TITLE: Bioaccessibility and uptake by Caco-2 cells of carotenoids from cereal-based products enriched with butternut squash (Cucurbita moschata L.)

AUTHORS: M. Rošul, N. Đerić, A. Mišan, M. Pojić, O. Šimurina, C. Halimi, M. Nowicki, B. Cvetković, A. Mandić, E. Reboul

Food Chemistry 385 (2022) 132595

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Bioaccessibility and uptake by Caco-2 cells of carotenoids from cereal-based products enriched with butternut squash (*Cucurbita moschata* L.)

Milana Rošul [a], Nataša Đerić [a], Aleksandra Mišan [a], Milica Pojić [a], Olivera Šimurina [a], Charlotte Halimi [b], Marion Nowicki [b], Biljana Cvetković [a], Anamarija Mandić [a], Emmanuelle Reboul [b,*]

[a] University of Novi Sad, Institute of Food Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[b] Aix-Marseille University, INRAE, INSERM, C2VN, Marseille, France

ARTICLE INFO

Keywords:
In vitro digestion
Micelles
Enterocytes
Bioavailability
Pumpkin

ABSTRACT

Enriching cereals-based products with bioactive compounds is a valuable strategy to improve product quality. We studied carotenoid bioaccessibility and intestinal uptake from a pumpkin-enriched porridge, cookies and sponge cakes by using *in vitro* digestion coupled with Caco-2 cell uptake. Among the carotenoids recovered in different products, α-carotene was the most important abundant one. However, lutein displayed a significantly higher bioaccessibility compared to α-carotene and β-carotene in baked products (up to 10.28% compared to 1.22% and 0.88%, respectively). α-Carotene was the only carotenoid recovered in Caco-2 cells after micelle incubation. Cookie micelles led to the highest percentage of α-carotene cell uptake (2.33% and 1.38% for cookies with butter and cookies with vegetable oil, respectively) compared to the other baked products, followed by dry pumpkin puree micelles (1.31%). Overall, our data show that both bioaccessiblity and cell uptake of carotenoids from cereal-based products are variable and highly depend on food formulation and structure.

1. Introduction

Pumpkin (*Cucurbita* spp.) is an important but underutilised vegetable crop cultivated worldwide at around 3 million hectares, yielding >27 thousand million tons. This large cultivation of pumpkin is primarily because of its affordable price, and high nutritional value ascribed to high levels of flavonoids, carotenoids, macro- and micro-elements in flesh (Hosen et al., 2021; Hussain et al., 2021). Among various pumpkin species, *Cucurbita moschata*, also known as "butternut squash" or "butternut pumpkin" is popular with consumers due to its sweetness and flavour (Abbas et al., 2020; Corrigan et al., 2000). The numerous saccharides in pumpkin are not only responsible for its sweet taste, but also for the antidiabetic effects, as protein-bound polysaccharides can increase the level of insulin and decrease blood glucose level (Dar et al., 2017). Furthermore, the high content in bioactive compounds such as carotenoids (α-carotene, β-carotene, lutein, zeaxanthin, violaxanthin, flavoxanthin, luteoxanthin) and polyphenols contribute to pumpkin's antioxidant properties and its health benefits.

The yellow-orange colour of pumpkin mainly originates from β-carotene (0.06 to 7.4 mg/100 g), α-carotene (0.03 to 7.5 mg/100 g), and lutein (from not detected to 17 mg/100 g), with concentrations of the carotenoids differing among varieties (Saini et al., 2015). Such high α- and β-carotene content is important as those carotenoids exhibit both antioxidant capacities and provitamin A activity (Ribeiro et al., 2015).

Vitamin A deficiency impacts up to 500 million women and children globally. It is caused by a poor quality diet and limited food availability. Vitamin A deficiency has a huge negative impact on the health of vulnerable people and wellbeing of the population in the developing countries (Ferruzzi et al., 2020). Cereal-based products are staple food in many low-income and nutritionally at-risk groups (Ferruzzi et al., 2020). Generally, cereals are a poor source of bioavailable carotenoids, and various biofortification and food-to-food fortification strategies have been proposed and implemented over the past 20 years to improve micronutrient density and bioavailability of staple cereal foods (Kruger

* Corresponding author at: Center for CardioVascular and Nutrition Research (C2VN), UMR 1263 INSERM/1260 INRA/Aix-Marseille Université, Faculté de Médecine la Timone, 27 boulevard Jean-Moulin, 13385 Marseille Cedex 5, France.
E-mail address: Emmanuelle.Reboul@univ-amu.fr (E. Reboul).

https://doi.org/10.1016/j.foodchem.2022.132595
Received 5 October 2021; Received in revised form 22 February 2022; Accepted 26 February 2022
Available online 28 February 2022

et al., 2020). Incorporating carotenoid-rich foods, such as pumpkin, into cereals could be a promising concept. Indeed, pumpkin puree can be used as an ingredient in formulations of sweets, beverages, and other products. Pumpkin powder (dried pumpkin puree) is also often used as an ingredient in different types of pastry (Provesi et al., 2011).

Heat and other processing conditions may influence food carotenoid content (Dini et al., 2013). The impact of the bioactive compounds highly depends on their bioaccessibility, defined as the total amount of bioactive compounds available for absorption during digestion. Carotenoid bioaccessibility from a food matrix is evaluated as the quantity of micellarized carotenoids after gastro-duodenal digestion (Reboul et al., 2006). The bioaccessibility of carotenoids depends on both food matrix structure, and food processing level and type (Fernández-García et al., 2012). It has been demonstrated that the bioavailability of carotenoids from the raw plants is relatively low. Ribeiro et al. (2015) estimated that the low efficiency of pumpkin carotenoid micellarization (0.4–3.3% for all-*E*-β-carotene; 0.3–3.9% for α-carotene) was mostly due to incomplete digestion of the vegetable matrix. Cells in pumpkin pulp, where protein-bound carotenoids are stored, display fibrous walls that are hard to break. Processing, especially thermal treatments, can improve the bioavailability of carotenoids from fruits and vegetables by disrupting cellular walls. During cooking or steaming of pumpkin pulp, carotenoids are well preserved and released from their cellular matrix, so carotenoids can later be micellarized into the lipid fractions (Carvalho et al., 2014). Therefore, the initial amount of carotenoids in raw pumpkin is not the main factor affecting carotenoid bioaccessibility. Lipids also promote the bioaccessibility of all dietary lipophilic compounds, and it is established that the co-ingestion of fats and oils enhances the micellarization of carotenoids from commonly consumed fruit- and vegetable-based products (Kopec & Failla, 2018).

Cookies, porridge, and sponge cakes are widely consumed cereal-based products. Enriching those products with carotenoids could improve product taste and appearance (e.g. colour), and could also increase the product functionality (de Souza Mesquita et al., 2020). New formulations containing bioactive compounds will consequently draw consumers' attention.

The aim of this study was to determine the most effective cereal-based product enriched with vacuum-dried pumpkin puree (porridge, cookies, and sponge cakes) regarding carotenoid bioaccessibility. The formulated cereal-based products differed in terms of fat type and fat content. The influence of the different food matrices was evaluated using an *in vitro* digestion model coupled to Caco-2 (TC-7 clone) intestinal cell experiments.

