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Effect of addition of rich sources of lipids on the bioaccessibility of lutein and β-carotene from raw baby spinach leaves

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RESEARCH ARTICLE

Abstract
The objective of the present study was to investigate the effect of the addition of lipids with different origin and unsaturation degree on the bioaccessibility of major carotenoids from raw baby spinach leaves. The standardized INFOGEST protocol of static in vitro digestion was applied to determine the bioaccessibility of lutein and β-carotene from baby spinach, before and after the addition of avocado pulp (5% and 10%) and sour cream (10% and 20%), as lipid sources. Baby spinach leaves, avocado fruits and sour cream were characterized in terms of carotenoid composition, lipid content and fatty acids profile by C30-HPLC-DAD and GC-MS. The bioaccessibility of lutein was lower (7.1%) than that of β-carotene (9.05%) from unsupplemented baby spinach leaves. Addition of avocado and sour cream determined a significant decrease of lutein bioaccessibility, especially in the case of high concentration of sour cream (20%). Oppositely, the bioaccessibility of β-carotene was increased by the addition of both lipid sources, at both concentrations, with a significant improvement for 10% sour cream, from 9.05% to 13.28%. Generally, the highest amount of lipid did not result in better bioaccessibility of carotenoids.

Keywords: lutein, β-carotene, bioaccessibility, fatty acids, INFOGEST

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INTRODUCTION
Over the time, it has been well documented that regular consumption of fruits and vegetables, especially those rich in fat-soluble bioactive compounds, contributes to human wellbeing and the prevention of numerous diseases, such as macular degeneration, cataract formation, cancer or cardiovascular diseases (Bohn et al. 2021). Carotenoids are a class of natural, fat-soluble pigments, responsible for the yellow, orange, and red colors of fruits, vegetables or animal products (Rodriguez-Concepcion et al. 2018). Even though there are over 750 carotenoids described, only 40 are commonly found in the human diet and the most important are lutein, lycopene, zeaxanthin, cryptoxanthin, α- and β-carotene (Bergantin et al. 2018). Lutein and zeaxanthin can be found naturally in the macula and have been shown to play an important role in the protection against the development of age-related macular degeneration and cataract formation, while β-carotene, α-carotene and β-cryptoxanthin are the most important vitamin A precursors in the human diet (Chung et al. 2019; Courraud et al. 2013). The most common sources of lutein and β-carotene in human diet are dark green leafy vegetables, and among them, the consumption of spinach showed both an increased concentration of lutein in plasma and an increase in macular pigment optical density (Rodriguez-Concepcion et al., 2018; Bohn et al., 2021).

Several mechanisms are involved in these beneficial effects, but the absorption route is identical for carotenoids and involves their release from the food matrix followed by the incorporation into the lipid fraction, formation of the mixed micelles and intestinal absorption (Kopec and Failla, 2018). The release of these compounds depends on several factors such as the chemical nature (polarity) of carotenoids, their deposition form in the food matrix, the composition of the food matrix and the degree of processing (Schweiggert and Carle 2017; Odorissi Xavier and Zerlotti Mercadante 2019).

The absorption of these carotenoids from ingested foods is sometimes inefficient and, in this direction, intensive research has been conducted. Nowadays, bioavailability and bioaccessibility studies are very important, as they are the first steps that must be taken into account before characterizing a compound's beneficial activity and properties. The term bioaccessibility represents the fraction of a fat-soluble compound which is transferred from the matrix into mixed micelles during digestion, while bioavailability generally indicates how much of a consumed nutrient or dietary constituent is accessible for utilization in physiological conditions, metabolism, or storage (Kopec and Failla 2018). In this regard, for carotenoid compounds it is estimated that only 5% to 50% are absorbed into micelles and reach the small intestine and circulatory system (Bohn et al. 2021). The different bioaccessibility percentages are generated by a sum of factors related to the food matrix and to the host. For example, lutein - a xanthophyll (more polar) has better absorption than lycopene - a carotene. The food matrix-related factors are, in general, linked to the initial processing of the matrix (heat treatment, lyophilization, physical or chemical treatments). Additionally, the presence of certain amounts of carotenoids and lipids and a smaller amount of dietary fibers and minerals can lead to better bioavailability (Bohn et al. 2021).

Among all the dietary factors that can influence the bioaccessibility of carotenoids, one of the most important is the lipid addition. Lipids are mandatory for the transfer of carotenoids from food matrix into lipid droplets, for micellization and for the intestinal absorption. The consumption of dietary lipids together with carotenoids has been helpful to promote carotenoid bioaccessibility and in vivo absorption. During digestion, the lipids present in the food matrix can facilitate the extraction and absorption of carotenoids because they represent the hydrophobic environment necessary for their better solubilization. The presence of lipids can also increase chylomicron and biliary secretion, leading to better micelle production (Desmarchelier and Borel, 2017).

In this study, we aimed to investigate the effect of both the amount of added lipids and the type of lipid on the bioaccessibility of the major carotenoids from baby spinach. In this regard, two types of lipid sources were chosen, namely avocado - a plant lipid source rich in monounsaturated fatty acids, and sour cream - an animal lipid source rich in saturated fatty acids. Moreover, prior to the in vitro digestion, all the food samples were characterized in terms of carotenoid and lipid compositions.

