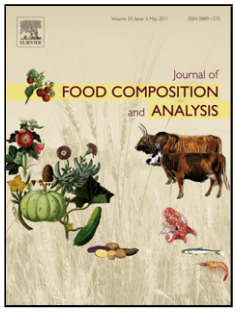

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Original Research Article

Bioaccessibility analysis of anthocyanins and ellagitannins from blackberry at simulated gastrointestinal and colonic levels

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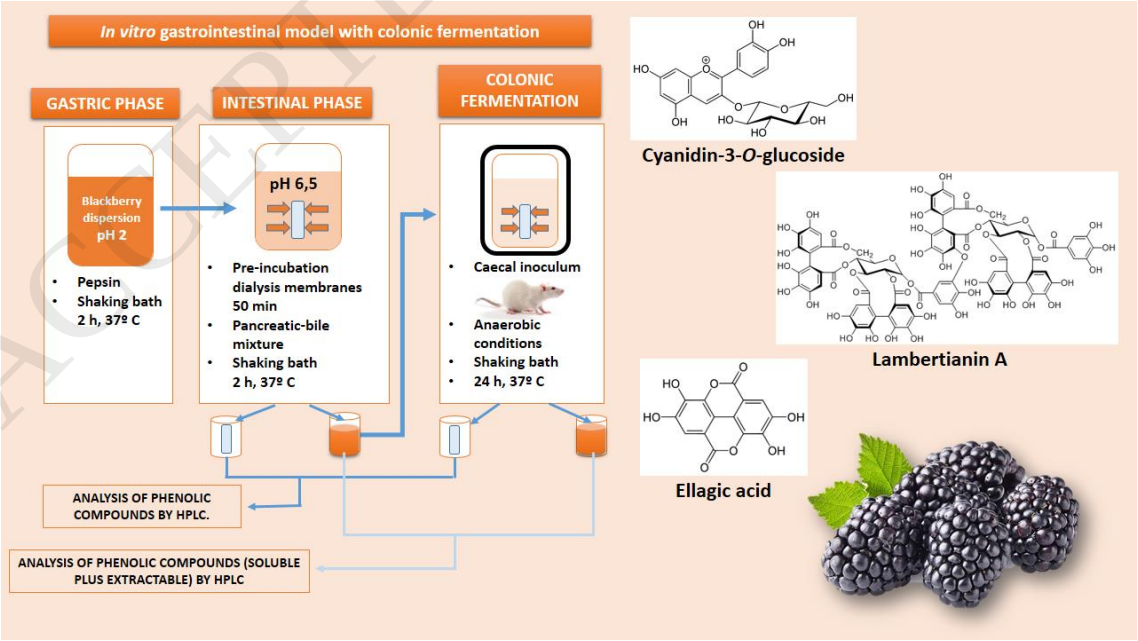
Graphical abstract



Highlights

- The *in vitro* total bioaccessibility of polyphenols from blackberry was investigated.
- Before starting the fermentation phase, dialysis membranes were novelly incorporated.
- The intestinal bioaccessibility of total polyphenols analyzed by HPLC was 2.2%.
- Only 1% of indigenous polyphenols was accessible after colonic fermentation.
- The total bioaccessibility of blackberry phenolic compounds was around 4%.

Abstract

The total bioaccessibility of the main phenolic compounds from blackberry fruit using an *in vitro* gastrointestinal model with colonic fermentation was investigated. The incorporation of a dialysis membrane before starting the fermentation phase was included with the aim of providing more correct bioaccessibility estimations at colonic level. Phenolic compounds in the raw material and in digested samples were analyzed by high-performance liquid chromatography. The intestinal bioaccessibility was 1.8% for the principal anthocyanin, cyanidin-3-*O*-glucoside, and less than 1% for the principal ellagitannins, lambertianin A and C. In contrast, this value was 14.9% for ellagic acid, due to its release from ellagitannin breakdown. The intestinal bioaccessibility of total polyphenols, calculated as the sum of indigenous phenolic compound contents, was 2.2%. After fermentation, only 1% of the indigenous individual polyphenols was potentially bioaccessible at colonic level. However, ellagic acid colonic bioaccessibility was around 10% due to the prevention of its further metabolization by dialysis process. Total bioaccessibility of the main blackberry

polyphenols, calculated as the sum of intestinal and colonic bioaccessibility, was around 3.3%, indicating that gastrointestinal and colonic conditions affected the amount of the indigenous polyphenols and, consequently, their bioaccessibility as intact structures.

Keywords: phenolic compounds; intestinal bioaccessibility; colonic bioaccessibility; dialysis; food analysis; food composition.

1. Introduction

Blackberries are recognized as a rich source of phenolic compounds, with human health benefits, due to their antioxidant and anti-inflammatory properties (Koca & Karedeniz, 2009; Zia-Ul-Haq et al., 2014). Research on blackberry composition has increased because they contain a unique and intense complement of polyphenolic compounds (Cuevas-Rodriguez et al., 2010). The major polyphenols in blackberries are anthocyanins, hydrolyzable tannins (gallo- and ellagitannins), flavonols, and flavan-3-ols, including proanthocyanidins (Mertz et al., 2007). The phenolic compound profile characterization, antioxidant and anti-inflammatory properties of three blackberry cultivars adapted to Argentina were studied by Van de Velde et al. (2016).

To exert their biological properties, these polyphenols have to be available to some extent in the target tissue (Saura-Calixto et al., 2007). Therefore, the biological properties of dietary polyphenols will depend on the quantity or fraction of them which is released from the food matrix in the gastrointestinal tract and becomes available for absorption in the gut (bioaccessibility), reaches the systemic circulation and is utilized (bioavailability) (Carbonell-Capella et al., 2014).

In-vivo feeding methods, using animal or human models, generally offer the most precise results and are considered as the “gold standard” for determining the bioaccessibility and bioavailability of phenolic compounds (Minekus et al., 2014).

Unluckily, analyzing the complex multistage process that occurs during the human or animal digestion is technically difficult, costly, and limited by ethical issues (Augustin et al., 2014; Minekus et al., 2014, Lucas-Gonzáles et al., 2018). Consequently, there is a real need of using *in-vitro* models that closely mimic the physiological processes occurring during human digestion (Minekus et al., 2014). Nowadays, there are different ways of simulating the bioaccessible fraction of foods at the intestinal level (Gayoso et al., 2016). The easiest approach is to analyze the resulting content of the entire intestinal fraction, just by its filtration and/or centrifugation to separate the soluble material (fraction available for uptake) (Bermúdez-Soto et al., 2007; Gayoso et al., 2016). In addition, dialysis has been also used for simulating the bioaccessible fraction of food and extracts (Etcheverry, et al., 2012). In the dialysis model, the dialyzable fraction represents the sample that goes through the semi-permeable membrane and is available for absorption; meanwhile the fraction outside the dialysis membrane represents the non-absorbable sample (Gayoso et al., 2016). Estimations of the *in-vitro* bioaccessibility of phenolic compounds from foodstuffs at the small intestine level are reported in literature by solubility (Tagliazucchi et al., 2010, Lopes-Neto et al., 2017), and by dialysis (Bouayed et al., 2012; Liang et al. 2012).