2. Materials and methods

2.1. Chemicals

All-E-β-carotene (>97% pure), lutein (>96% pure), α-carotene (>95% pure), retinyl acetate (>95% pure), sodium taurodeoxycholate, pancreatin (P7545; 8 × USP), $NaHCO_3$, NaCl, KCl, $CaCl_2$-$2H_2O$, K_2HPO_4, HCl, mucine, α-amylase from Bacillus subtilis, glucose, fructose and sucrose were purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France). Boric acid, silver nitrate, sodium thiosulphate, calcium carbonate, Luff-Schoorl Reagent, and acetonitrile were from Sigma-Aldrich (Steinheim, Germany). Sulphuric acid, sodium hydroxide petroleum ether, hydrogen chloride, chloroform, potassium iodide, phenolphthalein, and trichloromethane were of p.a. grade (Lach-Ner, Neratovice, Czech Republic). Ethanol (p.a. grade) was from Zorka Pharma-Hemija (Šabac, Serbia). Other solvents (HPLC grade) were from Carlo-Erba (Peypin, France). Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, non-essential amino-acids, penicillin/streptomycin, trypsin-EDTA (500 mg/L and 200 mg/L, respectively), phosphate-buffered saline (PBS), and Hanks' balanced salt solution (HBSS) were purchased from Life Technologies (Illkirch, France). Fetal bovine serum was from PAA (Vélizy Villacoublay, France).

Table 1
Formulation of cereal-based products enriched in dry pumpkin puree.

Products	Main ingredients (%, w/w)	Production procedure
Porridge	Vacuum dried pumpkin puree (30%) Sugar (26%) Pea protein isolate (20%) Extruded spelt (13%) Pea fibre (6%) Fat (coconut oil-powder form) (5%)	All ingredients were homogenized in a mixer (MR 2l, CHOPIN Technologies, Villeneuve-la-Garenne Cedex, France) during 30 min. The mixture was stored in polyethylene bags until analysis. Before use, porridge was rehydrated (instant porridge: water, 1:2, w/w) and left for up to 5 min.
Cookie with butter	Wheat flour (28%) Vacuum dried pumpkin puree (17%) Fat (butter) (17%) Sugar (16%) Wheat starch (11%) Water (10%) Soy lecithin (0.5%) Baking powder (0.5%)	All ingredients were mixed in a planetary mixer (Conti S.r.l., Bussolengo, Italy) during 10 min, refrigerated for 1 h 4 °C), sheeted to a thickness of 5 mm, and cut-out using cookie cutter to obtain 50 mm diameter circular shape dough pieces. The dough pieces were baked at 180 °C for 10 min in modular electric deck oven (MD/CO/S/B18; MAC-PAN, Thiene, Italy). After cooling to room temperature, the cookies were packed into polyethylene bags, sealed, and stored in airtight containers.
Cookie with vegetable oil	Wheat flour (28%) Vacuum dried pumpkin puree (17%) Fat (sunflower oil) (17%) Sugar (16%) Wheat starch (11%) Water (10%) Soy lecithin (0.5%) Baking powder (0.5%)	
Sponge cake with butter	Fresh whole eggs (20%) Fat (butter) (20%) Sugar (20%) Wheat flour (14%) Wheat starch (14%) Vacuum dried pumpkin puree (12%) Baking powder (0.4%)	The fresh whole eggs and sugar were mixed in the planetary mixer (Conti S.r.l., Bussolengo, Italy) at high speed, for 3 min. The sifted cake flour, wheat starch, and baking powder were gradually poured into the mixer at low speed, for 60 s. Then, the vacuum dried pumpkin puree and fat were poured into the bowl. Ingredients were mixed by hand utensil with a plastic scraper.
Sponge cake with vegetable oil	Fresh whole eggs (20%) Fat (sunflower oil) (20%) Sugar (20%) Wheat flour (14%) Wheat starch (14%) Vacuum dried pumpkin puree (12%) Baking powder (0.4%)	The dough was immediately deposited into silicone cake pans (10x5x3 cm) and baked at 200 °C for 30 min in a preheated modular electric deck oven (MD/CO/S/B18; MAC-PAN, Thiene, Italy). The sponge cakes were allowed to cool for one hour and then removed from the pans. The sponge cakes cooled at room temperature were packed in polypropylene bags, sealed, and stored in airtight containers.

2.2. Pumpkin and cereal-based product preparation

2.2.1. Pumpkin puree preparation and drying

Butternut pumpkins (*Cucurbita moschata* L.) were provided by the Institute of Field and Vegetable Crops, Novi Sad, Serbia. They were cultivated in the southern part of the Pannonian Plain, near Novi Sad. Pumpkins were harvested in October 2018 at full maturity, after 130 days of the growth period.

After washing with cold tap water, pumpkins were peeled, seeds and other inedible parts were removed, and the pulp was cut into cubes (1x1x1 cm). Pumpkin puree was then prepared by steaming the raw pumpkin pulp cubes in an autoclave (Tuttnauer 3870 ELV, Biomedis Laborservice GmbH, Gießen, Germany) at 121 °C, for 15 min, under the

pressure of 250 kPa.

Dried pumpkin puree (*P*1) was prepared in a preheated vacuum drier (BINDER VD 115, BINDER GmbH, Tuttlingen, Germany) in a thin layer on a tray at 60 °C until the moisture content reached 10% determined by rapid moisture analyzer (MB45, Ohaus Europe GmbH, Nänikon Switzerland).

Finally, the vacuum dried pumpkin puree was ground to a granulation below 80 μm using a sample mill (Knifetec 1095, Foss, Hilleroed Denmark), and used as an ingredient in porridge, cookies and sponge cake formulations.

2.2.2. Product formulation

Vacuum dried pumpkin puree was added to all formulations. All other ingredients: sugar (Sunoko, Novi Sad, Serbia), pea protein isolate and pea fiber (Vestkorn A/S, Holstebro, Denmark), extruded spelt (BioLitus, Djurdjevo, Serbia), coconut oil (Connoils, Big Bend, WI, USA), wheat flour (Danubius, Novi Sad, Serbia), butter (Imlek, Belgrade, Serbia), wheat starch (Fidelinka, Subotica, Serbia), soy lecithin (Sojaprotein, Bečej, Serbia), baking powder (dr Oetker, Wittlich, Germany), sunflower oil (Dijamant, Zrenjanin, Serbia), and fresh whole eggs (Vin Farm, Kulpin, Serbia) were purchased at a local store. Cookies and sponge cakes differed in the fat type and since the butternut pumpkin is sweet, less sugar was added to the formulation, compared to traditional cookies and sponge cakes (Table 1).