MATERIALS AND METHODS

Materials

Fresh baby spinach (Spinacia oleracea), fresh avocado (Persea americana, Cv. Hass), and sour cream were purchased from a local supermarket (Cluj-Napoca, Cluj, Romania). The bovine bile extract (B3883) and the enzymes used for the in vitro digestion were purchased from Sigma-Aldrich (Steinheim, Germany): α-Amylase from human saliva (A1031), pepsin from porcine gastric mucosa (P6887), pancreatin from porcine pancreas (P7545). Carotenoid standards of β-carotene and lutein were acquired from Extrasynthese (Lyon, France). All chemicals and reagents were of analytical or HPLC grade and ultrapure water (Milli-Q water purification system) was used throughout the experiments.

Methods

Samples preparation

Baby spinach was washed and cut into small pieces, to replicate mastication. The avocado pulp was smashed and homogenized using a mortar and a pestle. The samples were subjected to chromatographic analysis and in vitro digestion immediately after sample preparation.

Carotenoid extraction from fresh samples

Carotenoid extraction was conducted using the following protocol. Fresh samples of baby spinach (Spinacia oleracea) (3 g), 5g of fresh avocado (Persea americana, Cv. Hass), and 10 g of sour cream were subjected to extraction repeatedly using a mixture of methanol: ethyl acetate: petroleum ether (1:1:1, v/v/v) (Breithaupt and Schwack, 2000). The volume of the extraction mixture was 25 ml and the extraction time was 30 min for each step. The extraction was repeated three times, until the residue of baby spinach and avocado was colorless. For baby spinach and avocado, the extracts were combined, filtered, transferred into a separation funnel, and then washed with diethyl ether and water. Further, the ether phase (containing extracted carotenoids) was filtered through anhydrous sodium sulfate and evaporated to dryness, using a rotatory evaporator, at 35°C. The extraction of

carotenoids from sour cream was conducted using the same solvent mixture in a centrifuge tube. The procedure was repeated until the upper organic phase became colorless. The upper phases were filtered through anhydrous sodium sulfate and evaporated to dryness, to a rotary evaporator at 35°C. Sour cream extract and avocado extract were dissolved in diethyl ether (25 ml), and were saponified with 25 mL of 30% potassium hydroxide in methanol, at room temperature, for three hours. The mixture was washed with a saturated solution of sodium chloride and distilled water until alkali free and then evaporated to dryness. The residue was dissolved in methyl-tert-butyl-ether (MTBE), filtered through 0.22 μm PTFE filters in amber vials, and analyzed by HPLC.

Lipid extraction from fresh samples

Total lipids were extracted from 5g of fresh samples (baby spinach, avocado, sour cream) using chloroform/methanol (2:1, v/v) and quantified gravimetrically, as described by Folch et al. (1956).

Fatty acids analysis by GC-MS

The fatty acid composition of the samples was analyzed as fatty acid methyl esters (FAMEs) of the total lipids, produced by acid-catalyzed transesterification using 1% sulphuric acid in methanol (Christie 1989; Dulf et al. 2020). The samples were analyzed using a gas chromatograph (GC) coupled to a mass spectrometer (MS) (PerkinElmer Clarus 600 T GC-MS; PerkinElmer, Inc., Shelton, CT, U.S.A.) as previously described (Tudor et al. 2021). The samples (0.5μL) were injected into a 60m×0.25mm i.d., 0.25μm film thickness SUPELCOWAX 10 capillary column (Supelco Inc.). The operation conditions were the following: injector temperature 210 °C; helium carrier gas flow rate 0.8 ml/min; split ratio 1:24; oven temperature 140 °C (hold 2 min) to 220 °C at 7°C/min (hold 23 min); electron impact ionization voltage 70 eV; trap current 100 μA; ion source temperature 150°C; mass range 22−395 m/z (0.14 scans/s with an intermediate time of 0.02 s between the scans). The FAMEs identification was conducted by comparing their retention times with those of known standards (37 components FAME Mix, Supelco no. 47885-U), and the resulting mass spectra to those in the database (NIST MS Search 2.0). The amount of each fatty acid was expressed as area percentages calculated from the total area of identified FAMEs.

HPLC-DAD analysis of carotenoids from starting materials and from micellar fraction

Carotenoids were analyzed using an HPLC system (Shimadzu Corporation, Kyoto, Japan), equipped with a SPDM20A diode array detector and a YMC C30 reversed-phase column (250 × 4.6 mm i.d., 5 μm particle size). Carotenoids were separated using gradient elution with methanol/ methyl-tert-butyl-ether/water (83:15:2, v/v/v) as solvent A, and methyl-tert-butyl-ether /methanol/water (90:8:2, v/v/v) as solvent B. The gradient was: 0.01 min - 1% solvent B, 50 min - 55% B; 51 min - 60% B; 52 min - 60% B, 54.01 min - 60% B, 56 min - 1% B, 70 min - 1% B, followed by the column equilibration for 30 min. The flow rate was 0.8 mL/min and the injection volume was 30 μl. Carotenoids were identified based on their retention times and absorption spectra, compared with those of available standards and literature data. Quantification of β-carotene and lutein was performed using external eight-point calibration curves constructed in the range 1-100 μg/mL. The regression equations were: y = 481115x – 43279, correlation coefficient: R^2 = 0.9985 for β-carotene, and respectively y = 415811x - 17763 and R^2 = 0.9996, for lutein. The other compounds in food samples were determined (semi-quantification) using either the curve for lutein, either that of β-carotene.

