft foods (cream cheese and biscuits) were manually homogenized using a spatula to obtain a uniform consistency. Canned mackerel was gently broken into small pieces using a fork. Shelled walnuts were subjected to two different mechanical treatments to simulate different chewing conditions: rapid chewing (coarsely broken pieces) and thorough chewing (finely crushed particles). This preliminary comparison was introduced to verify whether oral particle-size reduction substantially influenced lipid release during digestion. Following this initial evaluation, the thorough-chewing condition was selected as the standardized condition for subsequent analytical characterization, in order to reduce variability associated with heterogeneous particle size and to allow more controlled comparison across food matrices.

At the end of digestion, the resulting digested foods were transferred into 50-ml Falcon® test tube and centrifuged at 4500*g* for 10 min at 4°C, obtaining a pellet and a BF (i.e., the aqueous/micellar phase operationally defined as the bioaccessible fraction), which was stored at −20° C degrees until further analyses. These conditions were selected in line with commonly used INFOGEST-based protocols to obtain a

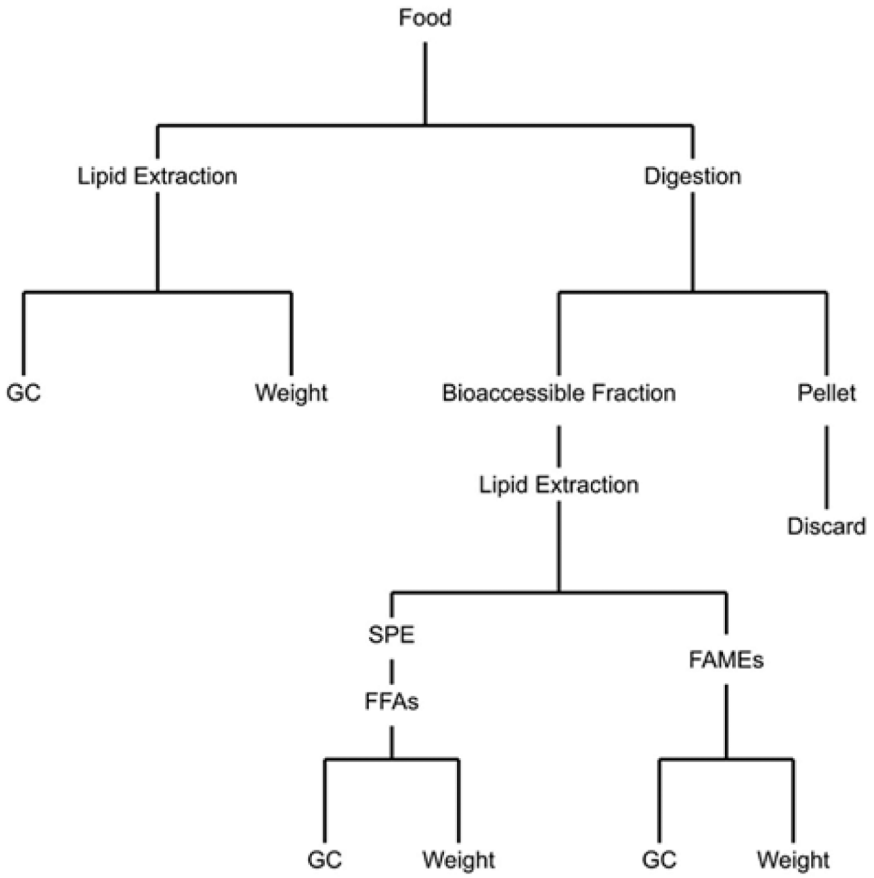

Fig. 1. Experimental Design.

reproducible operational separation of the aqueous phase, although they do not ensure a complete isolation of micellar structures.

