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

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18 **Abstract**

19 Enriching cereals-based products with bioactive compounds is a valuable strategy to improve
20 product quality. We studied carotenoid bioaccessibility and intestinal uptake from a pumpkin-
21 enriched porridge, cookies and sponge cakes by using *in vitro* digestion coupled with Caco-2 cell
22 uptake. Among the carotenoids recovered in different products, α-carotene was the most
23 important one. However, lutein displayed a significantly higher bioaccessibility compared to α-
24 carotene and β-carotene in baked products (up to 10.28% compared to 1.22% and 0.88%,
25 respectively). α-Carotene was the only carotenoid recovered in Caco-2 cells after micelle
26 incubation. Cookie micelles led to the highest percentage of α-carotene cell uptake (2.33% and
27 1.38% for cookies with butter and cookies with vegetable oil, respectively) compared to the other
28 baked products, followed by dry pumpkin puree micelles (1.31%). Overall, our data showed that
29 both bioaccessiblity and cell uptake of carotenoids from cereal-based products were variable and
30 highly depend on food formulation and structure.

31

32 Key words: *Cucurbita moschata*, carotenoids, bioaccessibility, *in vitro* digestion, Caco-2 cell
33 uptake.

34

1. Introduction

Pumpkin (*Cucurbita* spp.) is an important but underutilised vegetable crop cultivated worldwide at around 3 million hectares, yielding more than 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/100g), α-carotene (0.03 to 7.5 mg/100g), and lutein (from not detected to 17 mg/100g), 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 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

80 the lipid fractions (Carvalho et al., 2014). Therefore, the initial amount of carotenoids in raw
81 pumpkin is not the main factor affecting carotenoid bioaccessibility. Lipids also promote the
82 bioaccessibility of all dietary lipophilic compounds, and it is established that the co-ingestion of
83 fats and oils enhances the micellarization of carotenoids from commonly consumed fruit- and
84 vegetable-based products (Kopec and Failla, 2018).

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

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

94

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).

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 minutes, under the pressure of 250 kPa.

Dried pumpkin puree (P1) 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 proteins 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 is added to the formulation, compared to traditional cookies and sponge cakes (Table 1).

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

153 **2.3. Proximate composition**

154 The proximate composition of all samples was determined according to standard methods of the
155 Association of Official Analytical Chemists (AOAC, 2000): moisture content (No. 926.5), total
156 protein content (No. 950.36), total fat content (No. 935.38), and ash content (No. 930.22). Total
157 dietary fiber content was determined following the procedure given with the Megazyme enzyme
158 kit, K-TDFR-100A/K-TDFR-200A 12/15, which is a modified version of the American
159 Association for Clinical Chemistry (AACC) total dietary fiber method, No. 32-05.01. Sugar
160 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, 5µm (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 x 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 0.10-15.0 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-turax (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

184 (1M) and 0.5 mL of a pepsin solution (40 mg/mL in 0.1 M HCl) was added. The mixture was
185 incubated at 37 °C in a shaking water bath for 30 min. Later, the pH of the mixture was adjusted
186 to 6 (±0.02) with sodium bicarbonate (0.9M). Then, 3.25ml of a mixture of porcine bile extract
187 (39.08 mg/mL) and pancreatin (2.08 mg/mL) in 0.1M trisodium citrate (pH 6) was added. The
188 samples were incubated in an agitated water bath at 37 °C again for 30 min. The samples were
189 then placed into ice-cold water to stop the reaction. The obtained solution forms the digesta, so
190 500 µL of each digesta was placed in 2 mL Eppendorf tubes and stored at -80 °C until analysis.
191 Micelles were further separated by centrifugation at 2000 g for 1h at 10 °C. The supernatant was
192 collected from the centrifuge tube and filtered through a 0.80 µm cellulose filter (Millipore,
193 Molsheim, France). Then it was filtered through a 0.22 µm filter (Millipore, Molsheim, France)
194 and micellar fractions were poured into the Eppendorf tubes and stored at -80 °C until further
195 analysis. Carotenoid bioaccessibility was calculated as the percentage of carotenoids recovered
196 in mixed micelles relative to the initial amount of carotenoid in the cereal-based products at the
197 beginning of digestion.

198 **2.5. Uptake of carotenoids by Caco-2 cells**

199 Caco-2 (TC-7 clone) intestinal cells were maintained in flasks of 25 cm² with ventilated plugs in
200 a 10% CO_2 atmosphere at 37 °C and 90% humidity. Cells were cultured according to previous
201 published protocol (Malapert et al., 2018). After 21 days of growth, the cells were confluent,
202 differentiated, and ready to be used. The day before experimentation, the complete medium was
203 changed into serum-free medium. At the beginning of the experiment, cell monolayers were
204 washed twice with 1 mL phosphate-buffered saline (PBS) on the apical side and 2 mL on the
205 basolateral side. Cell monolayers were incubated with diluted micelles obtained from *in vitro*
206 digestion (1/4 dilution in DMEM) at 37 °C for 2 h for all samples. After the incubation period,

207 the media from each side of the membrane were harvested. Cell monolayers were washed twice
208 with 1 mL PBS to eliminate adsorbed carotenoids, scraped, and collected in 1 mL of PBS.
209 Absorbed carotenoids were estimated as carotenoids in scraped cells. All samples were stored at
210 -80 °C until the carotenoid extraction and HPLC analysis.

