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Bioaccessibility of Polyphenols from Plant Processing By-products of Black Carrot (*Daucus carota* L.)

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ABSTRACT

Plant processing by-products of black carrot represent an important disposal problem for the industry, however they are also promising sources of polyphenols, especially anthocyanins. The present study focused on the changes in polyphenols from black carrot, peel and pomace during *in vitro* gastrointestinal digestion. Total phenolic content (TPC), total monomeric anthocyanin content (TMAC) and total antioxidant capacity (TAC) were determined using spectrophotometric methods, whereas identification and quantification of polyphenols was carried out using UPLC-ESI-MS^E and HPLC-DAD, respectively. TPC, TMAC and TAC significantly decreased (23–82%) as a result of *in vitro* gastrointestinal digestion. Nevertheless the amount of pomace anthocyanins released at all stages of *in vitro* gastrointestinal digestion was higher than black carrot anthocyanins, suggesting that pomace may be a better source of bioaccessible anthocyanins. Overall, the current study highlighted black carrot by-products as substantial sources of polyphenols, which may be used to enrich food products.

KEYWORDS: anthocyanins, antioxidant capacity, black carrot, *Daucus carota* L., HPLC, *in vitro* gastrointestinal digestion, peel, phenolics, pomace, SCFA

INTRODUCTION

Black carrots originate from Turkey and the Middle and Far East, where they have been cultivated for at least 3000 years. They have an attractive bluish-purple color with high levels of anthocyanins and can serve as a natural food colorant due to their high heat, light and pH stability.[1] Apart from their colorant properties, anthocyanins may serve an important role in promoting health by reducing the risk of atherosclerosis and cancer, preventing inflammation, and acting as antioxidants.[2] Besides anthocyanins as the major polyphenols, black carrots also contain significant amounts of phenolic acids, including hydroxycinnamates and caffeic acid.[3]

As many fruits and vegetables, black carrots are seasonal and perishable, and difficult to preserve as a raw material. Therefore, they are processed into various products such as juice,[4] concentrate,[5] jam[6] and shalgam - i.e., traditional lactic acid fermented beverage -.[7] As a result of processing, large amounts of by-products including peel and pomace are generated. Black carrot pomace production in Turkey is estimated as 2,700 and 6,900 tons in 2009 and 2010, respectively. This trend is expected to continue and even to accelerate in upcoming years.[8] By-products of plant food processing represent a major disposal problem for the industry concerned, however they are also promising sources of bioactive compounds.[9] In fact, several by-products especially the ones from wine industry[10,11] are shown to be rich sources of polyphenols.

Bioaccessibility is defined as the amount of a food constituent that is released from a complex food matrix in the lumen of the gastrointestinal tract, and therefore could potentially be available for absorption into the body. Only polyphenols released from the food matrix by the action of digestive enzymes and bacterial microflora are bioaccessible in the gut and therefore potentially bioavailable.[12] *In vitro* methods simulating digestion processes are widely used to study the gastrointestinal behavior of

food components. Although human nutritional studies are still being considered as the "gold standard" for addressing diet related questions, *in vitro* methods have the advantage of being more rapid, less expensive, less labor intensive, and do not have ethical restrictions.[13] *In vitro* gastrointestinal digestion methods have already been used to study the release of polyphenols from by-products of grape,[14] mango,[15] pomegranate[16] and cauliflower.[17] Nevertheless, to the best of our knowledge, this is the first study that has focused on the changes in polyphenols from black carrot peel and pomace during *in vitro* gastrointestinal digestion.

Given the above, the aim of this study was to investigate the digestive stability of the total phenolics, total monomeric anthocyanins and some abundant phenolic acids and anthocyanins from black carrot and its by-products as well as changes in their antioxidant activity using an *in vitro* model that simulates the conditions in the stomach, small intestine and colon. In addition, short chain fatty acids (SCFAs) formed as a result of microbial metabolic activity were also monitored.

MATERIALS AND METHODS

Plant Material. Black carrot (*Daucus carota* L. spp. *sativus* var. *atrorubens* Alef.) and its industrial by-product pomace were collected in triplicate from an established processing plant (Erkonsatre Co.) in Konya, Turkey in April 2014. Peel was obtained manually from whole black carrots. All samples were ground to a fine powder in liquid nitrogen using a precooled grinder (IKA A11 basic, IKA-Werke GmbH & Co., Staufen, KG, Germany), and subsequently lyophilized (Christ Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) for 24 hours and transported in frozen state to Belgium, where they were stored at -20 °C until further analysis.

The flow chart of the black carrot juice processing line, where the pomace samples were collected, is provided in Supporting Information Figure S1.

Chemicals. For simulation of *in vitro* digestion, mucin, bovine serum albumin, pepsin, pancreatin, lipase and bile from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and urea from Chem-Lab NV (Zedelgem, Belgium) were purchased. The following standards were used for the quantification of major polyphenols: neochlorogenic acid (≥98%), cryptochlorogenic acid (≥98%), caffeic acid (≥98%) and cyanidin-3-*O*-glucoside (≥95%) from Sigma-Aldrich, and chlorogenic acid (≥98%) and ferulic acid (≥99%) from Fluka Chemie AG (Buchs, Switzerland).

Simulated *In Vitro* Gastrointestinal Digestion. The *in vitro* gastrointestinal digestion model applied in this study consisted of a three-step procedure, which sequentially simulated the digestion in the stomach, small intestine and colon. The composition of gastric, duodenal and bile solutions are presented in detail in Table 1. Briefly, 3 g of black carrot, peel and pomace samples were weighed into penicillin bottles and mixed with 47 mL of distilled water and 10 mL of gastric solution. The samples were acidified to pH 3 and incubated at 37 °C in a shaker (Edmund Bühler TH 15, Hechingen, Germany) at 100 rpm for 2 h. After gastric digestion, 15 mL aliquots were collected for each sample. Afterwards, the pH was increased to 7 with the addition of 30 mL duodenal and 15 mL bile solutions. The samples were incubated in a shaker at 37 °C, 100 rpm for another 4 h to complete the small intestinal phase of the *in vitro* gastrointestinal digestion. After the small intestine phase, again samples were collected. Following sampling, 30 mL of colon microbial suspensions from the descending colon compartment of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) were incubated into closed bottles for 48 h at 37 °C. The descending colon compartment of SHIME contained *in vitro* cultured microbiota that harbored a reproducible human

93 microbial community representative for the *in vivo* conditions, both in composition and
94 metabolic activity.[18,19] Samples were taken at 24 and 48 h with a needle that extends
95 beyond the rubber stoppers that seal off the incubation bottles. The blank (without the
96 added black carrot, peel and pomace samples) was also incubated under the same
97 conditions and used for the correction of interferences from the digestive fluids and
98 colon microbiota. Samples collected from stomach, small intestine and colon phases
99 were centrifuged at 3000 *g*, 4 °C for 10 min (Sigma Laboratory Centrifuge 4K15,
100 Osterode am Harz, Germany) and the supernatants were kept at -20 °C until further
101 analysis.

102 **Chemical Extraction of Undigested Samples.** For each sample, three
103 independent extractions were carried out as described previously.[20] 0.1 g of lyophilized
104 powder was treated with 5 mL of two different solvents: 75% aqueous methanol or
105 ethanol both containing 0.1% (v/v) formic acid. The treated samples were sonicated in
106 an ultrasonic bath (Elma S60H elmasonic, Singen, Germany) for 15 min and
107 subsequently centrifuged at 3000 *g*, 4 °C for 10 min (Sigma) and the supernatants were
108 collected. This extraction protocol was repeated once more for the pellet, and the two
109 supernatants were pooled to a final volume of 10 mL. Prepared extracts were stored at -
110 20 °C until analysis. Since methanol extracted significantly more total phenolics and
111 total monomeric anthocyanins than ethanol (Supporting Information Figure S2),
112 methanol was selected as the extraction solvent for all other subsequent analyses.