2.3. Lipid extraction and methyl-esterification

Lipids were extracted from 0.1 g ND foods and 800 µl of BF of digested food with two different methods (Bligh & Dyer, 1959; Folch et al., 1957). For the Bligh and Dyer method (Bligh & Dyer, 1959), samples were homogenized in 9 ml of methanol:chloroform (2:1), then stored at −20 °C overnight. After overnight storage 3 ml of chloroform was added and the solution was mixed by vortexing. Then, 3 ml of HCl 2.5 N was added and the sample was vortexed again. The solution was then transferred into a conical glass tube with a cap and centrifuged at 450*g* for 10 min at room temperature, thus forming two separate phases. The upper aqueous phase was removed with a Pasteur pipette, retaining the lower organic phase which was poured over a paper filter filled with a spatula of Na_2SO_4 into Pyrex tubes.

In the Folch method (Folch et al., 1957), samples were homogenized in 8 ml of 1:1 chloroform:methanol solution and incubated at 60°C for 20 min. Then the sample was cooled to room temperature and 4 ml of chloroform was added. The solution was vortexed, 4 ml of 0.1 M KCl was added and the sample was incubated at 4 °C overnight. The next day, the top aqueous phase was removed and the bottom organic phase was poured over a paper filter filled with a spatula of Na_2SO_4 into Pyrex tubes.

The Pyrex tubes from both extractions were placed into a thermo-block at 37 °C under nitrogen flow, bringing the sample to complete dryness without oxidizing the lipids, then the lipids were quantified by weighing using an analytical balance.

After weighing the lipids, the same methyl-esterification procedure (Stoffel et al., 1959) was used for all samples. After addition of 100 µl of IS (pentadecanoic acid, C:15), samples were put in a thermo-block at 37 °C under nitrogen flow and brought to dryness. Then, 500 µl of methanolic HCl 3 N was added, and after vortexing the capped Pyrex tubes were placed in an oven at 100 °C for 1 h. After allowing the samples to cool to room temperature, 2 ml of hexane and 2 ml of distilled water were added. The upper organic phase was taken out, transferred into glass truncated conical tubes and stored at −20°C until analysis. Just before gas chromatographic analysis, the conical tubes were placed in a thermo-block at 37° C under nitrogen flow until the organic phase completely evaporated. The sample was then resuspended in hexane via percolating it along the walls of the tube. The tube was closed with a cap and stored at −20 °C until injection, after taking the aliquot to make the injection the sample was again stored at −20 °C.

2.4. GC-FID

A 30 m × 0.20 mm i.d. × 0.20 µm film thickness MEGA 10 fused silica capillary column was used for FAMEs analysis at the gas chromatograph (GC), with an initial temperature of 50 °C, ramp 10 °C/min to 250 °C, hold 3 min, and the injector temperature was 250 °C. The detector was a flame ionization detector (FID) with hydrogen flow 32.0 ml/min and air flow 200 ml/min with a temperature of 255 °C and a split ratio of 5:1 was used. The peaks were identified by comparison of retention times with those of the authenticated Supelco 37 Component FAME Mix reference standard. Quantification was performed using pentadecanoic acid (C15) as an internal standard and peak area ratios; therefore, external calibration curves for individual fatty acids were not generated. Peak areas were determined using LabSolutions software (version 5.99). External calibration curves for individual fatty acids were not employed.

2.5. FFA separation

An adapted method based on the work of Kaluzny was developed for solid phase extraction of lipid samples (Devle et al., 2014; Kaluzny et al., 1985; Mulet-Cabero et al., 2017). This approach allows selective isolation of FFAs from other lipid classes, enabling their independent quantification. For SPE, Strata® NH_2 cartridges (55 μm, 70 Å, 500 mg/6 ml, Phenomenex, Torrance, CA, USA) were conditioned with 6 ml of hexane and kept at atmospheric pressure. Total lipids from the BF of digested food, previously resuspended in 3 ml of hexane, were then loaded onto the cartridge. To elute the glyceride fraction, 6 ml of chloroform were added. Following this step, a collection tube was placed under the cartridge, and FFAs were eluted using 6 ml of a solution prepared by mixing 50 ml of chloroform with 1 ml of glacial acetic acid and 1 ml of methanol. The FFAs fraction was then methyl-esterified and analyzed via gas chromatography as described above.