The pumpkin porridge formulation was directed towards supplementing the highest possible amounts of proteins and vacuum dried pumpkin puree, without compromising the sensory quality of the final product. The textural properties of the newly formulated pumpkin porridge (water absorption capacity, porridge firmness, overall acceptability) were not significantly changed compared to commercial high-protein porridge based on spelt (data not shown). The selection of coconut oil powder was guided by the fact that the instant porridge has a powdery consistency, must be available on the market and should possess health beneficial effects. The popularity of coconut oil consumption has increased in recent years, due to the various health claims associated with cardiovascular and brain protection (Jayawardena et al., 2020; Ramesh et al., 2021). Referring to Deb Mandal and Mandal (2011), coconut oil is rich in medium chain saturated fatty acids which are directly absorbed from the intestine and sent to the liver to be rapidly metabolized for energy production. Sunflower oil and butter were not available in powdered form, hence the formulation of porridge was made only with coconut oil powder. Cookie and sponge cake are traditionally prepared with butter or vegetable fat, and therefore the coconut oil was not selected as a fatty ingredient in these formulations.

2.3. Proximate composition

The proximate composition of all samples was determined according to standard methods of the Association of Official Analytical Chemists (AOAC, 2000): moisture content (No. 926.5), total protein content (No. 950.36), total fat content (No. 935.38), and ash content (No. 930.22). Total dietary fiber content was determined following the procedure given with the Megazyme enzyme kit, K-TDFR-100A/K-TDFR-200A 12/15, which is a modified version of the American Association for Clinical Chemistry (AACC) total dietary fiber method, No. 32–05.01. Sugar content was determined using HPLC system Agilent 1200 Series LC system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with evaporative light scattering detector (ELSD) using Zorbax carbohydrate analysis column 4.6x250 mm, 5 μm (Agilent Technologies Inc., Santa Clara, CA, USA). Isocratic elution with acetonitrile:water (75:25, v/v), at a flow rate of 1.0 mL/min was applied. The injection volume of the sample was 5 μL. ELSD parameters: temperature: 40 °C, pressure 380 kPa, gain: 2. The homogenized samples (1 g) were suspended in 30 mL of demineralized water, homogenized for 30 s at 10000 min^{-1} using an Ultra-Turrax T25 basic (IKA, Staufen, Germany). The supernatant was collected after centrifugation at 16.770 × g for 10 min (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany). The 1 mL of supernatant was diluted with the same volume of acetonitrile and filtered through 0.45-μm pore diameter RC filters (Millipore, Darmstadt, Germany) before sugars (fructose, glucose, and sucrose) were determined. LOQ for fructose, glucose, and sucrose were 316 ng, 301 ng and 301 ng, respectively. Repeatability, calculated on the basis of peak areas (%RSD) of fructose, glucose, and sucrose was 0.42, 0.21, 0.57, respectively. Response linearity (r^2) over the concentration range from 0.10 to 15.0 mg/mL of each sugar was 0.9999, 0.9979 and 0.9986, respectively.

2.4. In vitro digestion model

All samples were homogenized before testing. The *in vitro* digestion procedure was carried out according to the previous protocol, except that all volumes were divided by 2 (Malapert et al., 2018; Reboul et al., 2006). Briefly, 1.675 g of each sample was weighed and mixed with 8 mL of 0.9% NaCl solution. The mixture was homogenized by Ultra-turrax (Ika, Staufen, Germany) at 14000 rpm for 1 min. 0.625 mL of artificial saliva ($NaHCO_3$, NaCl, KCl, $CaCl_2{\cdot}2H_2O$, $K_2HPO_4{\cdot}3H_2O$, mucine, and α-amylase, pH = 7) was added and the mixture was incubated at 37 °C in a shaking water bath for 10 min. The pH was then adjusted to 4 (±0.02) by adding HCl (1 M) and 0.5 mL of a pepsin solution (40 mg/mL in 0.1 M HCl) was added. The mixture was incubated at 37 °C in a shaking water bath for 30 min. Later, the pH of the mixture was adjusted to 6 (±0.02) with sodium bicarbonate (0.9 M). Then, 3.25 mL of a mixture of porcine bile extract (39.08 mg/mL) and pancreatin (2.08 mg/mL) in 0.1 M trisodium citrate (pH 6) was added. The samples were incubated in an agitated water bath at 37 °C again for 30 min. The samples were then placed into ice-cold water to stop the reaction. The obtained solution forms the digesta, so 500 μL of each digesta was placed in 2 mL Eppendorf tubes and stored at −80 °C until analysis. Micelles were further separated by centrifugation at 2000 g for 1 h at 10 °C. The supernatant was collected from the centrifuge tube and filtered through a 0.80 μm cellulose filter (Millipore, Molsheim, France). Then it was filtered through a 0.22 μm filter (Millipore, Molsheim, France) and micellar fractions were poured into the Eppendorf tubes and stored at −80 °C until further analysis. Carotenoid bioaccessibility was calculated as the percentage of carotenoids recovered in mixed micelles relative to the initial amount of carotenoid in the cereal-based products at the beginning of digestion.

2.5. Uptake of carotenoids by Caco-2 cells

Caco-2 (TC-7 clone) intestinal cells were maintained in flasks of 25 cm^2 with ventilated plugs in a 10% CO_2 atmosphere at 37 °C and 90% humidity. Cells were cultured according to previous published protocol (Malapert et al., 2018). After 21 days of growth, the cells were confluent, differentiated, and ready to be used. The day before experimentation, the complete medium was changed into serum-free medium. At the beginning of the experiment, cell monolayers were washed twice with 1 mL phosphate-buffered saline (PBS) on the apical side and 2 mL on the basolateral side. Cell monolayers were incubated with diluted micelles obtained from *in vitro* digestion (1/4 dilution in DMEM) at 37 °C for 2 h for all samples. After the incubation period, the media from each side of the membrane were harvested. Cell monolayers were washed twice with 1 mL PBS to eliminate adsorbed carotenoids, scraped, and collected in 1 mL of PBS. Absorbed carotenoids were estimated as carotenoids in scraped cells. All samples were stored at −80 °C until the carotenoid extraction and HPLC analysis.

Carotenoid uptake was calculated as the quantity of carotenoid present in the harvested cells divided by the sum of the quantity of carotenoid remaining in the apical chamber and that present in the harvested cells.