113 **Spectrophotometric Assays.** Total phenolic content (TPC), total monomeric
114 anthocyanin content (TMAC) and total antioxidant capacity (TAC) assays were
115 performed using a Varian Cary 50 Bio UV-Visible spectrophotometer (Varian Inc., Palo
116 Alto, CA, USA).

TPC of samples was estimated using Folin-Ciocalteu reagent as described before.[21] One hundred microliters of extract was mixed with 0.75 mL of Folin-Ciocalteu reagent. The mixture was allowed to stand for 5 min and then 0.75 mL of 6% Na_2CO_3 solution was added to the mixture. After 90 min of incubation, the absorbance was measured at 725 nm. TPC was expressed as mg of gallic acid equivalent (GAE) per 100 g of dry weight (dw) of sample.

TMAC was determined according to the pH differential method.[22] Absorbance was measured at 520 and 700 nm in buffers at pH 1.0 (KCl, 0.025 M) and pH 4.5 (CH_3CO_2Na, 0.4 M) within 20-50 min of preparation and calculated using the following equation: TMAC = (A x MW x DF x 1000) / (ε x l), where $A = (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$, MW = molecular weight of cyanidin-3-*O*-glucoside (C3G) (449.2 g/mol), DF = dilution factor, ε = molar extinction coefficient of C3G (26900 L/(mol.cm)), and l = pathlength (cm). TMAC was expressed as mg of C3G per 100 g of dw of sample.

TAC was evaluated using three different assays: 2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Ion Reducing Antioxidant Power (FRAP) and Cupric Ion Reducing Antioxidant Capacity (CUPRAC). For DPPH assay, 100 µL of extract was mixed with 2 mL of 0.1 mM DPPH in methanol. After 30 min of incubation, absorbance was measured at 517 nm.[23] For FRAP assay, 900 µL of FRAP reagent (mixture of acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM $FeCl_3$ in proportions of 10:1:1, respectively) was combined with 100 µL of extract. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min.[24] For CUPRAC assay, 100 µL of extract was mixed with 1 mL of 10 mM $CuCl_2$, 7.5 mM neocuproine and 1 M $NH_4C_2H_3O_2$. Subsequently, 1 mL of distilled water was added to the mixture so as to bring the final volume to 4.1 mL. After 30 min of incubation at room temperature,

absorbance was measured at 450 nm.[25] For all TAC assays, results were expressed in terms of mg of Trolox® equivalent (TE) per 100 g of dw of sample.

For each spectrophotometric assay, samples were analyzed in triplicate and the average values were reported.

Identification of Anthocyanins and Phenolic Acids Using UPLC-ESI-MS^E. Before LC-MS analysis, samples from the *in vitro* gastrointestinal digestion were purified using a solid-phase extraction (SPE) method. Initially, 500 mg/4 mL C18 SPE cartridges (Grace Davison Discovery Sciences, Deerfield, IL, USA) were conditioned by rinsing with 6 mL formic acid/methanol (1:100, v/v) followed by 4 mL formic acid/MQ water (1:100, v/v). 1.5 mL aliquots of samples were acidified with 30 µL formic acid and centrifuged (Labnet Spectrafuge 16M, Labnet International Inc., Woodbridge, NJ, USA) at 16000 *g* for 10 min. Afterwards, the supernatants were loaded to activated cartridges which were subsequently washed with 5 mL formic acid/MQ water (1:100, v/v). Samples were eluted with formic acid/methanol (1:100, v/v) and then dried using nitrogen. Prior to LC-MS analysis, samples were redissolved in DMSO/MQ water (1:10, v/v) and filtered through 0.45-µm membrane filters.

LC-MS analysis was performed with a Waters Acquity UPLC system (Waters Co., Milford, MA, USA) connected to a Waters Synapt HDMS TOF mass spectrometer. For chromatographic separation, a Waters Acquity BEH C18 column (2.1 mm x 150 mm, 1.7 µm particle size) attached to a Waters VanGuard pre-column (2.1 mm x 5 mm) was used. Five microlitres of each filtered sample were injected into the system using formic acid/MQ water (1:1000, v/v; eluent A) and formic acid/methanol (1:1000, v/v; eluent B) at a flow rate of 250 µL/min. The gradient was as follows: 0 min, 5% B; 0-10 min, 5-15% B linear; 10-15 min, 15% B isocratic; 15-23 min, 15-95% B linear; 23-28 min, 95% B isocratic; 28-30 min, 95-5% B linear; 30-32 min, 5% B isocratic. The

column temperature was maintained at 40 °C, whereas the temperature of the autosampler was held at 10 °C. ESI-MS analysis was performed in both positive and negative mode. Collision energies of 6 V (for low energy) and 45 V (for high energy) were used for full-scan LC-MS in the m/z range 100-1500. Leucine enkephalin was used for online mass calibration. For the acquisition of the data, MassLynx 4.1 software (Waters Co.) was used.

Quantification of Anthocyanins and Phenolic Acids Using HPLC-DAD. Anthocyanins and phenolic acids were quantified following the method of Capanoglu et al.[20] Samples purified using SPE were passed through 0.45-μm membrane filters and injected into Thermo Dionex Ultimate 3000 HPLC (Thermo Fischer Scientific, Landsmeer, The Netherlands) coupled with diode array detector (DAD). A Grace Smart RP C18 column (250 x 4.6 mm, 5 μm) was used as the stationary phase. The following solvents with a flow rate of 1 mL/min and injection volume of 10 μL were used for spectral measurement at 312 and 520 nm: TFA/MQ water (1:1000, v/v; eluent A) and TFA/acetonitrile (1:1000, v/v; eluent B). The linear gradient was used as follows: 0 min, 5% B; 0-45 min, 35% B linear; 45-47 min, 75% B linear; 47-54 min 5% B. The content of anthocyanin glycosides were quantified using cyanidin-3-*O*-glucoside, whereas phenolic acids were quantified using their authentic standards. Matrix-match calibration curves were prepared by spiking blank control samples of *in vitro* gastrointestinal digestion at concentration levels from 0.1 to 100 ppm. Curves were plotted as the chromatographic peak area of the standards versus nominal concentrations. Slope, intercept, R^2, limit of detection (LOD) and limit of quantification (LOQ) of the standard curves for each stage of the digestion are provided in Supporting Information Table S1. All results were expressed as mg per 100 g dw of sample.

Analysis of Short Chain Fatty Acids (SCFAs) Using GC-FID. SCFAs were extracted from colon samples of *in vitro* gastrointestinal digestion and analyzed as described previously by De Weirdt et al.[26] Briefly, 500 µL of extracts were treated with 500 µL of H_2SO_4 (1:1, v/v) followed by the addition of 400 µL 2-methyl hexanoic acid as an internal standard. Subsequently, 0.4 g NaCl and 2 mL of diethyl ether were added and centrifuged at 3000 *g* for 3 min (Sigma). The supernatants were injected into a GC-2014 gas chromatograph (Shimadzu, 's-Hertogenbosch, the Netherlands), equipped with a capillary fatty acid-free EC-1000 Econo-Cap column (25 x 0.53 mm, 1.2 mM; Alltech, Laarne, Belgium), a flame ionization detector (FID) and a split injector. The temperature profile was set from 110 to 160 °C, with a temperature increase of 6 °C per min. Nitrogen was used as a carrier gas, the injection volume was 1 µL and the temperature of the injector and detector were maintained at 100 and 220 °C, respectively. Results were expressed as mg per L of extracts.

Statistical Analysis. All analyses were performed with three technical and three biological replicates. Data were subjected to statistical analysis using SPSS software (version 20.0, SPSS Inc., Chicago, IL, USA). Treatments were compared using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. $p < 0.05$ was considered significant. Slope, intercept, R^2, LOD and LOQ of the standard curves were calculated using the LINEST function in Microsoft Office Excel 2011 software (Microsoft Co., Redmond, WA, USA).