Simulated in vitro digestion model

The in vitro gastrointestinal digestion simulation was performed according to the INFOGEST method, described by Minekus et al. (2014). The in vitro digestion was performed on baby spinach samples without the addition of lipid sources (Simple Digestion, SD) and with the addition of lipids from avocado (5% and 10%) or sour cream (10% or 20%). The samples were subjected to an in vitro digestion protocol consisting of three phases: oral, gastric, and small intestinal phase.

Oral phase: 3 g of baby spinach with/without lipid addition, were mixed with 1.9 mL of simulated salivary fluid (SSF), 150 μL CaCl2 (0.03 M), 0.5 mL α-amylase in SSF (75 U/mL in final volume) and the volume was adjusted with ultrapure water, in order to achieve 6 mL final volume. The mixture was homogenized, using a vortex, for one minute, and then was incubated for 2 minutes, at 37°C, in a shaking water bath (Memmert GmbH + Co. KG, Schwabach, Germany).

Gastric phase: to the bolus obtained after the oral phase, were added 3.8 mL simulated gastric fluid (SGF), 30 μL CaCl2 (0.03 M), and 1 mL of porcine pepsin in SGF (2000 U/mL in final volume). The pH was adjusted to 3.0 with HCl (1 M) and the final volume was adjusted to 12 mL by adding ultrapure water. The mixture was vortexed for one minute, and after that, incubated for 2 hours at 37°C, in a shaking water bath, at 150 orbital shakes/min.

Small Intestinal phase: to the gastric chyme were added 5.6 mL simulated intestinal fluid (SIF), 240 μL CaCl2 (0.03 M), 2 mL of pancreatin in SIF (based on the lipase activity, 2000U/mL in the final volume), 2 mL bile salts in SIF (10 mM in final volume), and the pH was adjusted to 7 by adding NaOH (1M). The mixture was homogenized using a

vortex and ultrapure water was added in order to obtain a 24 mL final volume. The samples were incubated for 2 hours, at 37°C, in a shaking water bath, at 150 orbital shakes/min.

The digesta was centrifuged for 90 minutes at 4°C (10000 rpm; Eppendorf 5810 R, Hamburg, Germany) to obtain the micellar phase. Further, the carotenoid extraction was conducted using the following protocol: an aliquot of the micellar phase was mixed with hexane: acetone mixture (1:2, v/v) and centrifuged for 5 minutes (5000 rpm). After the centrifugation, the upper phase was collected and the digesta was subjected to two more extractions using as extraction solvent only hexane. The ratio between the micellar phase and the extraction solvent was 1:2. The final extract was filtered (0.22 μm PTFE filter), evaporated to dryness, and stored at -80°C until HPLC analysis.
All the experiments were performed in triplicate.

Carotenoid bioaccessibility

Carotenoid bioaccessibility (%) represents the percentage between the concentration of carotenoids in the micellar phase and the concentration of carotenoids found in the analyzed samples, and was calculated using the following formula:

$$\text{Bioaccessibility (\%)} = \frac{\text{Total carotenoid content in micelles}}{\text{Total initial carotenoid content}} \text{ x } 100$$

Where total carotenoid content in micelles refers to the amount of carotenoids extracted after digestion and total initial carotenoid content refers to the amount of carotenoids found in raw samples before digestion (Tudor et al. 2021). The results represent the average ± SD of three replicates.

Statistical analysis

All the analyses were performed in triplicate and the results were expressed as the mean ± standard deviation (SD). For the bioaccessibility study the statistical analysis was performed with the software GraphPad Prism version 6.07. Data represents the arithmetic mean and standard deviation (SD) of 3 different experiments. Statistically significant differences were determined by Ordinary one-way ANOVA Multiple Tukey comparison test and noted with different symbols to indicate a significant difference between the simple digesta (without lipid addition) and the digesta with avocado pulp and sour cream addition (extremely significant****; p = 0.0001 to 0.001, extremely significant ***; p = 0.001 to 0.01, very significant**; p = 0.01 to 0.05, non-significant – ns *) or to compare digesta with avocado pulp 5% with 10%, respectively digesta with sour cream 10% with 20% (extremely significant ###; p = 0.001 to 0.01, significant #; p ≥ 0.05, non-significant – ns).

RESULTS AND DISCUSSIONS

Carotenoid composition of baby spinach, avocado and sour cream

The carotenoid content for every raw sample, namely baby spinach leaves, avocado fruit, and sour cream was determined first using HPLC-PDA (Figure 1). It is known that dark green leafy vegetables, and especially spinach, are rich sources of lutein and β-carotene (Dias et al. 2021). Hass avocado has a high content of xanthophylls, with lutein and β-cryptoxanthin as predominant compounds (Dreher and Davenport 2013). Carotenoids are present in small amounts in milk and dairy products but they contribute to their sensorial properties. β-carotene is the major contributor to the carotenoid fraction in milk (90%), but lutein, zeaxanthin and β-cryptoxanthin were also reported, depending on various factors, mainly on dietary factors (Meléndez-Martínez et al. 2022).