Lipid bioaccessibility was operationally defined as the percentage of FFAs (expressed as methyl esters) quantified in the BF after digestion relative to the total FAs (as FAMEs) in the corresponding undigested food. This definition provides a conservative estimate of lipid bioaccessibility, as it does not account for all absorbable lipid species.

2.6. Statistical analysis

Statistical analysis was performed by using the Student's t test for pairwise comparisons, considering $p < 0.05$ as statistically significant. Normality was verified prior to applying parametric tests. All statistical tests were performed with Graphpad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California, USA).

3. Results

In preliminary experiments, two different methods were used for lipid extraction, which produced comparable results for mackerel and biscuits, while the Bligh & Dyer method showed slightly but significantly higher yields for cream cheese and shelled walnuts (Supplementary Table 1). Therefore, this method was used in subsequent experiments.

Each food was divided into two aliquots, one of which was used for lipid extraction and the other for *in vitro* digestion. Two slightly different *in vitro* digestion protocols were performed on walnuts, which differed from one another in the oral phase, simulating rapid, and more thorough chewing. After *in vitro* digestion, lipids were extracted from the BF and quantified by weight. The results were normalized to 100 g of corresponding ND food and are shown in Fig. 2.

In all foods, the amount of lipids recovered in the BF of the digesta was significantly lower than in the corresponding ND food, being about 70% for cream cheese and biscuits and approximately 55% in canned mackerel. In shelled walnuts, the degree of chewing had a significant impact on lipid recovery after digestion, which was less than 20% after rapid chewing and more than 40% after thorough chewing ($p < 0.0001$).

Lipids were extracted according to Bligh & Dyer (Bligh & Dyer, 1959), *and lipid content was quantified by weighing. Data are expressed as g/100 g of ND food, and are means ± SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing ND food and the corresponding BF: * p < 0.05; ** p < 0,01; ***p < 0,001*

An aliquot of lipids extracted from ND foods and corresponding BF was methyl-esterified and gas chromatographed, and FAMEs were quantified using pentadecanoic acid as IS. The total amount of FAMEs was normalized to 100 g of ND food. The recovery of FAMEs in the BF confirmed that the release from the food matrix after digestion was higher in cream cheese and biscuits (about 70% of the corresponding ND food) than in canned mackerel (about 40%). In walnuts, release from the food matrix depended on the degree of chewing, and it was about 15% and 50% after rapid and thorough chewing, respectively ($p < 0.0001$) (Fig. 3).

*Lipids were extracted, methyl-esterified and gas-chromatographed as reported in the Methods section. Data are expressed as g/100 g of ND food, and are means ± SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing the content of FAMEs in ND food and the corresponding BF of the digesta: ** p < 0,01; ***p < 0,001*

FFAs were separated from another aliquot of lipids extracted from the BF, and were quantified by weight—comparing them to the total lipids in the corresponding BF—and by gas chromatography, after methyl-esterification—comparing them to the total FAMEs in the corresponding BF. For nuts, the BF obtained after thorough chewing was used for subsequent analyses, as this condition had been selected as the standardized condition following the preliminary comparison reported above.

In all cases, to facilitate comparison, the results were normalized to 100 g of the corresponding ND food. Regardless of the type of quantification and comparison, the data indicate that in canned mackerel a high proportion of lipids released during digestion was recovered as FFAs, while in other foods the degree of FA release was lower and the recovery of FFAs was about 50% in walnut and even lower in cheese and biscuits (Fig. 4).