RESULTS AND DISCUSSION

Spectrophotometric assays. The impact of gastrointestinal digestion on total phenolic content (TPC), total monomeric anthocyanin content (TMAC) and total antioxidant capacity (TAC) of samples is shown in Table 2. For undigested samples,

although by-products contained respectively 10-28% and 12-31% lower TPC and TAC than black carrot, statistically there was no significant difference ($p > 0.05$). The lower TPC and TAC of pomace may be attributed to citric acid treatment, which previously has been shown to reduce the TPC and TAC of black carrots[5] as a result of low pH values, which may affect the sensitivity of the methods used. On the other hand, TMAC of pomace was found to be slightly higher (3%) than black carrots. This may be explained by the facilitated extraction of compounds through the macerated tissue, due to increased membrane permeability with applied temperature and/or release of bound compounds with the breakdown of cellular constituents as a consequence of applied temperature and enzyme treatment.[5] In the stomach, lower TPC (43-71%), TMAC (51-74%) and TAC (31-69%) were observed for all samples. Studies carried out on black mulberry[27] and purple tomato[28] also revealed lower TPC (28%), TMAC (35-82%) and TAC (28-79%) after gastric digestion. The small intestinal phase gave a further increase in TPC, TMAC and TAC of the by-products (<28-30, 6-12 and 26-46%, respectively) compared to amounts released in the stomach; however, the values were still lower compared to the undigested samples. A similar trend was also observed in case of apple polyphenols and the authors explained this increase after the small intestinal phase by the additional contact time of the food material with the intestinal fluids (plus 4 h) and/or the effect of intestinal digestive enzymes (lipase and pancreatin, with the latter also having amylase and protease activity) on the complex food matrix, facilitating the release of polyphenols bound to the matrix.[29] Furthermore, higher antioxidant activity could also be explained by the fact that enzyme action and pH change might generate new compounds with higher TAC than the ones found in the stomach. From the small intestine to the colon after 24 h of incubation, although mostly additional reductions in TPC (4-37%), TMAC (18-22%) and TAC (8-46%) occurred, there was no statistical

241 difference ($p > 0.05$). Further incubation in the colon (t = 48 h) resulted in varying
242 results, showing both increases and decreases. However, in general these variations
243 were statistically not significant ($p > 0.05$). Correlations between TPC, TMAC and
244 TAC were also determined. For both the undigested and digested samples, generally a
245 highly linear relationship was obtained between TPC and TAC, with the CUPRAC
246 assay being the highest with R^2 = 0.8873-0.9712. On the other hand, weak correlations
247 were obtained between TMAC and TAC, ranging between R^2 = 0.0068-0.6867. These
248 results imply that phenolic compounds other than anthocyanins may be the major
249 contributors to the antioxidant activity. The correlations within different TAC assays
250 were quite high, especially correlations between FRAP and CUPRAC assays (R^2 =
251 0.8577-0.9503). Taking into account the pH conditions of the TAC assays applied in
252 this study, it can be hypothesised that the FRAP assay conducted at pH 3.6 could be
253 more suitable for assessing antioxidant activity in the stomach, whereas DPPH and
254 CUPRAC assays could be more appropriate to evaluate TAC in the small intestine and
255 colon with a working pH of 7.0-8.0. In addition to the pH dependency, the chemical
256 structure of the polyphenol and interaction of polyphenols with other dietary
257 compounds released during digestion, e.g. dietary fiber or proteins, are also known to
258 affect the polyphenol accessibility, and thus the antioxidant activity.[29] Eventually, it is
259 recommended that at least two of the TAC assays should be combined to ensure a
260 reliable picture as was done in the current study.

261 **Anthocyanins.** LC-MS analysis of black carrots and by-products led to the
262 identification of five major anthocyanins. The detected anthocyanins were all cyanidin-
263 based with different sugar moieties, among them two were non-acylated (cyanidin-3-
264 xylosyl-glucosyl-galactoside and cyanidin-3-xylosyl-galactoside), and three were
265 acylated with sinapic acid (cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside), ferulic

266 acid (cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside) and *p*-coumaric acid (cyanidin-
267 3-xylosyl-coumaroyl-glucosyl-galactoside) (Table 3). The identification of each
268 anthocyanin was carried out in positive mode, based on the UV-visible characteristics,
269 MS and fragmentation pattern, as well as by comparing with the data already reported in
270 literature. The anthocyanin profile of black carrot was in agreement with those reported
271 previously.[1,5,30,31] The influence of *in vitro* gastrointestinal digestion on major
272 anthocyanins from black carrot and its by-products are presented in Table 4, whereas
273 representative chromatograms recorded at 520 nm corresponding to the anthocyanin
274 profile of black carrot at different stages of gastrointestinal digestion are displayed in
275 Figure 1. Among the undigested samples, pomace contained the highest amount of total
276 anthocyanins, which was consistent with the results obtained spectrophotometrically
277 (Table 2). The predominant anthocyanin was cyanidin-3-xylosyl-feruloyl-glucosyl-
278 galactoside for all undigested samples representing about 40-69% of the total
279 anthocyanins, whereas anthocyanins with acylated structure constituted 61-88% of the
280 total anthocyanins. It has been shown that acylated anthocyanins are more stable to pH
281 and temperature changes than non-acylated ones due to intramolecular copigmentation
282 effects of acylated anthocyanins.[32] Therefore, by-products from black carrot as sources
283 of acylated anthocyanins may provide the desirable stability for food applications. After
284 gastric digestion in the stomach, a significantly lower amount of anthocyanins was
285 observed (37-89%) for almost all samples ($p < 0.05$). Our findings were comparable
286 with the results of an *in vitro* study on purple figs, in which 35% cyanidin-3-rutinoside
287 was recovered after gastric digestion.[33] In the small intestine, while there was no
288 significant change in the anthocyanin content of black carrot and peel, additional loss of
289 anthocyanins (11-32%) was observed for pomace. The high loss of anthocyanins may
290 be related to the fact that they are metabolized to some non-colored forms, oxidized or

degraded into other chemicals, which may not be detected under the present conditions.[34] In addition, it has been reported that due to ionic interactions, approximately 65% of the anthocyanins from black carrots were bound to plant cell walls and overwhelmingly remain bound after *in vitro* gastric and small intestinal digestion.[35] The fermentation in the colon for 24 h resulted in elevated anthocyanin levels (up to 2 fold) compared to amounts released in the small intestine, whereas extra incubation (48 h) did not cause a significant difference ($p < 0.05$). The human gut lacks enzymes such as pectinases and cellulases allowing the transit of pectin and cellulose components through the gastrointestinal tract. Therefore, food matrix interactions between anthocyanins and these carbohydrate molecules may significantly enhance the bioaccessibility of anthocyanins in the colon, where these macrocomplexes are degraded by the residing microbiota.[36] The differences in the absorptivity of the black carrot anthocyanins in relation to cyanidin-3-glucoside might also be the cause of the increase in individual anthocyanin concentrations. Furthermore, the percent recovery of non-acylated anthocyanins in the colon (up to 3 fold) was found to be higher than acylated anthocyanins (up to 72%). An *in vivo* study conducted with 12 healthy volunteers consuming purple carrots revealed that plasma and urine recoveries of non-acylated anthocyanins were greater than recovery of acylated anthocyanins (8-10 and 11-14 fold, respectively).[37] The authors proposed that the acylations might have been cleaved to produce cyanidin-3-xylosyl-glucosyl-galactoside, which may also be valid for our study. Overall, the amount of pomace anthocyanins released at all stages of *in vitro* gastrointestinal digestion was higher than black carrot anthocyanins, suggesting that pomace may be a better source of bioaccessible anthocyanins.