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

214 **2.6. Extraction of carotenoids**

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

226 **2.7. HPLC Analysis of Carotenoids**

227 The HPLC system included a Dionex separation module (P680 HPLC Pump and ASI-100
228 Automated Sample Injector and a Dionex UVD340U photodiode array detector (ThermoFisher

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

238 **2.8. Statistical analysis**

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

247 ## 3. Results and discussion

248 Carotenoid absorption is a complex process consisting of digestive release and solubilization of
249 carotenoids by bile salt-lipid micelles in the gut lumen. It is already known that the transfer of
250 carotenoids to mixed micelles depends on the presence of dietary lipids and other physical and
251 chemical properties of the food matrix that affect the bioavailability of carotenoids (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 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 Figure 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/100g) 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 Table 2 and Table 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) (Figure 1). Regardless of its origin (American, Irish, Polish, or Dutch), the most abundant

296 fatty acid in butter is palmitic acid (C16:0), followed by myristic (C14:0), stearic (C18:0), and
297 oleic acid (C18:1n9) (Pustjens et al., 2017), while sunflower oil contains primarily unsaturated
298 fatty acids, polyunsaturated linoleic acid (18:2 *cis*-9,12), and monousaturated oleic acid (18:1
299 *cis*-9) (Alberio et al., 2016). As expected, a similar proximate lipid composition of these cookies
300 was obtained. However, butter cookies had a significantly lower content of lipids, which can be
301 explained by the fact that butter is a kind of a water-in-oil emulsion where water content can
302 reach above 20% (Rønholt et al., 2014), while sunflower oil contains less than 0.1% water (Pal et
303 al., 2015).

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

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

315

316 ### 3.4 Sponge cake preparation and characterization

317 Sponge cakes are usually made of wheat flour, eggs, and sugar. The other ingredients in the
318 formulation contribute to the formation of a complex hydrophilic colloid system of the sponge
319 cake batter, which solidifies during baking in a way to form a capillary-porous structure. During
320 the solidification process, starch gelatinization and protein denaturation occur (Goranova et al.,
321 2019). Our sponge cake contained 12% pumpkin powder, similar to the optimized formulation
322 presented by Hosseini Ghaboos et al. (2018), who found that the level of 10% pumpkin powder
323 supplementation is optimal for sponge cakes.

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

328 ### 3.5. Carotenoid bioaccessibility and cellular uptake

329 We also studied carotenoid bioaccessibility using an in vitro digestion model (Margier et al.,
330 2018; Margier et al., 2019; Reboul et al., 2006). Results are presented in Table 4. An example of
331 chromatogram of cookie digestat is given in Figure 2. Among the three carotenoids analysed in
332 the different samples, lutein displayed the highest bioaccessibility (3.88% to 10.28%), while α-
333 and β-carotene displayed the lowest bioaccessibility (0.18 to 1.22% and 0.01 to 0.88%,
334 respectively), which is consistent with previous data (Reboul et al., 2006). Indeed, xanthophylls
335 such as lutein contain at least one oxygen atom in their molecular structure, mostly in the form of
336 hydroxyl, methoxyl, carbonyl or epoxy groups. These oxygenated groups make xanthophylls
337 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 and 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 bioavailability (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 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

361 vegetal oil, respectively) compared to other baked products, followed by dry pumpkin puree
362 micelles (1.31%; Table 5). Conversely, porridge micelles led to the lowest α-carotene uptake.
363 Porridge formulation displays significant amounts of pea protein isolate that may contain other
364 bioactive compounds as well as pea fibre. The results of this study are consistent with the work
365 of Margier et al. (2019), showing that pulse bioactives and fibre can interact with fat-soluble
366 vitamin and carotenoid uptake by Caco-2 cells.

367 ## 4. Conclusion

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

376

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Tables

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 proteins 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 minutes.
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. 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.
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%)	

542 **Table 2. Proximate composition of cereal-based products**

Proximate composition (g/100g 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}±0.0	6.4^{b}±0.1	11.0^{c}±0.0	8.8^{d}±0.1	16.0^{e}±0.1	12.8^{f}±0.1
Ash value	6.5^{d}±0.1	2.7^{c}±0.0	1.6^{b}±0.0	1.6^{b}±0.1	1.3^{a}±0.1	1.4^{a}±0.1
Total proteins	10.0^{b}±0.0	20.5^{c}±0.1	5.1^{a}±0.0	5.1^{a}±0.0	5.7^{d}±0.0	5.8^{e}±0.0
Total lipids	0.8^{a}±0.0	1.2^{b}±0.0	15.6^{c}±0.1	19.9^{d}±0.0	19.4^{e}±0.2	22.2^{f}±0.1
Carbohydrates*	65.4^{a}±0.1	52.2^{b}±0.1	57.9^{c}±0.2	56.2^{d}±0.3	47.8^{e}±0.21	51.1^{f}±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}±0.1	17.0^{b}±0.1	8.8^{c}±0.0	8.4^{d}±0.0	9.8^{e}±0.0	6.7^{f}±0.0