These *in vitro* approaches that simulate gastrointestinal conditions are rapid, reproducible, safe, and do not have the same ethical restrictions as *in vivo* methods (You et al., 2010). However, they do not consider the fraction of phenolic compounds that are metabolized by the colonic microflora, or that could be potentially absorbed by the large intestine. These phenolic compounds reach the colon and they become fermentation substrates for bacterial microflora along with the non-digestible food constituents (Saura-Calixto et al., 2007).

Addressing these issues, there are some reported *in-vitro* studies where the intestinal digest is fermented with either a bacterial strain (Raimondi et al., 2009), ileostomy fluid (Knaup et al., 2007) or flora from freshly voided human (Aura et al., 2005) or animal feces (Saura-Calixto et al., 2007; Chitindingu et al., 2015; Gayoso et al., 2016; Low et al., 2016; Rocchetti et al., 2017) cultured in a suitable medium. In these *in-vitro* studies the colonic bioaccessibility (%) is estimated indirectly as the difference between the digested phenolic compounds before fermentation and the phenolic compounds of the residues obtained after fermentation. However, it is known that the plasma membrane is a selectively permeable barrier (Lodish et al., 2000). Thus, this approach, employing the whole pool of polyphenols to estimate the colonic bioaccessibility, does not discriminate between compounds of low and high molecular weight, and it may overestimate results. Moreover, the exposure of polyphenols to fecal microbiota and/or their secreted enzymes results in the production of lower molecular weight metabolites. These metabolites may be absorbed by the colon epithelium at the time they are produced, or subsequently they can be fermented to carbon dioxide (Saura-Calixto et al., 2007; Crozier et al., 2010). Thereby, the actual *in-vitro* approaches do not consider the possible further losses of metabolites from polyphenols to carbon dioxide. In this sense, the dialysis of phenolic compounds during their *in-vitro* fermentation may discriminate compounds on the basis of their molecular weight and also mimic the absorption of small molecular weight metabolites while they are being produced, avoiding their further metabolization.

In this work we investigated the total bioaccessibility of phenolic compounds from blackberry fruit using an *in-vitro* gastrointestinal model with colonic fermentation. We also proposed to incorporate a dialysis membrane before starting the colonic

fermentation phase with the aim of providing more correct bioaccessibility estimations of the indigenous phenolic compounds at the large intestine level.

2. Materials and methods

2.1. Chemical and reagents

Pepsin (Sigma P-7000), pancreatin (Sigma P-1750), bile extract (Sigma-B-8631), reference cyanidin-3-*O*-glucoside, and ellagic acid were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents were HPLC grade and obtained from Panreac Química S.L.U. (Castellar del Vallés, Barcelona, Spain). All other chemicals were reagent grade. Spectra/Pore® I dialysis tubing (cut-off 6000 to 8000 Da) was purchased from Fisher Scientific (Fairlawn, NJ).

2.2. Raw material

Blackberries (*Rubus fruticosus* L.) cultivar 'Black Satin' were collected from one planting at Coronda (31°58′00″S 60°55′00″W), Santa Fe, (Argentina) in January 2014. Fruits were transported 50 km directly from the field to the laboratory of the Instituto de Tecnología de Alimentos, FIQ, UNL, (Argentina). Samples were frozen at –80°C until lyophilization in a Flexi-Dry freeze dryer (SP Scientific, Gardiner, NY). The freeze-dried material was weighed and the dry matter content was estimated by difference in weight. Results were calculated on a fresh weight basis by considering the water content in the fruits and expressed as raw material (RM), according to Grace et al. (2014).

2.3. Phenolic compound analysis

2.3.1. Extraction of phenolic compounds from the raw material

The extraction procedure for the analysis of anthocyanins and other phenolic compounds by HPLC was conducted in triplicates according to Van de Velde et al. (2016). Briefly, 0.5 g of freeze-dried blackberries were placed into 15-mL centrifuge vials. Eight milliliters of 80:20 methanol: water (0.5% acetic acid) were added and the

mixture was sonicated (Ultrasonic Cleaner; Testlab, Buenos Aires, Argentina) for 10 min. The mixture was then centrifuged at 12,000 *g* for 20 min at 20 °C, and the resulting supernatant was collected into a 25-mL volumetric flask. The extraction of the pellet was repeated two more times and the combined extracts were brought to a final volume of 25 mL with the extraction solvent.

2.3.2. Re-extraction of phenolic compounds

Phenolic compounds from the residues of intestinal and fermentation digests (Section 2.4) that were not extracted and dissolved by the intestinal conditions were re-extracted by adding 10.0 mL of 80:20 methanol:water (0.5% acetic acid), as indicated by the raw material, according to Van de Velde et al. (2016). Then, the re-extracted samples were sonicated for 10 min and centrifuged at 12,000 *g* for 10 min at 20 °C. Supernatants were separated and used for analysis.

2.3.3. Phenolic compounds determination by high-performance liquid chromatography (HPLC)

HPLC analysis was conducted using an LC-20AT Prominence Liquid Chromatograph (LC) (Shimadzu Co., Kyoto, Japan) with a photodiode array detector and with LabSolution software as a controller and for data processing. Anthocyanin separation was performed according to Grace et al. (2013) with some modifications, using a reversed-phase Phenomenex Gemini column, 25 mm × 4.6 mm, with 5 μm particle size (Phenomenex, Torrance, CA) at 30 °C. The mobile phase consisted of 5% formic acid in water (**A**) and 100% methanol (**B**). The flow rate was constant during HPLC analysis at 1 mL/min with a step gradient of 10, 15, 20, 25, 30, 60, 10, and 10% of solvent **B** at 0, 5, 15, 20, 25, 40, 42, and 45 min, respectively. Quantification of anthocyanins was performed from the peak areas recorded at 520 nm, with reference to the external standard method calibration curve obtained with cyanidin-3-*O*-glucoside (0.05–1

mg/mL), and results were expressed as g/100 g RM. Phenolic compounds separation was performed according to Grace et al. (2014) with the same column used for the analysis of anthocyanins. The mobile phase consisted of 2% acetic acid in water (**A**) and 0.5% acetic acid in 50% acetonitrile in water (**B**). The flow rate was set at 1 mL/min with a step gradient of 10, 15, 25, 55, 100, and 10% of solvent **B** at 0, 13, 20, 50, 54, and 60 min, respectively. UV maximum absorption was recorded at 254 and 280 nm. Ellagic acid (0.025–0.5 mg/mL) was used as external standard, and results were expressed as g/100 g RM.