Phenolic Acids. Five major phenolic acids, namely neochlorogenic acid (3-*O*-caffeoylquinic acid), cryptochlorogenic acid (4-*O*-caffeoylquinic acid), chlorogenic acid

316 (5-*O*-caffeoylquinic acid), caffeic acid and ferulic acid were detected in black carrot and
317 by-products using LC-MS in negative mode (Table 3). Table 5 shows the impact of *in*
318 *vitro* gastrointestinal digestion on major phenolic acids present in black carrot and by-
319 products. In accordance with previous black carrot reports,[3,5,6] chlorogenic acid was
320 identified as the most abundant compound for all samples accounting for 71-84% of
321 total phenolic acids, whereas caffeoylquinic acids represented 83-89% of the phenolic
322 acids. Similar to anthocyanin results, for undigested samples the highest content of total
323 phenolic acids was determined in pomace. Especially the amounts of neochlorogenic
324 and caffeic acids were significantly higher (70% and 8-fold, respectively) than those
325 found in black carrot ($p < 0.05$). Some studies described the influence of processing on
326 the conversion of caffeoylquinic acids into other isomers. Besides isomerization,
327 caffeoylquinic acids can also undergo hydrolysis during processing, resulting in a
328 consequent increase in caffeic acid.[38] Accordingly, the higher neochlorogenic and
329 caffeic acid contents in pomace may be related to these factors. Gastric digestion in the
330 stomach significantly decreased the caffeoylquinic acid content of the samples (14-
331 89%) ($p < 0.05$). Previous studies have also noticed low stability of phenolic acids
332 under gastric conditions.[16,33] Pancreatic digestion in the small intestine caused further
333 loss of caffeoylquinic acids (3-35%). This additional loss could be associated with the
334 instability of the caffeoylquinic acids in aqueous solution. In addition, the effect of pH
335 (pH = 7) and the bile salts could also contribute to the yield with a higher loss.[39]
336 Moreover, phenolic acids from black carrots were shown to stay bound to plant cell
337 walls during *in vitro* gastric and small intestinal digestion.[35] On the other hand,
338 increases in ferulic acid content was observed as a result of small intestinal digestion.
339 This may be attributed to cleavage of the major anthocyanin cyanidin-3-xylosyl-
340 feruloyl-glucosyl-galactoside to ferulic acid. At the end of the *in vitro* fermentation in

the colon, 27% further decrease in the content of chlorogenic acid was observed for pomace, along with the formation of caffeic acid. This was an expected outcome of microbial fermentation since caffeic acid has been reported as the major product resulting from the hydrolysis of chlorogenic acid by colonic microbiota.[36]

SCFAs. To investigate whether the release of anthocyanins and phenolic acids in colon conditions can be correlated with changes in microbial activity, SCFAs as end products of fermentation have been investigated. Although there are other indicators of microbial activity (e.g. ammonium production), measurement of SCFA production is also often used as an indicator of microbial activity when studying colonic metabolism. SCFAs produced from black carrot, by-products and control (without the food matrix) samples during colonic fermentation comprised mainly of acetic, propionic and butyric acids (Figure 2). The proportional production of major SCFAs (acetate:propionate:butyrate) after 24 h were similar for black carrot (74:23:3), peel (74:22:4) and pomace (79:18:3). In a previous study, Aura et al.[40] also showed production of similar proportions of SCFAs at the end of a 24 h *in vitro* microbial fermentation of digested grape by a human faecal inoculum. After 48 h, butyric acid concentrations increased twice as much, whereas the changes in acetic and propionic acid were not significant ($p > 0.05$). At both time points, the total amount of SCFAs produced from pomace samples was significantly higher (15-24%) than black carrot and peel ($p < 0.05$). This is probably due to the higher sugar content of free sugars in the pomace compared to the peel and black carrot. The amount of acetate present in the colon after 24 h was correlated well with the amount of cyanidin-3-xylosyl-galactoside released ($R^2 = 0.8415$). Similarly, after 48 h a high correlation was obtained between acetate and ferulic acid ($R^2 = 0.8742$).

To the best of our knowledge, this is the first study that has focused on the changes in bioaccessible polyphenols from black carrot by-products during *in vitro* gastrointestinal digestion. The current study pointed out that black carrot peel and pomace provide considerable polyphenol intake, which are preserved to a certain degree after digestion; therefore they may be used to enrich food products. Even though the conclusions achieved with this static model used for the simulation of *in vitro* gastrointestinal digestion cannot directly estimate the human *in vivo* conditions, we suggest that this model is useful for studying the effect of the food matrix, enzymes and colon microbiota on polyphenol bioaccessibility. In future studies, to investigate the bioavailability of the phenolic compounds and their uptake mechanisms, cell culture systems simulating the epithelial barrier such as Caco-2 cells may be used.

ABBREVIATIONS

ANOVA: Analysis of Variance; **C3G**: Cyanidin-3-*O*-Glucoside; **CUPRAC**: Cupric Ion Reducing Antioxidant Capacity; **DMSO**: Dimethyl Sulfoxide; **DPPH**: 2,2-diphenyl-1-picrylhydrazyl; **FRAP**: Ferric Ion Reducing Antioxidant Power; **GAE**: Gallic Acid Equivalent; **GC-FID**: Gas Chromatography-Flame Ionization Detection; **HPLC-DAD**: High-Performance Liquid Chromatography-Diode Array Detection; **LC-MS**: Liquid Chromatography-Mass Spectrometry; **LOD**: Limit of Detection; **LOQ**: Limit of Quantification; **SCFA**: Short Chain Fatty Acid; **SHIME**: Simulator of the Human Intestinal Microbial Ecosystem; **SPE**: Solid-Phase Extraction; **TAC**: Total Antioxidant Capacity; **TE**: Trolox® Equivalent; **TFA**: Trifluoroacetic Acid; **TMAC**: Total Monomeric Anthocyanin Content; **TPC**: Total Phenolic Content; **UPLC-ESI-MS^{E}**: Ultra Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry.

SUPPORTING INFORMATION

Supporting Information Available: Flow chart outlining the steps involved in black carrot juice production, Total phenolic and total monomeric anthocyanin contents of black carrot and by-products extracted with different solvents, Standards used for the quantification of polyphenols during *in vitro* gastrointestinal digestion. This material is available free of charge via the Internet at http://pubs.acs.org."

REFERENCES

(1) Montilla, E.C.; Arzaba, M.R.; Hillebrand, S.; Winterhalter, P. Anthocyanin composition of black carrot (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) cultivars Antonina, Beta Sweet, Deep Purple, and Purple Haze. *J. Agric. Food Chem.* **2011**, *59*, 3385-3390.

(2) Kurilich, A.C.; Clevidence, B.A.; Britz, S.J.; Simon, P.W.; Novotny, J.A. Plasma and urine responses are lower for acylated vs nonacylated anthocyanins from raw and cooked purple carrots. *J. Agric. Food Chem.* **2005**, *53*, 6537-6542.

(3) Kammerer, D.; Carle, R.; Schieber, A. Characterization of phenolic acids in black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1331-1340.

(4) Khandare, V.; Walia, S.; Singh, M.; Kaur, C. Black carrot (*Daucus carota* ssp. *sativus*) juice: processing effects on antioxidant composition and color. *Food Bioprod. Process.* **2011**, *89*, 482-486.

(5) Suzme, S.; Boyacioglu, D.; Toydemir, G.; Capanoglu, E. Effect of industrial juice concentrate processing on phenolic profile and antioxidant capacity of black carrots. *Int. J. Food Sci. Technol.* **2014**, *49*, 819-829.

(6) Kamiloglu, S.; Pasli, A.A.; Ozcelik, B.; Van Camp, J.; Capanoglu, E. Influence of different processing and storage conditions on *in vitro* bioaccessibility of polyphenols in black carrot jams and marmalades. *Food Chem.* **2015**, *186*, 74-82.

(7) Turker, N.; Aksay, S.; Ekiz, H.I. Effect of storage temperature on the stability of anthocyanins of a fermented black carrot (*Daucus carota* var. L.) beverage: Shalgam. *J. Agric. Food Chem.* **2004**, *52*, 3807-3813.

