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Original article
Red Beetroot Thematic Issue

Red Beetroot Juice Phytochemicals Bioaccessibility: an *In Vitro* Approach

Ivelina Desseva[1], Magdalena Stoyanova[1], Nadezhda Petkova[2], Dasha Mihaylova[3]*

[1]*Department of Analytical Chemistry and Physical Chemistry, University of Food Technologies, 26 Maritza Blvd., Plovdiv, Bulgaria*
[2]*Department of Organic Chemistry and Inorganic Chemistry, University of Food Technologies, 26 Maritza Blvd., Plovdiv, Bulgaria*
[3]*Department of Biotechnology, University of Food Technologies, 26 Maritza Blvd., Plovdiv, Bulgaria*

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Beetroot, the cultivated form of *Beta vulgaris* subsp. *vulgaris*, is known for its various beneficial properties but more critical data about its bioactive compounds digestion is needed. In the present research, the bioaccessibility of phytochemicals in freshly prepared red beetroot juice was studied. Changes in total phenolics content, total flavonoids content, contents of betacyanins and betaxanthins, phenolic acids profile as well as the antioxidant activity were monitored before and after simulated gastrointestinal digestion. Several parameters that provide interrelated information about food quality were additionally evaluated, including oxalic acid and individual sugars content, total titratable acidity, and acetylcholinesterase inhibitory activity. Significant loss of contents of total phenolics and flavonoids measured after digestion resulted in the recovery of 27.07 and 36.4%, respectively. The same negative tendency was observed for betalains bioaccessibility. While nearly 27% of betaxanthins were present after the simulated digestion, almost all betacyanins (96.07%) have been lost. The HPLC analysis of phenolic acids of beetroot juice revealed the presence of chlorogenic, caffeic, *p*-coumaric, and sinapic acids. After digestion, a 2.5-fold higher concentration of chlorogenic acid was found, however caffeic and *p*-coumaric acids were no longer detected. The results concerning the antioxidant activity of digested juice were inexplicit. According to the DPPH assay, there was a complete recovery of antioxidant activity, while no activity was detected employing the ABTS assay. Following the cupric ion reducing antioxidant capacity (CUPRAC) and ferric-reducing antioxidant power (FRAP), approximately half of the initial activity was retained. Despite the losses, red beetroot remains a valuable source of biologically active substances. Better understanding of their transformation during digestion is further needed.

LIST OF ABBREVIATIONS

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AchE – acetylcholinesterase; ATCI – acetylthiocholine; BJ – beetroot juice; CUPRAC – cupric ion reducing antioxidant capacity; DAD – diode-array detection; DPPH – 2,2-diphenyl--1-picrylhydrazyl; DTNB – 5,5'-dithiobis(2-nitrobenzoic acid); dw – dry weight; FRAP – ferric-reducing antioxidant power; HPLC – high performance liquid chromatography; MW – molecular weight; PBS – phosphate-buffered saline; SGD – simulated gastrointestinal digestion; SGF – simulated gastric fluid; SIF – simulated intestinal fluid; TE – Trolox equivalent; TEAC – Trolox equivalent antioxidant capacity; TFC – total flavonoid content; TPC – total phenolics content.

INTRODUCTION

Beetroot (or red beet) is the cultivated form of *Beta vulgaris* subsp. *vulgaris* (conditiva) grown throughout the Americas, Europe, and Asia. Unlike *Beta vulgaris* subsp. *vulgaris* (altissima), known as sugar beet, conditiva subspecies are two times poorer in sucrose [Wruss *et al*., 2015]. Red beet is a root vegetable and known as a source of phenolic compounds, carotenoids, nitrates, vitamins, minerals and water-soluble pigments [Chhikara *et al*., 2019]. It is consumed regularly as part of an everyday diet and also is extensively used as a food coloring agent (E162) [Georgiev *et al*., 2010]. Red beetroot has gained popularity owing to its biological activity and potential utility as a health-promoting and disease-preventing functional food [Clifford *et al*., 2015]. Its intense red color is due to the presence of highly reactive nitrogen-containing pigments called betalains. They represent plant secondary metabolites that share some similar chemical properties, biological functions, and color spectrums with anthocyanins but these two groups of metabolites never coexist together in plants [Solovchenko *et al.,* 2018]. Betalains are a class of betalamic acid derivatives which are composed of betacyanins (red-violet colored substances) and betaxanthins (yellow-orange colored substances) [Gandía-Herrero *et al*., 2010]. Betalains are considered to induce extremely powerful antiradical and antioxidant effects [Gandía-Herrero *et al*., 2010]. In addition, many studies with laboratory animals demonstrated tumor-chemopreventive effects of red beetroot extracts [Kapadia & Rao, 2013]. Beetroot is also a rich source of phenolic acids and flavonoids as well as of other compounds, such as carotenoids and ascorbic acid, which may further increase its total antioxidant capacity [Clifford *et al*., 2015; Wootton-Beard & Ryan, 2011].