2.4. *In vitro* gastrointestinal digestion with colonic fermentation

The *in-vitro* method of Miller et al. (1981) was used to simulate the conditions of the gastric and intestinal phases of the digestive process. For the gastric phase, the pH of 2% blackberry dispersion samples was adjusted to 2 with 1 Eq/L HCl. Then, 0.4 mL of freshly prepared 16 % pepsin suspension (prepared in HCl 0.1 Eq/L) were added and the mixtures were incubated in a shaking bath at 37 ºC for 2 h (Figure 1). At the end of pepsin digestion, dialysis bags containing 10 mL $NaHCO_3$ buffer were placed in each beaker and incubated for 50 min in a shaking water bath at 37 ºC (Figure 1). $NaHCO_3$ buffer molarity was calculated to obtain a final pH of digest-dialysate (6.5 ± 0.2), according to Wolfgor et al. (2002). Factors taken into account to calculate buffer molarity were: buffer capacity of the food matrix (HCl mEq needed to reach pH 2.0), HCl mEq incorporated with pepsin solution (0.04 mEq), and mEq equivalent to titratable acidity defined as NaOH mEq required to reach a pH of 6.5. Titratable acidity was measured using 0.1 Eq/L NaOH on an extra blackberry water suspension. Equation 1 was used to calculate the molarity of $NaHCO_3$ buffer.

$$M = \frac{(Total\ mEq + f \times Total\ mEq)}{f \times V} \quad (1)$$

Where Total mEq = HCl mEq needed to reach pH 2.0 + HCl mEq added with pepsin + NaOH mEq to reach pH 6.5; $f = 0.71 = 10^{-pH}/10^{-pKa}$, pH = 6.5 and pKa = 6.35, and V = volume of $NaHCO_3$ solution in the dialysis bag (10.0 mL) .

Then, pancreatin-bile mixture (3.125 mL of 1.0% bile, 0.4% pancreatin solution in 0.1 Eq/L $NaHCO_3$) was added to each beaker, and the incubation continued for another 2 h. After that, the dialysis bags were removed and rinsed with water. Bag contents corresponding to "dialysates of the *in-vitro* intestinal phase" were transferred to tared flasks, weighed and frozen at –20 °C until analysis. Residues corresponding to "digests of the *in-vitro* intestinal phase" were weighed, and 2 aliquots (5 mL) were separated and transferred into 50.0-mL sterile flasks and frozen at –20 °C until the colonic fermentation phase. The rest of the intestinal digest was centrifuged at 12,000 *g* for 20 min at 20 °C. Supernatants were separated and frozen at –20 ° C until analysis. Residues were re-extracted subsequently as explained above.

Blanks were prepared by replacing the sample with water, and were subjected to the same digestive process.

To simulate the colonic fermentation phase, the *in-vitro* fermentation method described by Barry et al. (1995) and standardized by Goñi & Martín-Carrón (2000) was used with some modifications. Male Wistar rats (body weight 200 ± 5 g) fed with standard maintenance diet were supplied by the breeding Center at the Facultad de Bioquímica y Ciencias Biológicas (Universidad Nacional del Litoral, Santa Fe, Argentina). Rats were killed in a carbon dioxide chamber and fresh rat cecal content was used as inoculum. Ceca were removed through abdominal midline incisions. Rat cecal contents were scraped, weighed and added to a flask containing sterile anaerobic medium with thioglycolate as indicator ((Laboratorios Britania S.A., Argentina) to give a 100 g/L inoculum. The inoculum was mixed (2 min) in a Stomacher 80 Lab Blender (Seward

Medical, London, UK) and filtered (1 mm flesh) before use. Aliquots of intestinal digest were thawed and mixed with 8 mL of fermentation medium and 2 mL of inoculum. Then, dialysis bags with 10 mL of fermentation medium were placed in each beaker. Finally, fermentation beakers were placed in a GasPak™ EZ Anaerobe Container System in an oxygen-free CO_2 saturated atmosphere, and the whole set-up was placed in a shaking bath at 37 ºC for 24 h (Figure 1). Blanks containing no substrate or 6% raffinose were included in the experiment as zero and completely fermentable substrate, respectively. After incubation time, 2.5 mL 1 mol/L NaOH was used to stop the fermentation process. Bag contents corresponding to "dialysates of the *in vitro* fermentation phase" were transferred to tared flasks, weighed and frozen at –20 °C until analysis. Residues in each beaker after the colonic fermentation process corresponding to "digests of the *in vitro* fermentation phase" were weighed and centrifuged at 12,000 *g* for 20 min at 20 °C. Supernatants were separated and frozen at –20 ° C until analysis. Residues were re-extracted subsequently as explained above. The entire procedure was performed in quintupled samples.

2.5. Phenolic compound bioaccessibility

The intestinal bioaccessibility (IB %) was calculated with Equation 2 as the dialyzable fraction of phenolic compounds in relation to the phenolic compound content of the raw material (RM).

$$IB_{RM}\ (\%) = \frac{mg_{dialyzable\ polyphenols}}{mg_{polyphenols\ raw\ material}} \times 100 \quad (2)$$

The residual phenolic compounds of the intestinal digest (RID %) were calculated as the non-dialyzable fraction of phenolic compounds (soluble plus extractable) in relation to the phenolic compound content of the raw material (Equation 3).

$$RID\ (\%) = \frac{mg_{non-dialyzable\ polyphenols}}{mg_{\ polyphenols\ raw\ material}} \times 100 \quad (3)$$

The colonic bioaccessibility was calculated as the dialyzable fraction of phenolic compounds in relation to the phenolic compound content of the intestinal digest (soluble plus extractable), CB_{ID} % (Equation 4), and as the dialyzable fraction of phenolic compounds in relation to the phenolic compound content of the raw material, CB_{RM} % (Equation 5).

$$CB_{ID}\ (\%) = \frac{mg_{dialyzable\ polyphenols}}{mg_{\ polyphenols\ intestinal\ digest}} \times 100 \quad (4)$$

$$CB_{RM}\ (\%) = \frac{mg_{dialyzable\ polyphenols}}{mg_{\ polyphenols\ raw\ material}} \times 100 \quad (5)$$

The residual phenolic compounds of the colonic digest were calculated as the non-dialyzable fraction of phenolic compounds (soluble plus extractable) in relation to the phenolic compound content in the intestinal digest (soluble plus extractable): RCD_{ID} % (Equation 6), and in the raw material: RCD_{RM} % (Equation 7) .

$$RCD_{ID}\ (\%) = \frac{mg_{non-dialyzable\ polyphenols}}{mg_{\ polyphenols\ intestinal\ digest}} \times 100 \quad (6)$$

$$RCD_{RM}\ (\%) = \frac{mg_{non-dialyzable\ polyphenols}}{mg_{\ polyphenols\ raw\ material}} \times 100 \quad (7)$$

The total bioaccessibility (TB %) of phenolic compounds was calculated as the sum of IB_{RM} (%) and CB_{RM} (%).

2.6. Statistical analysis

All analyzes were performed in triplicate. Calculation of means and standard deviation was performed using the software STATGRAPHICS Centurion XV 15.2.06 (StatPoint Technologies, Inc., Warrenton, VA).