(8) Agcam, E.; Akyildiz, A. Effects of different solvents and acid concentrations on extraction of anthocyanins from black carrot pomace. *Food*. **2015**, *40*, 149-156.

(9) Schieber, A.; Stintzing, F.C.; Carle, R. By-products of plant food processing as a source of functional compounds-recent developments. *Trends Food Sci. Technol.* **2001**, *12*, 401-413.

(10) Kammerer, D.; Claus, A.; Carle, R.; Schieber, A. Polyphenol screening of pomace from red and white grape varieties (*Vitis vinifera* L.) by HPLC-DAD-MS/MS. *J. Agric. Food Chem.* **2004**, *52*, 4360-4367.

(11) Makris, D.P.; Boskou, G.; Andrikopoulos, N.K. Polyphenolic content and *in vitro* antioxidant characteristics of wine industry and other agri-food solid waste extracts. *J. Food Compos. Anal.* **2007**, *20*, 125-132.

(12) Saura-Calixto, F.; Serrano, J.; Goni, I. Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chem.* **2007**, *101*, 492-501.

(13) Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carriere, F.; Boutrou, R.; Corredig, M.; Dupont, D.; Dufour, C.; Egger, L.; Golding, M.; Karakaya, S.; Kirkhus, B.; Le Feunteun, S.; Lesmes, U.; Macierzanka, A.; Mackie, A.; Marze, S.; McClements, D.J.; Menard, O.; Recio, I.; Santos, C.N.; Singh, R.P.; Vegarud, G.E.; Wickham, M.S.J.; Weitschies, W.; Brodkorb, A. A standardised static *in*

439 *vitro* digestion method suitable for food - an international consensus. *Food Funct.* **2014**,
440 *5*, 1113-1124.
441 (14) Wang, D.; Williams, B.A.; Ferruzzi, M.G.; D'Arcy, B.R. Microbial metabolites,
442 but not other phenolics derived from grape seed phenolic extract, are transported
443 through differentiated Caco-2 cell monolayers. *Food Chem.* **2013**, *138*, 1564-1573.
444 (15) Blancas-Benitez, F.J.; Mercado-Mercado, G.; Quirós-Sauceda, A.E.; Montalvo-
445 González, E.; Gonzalez-Aguilar, G.A.; Sayago-Ayerdi, S.G. Bioaccesibility of
446 polyphenols associated with dietary fiber and *in vitro* kinetics release of polyphenols in
447 Mexican 'Ataulfo' mango (*Mangifera indica* L.) by-products. *Food Funct.* **2015**, *6*,
448 859-868.
449 (16) Mosele, J.I.; Macia, A.; Romero, M.P.; Motilva, M.J.; Rubio, L. Application of
450 *in vitro* gastrointestinal digestion and colonic fermentation models to pomegranate
451 products (juice, pulp and peel extract) to study the stability and catabolism of phenolic
452 compounds. *J. Funct. Foods*. **2015**, *14*, 529-540.
453 (17) Gonzales, G.B; Smagghe, G.; Mackie, A.; Grootaert, C.; Bajka, B.; Rigby, N.;
454 Raes, K.; Van Camp, J. Use of metabolomics and fluorescence recovery after
455 photobleaching to study the bioavailability and intestinal mucus diffusion of
456 polyphenols from cauliflower waste. *J. Funct. Foods*. **2015**, *16,* 403-413.
457 (18) Sánchez-Patán, F.; Barroso, E.; van de Wiele, T.; Jiménez-Girón, A.; Martín-
458 Alvarez, P.J.; Moreno-Arribas, M.V.; Martínez-Cuesta, M.C.; Peláez, C.; Requena, T.;
459 Bartolomé, B. Comparative *in vitro* fermentations of cranberry and grape seed
460 polyphenols with colonic microbiota. *Food Chem*. **2015**, *183*, 273-282.
461 (19) Van den Abbeele, P.; Grootaert, C.; Marzorati, M.; Possemiers, S.; Verstraete,
462 W.; Gérard, P.; Rabot, S.; Bruneau, A.; Aidy, S.E.; Derrien, M.; Zoetendal, E.;
463 Kleerebezem, M.; Smidt, H.; Van de Wiele, T. (2010). Microbial community

development in a dynamic gut model is reproducible, colon region specific, and selective for Bacteroidetes and Clostridium cluster IX. *Appl. Environ. Microbiol.* **2010**, *76*, 5237-5246.

(20) Capanoglu, E.; Beekwilder, J.; Boyacioglu, D.; Hall, R.; De Vos, R. Changes in antioxidant and metabolite profiles during production of tomato paste. *J. Agric. Food Chem.* **2008**, *56*, 964-973.

(21) Velioglu, Y.S.; Mazza, G.; Gao, L.; Oomah, B.D. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agric. Food Chem.* **1998**, *46*, 4113-4117.

(22) AOAC Int 2006. *Official methods of analysis of AOAC International, Official Method 2005.02.* Gaithersburg, MD.

(23) Kumaran, A; Karunakaran, R.J. Antioxidant and free radical scavenging activity of an aqueous extract of *Coleus aromaticus*. *Food Chem*. **2006**, *97*, 109-114.

(24) Benzie, I.F.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70-76.

(25) Apak, R.; Guclu, K.; Ozyurek, M.; Karademir, S.E. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *J. Agric. Food Chem.* **2004**, *52*, 7970-7981.

(26) De Weirdt, R.; Possemiers, S.; Vermeulen, G.; Moerdijk-Poortvliet, T.C.; Boschker, H.T.; Verstraete, W.; Van de Wiele, T. Human faecal microbiota display variable patterns of glycerol metabolism. *FEMS Microbiol Ecol.* **2010**, *74*, 601-611.

(27) Tomas, M.; Toydemir, G.; Boyacioglu, D.; Hall, R.; Beekwilder, J.; Capanoglu, E. The effects of juice processing on black mulberry antioxidants. *Food Chem*. **2015**, *186*, 277-284.

(28) Li, H.; Deng, Z.; Liu, R.; Loewen, S.; Tsao, R. Bioaccessibility, *in vitro* antioxidant activities and *in vivo* anti-inflammatory activities of a purple tomato (*Solanum lycopersicum* L.). *Food Chem.* **2014**, *159*, 353-360.

(29) Bouayed, J.; Hoffmann, L.; Bohn, T. Total phenolics, flavonoids, anthocyanins and antioxidant activity following simulated gastro-intestinal digestion and dialysis of apple varieties: Bioaccessibility and potential uptake. *Food Chem.* **2011**, *128*, 14-21.

(30) Algarra, M.; Fernandes, A.; Mateus, N.; de Freitas, V.; da Silva, J.C.E.; Casado, J. Anthocyanin profile and antioxidant capacity of black carrots (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) from Cuevas Bajas, Spain. *J. Food Compos. Anal.* **2014**, *33*, 71-76.

(31) Kamiloglu, S.; Pasli, A.A.; Ozcelik, B.; Van Camp, J.; Capanoglu, E. Colour retention, anthocyanin stability and antioxidant capacity in black carrot (*Daucus carota*) jams and marmalades: Effect of processing, storage conditions and *in vitro* gastrointestinal digestion. *J. Funct. Foods*. **2015**, *13*, 1-10.

(32) Kammerer, D.; Carle, R.; Schieber, A. Quantification of anthocyanins in black carrot extracts (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) and evaluation of their color properties. *Eur. Food Res. Technol.* **2004**, *219*, 479-486.

(33) Kamiloglu, S.; Capanoglu, E. Investigating the *in vitro* bioaccessibility of polyphenols in fresh and sun-dried figs (*Ficus carica* L.). *Int. J. Food Sci. Technol.* **2013**, *48*, 2621-2629.

(34) Pérez-Vicente, A.; Gil-Izquierdo, A.; Garcia-Viguera, C. *In vitro* gastrointestinal digestion study of pomegranate juice phenolic compounds, anthocyanins, and vitamin C. *J. Agric. Food Chem.* **2012**, *50*, 2308-2312.