Analyzing the carotenoid content for baby spinach leaves, we observed that lutein was the major carotenoid (8407.4 ± 143.1 μg/100g F.W.), followed by all-trans-β-carotene (2848.8 ± 40.7 μg/100g F.W.). Other carotenoids were neoxanthin, violaxanthin and cis-β-carotene, while α-carotene was present only in traces. This profile is consistent with the well-known compositions of plant tissues (Britton et al. 1995). Similar to our findings were the results of Biehler et al. (2011) who reported values between 3400±20 μg/100g F.W. and 6000 μg/100g F.W. for lutein, and between 3400±20 μg/100g F.W. and 5900 μg/100g F.W. for β-carotene. Eriksen et al. (2017) adapted an in vitro protocol for determination of liberation and bioaccessibility of carotenoids from differently processed spinach and found the lutein and β-carotene content from raw spinach to be 6000±11 μg/100g F.W. and 1108±11 μg/100g F.W. respectively. Another study evaluating the intake of carotenoids in Luxemburg meals, reported a content of 4866±387 μg/100g F.W. lutein for spinach (Biehler et al. 2012). Even though spinach is one of the most important sources of lutein and β-carotene, it is still not very clear how different preparation conditions and procedures can affect lutein liberation from food. There were many effects reported depending on thermal treatment, preparation methods, and the complexity of the food matrix, and those factors can contribute to different concentrations of lutein and β-carotene in baby spinach leaves extracts (Eriksen et al., 2016; Eriksen et al., 2017; Chung et al. 2019;).

Avocado is one of the most appreciated and consumed fruits among the tropical and subtropical varieties, due to its chemical composition and sensorial properties. It has high nutritional value especially because of its fiber and

lipid composition, but also because of its low sugar content (Galvão et al. 2014). Avocado pulp has important quantities of proteins, carbohydrates, dietary fibers, vitamins, and minerals, but the most important feature is its high content of unsaturated fatty acids (>70%).

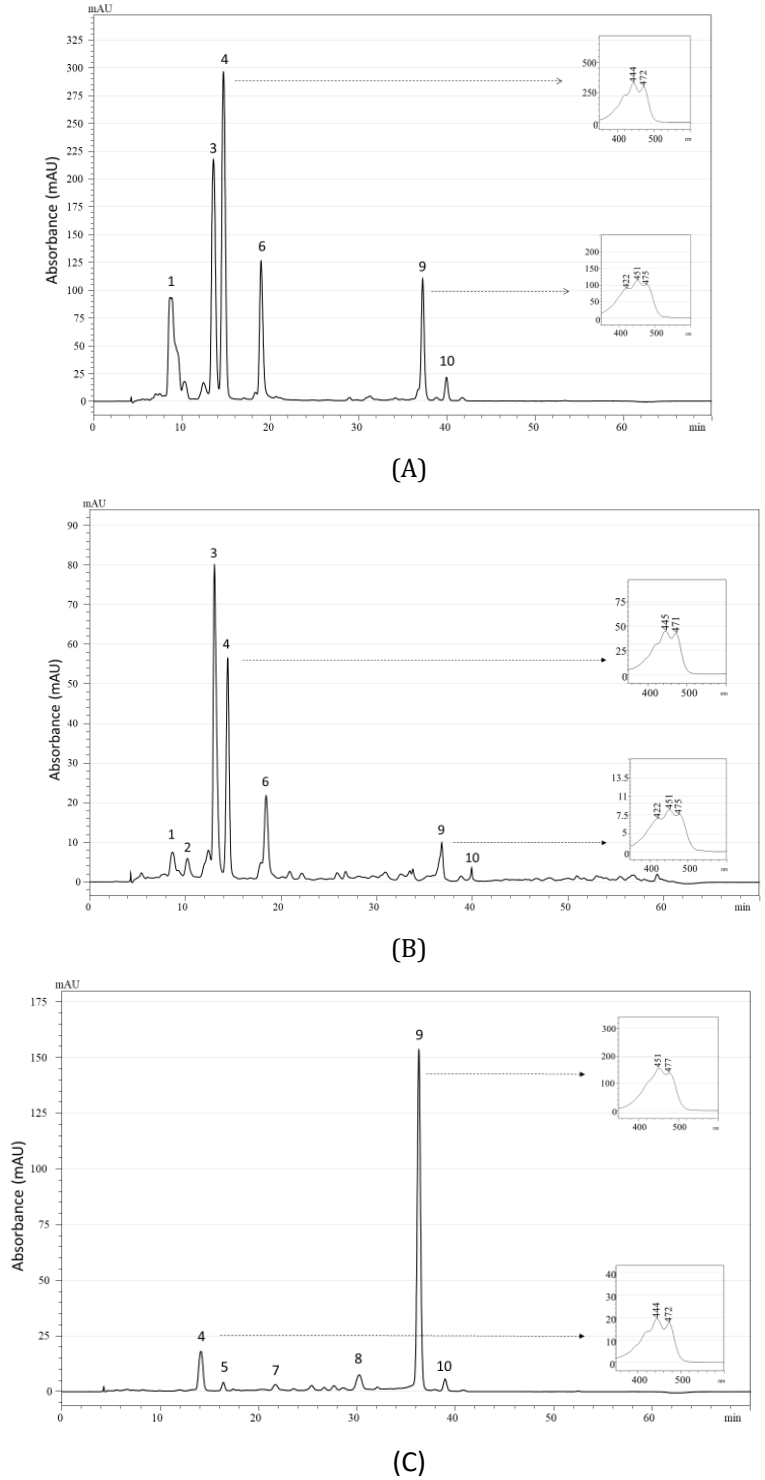

Figure 1. HPLC-DAD chromatogram of carotenoids from baby spinach leaves (A), avocado pulp (B) and sour cream (C). Peak identification: 1-neoxanthin+violaxanthin; 2-cis-lutein; 3-chlorophyll a; 4-all-trans-lutein; 5-zeaxanthin; 6-chlorophyll b; 7-β-cryptoxanthin; 8-cis-β-carotene; 9-all-trans-β-carotene; 10-cis-β-carotene.