3. Results and discussion

3.1. Analysis of phenolic compounds in blackberry fruits

The phenolic compound profile in the blackberry variety 'Black Satin' is presented in Table 1. The polyphenolic profile of this blackberry cultivar was previously characterized by Van de Velde et al. (2016), based on LC retention times, UV-Vis, MS, MS^2 spectra and by comparison with available references and reported literature (Cho et al., 2004; Fan-Chiang and Wrolstad, 2005; Jordheim et al., 2011). Figure 2A shows the anthocyanin chromatogram recorded at 520 nm obtained after the HPLC separation of the raw material. Cyanidin-3-*O*-glucoside (peak **1**) was the primary anthocyanin detected in the samples, representing more than 84% of the total anthocyanins quantified by HPLC (Table 1). This finding was in agreement with the reports for other blackberry cultivars (Mertz et al., 2007; Cuevas-Rodriguez et al., 2010). Peak **2** was identified as cyanidin-3-*O*-xyloside and peaks **3** and **4** were identified as acylated derivatives of cyanidin-3-*O*-glucoside. Peak **3** was assumed to be cyanidin-3-*O*-(6-*O*-malonyl-glucoside), and peak **4** as cyanidin-3-*O*-*β*-(6''-(3-hydroxy-3-methylglutaroyl)-glucoside (Figure 2A). These acylated anthocyanins represented near to 11% of the total anthocyanins quantified by HPLC (Table 1). Figure 2B shows the chromatogram obtained (254 nm) after the phenolic compound separation by HPLC. Apart from the anthocyanin peaks, two main compounds (peak **5** and **6**) of maximum absorption at 240 nm were identified as ellagitannin derivatives (Van de Velde et al., 2016). Ellagitannins are hydrolysable tannins since they are esters of hexahydroxydiphenic acid (HHDP: 6, 6´-dicarbonyl-2,2´,3,3´,4,4´-hexahydroxybiphenyl moiety) and a polyol, usually glucose, and in some cases gallic acid (Häkkinen et al., 1999). Peak **5** was identified as a trimer of galloyl-bis-HHDP-glucose (lambertianin C), and peak **6** was identified as a dimer of galloyl-bis-HHDP-glucose (sanguiin H-6/lambertianin A). Sanguiin H-6 was the principal hydrolysable tannin found in the samples, representing more than 14% of the total phenolic compounds calculated as the sum of the individual phenolic

compound contents analyzed by HPLC (Table 1). Free ellagic acid was also identified (peak 7) in the samples (Figure 2B), as reported by other authors in blackberry fruit (Mertz et al., 2007; Hager et al., 2010).

Other phenolic compounds were identified and quantified in the samples at lower concentrations (Van de Velde et al., 2016). However, the bioaccessibility of the main phenolic compounds occurring in blackberry fruit at higher concentrations will be described and analyzed in this work.

3.2. Estimation of the intestinal bioaccessibility of phenolic compounds in blackberry fruits

The intestinal bioaccessibility (IB %) of blackberry phenolic compounds estimated as the fraction of polyphenols that could dialyze after the gastrointestinal digestion and may potentially be taken up by the enterocytes was analyzed. Moreover, the fraction of polyphenols that were not dialyzed and remains in the intestinal digest, representing the residual phenolic compounds in the intestinal digest (RID %) was also estimated. For the calculation of this fraction, total phenolic compounds already present in the soluble digest media and the extractable phenolic compounds obtained after the re-extraction of the digest pellet in 80:20 methanol: water (0.5% acetic acid) were taken into account. The non-bioaccessible phenolic compounds, which will not pass through the intestinal barrier, may have some antioxidant effects on the small intestine since they are, at least in part, soluble in the digest media (Saura-Calixto et al., 2007). The intestinal bioaccessibility (IB_{RM} %) results of individual phenolic compounds analyzed by HPLC are shown in Table 2.

IB_{RM} of cyanidin-3-*O*-glucoside, the principal anthocyanin detected and quantified in blackberry fruit was less than 2% (Table 2). Even though concentrations of other anthocyanins analyzed in the samples were lower (Table 1), their IB % values were in

the same range as that obtained for cyanidin-3-*O*-glucoside (Table 2). Thus, a very low bioaccessibility of intact anthocyanins after the gastrointestinal process can be inferred. Figure 3A shows the chromatograms obtained when anthocyanins were analyzed in the intestinal digests and the dialyzed fractions. Chromatogram scale of "*y*" axis in Figure 3 was amplified 1000 times with respect to Figure 2 for better peak appreciation. As it is shown, the anthocyanin profile among the different digested samples did not experience changes at 520 nm in relation to the chromatogram obtained for the raw material (Figure 2A). However, it is evident that the four peaks experienced a great reduction, especially in the intestinal digest sample, after pancreatic digestion (Figure 3A). Liang et al. (2012) reported that only 0.34% of total anthocyanins from mulberry fruit, composed of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, were dialyzed throughout the membranes in an *in-vitro* gastrointestinal digestion model and, in other words, would be bioavailable in the small intestine. According to these authors, the low recovery of anthocyanins could be partially due to the transformation of the colored anthocyanin flavylium cation to the colorless chalcone pseudobase at the pH of the intestinal environment (pH 7.5–8.0), and theses colorless anthocyanins are not detected at the analysis detection wavelength (520 nm). The colored flavylium cation form predominates at pH 2 or below, but at pH>5 anhydrobases become progressively more stable and increasingly formed as pH increases, when ring fission occurs with formation of ionized chalcones. Therefore, the pH value reached after the intestinal digestion (~ 7.0) could explain the low recovery of intact anthocyanins obtained, and the concomitant low amount of the dialyzable fraction (Table 2).

According to Liang et al. (2012), the reason for the high loss of anthocyanins at intestinal level remains not quite clear, although the metabolic breakdown to some non-colored forms, oxidization, or degradation into other chemicals, which were not evident

under the present conditions, could be involved. In addition, according to Czank et al. (2013) and de Ferrars et al. (2014), the identification of anthocyanin-derived phenolic acids in the blood within 30 to 90 minutes of consumption suggests deglycosylation and chemical degradation that can occur in the upper small intestine.

The IB_{RM} of total anthocyanins calculated as the sum of the individual anthocyanin contents analyzed by HPLC was around 2% (Table 2). In agreement with our findings, studies on healthy subjects consuming anthocyanin-rich foods established that < 2% of the total anthocyanin dose is absorbed intact, as estimated from the amount of anthocyanin and conjugates measured either in urine (Azzini et al., 2010; Ludwig et al., 2015) or in blood (Mullen et al., 2008).

In regard to IB_{RM} of the ellagitannins lambertianin A and C, these values were less than 1%, and the amount that remained in the intestinal digest was less than 5 % (Table 2). In contrast with this situation, free ellagic acid bioaccessibility was around 15%, and the amount that remained in the intestinal digest was near to 75%. Figure 3B shows the chromatograms obtained for phenolic compounds at 254 nm in the samples obtained in the intestinal digests and dialyzed fractions. The occurrence of new peaks at the first 15 min of the chromatogram are presented, likely to be small molecules originated from phenolic compounds breakdown, which were not analyzed. Furthermore, peaks **5** and **6**, corresponding to ellagitannins, experienced a great reduction after intestinal digestion. On the contrary, ellagic acid (peak **7**) increased after the intestinal digestion and, as expected, in the dialyzed fraction. It is known that ellagitannins are hydrolyzable tannins, whose absorption begins in the small intestine, particularly in the jejunum, after hydrolyzing to ellagic acid (Giampieri et al., 2012). Therefore, the high recovery of ellagic acid obtained in the intestinal digest was due, in part, to the release from the breakdown of ellagitannins.