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

546 **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	$7.18^{a,b} \pm 0.82$	$5.58^{a} \pm 0.60$	$0.71^{a,b} \pm 0.06$

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

550 **Table 4. Carotenoid bioaccessibility in relation to their initial quantity in cereal-based**
551 **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.	$9.15^{b,\$} \pm 1.84$

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

555

556 **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	$0.72^{a} \pm 0.29$

557 Mean value ± standard deviation (n=6). Values followed by the same letter in the column are not

558 significantly different ($p>0.05$)

559

560

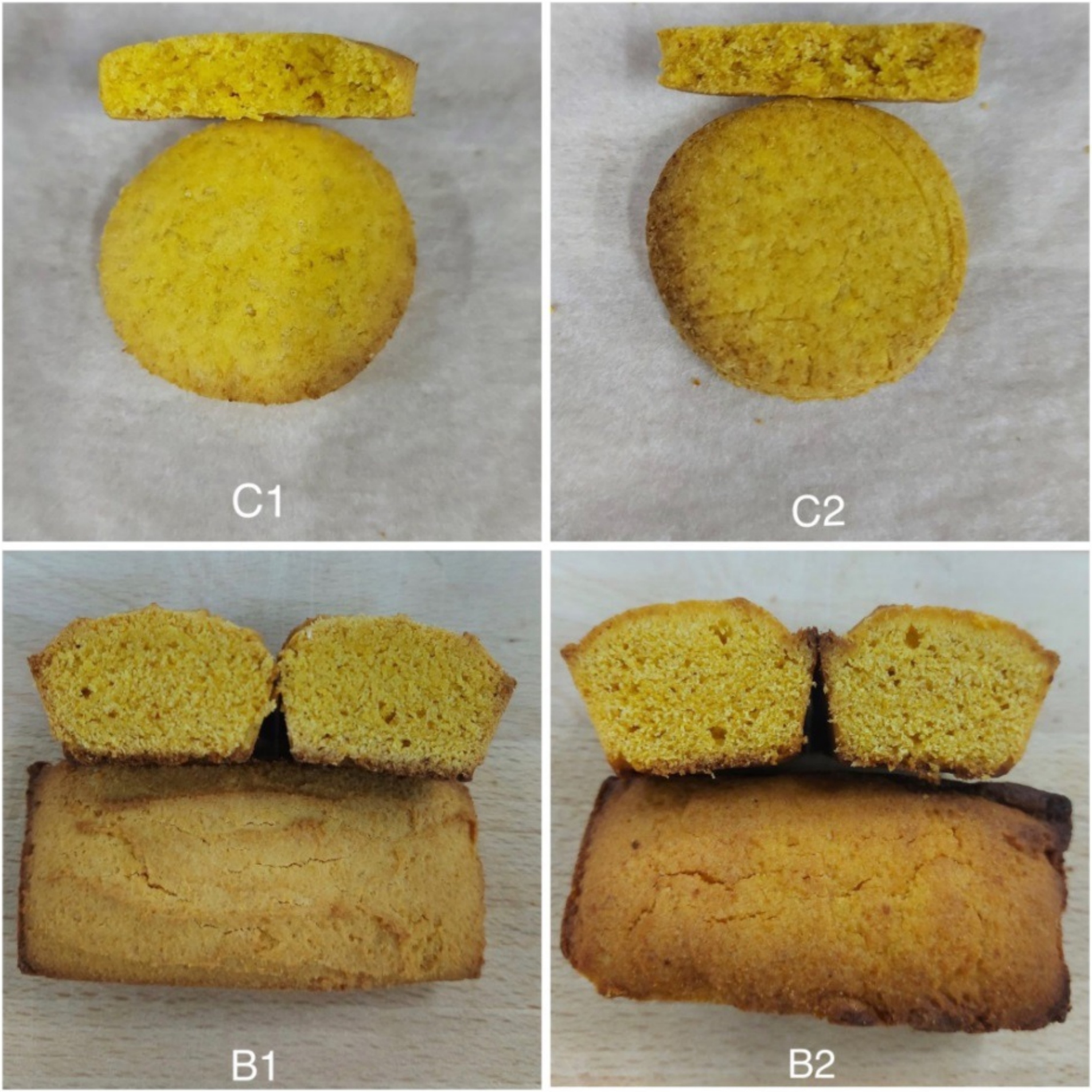
561 **Figure captions**

562 **Figure 1.** Cross section of samples: C1-cookie with butter, C2-cookie with vegetable oil, B1-
563 sponge cake with butter, B2-sponge cake with vegetable oil. Average size of cookie (mm):
564 ø=54.2, hight, h=9.35 mm. Average size of sponge cake (mm): length:width:hight, 100:50:35.

565

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

569


C1
C2
B1
B2