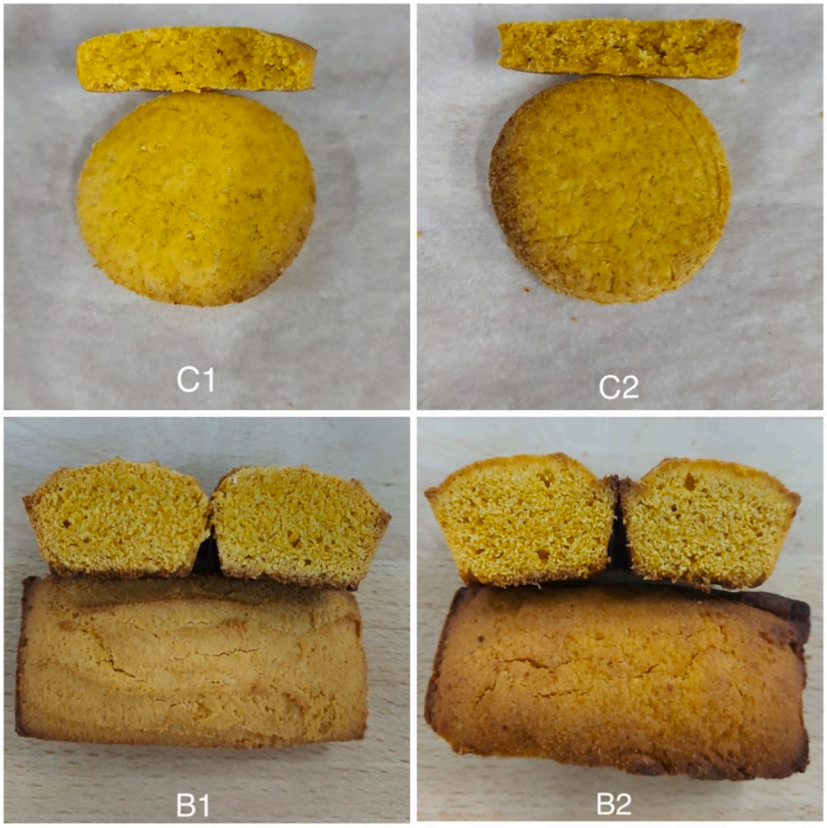

Fig. 1. Cross section of samples: C1-cookie with butter, *C*2-cookie with vegetable oil, B1-sponge cake with butter, B2-sponge cake with vegetable oil. Average size of cookie (mm): ø=54.2, hight, h = 9.35 mm. Average size of sponge cake (mm): length:width:hight, 100:50:35.

2.6. Extraction of carotenoids

Sample preparation was carried out under the dim light to avoid the destruction of the carotenoids, as they are photosensitive. For solid samples, approximately 40 mg of each product (cookies, sponge cake, and porridge) were weighed in tubes and 450 μL of distilled water were added. For aqueous samples (digestats, micelles, samples from cell experiments), a volume of 500 μL was used. Five hundred μL of retinyl acetate (internal standard) in ethanol were then added, followed by the addition of 2 mL of *n*-hexane and the whole content was homogenized on a vortex (10000 rpm, 10 min) and centrifuged (1400 g, 10 min, 4 °C). The upper phase from the tube was collected and the lower phase was extracted in the same way with hexane. The two upper phases were merged in the same tube and dried under nitrogen. Residues were then dissolved in 200 μL of methanol/dichloromethane (65/35, v/v) and transferred to the vials for HPLC. A volume of 180 μL was used for HPLC analysis.

2.7. HPLC analysis of carotenoids

The HPLC system included a Dionex separation module (P680 HPLC Pump and ASI-100 Automated Sample Injector and a Dionex UVD340U photodiode array detector (ThermoFisher Scientific, Villebon sur Yvette, France). Carotenoids were separated using 250 × 4.6 mm i.d. YMC C30 column, kept at 35 °C. The mobile phase was a gradient of methanol, methyl-*tert*-butyl ether, and water and it was set up according to Gleize et al. (2012). Carotenoids were detected at 450 nm and retinyl palmitate was detected at 325 nm. Molecules were identified by retention time and spectral analysis (from 200 to 600 nm) in comparison to pure standards (α-carotene, β-carotene and lutein) using Chromeleon 6.8 (ThermoFisher Scientific, Villebon sur Yvette, France). Based on the extraction methods and the UV limit of quantification (signal-to-noise ratio > 5), it was possible to quantify carotenoids down to 0.001 μg. Other characteristics and performances of the method are described in Gleize et al. (2012).

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the significant differences of the applied treatments. ANOVA was followed by Fisher's least significant difference test, where the differences between means at the 5 % level ($p < 0.05$) were considered significant. Statistical analysis was performed using Statistica13 (StatSoft, Tulsa, OK, USA). All analyses were performed on sample replicates derived from independent samples. Number of sample replicates (n) was as follows: proximate composition and carotenoids content of cereal-based products (n = 4), carotenoid bioaccessibility using an *in vitro* digestion model (n = 8), and α-carotene uptake by Caco-2 cells (n = 6).

3. Results and discussion

Carotenoid absorption is a complex process consisting of digestive release and solubilization of carotenoids by bile salt-lipid micelles in the gut lumen. It is already known that the transfer of carotenoids to mixed micelles depends on the presence of dietary lipids and other physical and chemical properties of the food matrix (Ferruzzi et al., 2020). The suitability of three types of cereal-based products with relatively high content of lipids and long shelf-life (porridge, cookies, and sponge cakes) as carrier products was tested.

Porridge, cookies, and sponge cakes were prepared with different types of fats, because the degree of saturation and the length of fatty acyl chains in lipids must be considered when assessing the bioaccessibility of carotenoids. Most of the studies suggest that the longer the acyl chain length is, the better efficiency of micellarization of β-carotene and the

Table 2
Proximate composition of cereal-based products.

Proximate composition (g/100 g FW)						
Sample	**Dry pumpkin puree#**	**Porridge with dry pumpkin puree#**	**Cookie with butter**	**Cookie with vegetable oil**	**Sponge cake with butter**	**Sponge cake with vegetable oil**
Moisture	$9.6^{a} \pm 0.0$	$6.4^{b} \pm 0.1$	$11.0^{c} \pm 0.0$	$8.8^{d} \pm 0.1$	$16.0^{e} \pm 0.1$	$12.8^{f} \pm 0.1$
Ash value	$6.5^{d} \pm 0.1$	$2.7^{c} \pm 0.0$	$1.6^{b} \pm 0.0$	$1.6^{b} \pm 0.1$	$1.3^{a} \pm 0.1$	$1.4^{a} \pm 0.1$
Total proteins	$10.0^{b} \pm 0.0$	$20.5^{c} \pm 0.1$	$5.1^{a} \pm 0.0$	$5.1^{a} \pm 0.0$	$5.7^{d} \pm 0.0$	$5.8^{e} \pm 0.0$
Total lipids	$0.8^{a} \pm 0.0$	$1.2^{b} \pm 0.0$	$15.6^{c} \pm 0.1$	$19.9^{d} \pm 0.0$	$19.4^{e} \pm 0.2$	$22.2^{f} \pm 0.1$
Carbohydrates*	$65.4^{a} \pm 0.1$	$52.2^{b} \pm 0.1$	$57.9^{c} \pm 0.2$	$56.2^{d} \pm 0.3$	$47.8^{e} \pm 0.21$	$51.1^{f} \pm 0.2$
***glu + fru + sacc**	43.5 ± 0.1	31.8 ± 0.1	18.3 ± 0.2	19.5 ± 0.2	19.4 ± 0.2	20.3 ± 0.2
***starch**	21.9 ± 0.0	20.4 ± 0.0	39.6 ± 0.0	36.7 ± 0.0	28.4 ± 0.0	30.8 ± 0.0
Total fiber	$7.7^{a} \pm 0.1$	$17.0^{b} \pm 0.1$	$8.8^{c} \pm 0.0$	$8.4^{d} \pm 0.0$	$9.8^{e} \pm 0.0$	$6.7^{f} \pm 0.0$

Mean value ± standard deviation (n = 4). Values followed by the same letter in the row are not significantly different ($p > 0.05$). #Proximate composition determined in samples without the addition of water.

Table 3
Carotenoid content of cereal-based products.