*FFAs were separated by SPE as described in the Methods section, and quantified by weight (left panel) or by gas-chromatography after methyl-esterification (right panel). Data are expressed as g/100 g of ND food, and are means ± SD of 3 biological replicates. Statistical analysis was by the Student's t test: * p < 0.02; ** p < 0,01; ***p < 0,001.*

Bioaccessibility was then calculated as

$$\frac{FFAs\ (as\ Methyl\ Esters)\ in\ digesta}{Total\ FAMEs\ in\ ND\ food} \times 100$$

and it was in the order mackerel> cheese = walnuts> biscuits (Table 1). A comparison of bioaccessibility values calculated using different analytical approaches is reported in Supplementary Table 2.

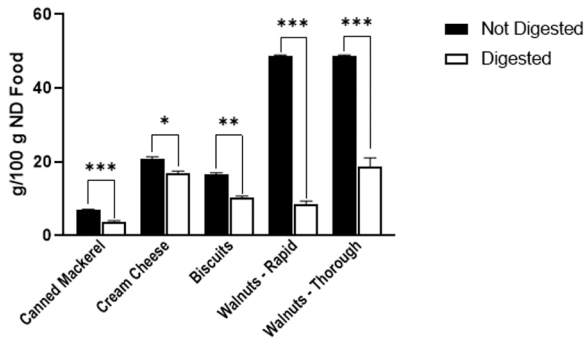

Fig. 2. Lipid content in ND and in the BF of digested foods.

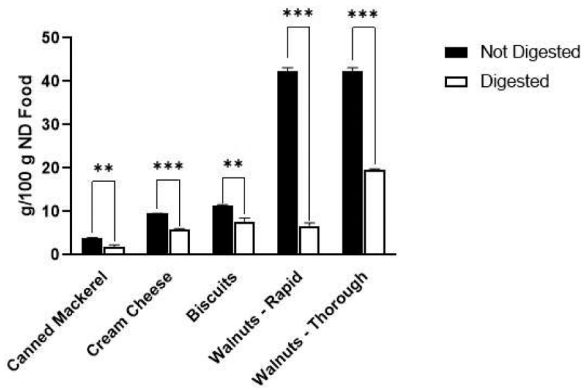

Fig. 3. Total FAME content in ND and in the BF of digested foods.

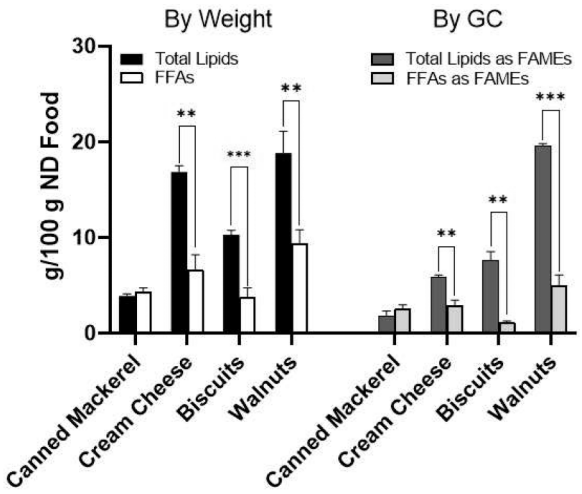

Fig. 4. Quantification of FFAs in the BF of digested foods.

Table 1
Lipid bioaccessibility.

Sample	Bioaccessibility (%)
Canned mackerel	67.6 ± 7.4
Cream cheese	29.7 ± 6.3
Biscuits	9.9 ± 1.5
Shelled walnuts	25.2 ± 5.8

Lipid bioaccessibility was calculated as the percentage of FFAs (as methyl esters) in the digested food compared to the total FAMEs in the corresponding ND food. Data are means ± SD of 3 biological replicates.

Given that the bioaccessibility values were well below 100% and different in the examined foods, we evaluated whether FA composition differed between the undigested samples and the corresponding FFA fraction. Therefore, the total FA composition (as methyl-esters) of the ND samples and of the corresponding FFA fraction in the BF obtained after digestion were evaluated.