(35) Padayachee, A.; Netzel, G.; Netzel, M.; Day, L.; Mikkelsen, D.; Gidley, M.J. Lack of release of bound anthocyanins and phenolic acids from carrot plant cell walls

514 and model composites during simulated gastric and small intestinal digestion. *Food*
515 *Funct.* **2013**, *4*, 906-916.
516 (36) Correa-Betanzo, J.; Allen-Vercoe, E.; McDonald, J.; Schroeter, K.; Corredig,
517 M.; Paliyath, G. Stability and biological activity of wild blueberry (*Vaccinium*
518 *angustifolium*) polyphenols during simulated *in vitro* gastrointestinal digestion. *Food*
519 *Chem.* **2014**, *165*, 522-531.
520 (37) Kurilich, A.C.; Clevidence, B.A.; Britz, S.J.; Simon, P.W.; Novotny, J.A.
521 Plasma and urine responses are lower for acylated vs nonacylated anthocyanins from
522 raw and cooked purple carrots. *J. Agric. Food Chem.* **2005**, *53*, 6537-6542.
523 (38) Van Boekel, M.; Fogliano, V.; Pellegrini, N.; Stanton, C.; Scholz, G.; Lalljie, S.;
524 Somoza, V.; Knorr, D.; Jasti, P.R.; Eisenbrand, G. A review on the beneficial aspects of
525 food processing. *Mol. Nutr. Food Res.* **2010**, *54*, 1215-1247.
526 (39) Vallejo, F.; Gil-Izquierdo, A.; Pérez-Vicente, A.; García-Viguera, C. *In vitro*
527 gastrointestinal digestion study of broccoli inflorescence phenolic compounds,
528 glucosinolates, and vitamin C. *J. Agric. Food Chem.* **2004**, *52*, 135-138.
529 (40) Aura, A.M.; Mattila, I.; Hyötyläinen, T.; Gopalacharyulu, P.; Cheynier, V.;
530 Souquet, J.M.; Bes, M.; Le Bourvellec, C.; Guyot, S.; Orešič, M. Characterization of
531 microbial metabolism of Syrah grape products in an *in vitro* colon model using targeted
532 and non-targeted analytical approaches. *Eur. J. Nutr.* **2013**, *52*, 833-846.
533

534 **NOTE**

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FIGURE CAPTIONS

Figure 1. HPLC chromatograms (DAD, recorded at 520 nm) of black carrot anthocyanins during *in vitro* gastrointestinal digestion**.** Numbers refer to the major anthocyanins identified: (1) cyanidin-3-xylosyl-glucosyl-galactoside (cyd-3-xyl-gluc-gal); (2) cyanidin-3-xylosyl-galactoside (cyd-3-xyl-gal) (3) cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside (cyd-3-xyl-sin-gluc-gal); (4) cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside (cyd-3-xyl-fer-gluc-gal); (5) cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside (cyd-3-xyl-coum-gluc-gal).

Figure 2. Short chain fatty acids (SCFAs) produced during colon fermentation. Data represent average values ± standard deviation of three independent batches. Different letters above bars within each time point represent statistically significant differences between samples ($p < 0.05$), whereas differences between time points (24 and 48 h) are indicated as $*p < 0.05$.

Table 1. Composition of Gastric, Duodenal and Bile Solutions Applied During *In Vitro* Gastrointestinal Digestion[a]

Constituent (g/L)	Gastric solution pH 3	Duodenal solution pH 7	Bile solution pH 8
Salts			
NaCl	2.75	7.01	5.26
NaH_2PO_4	0.35	-	-
KCl	0.82	0.56	0.38
$CaCl_2.2H_2O$	0.40	0.20	0.22
$NaHCO_3$	-	5.61	5.79
KH_2PO_4	-	0.08	-
$MgCl_2.6H_2O$	-	0.50	-
Host factors			
Urea	0.09	0.10	0.25
Mucin	0.35		
Bovine serum albumin	1.00	1.00	1.80
Pepsin	1.00	-	-
Pancreatin	-	3.00	-
Lipase	-	0.50	-
Bile	-	-	6.00

[a]All solutions were prepared with distilled water to a total volume of 1 L. The enzymes and other compounds assigned in the table were mixed just before use, and pH was adjusted using 1 M NaOH or concentrated HCl in cases where it was necessary.

Table 2. Changes in the Total Phenolic Content (TPC), Total Monomeric Anthocyanin Content (TMAC) and Total Antioxidant Capacity (TAC) of the Bioaccessible Fraction of Black Carrot and By-products During *In Vitro* Gastrointestinal Digestion[a]

Sample	Undigested	Stomach	Small Intestine	Colon t = 24h	Colon t = 48h
			TPC (mg GAE/100 g dw)		
Black Carrot	5743.0 ± 910.8^{aA}	3296.0 ± 751.5^{abA}	2768.5 ± 711.8^{bA}	2653.0 ± 697.2^{bA}	3669.3 ± 964.4^{abA}
Peel	5170.1 ± 620.4^{aA}	2234.4 ± 351.2^{bAB}	3761.2 ± 796.3^{abA}	3218.6 ± 345.7^{bA}	3136.7 ± 214.0^{bA}
Pomace	4151.3 ± 224.8^{aA}	1202.9 ± 142.6^{dB}	2987.2 ± 223.0^{bA}	1883.3 ± 293.0^{cdA}	2215.8 ± 577.9^{bcA}
			TMAC (mg C3G/100 g dw)		
Black Carrot	1653.8 ± 183.1^{aA}	804.0 ± 62.8^{bA}	337.5 ± 54.9^{cB}	451.1 ± 38.7^{cA}	304.6 ± 50.9^{cC}
Peel	1221.1 ± 236.2^{aA}	324.3 ± 36.0^{bB}	470.0 ± 64.0^{bAB}	367.9 ± 32.3^{bA}	577.9 ± 13.9^{bB}
Pomace	1703.4 ± 164.1^{aA}	446.1 ± 52.3^{cB}	545.6 ± 54.3^{bcA}	449.7 ± 58.9^{cA}	730.0 ± 69.8^{bA}
			TAC (mg TE/100 g dw)		
DPPH (2,2-diphenyl-1-picrylhydrazyl)					
Black Carrot	7198.7 ± 1027.3^{aA}	3583.3 ± 469.3^{bA}	3095.7 ± 1136.6^{bA}	2686.4 ± 605.8^{bAB}	2443.6 ± 630.1^{bA}
Peel	4943.9 ± 1316.3^{abA}	2702.5 ± 260.1^{bB}	4995.6 ± 1188.6^{aA}	3750.8 ± 450.8^{abA}	3058.9 ± 338.7^{abA}
Pomace	5247.4 ± 770.4^{aA}	1868.8 ± 151.5^{cC}	4064.5 ± 300.0^{abA}	2187.9 ± 518.6^{cB}	2534.5 ± 903.8^{bcA}
FRAP (Ferric Ion Reducing Antioxidant Power)					
Black Carrot	9257.2 ± 1537.0^{aA}	4605.6 ± 1231.4^{bA}	3548.1 ± 1170.4^{bA}	3183.8 ± 1094.8^{bA}	3489.3 ± 629.1^{bA}
Peel	7641.7 ± 913.0^{aA}	2719.6 ± 272.9^{cAB}	4761.2 ± 1055.1^{bA}	3468.2 ± 533.8^{bcA}	3818.4 ± 389.3^{bcA}
Pomace	6937.5 ± 587.7^{aA}	2167.1 ± 250.5^{cB}	4372.3 ± 695.8^{bA}	2893.9 ± 617.3^{bcA}	3197.5 ± 803.0^{bcA}
CUPRAC (Cupric Ion Reducing Antioxidant Capacity)					
Black Carrot	17426.1 ± 2935.5^{aA}	11992.6 ± 1474.7^{abA}	7817.9 ± 2291.5^{bA}	7195.6 ± 1828.3^{bAB}	7647.3 ± 1301.4^{bA}
Peel	15324.0 ± 1802.4^{aA}	7311.3 ± 944.9^{bB}	11343.5 ± 2655.6^{abA}	9250.8 ± 961.8^{bA}	8583.1 ± 337.0^{bA}
Pomace	12960.7 ± 852.6^{aA}	4447.4 ± 785.1^{cB}	8919.1 ± 959.5^{bA}	5542.2 ± 1012.1^{bcB}	6924.4 ± 2066.4^{bcA}

[a]The data presented in this table consist of average values ± standard deviation of three independent batches. TPC, TMAC and TAC are expressed as mg of gallic acid equivalent (GAE), cyanidin-3-*O*-glucoside equivalent (C3G) and Trolox® equivalent (TE) per 100 g of dry weight (dw) of sample, respectively. Different capital letters in the columns or small letters in the rows represent statistically significant differences ($p < 0.05$).