The estimation of the IB_{RM} of total polyphenols calculated as the sum of the contents of the individual polyphenols was around 2% (Table 2). Thus, it is inferred that only a few part of the initial phenolic compounds of blackberry samples could be dialyzed throughout the membrane and be potentially bioaccessible. Moreover, the IB_{RM} % of total anthocyanins represented more than 86% of the IB_{RM} of total phenolic compounds analyzed by HPLC (Table 2), suggesting that anthocyanins represent most of the intact bioaccessible phenolic compounds analyzed in blackberry fruit. On the other hand, the residual polyphenol fraction in the intestinal digest (RID %) was less than 5% (Table 2), indicating that most of the main individual phenolic compounds from blackberry fruit were degraded by the gastrointestinal conditions.

3.3. Estimation of the colonic bioaccessibility of phenolic compounds in blackberry fruits

The unabsorbed polyphenols, which remain in the intestinal digest, are transported to the colon, where they could be absorbed by the epithelium, released and metabolized by colonic bacteria before being absorbed, or excreted without further metabolism (Crozier et al., 2010; Low et al., 2016). In this approach, the non-dialyzed phenolic compounds of the intestinal digests were subjected to rat fecal microbiota fermentation. During this metabolization, a simultaneous selective dialysis of phenolic compounds was achieved, a process which mimics the colon epithelium absorption.

Table 3 shows the colonic bioaccessibility of the main blackberry polyphenols in relation to the phenolic compounds of the intestinal digest (CB_{ID}) and in relation to the phenolic compounds of the raw material (CB_{RM}). Figure 4A shows the chromatograms obtained at 520 nm for anthocyanins in the colonic digests and in the dialyzed fractions. As shown, only cyanidin-3-*O*-glucoside and cyanidin-3-*O*-β-(6"-(3-hydroxy-3-methylglutaryl)-glucoside were detected in these samples at a very low concentration

near to the LOD of the method (0.01 mg/mL). As was expected, their CB_{ID} % and CB_{RM} % results represented insignificant amounts in relation to the high content of anthocyanins contributed to the diet by blackberries (Tables 1 and 3). For its part, the CB_{ID} and CB_{RM} results of total anthocyanins calculated as the sum of the individual anthocyanin contents analyzed by HPLC were 2.3 and 0.03%, respectively (Table 3).

Aura et al. (2005) studied the *in-vitro* metabolism of anthocyanins by human gut microflora. According to their results, most of the anthocyanin glycosides disappeared within the first hour in the presence of 5 % fecal slurry containing active gut microflora, and only traces of the different anthocyanins were still detectable after an hour. They reported that anthocyanins were transformed by bacteria in the colon to smaller phenolic compounds or conjugates of the aglycone. The almost insignificant amounts of intact anthocyanins obtained herein in the colonic digest after fermentation agree with these findings (Table 3). It seems that bacterial metabolism involves the cleavage of glycosidic linkages and breakdown of the anthocyanidin heterocycle, protocatechuic acid being one of the most likely major degradation products from anthocyanins having a 3,4-dihydroxy B-ring (Aura et al., 2005; Williamson & Clifford, 2010).

Figure 4B shows the chromatograms obtained at 254 nm for phenolic compounds in the colonic digests and in the dialyzed fractions. The occurrence of new low-molecular-weight metabolites at the first 15 min of the chromatogram is clear.

The CB_{ID} % of free ellagic acid was around 10%, and its CB_{RM} % resulted near to 13% (Table 3), suggesting a considerable accessibility of this compound at the colonic level. As presented before, free ellagic acid results, in part, from ellagitannin breakdown during intestinal digestion (Table 2). The absence of lambertianin A and C in the colonic digests after fermentation (Table 3, Figure 4B), suggests the hydrolysis of these compounds to ellagic acid also at this level. However, the non-detectable amount of

ellagic acid in the residue digest after fermentation indicates a likely further metabolization of this compound to small molecules that were not analyzed in this study (Table 3). According to these results, the incorporation of a dialysis membrane at the colonic level avoided the complete metabolic breakdown of ellagic acid by the colonic bacteria. Thus, the ellagic acid fraction that dialyzed during the colonic fermentation allowed an estimation of its *in vitro* bioaccessibility at the large intestine level.

Cerdá et al. (2003) evaluated the bioavailability and metabolism of punicalagin (ellagitannin of pomegranate juice), in a rat model. Only 3–6% of the ingested punicalagin was detected as such or as its metabolites in urine and feces, because most of this ellagitannin is converted to undetectable metabolites (i.e. CO_2) or accumulated in non-analyzed tissues. According to Larrosa et al. (2006), the human colonic microflora metabolized the dietary ellagic acid and the related ellagitannins to urolithins A and B. Both urolithins could be potentially considered as enterophytoestrogens, that is, gut microflora-derived metabolites with estrogenic/antiestrogenic activities derived from plant food constituents.

The fraction of total phenolic compounds bioaccessible after colonic fermentation, which were originally present in the intestinal digest was around 13% when total phenolic compound content was calculated as the sum of the intact individual phenolic compound contents analyzed by HPLC (Table 3). The colonic bioaccessibility of total phenolic compounds calculated in relation to the original polyphenol content of the raw material (CB_{RM}) was only 1% (Table 3). Evidently, only 1% of the originally individual phenolic compounds of blackberry were recovered in their indigenous forms after colonic fermentation.

In the same way, fractions of indigenous phenolic compounds that remained in the colonic residues (RCD_{ID} % and RCD_{RM} %) after fermentation were less than 0.2%

(Table 3), suggesting the further metabolization of phenolic compounds that could not be dialyzed during the colonic phase to low-molecular-weight metabolites or carbon dioxide.

In agreement, Rocchetti et al. (2017) studied the changes over the polyphenolic profile of gluten-free pasta after a simulated *in-vitro* gastrointestinal digestion, including colonic fermentation. Authors revealed that the large intestine microbiota metabolic activity resulted in a large modification of the phenolic compound profile, with a degradation of several chemical classes and a parallel formation of new metabolites. Flavonoids, hydroxycinnamics and lignans were degraded over time, with a concurrent increase in low-molecular-weight phenolic acids (hydroxybenzoic acids), alkylphenols, hydroxybenzoketones and tyrosols.

3.4. Estimation of the total bioaccessibility of phenolic compounds in blackberry fruits

Table 4 presents the estimation of the total bioaccessibility (TB %) of phenolic compounds in blackberry fruit as the sum of IB_{RM} (%) plus CB_{RM} (%). According to the results, only 2% of the total anthocyanins calculated as the sum of the intact individual anthocyanin contents detected by HPLC could be potentially accessible after the fruit intake.