Carotenoid content (ng/mg FW)			
Sample	α-carotene	β-carotene	Lutein
Dry pumpkin puree*	$17.66^{e} \pm 1.61$	$12.76^{c} \pm 1.02$	$1.30^{c} \pm 0.03$
Porridge with dry pumpkin puree*	$5.63^{a} \pm 2.18$	$4.48^{a} \pm 1.70$	$0.85^{b} \pm 0.31$
Cookie with butter	$10.36^{c} \pm 1.02$	$8.32^{b} \pm 0.75$	$0.82^{a} \pm 0.09$
Cookie with vegetable oil	$12.82^{d} \pm 1.44$	$10.28^{d} \pm 1.14$	$1.04^{a,c} \pm 0.11$
Sponge cake with butter	$8.92^{b,c} \pm 0.17$	$7.70^{b} \pm 0.15$	$0.84^{a} \pm 0.04$
Sponge cake with vegetable oil	$7.18^{a,b} \pm 0.82$	$5.58^{a} \pm 0.60$	$0.71^{a,b} \pm 0.06$

Mean value ± standard deviation (n = 4). Values followed by the same letter in the column are not significantly different ($p > 0.05$). * Carotenoid content determined in samples without the addition of water.

uptake of β-carotene by Caco-2 cells (Huo et al., 2007) are. Unsaturated fatty acids, particularly mono-unsaturated fatty acids, likely promote carotenoid bioaccessibility (Yuan et al., 2018). Therefore, carotenoid bioaccessibility from formulated products containing either butter or sunflower oil (cookies and sponge cakes), or coconut oil (porridge) was compared. The final products are presented in Fig. 1.

3.1. Pumpkin powder preparation and characterization

Pumpkin pulp can be processed and stabilized in many ways. Pumpkin powders are often used as ingredients in different types of cereal-based products (Provesi et al., 2011), as they have higher storage and transportation advantages over fresh pumpkins due to their longer shelf lives.

The proximate composition of the obtained pumpkin powder (Table 2) was similar to the composition of the pumpkin powder reported by Bhat and Anju (2013), except that the protein content was higher and that total carbohydrates were lower in this study. Although determined differently, the total carotenoid content reported by these authors (7.3 mg/100 g) was comparable with our results (Table 3). The main carotenoid in the pumpkin powder was α-carotene.

3.2. Porridge preparation and characterization

The proximate composition of the high-protein porridge and its carotenoids content are given in Tables 2 and 3. Since porridge contains 30% of pumpkin powder, the obtained values for α- and β-carotene content were slightly lower than expected, and corresponded to 25.76% and 28.44% of pumpkin flour addition, respectively. These losses could be due to oxidation of these carotenoids during the production process.

Compared to the other baked products and dry pumpkin puree, porridge had a lower amount of carotenoids (α-carotene, β -carotene and lutein) (Table 3). Comparing the expected carotenoid amounts with the measured carotenoid amounts in the final products highlighted that thermal treatments during the processing led to a small decrease in carotenoid content. Other than temperature, a reason for the loss of carotenoids can be oxidation during manipulation, such as exposure to oxygen during product packaging (Rodriguez-Amaya, 1999).

3.3. Cookie preparation and characterization

Cookies are cereal-based products characterized by a high content of sucrose and fat, and low water content. Changes in a formulation such as fibre incorporation, sucrose and fat replacements generate changes in dough rheological properties, which may result in excessive adhesion to work surfaces and changes in baked product shape, colour, density, and texture, that could decrease consumer acceptability of the product (Canalis et al., 2020). In this work, the optimization of the formulation was made to enable maximum pumpkin powder incorporation without compromising sensory and textural properties, characteristic tender but snapping texture, and uniform surface-cracking pattern of the final products (Canalis et al., 2020). Two similar formulations of cookies were made differing in the type of lipids, butter (C1) and sunflower oil (*C2*) (Fig. 1). Regardless of its origin (American, Irish, Polish, or Dutch), the most abundant fatty acid in butter is palmitic acid (C16:0), followed by myristic (C14:0), stearic (C18:0), and oleic acid (C18:1n9) (Pustjens et al., 2017), while sunflower oil contains primarily unsaturated fatty acids, polyunsaturated linoleic acid (18:2 *cis*-9,12), and monounsaturated oleic acid (18:1 *cis*-9) (Alberio et al., 2016). As expected, a similar proximate lipid composition of these cookies was obtained. However, butter cookies had a significantly lower content of lipids, which can be explained by the fact that butter is a kind of a water-in-oil emulsion where water content can reach above 20% (Rønholt et al., 2014), while sunflower oil contains less than 0.1% water (Pal, Patra, Sahoo, Bakhara, & Panda, 2015).

As for the carotenoid composition, contents of α-carotene, β-carotene, and lutein were higher than expected. Additional carotenoids were probably contributing from other raw materials such as soy lecithin (Bot et al., 2021). Additionally, cookies with butter (C1) had a significantly lower content of carotenoids than cookies with vegetable oil (*C2*).

Content of β-carotene in cookies, made by substituting wheat flour with pumpkin flour in different percentages, has been previously investigated by Pongjanta et al. (2006). In that study pumpkin powder was substituted at levels from 10 to 50% of all-purpose flour. The formulation contained margarine, sugar, eggs, water, skim milk powder, baking powder, and salt. Cookies made from 20% substituted pumpkin flour, contained 2.0 ng/mg FW of β-carotene. Our cookies, which contained 17% pumpkin flour, had higher amount of β-carotene − 8.32 ± 0.75 ng/mg FW with butter and 10.28 ± 1.14 ng/mg FW with vegetable oil.

Table 4
Carotenoid bioaccessibility in relation to their initial quantity in cereal-based products (%).

Sample	α-carotene	β-carotene	Lutein
Dry pumpkin puree	$1.22^{c, \#} \pm 0.57$	$0.88^{a, \#} \pm 0.55$	$10.28^{b, \$} \pm 4.08$
Porridge with dry pumpkin puree	$0.33^{a,b, \#} \pm 0.20$	$0.16^{b, \#} \pm 0.18$	$3.88^{a, \$} \pm 1.87$
Cookie with butter	$0.49^{b, \#} \pm 0.26$	$0.20^{b, \#} \pm 0.29$	$7.73^{b, \$} \pm 3.26$
Cookie with vegetable oil	$0.18^{a, \#} \pm 0.08$	$0.07^{b, \#} \pm 0.10$	$4.79^{a, \$} \pm 1.82$
Sponge cake with butter	$0.20^{a, \#} \pm 0.12$	$0.01^{b, \#} \pm 0.04$	$4.62^{a, \$} \pm 1.02$
Sponge cake with vegetable oil	$0.34^{a,b, \#} \pm 0.08$	n.d.	$9.15^{b, \$} \pm 1.84$

Mean value ± standard deviation (n = 8). Values followed by the same letter (a, b,c) in the column are not significantly different ($p > 0.05$). Values followed by the same symbol (#, $) in the row are not significantly different ($p > 0.05$). n.d. - not detected.