Table 3. Mass Spectrometric Data and Identification of the Polyphenols Detected in the Black Carrot and By-products by LC-MS

Retention time (min)	Identity	λ (nm)	Mass (m/z)	MS^2 main fragment (m/z)
Anthocyanins				
17.57	Cyanidin-3-xylosyl-glucosyl-galactoside	500	(+) 743	287
18.34	Cyanidin-3-xylosyl-galactoside	500	(+) 581	287
18.98	Cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside	500	(+) 949	287
19.61	Cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside	500	(+) 919	287
19.67	Cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside	500	(+) 889	287
Phenolic Acids				
8.97	Neochlorogenic acid	312	(-) 353	191
11.85	Cryptochlorogenic acid	312	(-) 353	191
12.40	Chlorogenic acid	312	(-) 353	191
15.42	Caffeic acid	312	(-) 179	135
21.76	Ferulic acid	312	(-) 193	178

Table 4. Changes in the Anthocyanins of the Bioaccessible Fraction of Black Carrot and By-products During *In Vitro* Gastrointestinal Digestion[a]

Sample	Undigested	Stomach	Small Intestine	Colon t = 24h	Colon t = 48h
			cyanidin-3-xylosyl-glucosyl-galactoside (mg C3G/100 g dw)		
Black Carrot	44.1 ± 7.4^{aB}	11.0 ± 3.1^{bB}	12.4 ± 5.5^{bB}	33.1 ± 11.5^{aB}	33.2 ± 8.2^{aB}
Peel	10.5 ± 4.8^{bC}	13.8 ± 3.2^{bB}	10.9 ± 2.0^{bB}	31.7 ± 13.0^{aB}	32.0 ± 2.2^{aB}
Pomace	81.0 ± 8.7^{aA}	50.8 ± 6.7^{bA}	24.6 ± 4.4^{cA}	82.4 ± 3.9^{aA}	96.2 ± 12.9^{aA}
			cyanidin-3-xylosyl-galactoside (mg C3G/100 g dw)		
Black Carrot	176.9 ± 54.9^{aB}	19.2 ± 2.5^{bB}	19.2 ± 8.3^{bB}	41.2 ± 13.8^{bB}	44.9 ± 17.2^{bB}
Peel	75.8 ± 30.7^{aB}	23.2 ± 6.8^{bB}	11.1 ± 1.1^{bB}	44.8 ± 20.5^{abB}	52.6 ± 14.8^{abB}
Pomace	507.8 ± 38.2^{aA}	165.1 ± 31.4^{bA}	64.3 ± 10.7^{cA}	179.8 ± 4.2^{bA}	220.4 ± 41.6^{bA}
			cyanidin-3-xylosyl-sinapoyl-glucosyl-galactoside (mg C3G/100 g dw)		
Black Carrot	282.2 ± 92.1^{aA}	31.7 ± 7.7^{bB}	22.9 ± 10.4^{bB}	24.3 ± 12.0^{bB}	27.1 ± 9.7^{bB}
Peel	96.9 ± 22.3^{aB}	29.5 ± 9.1^{bB}	12.2 ± 1.9^{bB}	26.6 ± 8.6^{bB}	26.6 ± 5.3^{bB}
Pomace	250.6 ± 34.7^{aA}	110.8 ± 10.9^{bA}	75.5 ± 15.1^{bA}	82.9 ± 13.2^{bA}	66.1 ± 10.6^{bA}
			cyanidin-3-xylosyl-feruloyl-glucosyl-galactoside (mg C3G/100 g dw)		
Black Carrot	844.9 ± 181.1^{aA}	110.8 ± 35.6^{bB}	50.4 ± 20.1^{bB}	62.8 ± 28.5^{bB}	46.1 ± 19.9^{bB}
Peel	519.5 ± 62.7^{aB}	98.2 ± 29.3^{bB}	41.2 ± 4.6^{bB}	70.5 ± 30.3^{bB}	49.7 ± 13.4^{bAB}
Pomace	610.6 ± 43.0^{aAB}	265.2 ± 37.5^{bA}	198.5 ± 16.6^{bcA}	143.3 ± 0.6^{cdA}	86.5 ± 11.1^{dA}
			cyanidin-3-xylosyl-coumaroyl-glucosyl-galactoside (mg C3G/100 g dw)		
Black Carrot	97.5 ± 3.5^{aA}	35.0 ± 15.9^{bA}	19.6 ± 6.8^{bA}	17.9 ± 5.7^{bA}	31.0 ± 14.0^{bA}
Peel	46.5 ± 9.1^{aC}	28.4 ± 11.6^{abA}	13.8 ± 3.4^{bA}	33.3 ± 18.2^{abA}	31.1 ± 11.6^{abA}
Pomace	77.4 ± 5.1^{aB}	38.3 ± 6.4^{bA}	25.2 ± 6.3^{cA}	17.5 ± 1.1^{cA}	24.3 ± 2.8^{cA}

[a]The data presented in this table consist of average values ± standard deviation of three independent batches. Different capital letters in the columns or small letters in the rows represent statistically significant differences ($p < 0.05$).

Table 5. Changes in the Phenolic Acids of the Bioaccessible Fraction of Black Carrot and By-products During *In Vitro* Gastrointestinal Digestion[a]