The composition of ND canned mackerel was consistent with the Food Composition Database (BDA – Banca Dati di composizione degli Alimenti per studi Epidemiologici in Italia) and showed a very high concentration of n−3 fatty acids, mainly docosahexaenoic acid (DHA). No significant differences in the percentage composition were detected between ND mackerel and the FFA fraction obtained after *in vitro* digestion (Table 2), and the n−6/n−3 PUFA ratio remained 0.1:1.

The percentage FA composition of the cream cheese (Table 3) was also comparable to that reported in the Food Composition Database (BDA – Banca Dati di composizione degli Alimenti per studi Epidemiologici in Italia). In ND cheese, SFAs were the main components, particularly palmitic acid. In the FFA fraction obtained after *in vitro* digestion, a significant increase in palmitic and stearic acids was detected, which led to an increase in the total SFA content (Table 3). In both ND cheese and the corresponding FFA fraction, the concentration of n−6 PUFA was higher than that of n−3 PUFA, with a ratio ranging from 5.9:1 (ND cheese) to 5.6:1 (FFA fraction).

Similarly, the percentage content of myristic and stearic acids was significantly higher in the FFA fraction than in ND biscuits, but the content of SFA did not significantly change (Table 4). A significant increase in α-linolenic acid was also observed in the FFA fraction, resulting in a reduction of the n−6/n−3 PUFA ratio from 9.1:1 (ND biscuits) to 7.7:1.

Finally, the percentage FAs composition of ND walnuts was similar to that reported in the Food Composition Database (BDA – Banca Dati di composizione degli Alimenti per studi Epidemiologici in Italia), and confirmed the prevalence of polyunsaturated fatty acids, particularly linoleic acid (Table 5). In the FFA fraction, a significant increase in the SFAs - both palmitic and stearic acids - was observed, while the total amount of PUFAs was slightly reduced due to a decrease of linoleic acid percent content. The n−6/n−3 ratio remained nearly constant (around 4:1), indicating that digestion did not markedly alter the balance between the two classes of PUFAs.

Table 2
Fatty acid (as methyl esters) percent composition of ND canned mackerel and of the corresponding FFA fraction obtained after in vitro digestion.

	Not digested (mol/100 mol)	FFA fraction (mol/100 mol)
SFA	37.33 ± 1.17	41.62 ± 2.42
Myristic	4.26 ± 0.09	3.97 ± 0.2
Palmitic	24.61 ± 0.58	26.09 ± 1.8
Stearic	8.46 ± 0.53	11.57 ± 1.91
MUFA	20.06 ± 0.58	19.26 ± 2.39
Palmitoleic	4.58 ± 0.32	4.24 ± 0.52
Oleic	13.89 ± 0.38	13.55 ± 1.59
Gadoleic	1.60 ± 0.15	1.47 ± 0.31
PUFA	30.12 ± 0.87	27.41 ± 1.84
Linoleic	2.37 ± 0.1	2.20 ± 0.17
α-Linolenic	1.22 ± 0.08	1.22 ± 0.11
Eicosapentaenoic acid	6.28 ± 0.23	5.81 ± 0.27
Docosapentaenoic acid	1.62 ± 0.09	1.39 ± 0.23
Docosahexaenoic acid	18.72 ± 0.63	16.79 ± 1.45
n−6 PUFA	2.37 ± 0.1	2.20 ± 0.17
n−3 PUFA	27.84 ± 0.90	25.20 ± 1.81

Lipids were extracted, methyl-esterified and gas-chromatographed as reported in the Methods section. Data are expressed as mol/100 mol, and are means ± SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing ND mackerel to the FFA fraction obtained after in vitro digestion: *p < 0.02. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Table 3
Fatty acid (as methyl esters) percent composition of ND cream cheese and of the corresponding FFA fraction obtained after in vitro digestion.