Along with the major components, avocado also contains carotenoids, alkaloids, phytosterols, and tocopherols (Salazar-Lopez et al. 2020). In our study, carotenoid extraction was carried out from the whole pulp. All three

sections of the pulp (yellow, pale green, and dark green pulp) were mixed together and the mixture was subjected to carotenoid extraction and analysis. The total carotenoid content was 182.04 ± 12 μg/100g F.W. Lutein and β-carotene were the carotenoids of interest in our avocado samples, reaching the concentration of 102.3 ± 2.7 μg/100g F.W. for lutein and 33.7 ± 0.8 μg/100g F.W for β-carotene. Several studies have shown that lutein is the most abundant carotenoid in the dark green, pale green and yellow pulp avocado sections (Lu et al. 2009; Cervantes-Paz et al. 2020; Ramos-Aguilar et al. 2021). Comparing the phytochemical and nutritional composition of creole avocado and Haas avocado, Ramos-Aguilar et al. (2021) obtained similar concentrations of lutein in the pulp of both types of fruits and the reported value for Hass avocado lutein content was 8.5 μg/g. This value is higher than our value but it must be taken into account that it was expressed on dry weight basis. A much higher content of lutein can be explained by the ripening and harvesting processes, knowing that the presence of ethylene can activate metabolic reactions such as carotenoid synthesis. The accumulation of carotenoids in Californian Haas avocado is influenced by the geographic origin and by the season, being the highest in fruits harvested in September, with lutein as major pigment from 6-8.42 μg/g (Lu et al. 2009). Cervantes-Paz et al. (2020) observed a sharp decrease for all carotenoids during ripening of Hass avocado fruits, e.g., for lutein from 78 μg/ml oil at day 0 to 0.4 μg/ml after 16 days.

Among other ways of consumption, spinach leaves can also be eaten together with sour cream, raw or cooked. This association might have beneficial effects on the release and bioaccessibility of carotenoids from the baby spinach leaves. For our analyzed samples, the total carotenoid content was 74.5±28.5 μg/100g F.W. and the major carotenoid was β-carotene (44.5±0.7 μg/100g F.W.), but also, very small amounts of lutein were found (4.7±0.5 μg/100g F.W.). There are few studies about the carotenoid content of sour cream, but Biehler et al. (2012) reported a total carotenoid content of 0.03 mg/100g product with 30 % fat. As the carotenoid content of sour cream is very low, it does not influence the total carotenoid content, but due to its lipid composition, it can influence the liberation and the bioaccessibility of carotenoids from baby spinach leaves.

Baby spinach leaves were chosen as the main food matrix because of their high content of lutein and β-carotene, the two major carotenoids that we investigated, and also because spinach is widely consumed all over the world, both as raw and processed food. At the same time, even though spinach is a rich source of carotenoids, numerous studies have shown that the bioaccessibility of those carotenoids was very low when consumed raw. Because it is known that the addition of a lipid source can increase carotenoid bioavailability and bioaccessibility, we chose two different lipid sources. Avocado was selected because it is a widely consumed fruit all over the world, with a high content of healthy fatty acids. Sour cream is a dairy product, a source of animal origin lipids, which is often associated with the cooking of baby spinach. The total carotenoid content and the major carotenoids for all matrices is presented in Table 1.

Table 1. Carotenoid content (μg /100g) * of fresh samples

Sample	β-carotene	Lutein	Total carotenoids
Baby spinach	2848.8 ± 40.7	8407.4 ± 143.1	16104.4 ± 291
Avocado	33.7± 0.8	102.3 ± 2.7	182.04 ±12
Sour cream	44.5 ± 0.7	4.7 ± 0.5	75.1±3

*Mean ± SD (n = 3)

Fatty acids profile and total lipids in baby spinach, avocado and sour cream

Although lipids constitute a minor fraction of baby spinach leaves, they contain, as all photosynthetic tissue, a high proportion of polyunsaturated fatty acids. In the analyzed samples, the total lipid represented 0.25 g/100g F.W. The major fatty acids were α-linolenic (84.1%) and palmitic acid (11%) and small amounts of linoleic, oleic, and stearic acids were also identified. Polyunsaturated fatty acids (PUFA) represented the major class, with more than 88% of total fatty acids, while saturated fatty acids (SFA) were 11%. Even though the fatty acid composition of baby spinach is not as well documented, it is known that the lipid fraction of spinach contains mainly unsaturated fatty acids, such as α-linolenic acid, oleic acid, linoleic acid, and only a small percentage of saturated fatty acids (capric, myristic, stearic acid, etc.) (Elvira-Torales et al. 2019). Avocado samples had a total lipid content of 10.8 g/100g lipids and the most important acid was oleic acid (52.8%). In addition to oleic acid, were also identified palmitic (23.7%) and linoleic acids (11.7%). In much smaller quantities were found palmitoleic and vaccenic acids, which also belong to monounsaturated fatty acids (MUFA). Avocado is an important source of MUFA (63.3%) but with lower proportion of polyunsaturated fatty acids.

Saturated fatty acid values are higher than of the baby spinach, reaching 24%. According to the literature data, the lipid composition is very similar in the lipids extracted from fresh fruit pulp to the oil obtained from the fruit pulp, but the composition can slightly change during the ripening process. Even in this situation, avocado remains a valuable source of monounsaturated fatty acids (72.8%) with low contents of polyunsaturated fatty acids (17.8%)

and saturated fatty acids (17.8%). The most abundant fatty acid remains oleic acid with values between 53-60%, depending on the ripening stage, followed by palmitic (15-17%), linoleic (11-17%), and also small quantities of linolenic, stearic, and myristic acids (Lu et al. 2009; Cervantes-Paz et al. 2020).