3.4. Sponge cake preparation and characterization

Sponge cakes are usually made of wheat flour, eggs, and sugar. The other ingredients in the formulation contribute to the formation of a complex hydrophilic colloid system of the sponge cake batter, which solidifies during baking in a way to form a capillary-porous structure. During the solidification process, starch gelatinization and protein denaturation occur (Goranova et al., 2019). Our sponge cake contained 12% pumpkin powder, similar to the optimized formulation presented by Hosseini Ghaboos et al. (2018), who found that the level of 10% pumpkin powder supplementation was optimal for sponge cakes.

Contrary to cookies, sponge cake with vegetable oil (B2) had significantly lower content of α-carotene and β-carotene than sponge cake with butter (B1). This can be explained by lower oxidative stability of sunflower oil than butter in the sponge cake formulation, leading to higher utilization of carotenoids as antioxidants, probably during baking.

3.5. Carotenoid bioaccessibility and cellular uptake

We then studied carotenoid bioaccessibility using an *in vitro* digestion model (Margier et al., 2018; Margier et al., 2019; Reboul et al., 2006). Results are presented in Table 4. An example of chromatogram of cookie digestat is given in Fig. 2. Among the three carotenoids analysed in the different samples, lutein displayed the highest bioaccessibility (3.88% to 10.28%), while α- and β-carotene displayed the lowest bioaccessibility (0.18 to 1.22% and 0.01 to 0.88%, respectively), which is consistent with previous data (Reboul et al., 2006). Indeed, xanthophylls such as lutein contain at least one oxygen atom in their molecular structure, mostly in the form of hydroxyl, methoxyl, carbonyl or epoxy groups. These oxygenated groups make xanthophylls more polar than carotenes such as α- or β-carotene, and enable a more efficient solubilization of xanthophylls in the aqueous, surfactant-rich environment of the digestive phases, particularly in the intestinal phase containing bile salts (Chacón-Ordóñez et al., 2019).

Despite the absence of fat, the bioaccessibility of carotenoids from dry pumpkin puree was higher than in the baked products. This result is surprising as fat is supposed to promote carotenoid transfer to mixed micelles (Kopec & Failla, 2018). We suspected that carotenoid extraction from dry pumpkin puree was not as efficient as for the other baked products, which led to underestimation of puree carotenoid content. It is also possible that carotenoids from pumpkin puree were less degraded during the *in vitro* digestion process, due to higher concentrations of antioxidants in the mixture (carotenoids themselves, but also polyphenols). Another surprising result is that lutein bioaccessibility from cookies with butter, and from sponge cake with vegetable oil was ~ 2-fold higher than lutein bioavailability from cookies with vegetal oil and from sponge cake with butter. Butter was previously shown to enhance xanthophyll bioaccessibility (Gleize et al., 2013), hence the lower bioaccessibility of lutein from sponge cake with butter compared to the same product made with vegetal oil was unexpected. However, different food formulation can lead to modification in food matrix structure, which is another independent parameter that can impact lutein bioaccessibility (Hiolle et al., 2020). Further studies using multi-criteria approaches are needed to understand the interactions occurring between parameters such as fat content and food structure (Gleize et al., 2020).

Mixed micelles obtained from these *in vitro* digestions were diluted in cell culture medium. The dilution was necessary to avoid cell

Table 5
α-Carotene uptake by Caco-2 cells.

Sample	α-carotene uptake %
Dry pumpkin puree	$1.31^{a,b} \pm 0.78$
Porridge with dry pumpkin puree	$0.69^{a} \pm 0.20$
Cookie with butter	$2.33^{c} \pm 0.59$
Cookie with vegetable oil	$1.38^{b} \pm 0.84$
Sponge cake with butter	$0.77^{a,b} \pm 0.18$
Sponge cake with vegetable oil	$0.72^{a} \pm 0.29$

Mean value ± standard deviation (n = 6). Values followed by the same letter in the column are not significantly different ($p > 0.05$).

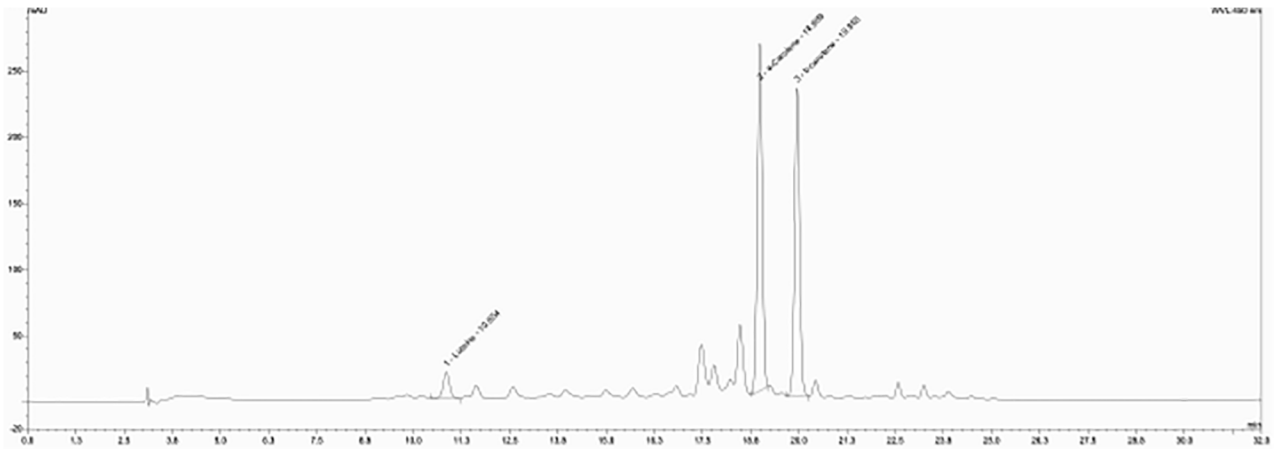

Fig. 2. Chromatogram of a digestat obtained after cookie *in vitro* digestion. HPLC analysis of carotenoid was performed at 450 nm. Internal standard (retinyl acetate) was simultaneously measured at another wavelength (325 nm).

cytotoxicity (data not shown). Diluted micelles were incubated on cells to assess carotenoid apical uptake. Conversely to α-carotene, lutein and β-carotene were not detectable in cells after 2 h of incubation. Cookie micelles led to the highest percentage of α-carotene cell uptake (2.33% and 1.38% for cookies with butter and cookies with vegetal oil, respectively) compared to other baked products, followed by dry pumpkin puree micelles (1.31%; Table 5). Conversely, porridge micelles led to the lowest α-carotene uptake. Porridge formulation displays significant amounts of pea protein isolate that may contain other bioactive compounds as well as pea fibre. The results of this study are consistent with the work of Margier et al. (2019), showing that pulse bioactives and fibre can interact with fat-soluble vitamin and carotenoid uptake by Caco-2 cells.

4. Conclusion

This study highlighted the importance of the *in vitro* digestion model as a tool for understanding the impact of product types and their nutritional composition on its potential bioaccessibility. α-Carotene was the main carotenoid in raw pumpkin, but lutein exhibited the highest bioaccessibility in porridge, cookies and sponge cakes. Our data show that both bioaccessiblity and cell uptake of carotenoids from cereal-based products are highly variable and depend on food formulation and food structure. Additional work is needed to further optimize carotenoid bioaccessibility, with the emphasis on novel techniques in food processing that would minimize the loss of these beneficial compounds.