As presented in Table 4, the TB % of total phenolic compounds was 3.3%, calculated as the sum of the intact individual phenolic compound contents. As discussed above, it is evident that the digestion conditions and colonic bacteria transformed the indigenous phenolic compounds from blackberry to smaller polyphenolic metabolites, evidenced at the beginning of chromatograms (Figure 3B and 4B). According to Crozier et al. (2010) the health properties of phenolic compounds are not only due to their intact structures but also to their low-molecular weight metabolites. Kay et al., (2017) highlighted the

importance of colon-derived phenolic and aromatic ring-fission products in flavonoid bioavailability. When the various metabolites and catabolites are taken into account, it is evident that anthocyanins and flavanones are much more bioavailable than previously estimated. Moreover, the gut-derived catabolites from phenolic compounds can often exert higher bioactivity than their precursor flavonoid structures (Kay et al. 2017).

The potential dialysis of these smaller bioactives released during the colonic fermentation may be protected from subsequent biotransformation to carbon dioxide, giving a more correct estimation of colonic bioaccessibility of phenolic compounds. Further research using HPLC-MS analysis will allow us to determine the different metabolites originating during the metabolization of phenolic compounds, to give even more reliable *in-vitro* bioaccessibility results.

4. Conclusions

Systemic effects of dietary polyphenols are dependent on their bioaccessibility in the gastrointestinal tract, and then, on the further bioavailability to the target tissues. The gastrointestinal and colonic conditions affected the amount of the indigenous polyphenols and, consequently, their bioaccessibility as intact structures. Total bioaccessibility of phenolic compounds from blackberry was estimated to be 3.3%, as the sum of the individual phenolic compound contents analyzed by HPLC. Bioaccessibility of total anthocyanins was 2%, these compounds representing most of the intact bioaccessible phenolic compounds analyzed in blackberry. These *in vitro* bioaccessibility results agree with those reported in human studies.

The incorporation of dialysis membranes before starting the colonic fermentation phase allowed the simultaneous dialysis of polyphenols while they were being metabolized by the colon microflora, avoiding their further fermentation to carbon dioxide.

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6. Conflict of Interest

The authors declare no conflict interest.

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Figure Captions:

Figure 1: *In vitro* gastrointestinal model with colonic fermentation.

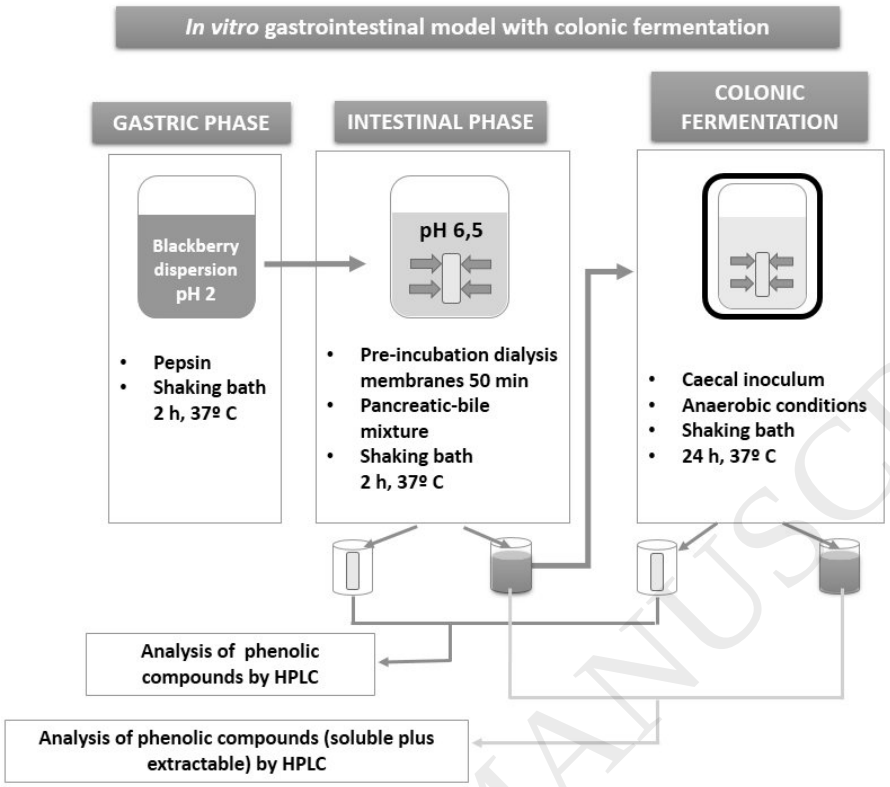
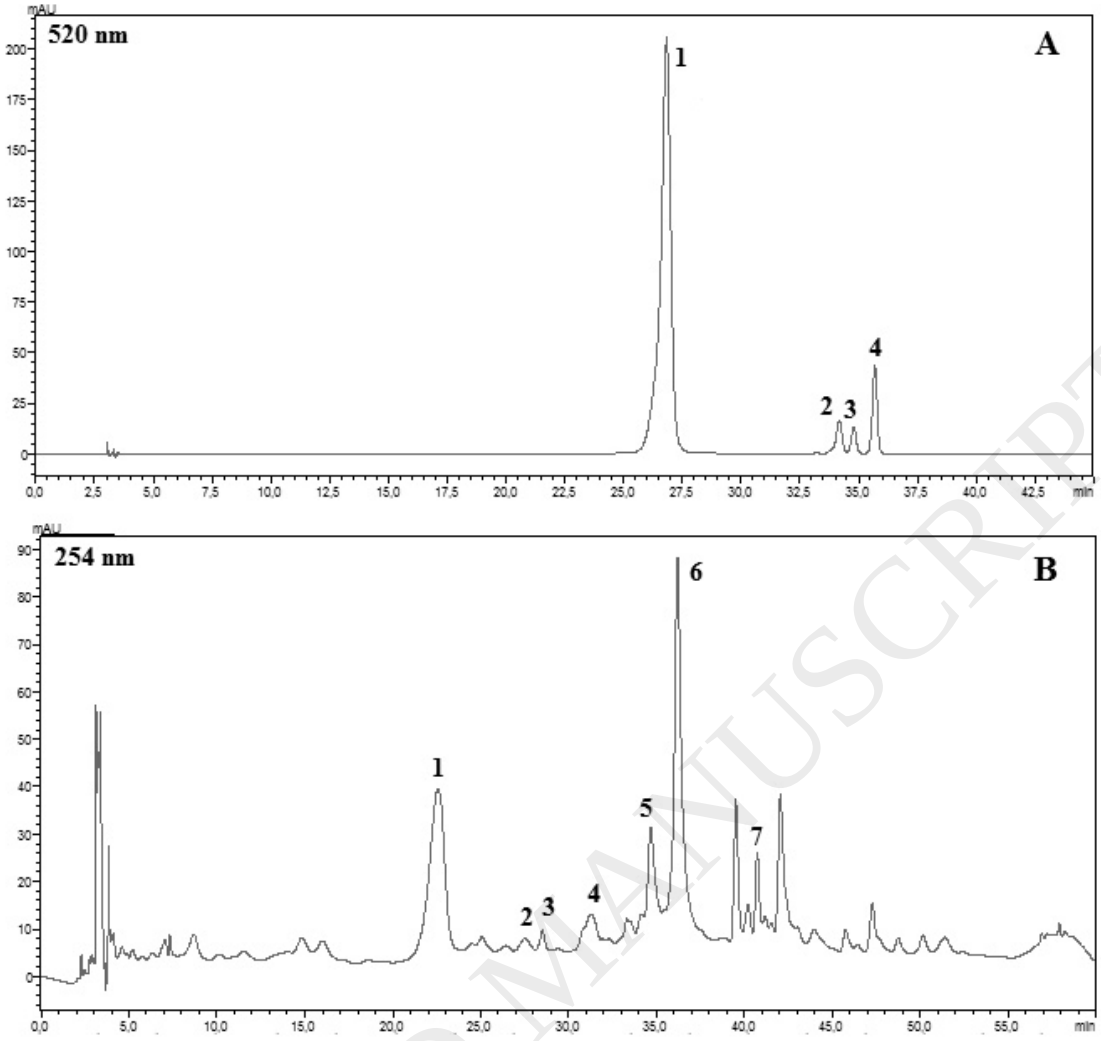