	Not digested (mol/100 mol)	FFA fraction (mol/100 mol)
SFA	60.49 ± 0.43	63.22 ± 0.68**
Lauric	3.13 ± 0.03	2.58 ± 0.23*
Myristic	11.6 ± 0.03	12.21 ± 0.51
Palmitic	35.97 ± 0.24	37.82 ± 0.28**
Stearic	9.79 ± 0.01	10.53 ± 0.29*
MUFA	29.37 ± 0.54	28.74 ± 0.42
Myristoleic	1.55 ± 0.03	1.54 ± 0.05
Palmitoleic	2.49 ± 0.005	2.29 ± 0.46
Oleic	25.33 ± 0.4	24.91 ± 0.26
PUFA	3.33 ± 0.08	3.19 ± 0.25
Linoleic	2.85 ± 0.05	2.71 ± 0.18
α-Linolenic	0.48 ± 0.01	0.48 ± 0.03
n−6 PUFA	2.85 ± 0.05	2.71 ± 0.18
n−3 PUFA	0.48 ± 0.01	0.48 ± 0.03

Lipids were extracted, methyl-esterified and gas-chromatographed as reported in the Methods section. Data are expressed as mol/100 mol, and are means ± SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing ND cream cheese to the corresponding FFA fraction obtained after in vitro digestion: *p < 0.05; **p < 0.01. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

4. Discussion

Accurate assessment of lipid bioaccessibility may benefit from distinguishing between lipid release from the food matrix and the fraction present as FFAs after digestion. In many *in vitro* studies, bioaccessibility is inferred from total fatty acids recovered in the BF after digestion, without discriminating between free and esterified lipid species. The present study suggests that this approach may lead to overestimation, whereas selective quantification of FFAs provides a more conservative

Table 4
Fatty acid (as methyl esters) percent composition of ND biscuits and of the corresponding FFA fraction obtained after in vitro digestion.

	Not digested (mol/100 mol)	FFA fraction (mol/100 mol)
SFA	41.85 ± 0.48	43.89 ± 2.43
Myristic	6.79 ± 0.1	5.9 ± 0.17**
Palmitic	24.35 ± 0.32	25.51 ± 1.21
Stearic	10.71 ± 0.4	12.48 ± 0.63*
MUFA	41.76 ± 0.13	43.56 ± 1.24
Oleic	41.76 ± 0.13	43.56 ± 1.24
PUFA	7.56 ± 0.12	7.50 ± 0.09
Linoleic	6.81 ± 0.1	6.64 ± 0.1
α-Linolenic	0.75 ± 0.01	0.86 ± 0.02***
n−6 PUFA	6.81 ± 0.1	6.64 ± 0.1
n−3 PUFA	0.75 ± 0.01	0.86 ± 0.02***

Lipids were extracted, methyl-esterified and gas-chromatographed as reported in the Methods section. Data are expressed as mol/100 mol, and are means ± SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing ND biscuits to the corresponding FFA fraction obtained after in vitro digestion: *$p < 0.02$; ** $p < 0.01$; ***$p < 0.001$. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Table 5
Fatty acid (as methyl esters) percent composition of ND walnuts and of the corresponding FFA fraction obtained after in vitro digestion.

	Not digested (mol/100 mol)	FFA fraction (mol/100 mol)
SFA	10.45 ± 0.78	14.1 ± 1.11*
Palmitic	7.13 ± 0.56	9.40 ± 0.55*
Stearic	3.33 ± 0.22	4.70 ± 0.37*
MUFA	12.96 ± 0.39	12.62 ± 0.16
Oleic	12.96 ± 0.39	12.62 ± 0.16
PUFA	73.75 ± 0.48	70.21 ± 1.04*
Linoleic	58.99 ± 0.97	55.45 ± 1.23*
α-Linolenic	14.77 ± 0.55	14.76 ± 0.38
n−6 PUFA	58.99 ± 0.79	55.45 ± 1.23*
n−3 PUFA	14.77 ± 0.45	14.76 ± 0.38

Lipids were extracted, methyl-esterified and gas-chromatographed as reported in the Methods section. Data are expressed as mol/100 mol, and are means ± SD of 3 biological replicates. Statistical analysis was by the Student's t test, comparing ND walnuts to the corresponding FFA fraction obtained after in vitro digestion: *$p < 0.05$. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids

operational estimate.