CRediT authorship contribution statement

Milana Rošul: Investigation, Formal analysis. **Nataša Đerić:** Investigation, Formal analysis. **Aleksandra Mišan:** Funding acquisition, Writing – review & editing. **Milica Pojić:** Data curation, Writing – review & editing. **Olivera Šimurina:** Formal analysis, Resources. **Charlotte Halimi:** Investigation, Methodology. **Marion Nowicki:** Investigation, Methodology. **Biljana Cvetković:** Formal analysis. **Anamarija Mandić:** Conceptualization, Resources, Validation, Methodology, Supervision, Writing – original draft. **Emmanuelle Reboul:** Conceptualization, Resources, Validation, Methodology, Supervision, Writing – original draft.

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.

Acknowledgements

This research was funded by:

- The Ministry of Education, Science and Technological Development of the Republic of Serbia (Contract No: 451-03-9/2022-14/200222), The Science Fund of the Republic of Serbia, PROMIS, Grant No. 6060592, DEStiny;
- The French Ministry of higher education, research and innovation (Exchange program Hubert Curien Pavle Savić 2018-2019),
- COST Action "European network to advance carotenoid research and applications in agro-food and health" EUROCAROTEN, CA15136, www.eurocaroten.eu, http://www.cost.eu/COST_Actions/ca/CA15136) supported by COST.

References

Abbas, H. M. K., Huang, H. X., Yang, Y. F., Xie, Y. H., Zou, J. F., Xue, S. D., & Zhong, Y. J. (2020). Characterization of Starch in Cucurbita moschata Germplasms throughout Fruit Development. *Journal of Agricultural and Food Chemistry, 68*(36), 9690–9696. https://doi.org/10.1021/acs.jafc.0c03181

Alberio, C., Izquierdo, N. G., Galella, T., Zuil, S., Reid, R., Zambelli, A., & Aguirrezábal, L. A. (2016). A new sunflower high oleic mutation confers stable oil grain fatty acid composition across environments. *European Journal of Agronomy, 73*, 25–33. https://doi.org/10.1016/j.eja.2015.10.003

AACC total dietary fiber (TDF). Method, 32-05.01. Approved methods of the American Association of Cereal Chemists (11th. Edition). (2009). Codex Alimentarius.

AOAC. (2000). *Official Methods of Analysis of the Association of Official of Analitycal Chemists. Association of the Official Analytical Chemists*. VA, USA: Inc.

Bhat, M. A., & Anju, B. (2013). Study on physico-chemical characteristics of pumpkin blended cake. *Journal of Food Processing and Technology, 4*(9), 4. https://doi.org/10.4172/2157-7110.1000262

Bot, F., Cossuta, D., & O'Mahony, J. A. (2021). Inter-relationships between composition, physicochemical properties and functionality of lecithin ingredients. *Trends in Food Science & Technology, 111*, 261–270. https://doi.org/10.1016/j.tifs.2021.02.028

Canalis, M. B., Baroni, M. V., León, A. E., & Ribotta, P. D. (2020). Effect of peach puree incorportion on cookie quality and on simulated digestion of polyphenols and antioxidant properties. *Food Chemistry, 333*, 127464. https://doi.org/10.1016/j.foodchem.2020.127464

Carvalho, L. M. J., Smiderle, L. A. S. M., Carvalho, J. L. V., Cardoso, F. S. N., & Koblitz, M. G. B. (2014). Assessment of carotenoids in pumpkins after different home cooking conditions. *Food Science and Technology, 34*, 365–370. https://doi.org/10.1590/fst.2014.0058

Chacón-Ordóñez, T., Carle, R., & Schweiggert, R. (2019). Bioaccessibility of carotenoids from plant and animal foods. *Journal of the Science of Food and Agriculture, 99*(7), 3220–3239. https://doi.org/10.1002/jsfa.9525

Corrigan, V. K., Irving, D. E., & Potter, J. F. (2000). Sugars and Sweetness in Buttercup Squash. *Food Quality and Preference, 11*, 313–322. https://doi.org/10.1016/S0950-3293(99)00077-4

Dar, A. H., Sofi, S. A., & Rafiq, S. (2017). Pumpkin the functional and therapeutic ingredient: A review. *International Journal of Food Science Nutrition, 2*, 165–170.

de Souza Mesquita, L. M., Neves, B. V., Pisani, L. P., & de Rosso, V. V. (2020). Mayonnaise as a model food for improving the bioaccessibility of carotenoids from Bactrisgasipaes fruits. *LWT, 122*, 109022. https://doi.org/10.1016/j.lwt.2020.109022

Deb Mandal, M., & Mandal, S. (2011). Coconut (Cocos nucifera L.: Arecaceae): In health promotion and disease prevention. *Asian Pacific Journal of Tropical Medicine, 4*(3), 241–247. https://doi.org/10.1016/S1995-7645(11)60078-3

Dini, I., Tenore, G. C., & Dini, A. (2013). Effect of industrial and domestic processing on antioxidant properties of pumpkin pulp. *LWT-Food Science and Technology, 53*(1), 382–385. https://doi.org/10.1016/j.lwt.2013.01.005

Fernández-García, E., Carvajal-Lérida, I., Jarén-Galán, M., Garrido-Fernández, J., Pérez-Gálvez, A., & Hornero-Méndez, D. (2012). Carotenoids bioavailability from foods: From plant pigments to efficient biological activities. *Food Research International, 46*(2), 438–450. https://doi.org/10.1016/j.foodres.2011.06.007

Ferruzzi, M. G., Kruger, J., Mohamedshah, Z., Debelo, H., & Taylor, J. R. (2020). Insights from in vitro exploration of factors influencing iron, zinc and provitaminA carotenoid bioaccessibility and intestinal absorption from cereals. *Journal of Cereal Science, 96*, 103126. https://doi.org/10.1016/j.jcs.2020.103126

Gleize, B., Steib, M., André, M., & Reboul, E. (2012). Simple and fast HPLC method for simultaneous determination of retinol, tocopherols, coenzyme Q10 and carotenoids in complex samples. *Food Chemistry, 134*(4), 2560–2564. https://doi.org/10.1016/j.foodchem.2012.04.043

Gleize, B., Hiolle, M., Meunier, N., Pereira, B., Richard, R., Savary-Auzeloux, I., … Reboul, E. (2020). Food Structure Modulates the Bioavailability of Triglycerides and Vitamin D, and Partly That of Lutein: A Randomized Trial with a Crossover Design in Adults. *Molecular Nutrition & Food Research, 64*(21), e2000228. https://doi.org/10.1002/mnfr.202000228

Gleize, B., Tourniaire, F., Depezay, L., Bott, R., Nowicki, M., Albino, L., … Borel, P. (2013). Effect of type of TAG fatty acids on lutein and zeaxanthin bioavailability. *British Journal of Nutrition, 110*(1), 1–10. https://doi.org/10.1017/S0007114512004813

Goranova, Z., Marudova, M., & Baeva, M. (2019). Influence of functional ingredients on starch gelatinization in sponge cake batter. *Food Chemistry, 297*, 124997. https://doi.org/10.1016/j.foodchem.2019.124997

Hiolle, M., Lechevalier, V., Floury, J., Boulier-Monthéan, N., Prioul, C., Dupont, D., & Nau, F. (2020). In vitro digestion of complex foods: How microstructure influences food disintegration and micronutrient bioaccessibility. *Food Research International, 128*, 108817. https://doi.org/10.1016/j.foodres.2019.108817

Hosen, M., Rafii, M. Y., Mazlan, N., Jusoh, M., Oladosu, Y., Chowdhury, M. F. N., … Khan, M. M. H. (2021). Pumpkin (Cucurbita spp.): A Crop to Mitigate Food and Nutritional Challenges. *Horticulturae, 7*, 352. https://doi.org/10.3390/horticulturae7100352

Hosseini Ghaboos, S. H., Seyedain Ardabili, S. M., & Kashaninejad, M. (2018). Physico-chemical, textural and sensory evaluation of sponge cake supplemented with pumpkin flour. *International Food Research Journal, 25*(2), 854–860.