Among all the analyzed samples, the one with the highest content of fat was sour cream, with a total of 24.5 g lipids/100 g product. Sour cream is a matrix rich in saturated fatty acids, with a total content of over 77%, compared to unsaturated fatty acids, namely MUFA and PUFA, which represent 22%, and respectively, below 1%. The representative fatty acids found in sour cream samples were palmitic (36%), stearic (15%) myristic (13%), as well as small quantities of medium and short chain saturated fatty acids (SMCFA), C4-C12. The only unsaturated fatty acid present in significant amount was oleic acid (22%). This profile is in line with literature data on the composition of dairy products and sour cream (Jensen 2002; Izso et al. 2020). The total lipid contents and the fatty acid profiles of the analyzed samples are presented in Table 2.

Table 2. Fatty acid profiles of investigated matrices (% of total fatty acids)

Fatty acids composition (% of total) *	Baby spinach	Avocado	Sour cream
Butyric acid (4:0)	nd	nd	0.88 ± 0.09
Caproic acid (6:0)	nd	nd	1.53 ± 0.10
Caprylic acid (8:0)	nd	nd	1.53 ± 0.10
Capric acid (10:0)	nd	nd	4.18 ± 0.39
Lauric acid (12:0)	nd	nd	4.19 ± 0.35
Myristic acid (14:0)	nd	nd	13.29 ± 0.32
Pentadecanoic acid (15:0)	nd	nd	0.18 ± 0.07
Palmitic acid (16:0)	10.92 ± 0.41	23.72 ± 0.25	36.09 ± 0.54
Palmitoleic acid (16:1 n-9)	nd	6.46 ± 0.52	nd
Stearic acid (18:0)	0.22 ± 0.06	0.50 ± 0.11	15.47 ± 0.21
Oleic acid (18:1 n-9)	0.56 ± 0.14	52.83 ± 1.31	21.93 ± 0.34
Vaccenic acid (18:1 n-7)	nd	4.04 ± 0.37	nd
Linoleic acid (18:2 n-6)	4.19 ± 0.31	11.67 ± 0.39	0.38 ± 0.10
Linolenic acid (18:3 n-3)	84.10 ± 2.82	0.71 ± 0.10	0.35 ± 0.11
Arachidic acid (20:0)	nd	0.07 ± 0.03	nd
Behenic acid (22:0)	nd	nd	nd
Σ SFA	11.14 ± 0.44	24.29 ± 0.26	77.34 ± 1.74
Σ MUFA	0.56 ± 0.14	63.32 ± 2.60	21.93 ± 0.66
Σ PUFA	88.3 ± 3.24	12.38 ± 0.42	0.73 ± 0.13
Σ SMCFA	0.00	0.00	12.31
Σ LCFA	100	100	87.69
PUFAs/SFAs	7.93	0.50	0.01
Total lipid content g/100 g F.W.	0.25 ± 0.03	10.8 ± 1.02	24.5 ± 2.12

*Mean ± SD (n = 3); ND = Not Detected; SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids; SMCFA – short and medium chain fatty acids (C4-C12); LCFA – long chain fatty acids (C14-C22)

Bioaccessibility of lutein and β-carotene from baby spinach without and with lipid addition

In order to exert their beneficial effects, carotenoids must be released from the matrix and absorbed into the mixed micelles. Although many sources' rich in carotenoids have been identified, several studies underlined their reduced bioavailability, which ranged between 5 to 30% (Odorissi Xavier and Zerlotti Mercadante 2019). In this regard, there are numerous studies that aim to identify the major factors and conditions which influence the absorption of carotenoids. Different strategies have been developed to improve the bioavailability of carotenoids, of which the addition of lipids is of particular interest due to the lipophilic nature of carotenoids. The studies that investigated the influence of lipid addition on the micellization of carotenoids have shown that both the type of the fatty acids found in the matrix (saturated or unsaturated fatty acids) and the quantities of the added lipids can influence carotenoid bioaccessibility (Goltz et al. 2012). Furthermore, some research has emphasized the fact that

the addition of a lipid matrix rich in monounsaturated fatty acids leads to better absorption of carotenoids compared to matrices rich in polyunsaturated fatty acids (de Abreu-Martins et al. 2020).

The aim of this study was to determine whether the addition of two different types and amounts of lipids can impact the bioaccessibility of lutein and β-carotene from baby spinach. Therefore, we studied the bioaccessibility of the two carotenoids with the addition of 5% and 10% avocado pulp, and respectively, 10% and 20% of sour cream. In order to obtain reliable results and to be able to compare them with the existing literature in the field, we have chosen to apply the standardized INFOGEST protocol (Minekus et al. 2014).

The lutein micellization percentage for the control sample (baby spinach leaves without lipid addition) subjected to in vitro digestion was 7.1%. Previous studies reported the lutein micellization to be ranging from 4.48% to 22.55% for the spring season, and from 9.39% to 30.59% for the fall season. The differences were generated by multiple factors such as spinach genotype, harvesting methods and conditions (temperature, soil, etc.), storage and processing methods (Hayes et al. 2021). In addition to these factors, when the obtained results are analyzed, the in vitro digestion protocol used must also be taken into account. There are several parameters that can generate different results, among them the origin of the used enzyme (rabbit, porcine, human), as the enzymatic activity is different depending on the source of the enzyme. The pH in different stages of the digestion, the minerals used or the presence of other compounds (phospholipids, individual enzymes, etc.) and the type of bile salts can also influence the results (Minekus et al. 2014).