Figure 2: Reversed-phase HPLC chromatograms of anthocyanins separation at 520 nm (**A**) and phenolic compound separation at 254 nm (**B**) in blackberries var. 'Black Satin' Peak identification: (**1**) cyanidin-3-*O*-glucoside, (**2**) cyanidin-3-xyloside, (**3**) cyanidin-3-*O*-(6-*O*-malonyl glucoside), (**4**) cyaniding-3-*O*-*β*-(6''-(3-hydroxy-3-methylglutaroyl)-glucoside), (**5**) lambertianin C, (**6**) lambertianin A, (**7**) ellagic acid.



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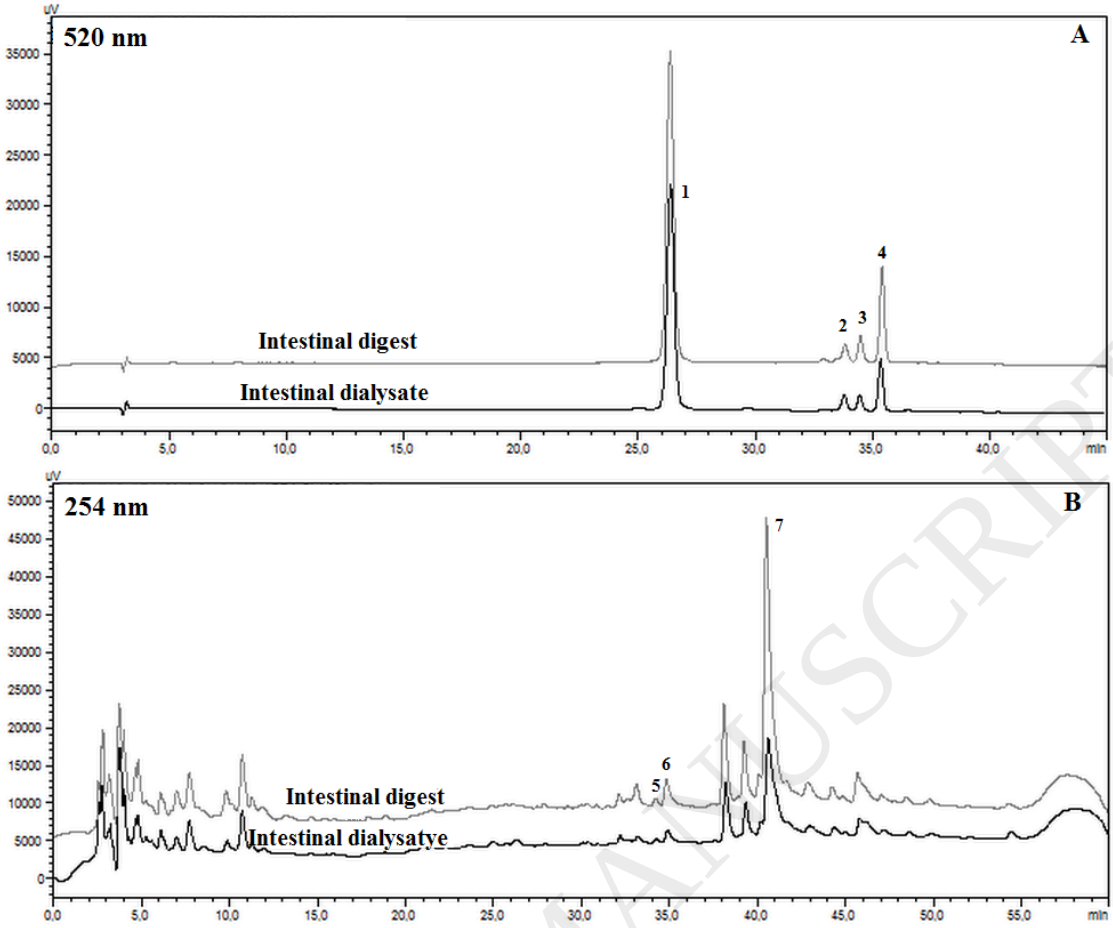

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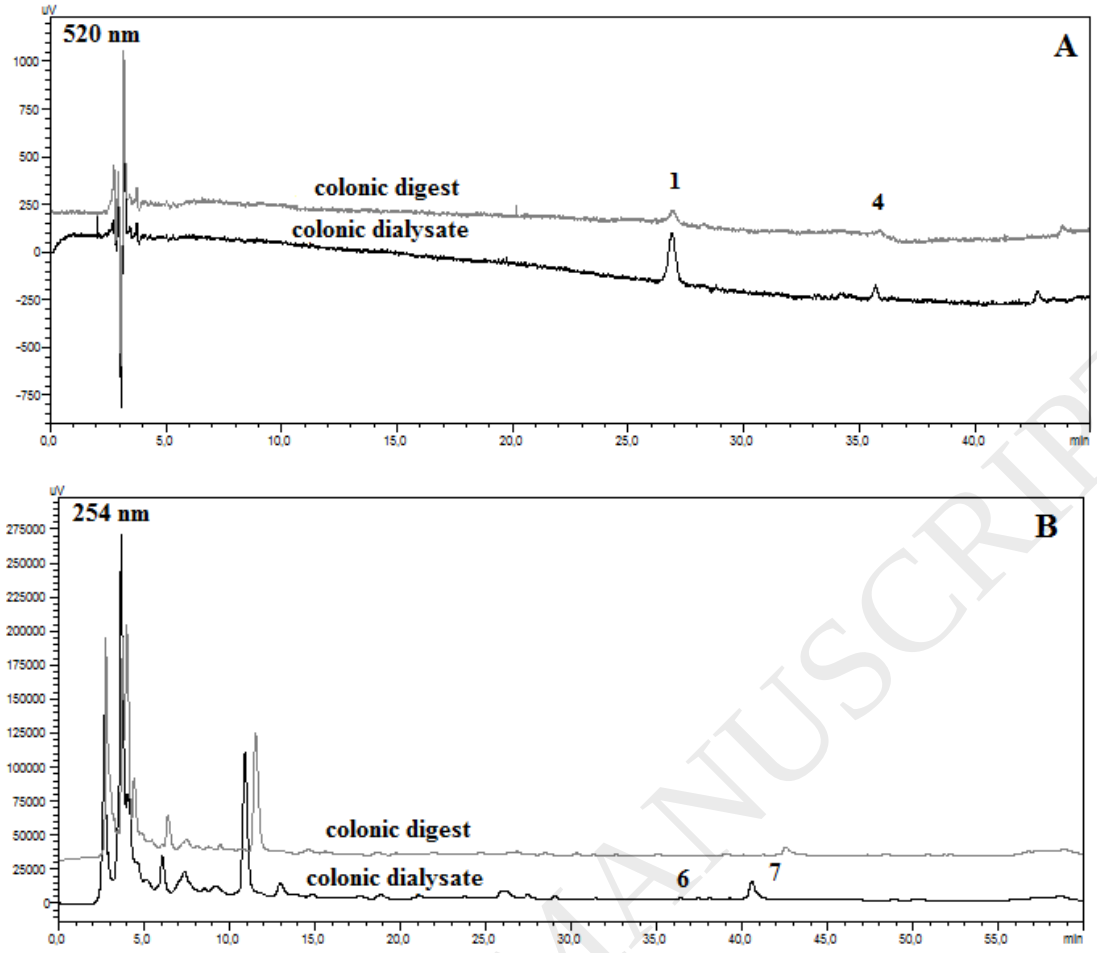