It should be noted that the operational definition of the BF depends on the centrifugation conditions applied. Higher centrifugation speeds and longer times may result in a more selective isolation of micellar structures. Therefore, the values reported in this study should be interpreted within the framework of the applied protocol, as they represent an operational rather than a strict separation of micellar species. This aspect should be considered when comparing results across studies adopting different separation conditions.

The availability of different analytical outputs in the present study allows a direct comparison between alternative operational definitions of lipid bioaccessibility (Supplementary Table 2). In the literature, reported values of fatty acid release (as total FAMEs) during *in vitro* digestion typically range between approximately 30% and 70%, depending on lipid type, matrix structure, and digestion conditions (Pereira et al., 2023). Differences in analytical methodologies contribute to variability in reported values (Devle et al., 2014; Montebugnoli et al., 2024; Mulet-Cabero et al., 2017; Tormási & Abrankó, 2021). In this context, the values obtained in the present study fall within previously reported ranges.

However, when calculated using selective quantification of FFAs, bioaccessibility values were generally lower than those obtained based on total lipids or total FAMEs in the BF, although this trend was less evident in mackerel. This is consistent with the view that conventional FAME-based approaches may overestimate the fraction of lipids present as FFAs after digestion. Previous studies have highlighted that partially hydrolyzed lipids such as MAGs and DAGs may be present in the BF (Tormási & Abrankó, 2021), and therefore FFA-based values should more cautiously be interpreted as FFA release or release ratio rather than as a comprehensive measure of lipid bioaccessibility.

In the present study, lipid release from the matrix and the fraction of lipids recovered as FFAs were not directly aligned. A substantial proportion of lipids was recovered in the BF after digestion in most foods; however, except for mackerel, a considerable fraction was not recovered as FFAs, suggesting that part of these lipids may have remained in esterified form. This further highlights the importance of clearly defining analytical approaches when estimating lipid bioaccessibility.

Differences in lipid release and FFA formation were observed across the tested foods, suggesting an association with matrix-specific structural and compositional characteristics. The discrepancy between total FAME-based estimates and selectively quantified FFAs was particularly pronounced in biscuits, suggesting that matrix-dependent factors may influence the relative abundance of partially hydrolyzed lipid species within the bioaccessible fraction. Although the present study did not directly characterize lipid classes such as MAGs and DAGs, these observations are consistent with the possibility that structural interactions within complex food matrices may limit complete lipid hydrolysis despite substantial lipid release from the matrix.

These observations are consistent with previous studies reporting that physical organization, lipid distribution, and particle size can influence digestion dynamics (Bohn et al., 2018; Kupikowska-Stobba et al., 2025).

For example, differences in micro- and macrostructure influence lipid digestion in dairy matrices (Schmidt et al., 2020), while particle size reduction plays a key role in lipid accessibility in nuts (Swackhamer et al., 2019), highlighting the marked effect of chewing on lipid release (Martínez-Sánchez et al., 2024). In contrast, in mackerel we observed both a relatively high lipid release and FFA formation, consistent with a more accessible lipid organization within the matrix. This is in line with the already observed high lipid bioaccessibility of fish matrices, consistent with their structural characteristics (Costa et al., 2016; García-Arias et al., 2003).

Although these observations support the role of food structure in shaping digestion dynamics beyond compositional data (Forde & Decker, 2022; McClements, 2023), the heterogeneity of the selected foods does not allow mechanistic conclusions. Rather, the results illustrate how both matrix characteristics and analytical definitions contribute to variability in bioaccessibility estimates.