The addition of avocado pulp to baby spinach leaves resulted in a significant reduction of lutein bioaccessibility, regardless of the used concentration (5% and 10%). The micellization percentage was 3.56% for 5% pulp respectively 4.54% for 10% avocado pulp addition (Figure 2-A).

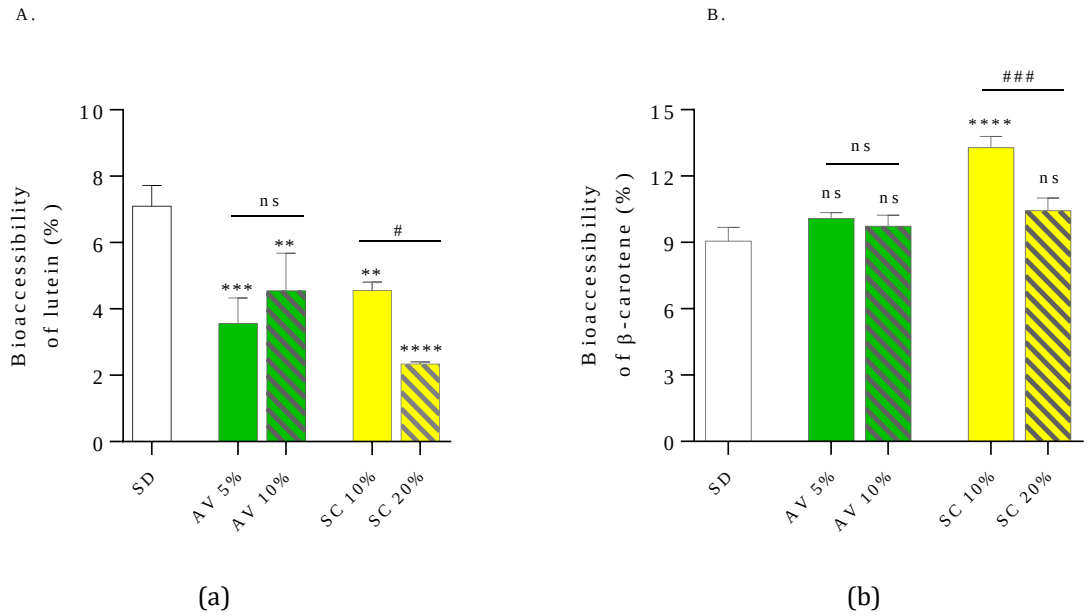

Figure 2. Bioaccessibility of lutein (A) and β-carotene (B) - different simbols indicate a significant difference between the simple digesta and the digesta with avocado pulp and sour cream determined by Ordinary one-way ANOVA Multiple Tukey comparison test: extremely significant****; p = 0.0001 to 0.001, extremely significant ***; p = 0.001 to 0.01, very significant**; p = 0.01 to 0.05, non-significant – ns; to compare digesta with avocado pulp 5% with 10%, respectively digesta with sour cream 5% with 10% -different simbols were used to indicated the significant difference by Ordinary one-way ANOVA Multiple Tukey comparison test: extremely significant ###; p = 0.001 to 0.01, significant #; p ≥ 0.05, non-significant – ns. (SD- simple digesta; AV 5% - digesta with avocado pulp 5%; AV 10% - digesta with avocado pulp 10%; SC 5% - digesta with sour cream 5%; SC 10% - digesta with sour cream 10%).

Although the addition of 10% avocado led to a higher bioaccessibility compared to the 5% addition, the differences were not statistically significant. In the case of the addition of 10% sour cream to the baby spinach leaves subjected to digestion, the lutein micellization decreased to 4.55%. Against our expectations, the increase in the percentage of cream from 10% to 20% led to an even greater and significant decrease in the bioaccessibility, its value becoming 3 times lower than that of the control sample. As far as we know, the effect of the addition of sour cream on the bioaccessibility of carotenoids from the spinach has not been studied. In their study, Corte-Real et al. (2018) investigated the way magnesium ions can affect the bioaccessibility of lutein and β-carotene from frozen

spinach, depending on the intestinal bile and pancreatic enzyme concentrations, and on the type of lipid (canola oil and coffee creamer with 10% fat). The bioaccessibility of lutein was very low, ranging between 0.06 and 3.68% in the absence of magnesium ions, with the highest value for coffee creamer addition and 8mM bile. Odorissi Xavier et al. (2014) studied the effect of fat content from whole and semi-skimmed milk and their corresponding yogurts, on the bioaccessibility of lutein esters added to dairy products, and the results also showed an improvement of the lutein bioaccessibility. However, esterified lutein is much more hydrophobic than free (unesterified) lutien, which can explain the different tendency.

Comparing the effect of the addition of sour cream and avocado on lutein bioaccessibility, according to their fatty acid compositions, we can conclude that a higher content of saturated fatty acids leads to a higher percentage of micellization than a high content of unsaturated fatty acids.