Table 1. Phenolic compound content in blackberry variety 'Black Satin'

peak	phenolic compound	content (mg/100 g RM)
	anthocyanins	
1	cyanidin-3-*O*-glucoside	189 ± 0.9
2	cyanidin-3-*O*-xyloside	9.3 ± 0.1
3	cyanidin-3-*O*-(6-*O*-malonyl glucoside)	5.9 ± 0.1
4	cyanidin-3-*O*-*β*-(6''-(3-hydroxy-3-methylglutaroyl)-glucoside)	18.7 ± 0.2
total anthocyanins[a]		222 ±0.9
	ellagitannins	
5	lambertianin C	11.0 ± 0.7
6	lambertianin A	39.8 ± 0.2
	total ellagitannins	50.8 ± 0.8
	free ellagic acid	
7	ellagic acid	11.8 ±0.1
total phenolic compounds[b]		285 ±0.9

RM: raw material. $n = 3$

a: Total anthocyanins calculated as the sum of individual anthocyanin contents analyzed by HPLC.

b: Total phenolic compounds calculated as the sum of individual phenolic contents analyzed by HPLC.

Table 2. Estimation of the intestinal bioaccessibility (IB %) of phenolic compounds considering the polyphenolic compound profile of blackberry 'Black Satin'

peak	phenolic compound	IB_{RM} (%)	RID (%)
	anthocyanins		
1	cyanidin-3-*O*-glucoside	1.8 ± 0.7	1.1 ± 0.1
2	cyanidin-3-*O*-xyloside	1.7 ±0.7	0.8 ± 0.5
3	cyanidin-3-*O*-(6-O-malonylglucoside)	2.8 ± 0.9	3.0 ± 0.9
4	cyanidin-3-*O*-β-(6''-(3-hydroxy-3-methylglutaryl)-glucoside	3.2 ± 0.8	4.0 ± 0.9
total anthocyanins[a]		1.9 ± 0.7	1.4 ± 0.8
	ellagitannins		
5	lambertianin C	1.0 ± 0.1	5.0 ± 0.4
6	lambertianin A	0.5 ± 0.1	2.0 ± 0.4
	free ellagic acid		
7	ellagic acid	14.9 ±0.9	74.9 ±0.9
total phenolic compounds[b]		2.2 ± 0.7	4.7 ± 0.5

IB_{RM}: intestinal bioaccessibility of phenolic compounds in relation to the phenolic compound content of the raw material (RM); RID: residual phenolic compounds of the intestinal digest in relation to the phenolic compound content of the raw material (RM). $n = 5$.

a: Total anthocyanins calculated as the sum of the individual anthocyanin contents analyzed by HPLC.

b: Total phenolic compounds calculated as the sum of the individual phenolic compound contents analyzed by HPLC.

Table 3. Estimation of the colonic bioaccessibility (CB %) of phenolic compounds considering the polyphenolic compound profile of blackberry 'Black Satin'

peak	phenolic compound	CB_{ID} (%)	RCD_{ID} (%)	CB_{RM} (%)	RCD_{RM} (%)
	anthocyanins				
1	cyanidin-3-*O*-glucoside	2.0 ± 0.5	0.6 ± 0.1	0.03 ± 0.01	0.02 ± 0.01
2	cyanidin-3-*O*-xyloside	-	-	-	-
3	cyanidin-3-*O*-(6-O-malonylglucoside)	-	-	-	-
4	cyanidin-3-*O*-β-(6''-(3-hydroxy-3-methylglutaryl)-glucoside	0.4 ± 0.1	0.04 ± 0.01	0.06 ± 0.02	0.02 ± 0.01
total anthocyanins[a]		2.3 ± 0.5	0.64 ± 0.08	0.03 ± 0.01	0.02 ± 0.01
	ellagitannins				
5	lambertianin C	0.2 ± 0.1	-	0.2 ± 0.02	-
6	lambertianin A	0.040 ± 0.001	-	0.010 ± 0.001	-
	free ellagic acid				
7	ellagic acid	10.0 ±1.0	0.080 ±0.004	13.0 ±1.0	0.12 ±0.01
total phenolic compounds[b]		13.23 ± 0.01	0.17 ± 0.01	1.08 ± 0.02	0.010 ± 0.001

CB_{ID}: colonic bioaccessibility of phenolic compounds in relation to the phenolic compound content of the intestinal digest; RCD_{ID}: residual phenolic compounds of the colonic digest in relation to the phenolic compound content of the intestinal digest; CB_{RM}: colonic bioaccessibility of phenolic compounds in relation to the phenolic compound content of the raw material (RM). RCD_{RM}: residual phenolic compounds of the colonic digest in relation to the phenolic compound content of the raw material (RM). $n = 5$

a: Total anthocyanins calculated as the sum of the individual anthocyanin contents analyzed by HPLC.

b: Total phenolic compounds calculated as the sum of the individual phenolic compounds contents analyzed by HPLC.

Table 4. Estimation of the total bioaccessibility (TB. %) of phenolic compounds in blackberry 'Black Satin'

item	total anthocyanins	total phenolic compounds
TB[a] %	2.0 ± 0.4	3.3 ± 0.4

a: Total bioaccessibility calculated as the sum of the intestinal bioaccessibility (IB_{RM}) and the colonic bioaccessibility (CB_{RM}) of phenolic compounds in relation to the phenolic content of the raw material (RM). $n = 5$.