Sample	Undigested	Stomach	Small Intestine	Colon t = 24h	Colon t = 48h
			Neochlorogenic acid (mg/100 g dw)		
Black Carrot	9.5 ± 3.5^{aB}	4.4 ± 1.0^{bAB}	1.6 ± 1.1^{bB}	3.7 ± 0.5^{bA}	5.1 ± 0.4^{abA}
Peel	6.8 ± 0.9^{aB}	3.3 ± 1.2^{bcB}	0.9 ± 0.4^{cB}	3.3 ± 0.9^{bcA}	5.3 ± 0.8^{abA}
Pomace	16.2 ± 0.9^{aA}	7.0 ± 1.3^{bA}	4.3 ± 0.4^{cdA}	2.9 ± 0.7^{dA}	5.7 ± 0.4^{bcA}
			Cryptochlorogenic acid (mg/100 g dw)		
Black Carrot	33.8 ± 18.3^{aA}	7.6 ± 2.6^{bB}	4.1 ± 0.4^{bC}	8.6 ± 1.1^{bB}	9.6 ± 0.7^{bB}
Peel	23.7 ± 5.7^{aA}	9.2 ± 2.8^{bAB}	6.1 ± 0.9^{bB}	9.3 ± 0.3^{bB}	10.7 ± 0.4^{bB}
Pomace	16.7 ± 2.1^{aA}	14.3 ± 1.9^{abA}	11.0 ± 0.5^{bA}	16.8 ± 0.9^{aA}	16.2 ± 1.2^{aA}
			Chlorogenic acid (mg/100 g dw)		
Black Carrot	335.5 ± 141.6^{aAB}	37.0 ± 11.0^{bB}	26.0 ± 12.0^{bB}	39.6 ± 4.7^{bA}	25.8 ± 0.7^{bA}
Peel	170.2 ± 60.0^{aB}	43.0 ± 16.3^{bB}	34.5 ± 4.1^{bB}	35.3 ± 12.3^{bA}	27.0 ± 4.0^{bA}
Pomace	475.7 ± 43.4^{aA}	277.1 ± 27.3^{bA}	156.9 ± 6.3^{cA}	51.4 ± 8.2^{dA}	26.1 ± 7.1^{dA}
			Caffeic acid (mg/100 g dw)		
Black Carrot	2.2 ± 0.5^{cB}	8.9 ± 2.2^{abA}	5.9 ± 0.5^{bcA}	12.8 ± 1.2^{aB}	11.8 ± 2.4^{aB}
Peel	2.0 ± 0.2^{bB}	8.8 ± 2.9^{abA}	5.4 ± 0.8^{bA}	16.1 ± 5.1^{aB}	14.4 ± 2.5^{aB}
Pomace	19.1 ± 2.2^{bcA}	8.8 ± 0.8^{cA}	4.3 ± 0.9^{cA}	28.6 ± 4.0^{bA}	46.1 ± 12.3^{aA}
			Ferulic acid (mg/100 g dw)		
Black Carrot	63.3 ± 13.7^{bA}	68.5 ± 15.3^{bA}	98.4 ± 7.4^{abA}	93.7 ± 23.1^{abAB}	108.0 ± 1.0^{aAB}
Peel	38.2 ± 7.6^{bB}	52.8 ± 11.3^{bA}	79.0 ± 15.3^{abA}	128.7 ± 49.8^{aA}	129.7 ± 21.6^{aA}
Pomace	41.5 ± 7.3^{bAB}	24.8 ± 1.1^{bB}	34.0 ± 4.2^{bB}	32.2 ± 3.9^{bB}	72.1 ± 16.5^{aB}

[a]The data presented in this table consist of average values ± standard deviation of three independent batches. Different capital letters in the columns or small letters in the rows represent statistically significant differences ($p < 0.05$).

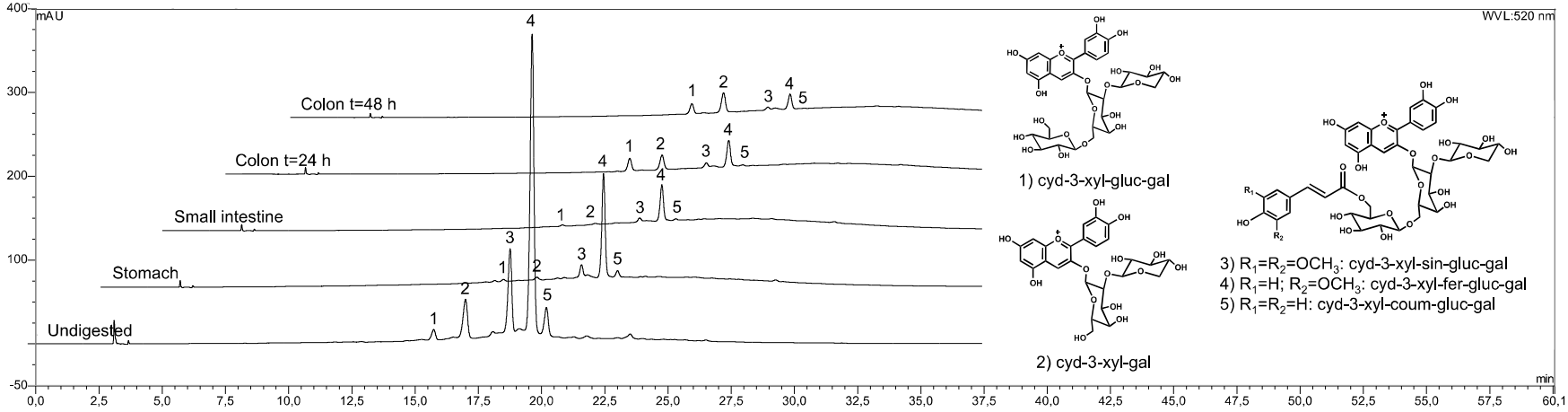

mAU
WVL:520 nm
Colon t=48 h
Colon t=24 h
Small intestine
Stomach
Undigested
min
1) cyd-3-xyl-gluc-gal
2) cyd-3-xyl-gal
3) R1=R2=OCH3: cyd-3-xyl-sin-gluc-gal
4) R1=H; R2=OCH3: cyd-3-xyl-fer-gluc-gal
5) R1=R2=H: cyd-3-xyl-coum-gluc-gal

Figure 1

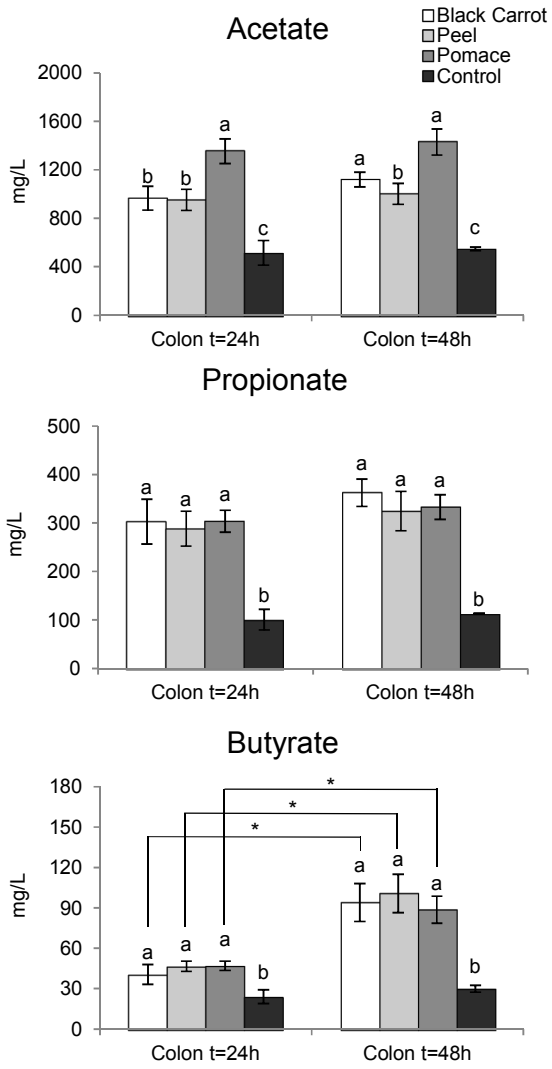
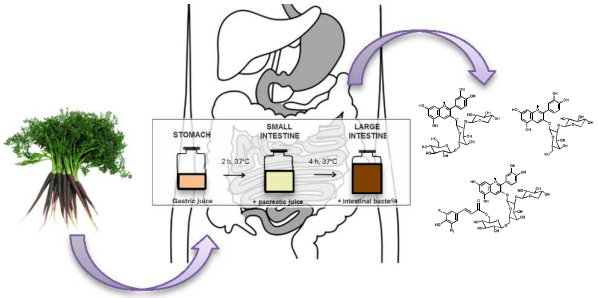

Acetate
Black Carrot
Peel
Pomace
Control
mg/L
2000
1600
1200
800
400
0
a
b
b
a
a
b
c
c
Colon t=24h
Colon t=48h
Propionate
mg/L
500
400
300
200
100
0
a
a
a
b
a
a
a
b
Colon t=24h
Colon t=48h
Butyrate
mg/L
180
150
120
90
60
30
0
*
*
*
a
a
a
b
a
a
a
b
Colon t=24h
Colon t=48h

Figure 2

TOC Graphic


STOMACH
SMALL INTESTINE
LARGE INTESTINE
2 h, 37°C
4 h, 37°C
Gastric juice
+ pancreatic juice
+ intestinal bacteria