Regarding β-carotene, all the tested lipid matrices showed positive results, determining an enhancement of micellization, for all tested concentrations (5%, 10%, and 20%). After the in vitro digestion, the β-carotene bioaccessibility for the control sample was 9.05 %. Addition of avocado at 5% determined an increase of β-carotene bioaccessibility to 10.08 %, while in the case of 10% addition the value was slightly lower (9.72%) (Fig. 2-B). Even though the absolute values were higher, they were not statistically significant compared to the control sample. In a human study, the consumption of tomatoes and carrots together with avocado showed a marked increase in the absorption of β-carotene but also on its conversion rate to vitamin A (Kopec et al. 2014). Nevertheless, better results were observed after the addition of sour cream. At 10% sour cream, the micellization of β-carotene increased to 13.28% and at 20%, the bioaccessibility was higher (10.42%) than for the non-supplemented spinach, but lower than that recorded for 10% sour cream. Even though the initial lutein concentration was almost three times higher than that of the β-carotene, the concentrations of the two carotenoids in the micellar fraction were much closer, e.g., the equivalent of 381 μg/100g for lutein and 276 μg/100g for β-carotene, when 10% of avocado pulp was added. Moreover, for 10% sour cream addition, the corresponding concentrations were 382 μg/100g for lutein and 378 μg/100g for β-carotene. These changes can be attributed to an increased level of added lipids, which can negatively affect the micellization of lutein. It was previously shown that lutein can reach saturation with only 1% lipids and, the fact that we used concentrations of 5%, 10%, and 20%, could lead to an oversaturation of the micelles, negatively affecting the bioaccessibility (Mashurabad et al. 2017).

Schweiggert et al. (2012) found significant differences on the bioaccessibility of β-carotene from various sources (mango>papaya>tomato>carrots), depending on the type of chromoplasts. Moreover, the addition of sunflower oil (0.5; 1; 2.5%) slightly, although not significantly, increased the relative bioaccessibility of β-carotene from all tested food, without changing the above-mentioned hierarchy. In the case of lycopene, which is also a hydrocarbon, the addition of oil (up to 2.5%) increased the bioaccessibility of lycopene from tomato (3.3-fold), while the further increase of oil concentration (5% and 15%) did not change the bioaccessibility. In the same study, the bioaccessibility of lutein from carrots was significantly increased by addition of 1% sunflower oil. This demonstrated that the structure of the carotenoids, the structure of the chromoplast and the amount of oil can all influence the bioaccessibility of carotenoids.

For lutein, the presence of saturated fatty acids showed better efficiency regarding the influence on the micellization process as against the presence of monounsaturated fatty acids, even though both the addition of sour cream and avocado led to a decrease in bioaccessibility, compared to the control sample. After increasing the concentration of avocado pulp, an increase in the bioaccessibility of lutein was observed, but the change was insignificant. However, in the case of the addition of sour cream, an increase in the added concentration led to a very significant negative change in terms of micellization percentage between the two concentrations.

When analyzing the effect of the presence of fatty acids on β-carotene bioaccessibility, we observed the fact that both saturated and unsaturated fatty acids registered positive results on the micellization process, although significant only for 10% sour cream addition. When the lipid matrices used in this study were chosen, an important criterion was the fat content and the fatty acid profile. Hence, the lipid matrices had a very different profile of fatty acids, namely avocado pulp had a high content of monounsaturated fatty acids, and sour cream was a rich source of saturated fatty acids. After a comparison among the two types of fatty acids, better results were obtained, as in the case of lutein, in the presence of saturated fatty acids (sour cream). At the same time, with the increase in the concentration of sour cream from 10% to 20% the bioaccessibility decrease was significant. It should be also noted that sour cream contains around 12% short and medium chain fatty acids, a category which is missing in avocado. As suggested by Yuan et al. (2018), the presence of medium-chain saturated fatty acids could lead to the formation of smaller mixed micelles, with a higher surface, which could promote the incorporation of carotenoids. Additionally, the particular form in which lipids are present in milk, as milk fat globule membrane containing also phospholipids and proteins with emulsifying properties, might explain the better results obtained with sour cream (Jensen 2002; Bezelgues et al. 2009).

The protocol applied in this study used the concentration of bile salts recommended by the INFOGEST protocol, 10 mM. Moreover, the concentration of pancreatin was adjusted in order to have a high lipase activity, appropriate for the addition of lipids. However, we cannot exclude an incomplete hydrolysis of the lipid fraction, which can

influence the micellization process and the overall bioaccessibility of carotenoids. As the concentration of pancreatin cannot be further increased due to solubility issues, another approach would be to use individual enzymes (gastric lipase, intestinal lipase).

CONCLUSIONS

Lutein and β-carotene bioaccessibility were determined from fresh unprocessed baby spinach leaves, without and with addition of two lipid sources. The results obtained in our study suggest that the bioaccessibility of both carotenoids without lipid addition is low (under 10%). A higher bioaccessibility was recorded for β-carotene than for lutein in the experimental conditions used and for this specific matrix. The effect of lipid addition was different on the two investigated pigments. While in the case of lutein the lipid addition had a negative impact for both lipid sources, in the case of β-carotene the addition of lipids had a positive effect, especially when saturated animal origin fat was used, at lower concentration. We can conclude that the lipid addition is more beneficial for the nonpolar carotenes than for the xanthophylls from spinach, regardless the type of lipid. At the same time, the addition of a high amount of lipids (10% avocado, 20% cream) generally had a negative impact on the bioaccessibility of investigated carotenoids. Further studies are needed in order to elucidate the interaction between carotenoids from spinach with other components of the matrix and with the added lipids, including the determination of the extent of the lipolysis and the size of the micelles.

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Conflicts of Interest

The authors declare that they do not have any conflict of interest

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