Previous studies have often quantified total FAMEs in the BF after digestion to estimate fatty acid bioaccessibility (Costa et al., 2016; Solomando et al., 2020, 2024; Swackhamer et al., 2019) implicitly assuming complete hydrolysis of triglycerides and other esterified lipids. Although dietary triglycerides are generally extensively digested, the extent and kinetics of fatty acid release may vary depending on matrix organization and digestive conditions, and our results suggest that the assumption of their complete hydrolysis may not hold under all conditions. In most of the tested foods, a substantial proportion of lipids released from the matrix was not recovered as FFAs. This methodological discrepancy may partially contribute to variability in reported bioaccessibility values and supports the relevance of clearly defining the analytical approach used.

The qualitative composition of the BF also differed from that of the corresponding undigested foods. In mackerel, the profile of the FFA fraction closely resembled that of the original matrix. In fish-based matrices, previous *in vitro* digestion studies reported that bioaccessible FA profiles were broadly similar to initial profiles (Gomes et al., 2019). In contrast, in the other foods, an enrichment in SFA was observed, and in walnuts a reduction in PUFAs was detected. Since the bioaccessibility of specific fatty acids may be influenced by their position on

triglycerides and by matrix interactions (Ye et al., 2019), these compositional shifts may be associated with differential fatty acid release during digestion. This underlines that nutritional evaluation based solely on pre-digestion fatty acid profiles may not fully reflect the fraction effectively available for absorption.

Limitations of the study should be acknowledged. The standardized static INFOGEST protocol used here does not include gastric lipase, which may have led to a slight underestimation of lipid hydrolysis. However, in adults, gastric hydrolysis contributes only marginally to overall triglyceride digestion. A further limitation is that lipid bioaccessibility was operationally defined on the basis of FFAs only. Since other absorbable lipid species such as MAGs were not quantified, the reported values should be considered a conservative estimate rather than a complete assessment of lipid bioaccessibility. Although the concentration of MAGs in the micellar fraction is generally low (Costa et al., 2016; Montebugnoli et al., 2024), a more comprehensive assessment would ideally include the quantification of all lipolytic products, whose relative abundance depends on interfacial and enzymatic conditions (Mu & Høy, 2004; Sarkar et al., 2019).

The present study should therefore be interpreted as a methodological contribution aimed at refining the estimation of lipid bioaccessibility. By highlighting how different analytical definitions lead to different quantitative outcomes, this work underscores the need for greater clarity and standardization in the assessment of lipid bioaccessibility. More broadly, the present results indicate that chemical composition alone does not necessarily reflect the fraction of lipids that becomes bioaccessible during digestion. The digestive process modifies food structure, and the composition of the bioaccessible fraction may differ from that of the undigested food. These findings emphasize the importance of complementing compositional data with digestion-based approaches when evaluating the nutritional relevance of dietary lipids.

5. Conclusions

The present study shows that lipid bioaccessibility estimates strongly depend on the analytical approach used. Selective quantification of FFAs provides a more conservative operational estimate compared to conventional total FAME-based methods, which may overestimate the fraction of lipids present as FFAs after digestion.

These findings highlight the importance of clearly defining and reporting the analytical approach used to assess lipid bioaccessibility. More broadly, they support the need for greater methodological clarity and standardization in digestion studies to enable meaningful comparison across different food matrices. In addition, the results indicate that chemical composition alone does not fully reflect the fraction of lipids that becomes bioaccessible during digestion, emphasizing the importance of integrating digestion-based metrics in food evaluation.

CRediT authorship contribution statement

Nicholas S. Rivera: Writing – review & editing, Validation, Methodology, Formal analysis. **Thomas Montebugnoli:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **Alessandra Bordoni:** Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alessandra Bordoni reports financial support was provided by Italian Ministry of University and Research. If there are other authors, they 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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2026.109303.

Data availability

The original data presented in the study are openly available in Zenodo at: https://doi.org/10.5281/zenodo.20393533

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