Huo, T., Ferruzzi, M. G., Schwartz, S. J., & Failla, M. L. (2007). Impact of fatty acyl composition and quantity of triglycerides on bioaccessibility of dietary carotenoids. *Journal of Agricultural and Food Chemistry, 55*(22), 8950–8957. https://doi.org/10.1021/jf071687a

Hussain, A., Kausar, T., Din, A., Murtaza, M. A., Jamil, M. A., Noreen, S., … Ramzan, M. A. (2021). Determination of total phenolic, flavonoid, carotenoid, and mineral contents in peel, flesh, and seeds of pumpkin (Cucurbita maxima). *Journal of Food Processing and Preservation, 45*, e15542. https://doi.org/10.1111/jfpp.15542

Jayawardena, R., Swarnamali, H., Lanerolle, P., & Ranasinghe, P. (2020). Effect of coconut oil on cardio-metabolic risk: A systematic review and meta-analysis of interventional studies. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews, 14*(6), 2007–2020. https://doi.org/10.1016/j.dsx.2020.09.033

Kopec, R. E., & Failla, M. L. (2018). Recent advances in the bioaccessibility and bioavailability of carotenoids and effects of other dietary lipophiles. *Journal of Food Composition and Analysis, 68*, 16–30. https://doi.org/10.1016/j.jfca.2017.06.008

Kruger, J., Taylor, J. R., Ferruzzi, M. G., & Debelo, H. (2020). What is food-to-food fortification? A working definition and framework for evaluation of efficiency and implementation of best practices. *Comprehensive Reviews in Food Science and Food Safety, 19*(6), 3618–3658. https://doi.org/10.1111/1541-4337.12624

Margier, M., Buffière, C., Goupy, P., Remond, D., Halimi, C., Caris-Veyrat, C., … Reboul, E. (2018). Opposite effects of the spinach food matrix on lutein bioaccessibility and intestinal uptake lead to unchanged bioavailability compared to pure lutein. *Molecular Nutrition & Food Research, 62*(11), 1800185. https://doi.org/10.1002/mnfr.201800185

Margier, M., Antoine, T., Siriaco, A., Nowicki, M., Halimi, C., Maillot, M., … Reboul, E. (2019). The Presence of Pulses within a Meal can Alter Fat-Soluble Vitamin Bioavailability. *Molecular Nutrition & Food Research, 63*(11), 1801323. https://doi.org/10.1002/mnfr.201801323

Malapert, A., Tomao, V., Margier, M., Nowicki, M., Gleize, B., Dangles, O., & Reboul, E. (2018). β-Cyclodextrin does not alter the bioaccessibility and the uptake by caco-2 cells of olive by-product phenolic compounds. *Nutrients, 10*(11), 1653. https://doi.org/10.3390/nu10111653

Pal, U. S., Patra, R. K., Sahoo, N. R., Bakhara, C. K., & Panda, M. K. (2015). Effect of refining on quality and composition of sunflower oil. *Journal of Food Science and Technology, 52*(7), 4613–4618. https://doi.org/10.1007/s13197-014-1461-0

Pongjanta, J., Naulbunrang, A., Kawngdang, S., Manon, T., & Thepjaikat, T. (2006). Utilization of pumpkin powder in bakery products. *Songklanakarin Journal of Science and Technology, 28*(1), 71–79.

Provesi, J. G., Dias, C. O., & Amante, E. R. (2011). Changes in carotenoids during processing and storage of pumpkin puree. *Food Chemistry, 128*(1), 195–202. https://doi.org/10.1016/j.foodchem.2011.03.027

Pustjens, A. M., Boerrigter-Eenling, R., Koot, A. H., Rozijn, M., & Van Ruth, S. M. (2017). Characterization of retail conventional, organic, and grass full-fat butters by their fat contents, free fatty acid contents, and triglyceride and fatty acid profiling. *Foods, 6*(4), 26. https://doi.org/10.3390/foods6040026

Ramesh, S. V., Krishnan, V., Praveen, S., & Hebbar, K. B. (2021). Dietary prospects of coconut oil for the prevention and treatment of Alzheimer's disease (AD): A review of recent evidences. *Trends in Food Science & Technology, 112*, 201–211. https://doi.org/10.1016/j.tifs.2021.03.046

Reboul, E., Richelle, M., Perrot, E., Desmoulins-Malezet, C., Pirisi, V., & Borel, P. (2006). Bioaccessibility of carotenoids and vitamin E from their main dietary sources. *Journal of Agricultural and Food Chemistry, 54*(23), 8749–8755. https://doi.org/10.1021/jf061818s

Ribeiro, E. M. G., Chitchumroonchokchai, C., de Carvalho, L. M. J., de Moura, F. F., de Carvalho, J. L. V., & Failla, M. L. (2015). Effect of style of home cooking on retention and bioaccessibility of pro-vitamin A carotenoids in biofortified pumpkin (Cucurbita moschata Duch.). *Food Research International, 77*, 620–626. https://doi.org/10.1016/j.foodres.2015.08.038

Rodriguez-Amaya, D. B. (1999). Changes in carotenoids during processing and storage of foods. *Archivos latinoamericanos de nutrición, 49*(3 Suppl 1), 38S–47S.

Rønholt, S., Madsen, A. S., Kirkensgaard, J. J., Mortensen, K., & Knudsen, J. C. (2014). Effect of churning temperature on water content, rheology, microstructure and stability of butter during four weeks of storage. *Food Structure, 2*(1–2), 14–26. https://doi.org/10.1016/j.foostr.2014.07.001

Saini, R. K., Nile, S. H., & Park, S. W. (2015). Carotenoids from fruits and vegetables: Chemistry, analysis, occurrence, bioavailability and biological activities. *Food Research International, 76*, 735–750. https://doi.org/10.1016/j.foodres.2015.07.047

Yuan, X., Liu, X., McClements, D. J., Cao, Y., & Xiao, H. (2018). Enhancement of phytochemical bioaccessibility from plant-based foods using excipient emulsions: Impact of lipid type on carotenoid solubilization from spinach. *Food & Function, 9*(8), 4352–4365. https://doi.org/10.1039/C8FO01118D