* Corresponding Author: Tel.: +359 886 947242;
E-mail: ivelina_hristova_vn@abv.bg (I. Desseva)

Till recently, not many studies have been focused on the transformations that occur during food digestion. This is a complex process with many factors involved. So far, *in vivo* studies are not an option due to higher experimental cost and serious ethical considerations [Sengul *et al*., 2014]. Therefore, more and more efforts are put into the development of reliable methods for *in vitro* gastrointestinal digestion evaluation [Minekus *et al*., 2014]. Although, *in vitro* methods have serious disadvantages, they can serve as preliminary test for the bioaccessibility and bioavailability of compounds from the food matrix and as a starting point for further investigations.

Several studies are available so far for beetroot phytochemicals digestibility [Dalmau *et al*., 2019; Guldiken *et al*., 2016; Tesoriere e*t al*., 2008]. The processing method and the interactions with the food matrix are considered as the two external factors that significantly influence the actual bioaccessibility and bioavailability of ingested phenolics [Shahidi & Peng, 2018]. Overall, a limited recovery for different beetroot products is reported that could be moderated by the pretreatment conditions [Dalmau *et al*., 2019; Guldiken *et al*., 2016]. So far, a lack of critical data about beetroot juice digestibility is noticeable.

In this regard, the aim of the current study was to evaluate the bioaccessibility of phytochemicals in freshly prepared beetroot juice (BJ) by using *in vitro* model simulating gastrointestinal digestion (SGD). No extraction method was applied in order to simulate real conditions of juice consumption. The loss of phytochemicals and their corresponding activity and/or recovery of such were also calculated. HPLC-DAD analysis of individual polyphenols, before and after SGD, was performed as well.

MATERIALS AND METHODS

Chemicals and instruments

The red beetroot used in this study was with Bulgarian origin (Plovdiv region), vintage 2018. The beetroot juice was purchased from a local fresh fruit juice shop where it was freshly cold-pressed on a slow-turn juicer. The BJ was then immediately subjected to analysis. Three independent samples were made and tested from the same beet material and the results are presented as mean. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described by Minekus *et al*. [2014].

All reagents used in this study were of analytical grade and purchased from Merck Chemicals (Germany) and Sigma-Aldrich (Germany).

All spectrophotometric measurements were performed on SPECTROstar Nano Microplate Reader (BMG LABTECH, Ortenberg, Germany), and all HPLC assays were performed on Elite LaChrome (Hitachi, Tokyo, Japan) HPLC system equipped with DAD and ELITE LaCHrome (Hitachi, Tokyo, Japan) software.

In vitro gastrointestinal digestion procedure

The assay was performed according to the procedures described by Minekus *et al*. [2014] with minor modifications. Only gastric and intestinal phase were included.

Gastric phase

BJ (5 mL) was mixed with 3.62 mL of a porcine pepsin stock solution (pepsin from porcine gastric mucosa, P7000, Sigma-Aldrich; 5520 U/mL made up in SGF electrolyte stock solution), 2.5 μL of 0.3 M $CaCl_2$ and 132 μL of phospholipids (0.17 mM in the final digestion mixture). The pH of the mixture was corrected with 1 M HCl to the value of 3.0 and the volume of the mixture was made up to 10 mL with distilled water. The mixture was then incubated at 37°C with constant shaking in a shaking water bath for 2 h. The pH was regularly checked and re-adjusted with 1 M HCl when needed.

Intestinal phase

Gastric chyme (10 mL) was mixed with 8 mL of a pancreatin solution (pancreatin from porcine pancreas, P1750, Sigma-Aldrich; 1.72 U/mL made up in SIF electrolyte stock solution based on trypsin activity), 1.9 mL of fresh bile extract (160 mM fresh bile salts in final mixture, Sigma-Aldrich), 20 μL of 0.3 M $CaCl_2$, 1 M NaOH to reach pH 7.0, and water to 20 mL total volume. The mixture was then incubated at 37°C in a shaking water bath for 2 h. The pH was regularly checked and re-adjusted with 1 M NaOH during the process, if needed.

For the blank sample, water was used instead of BJ. The values obtained for blanks were subtracted from the sample values for each analysis. The digestion sample was then centrifuged and stored at -20°C till further analysis, but no longer than for 7 days.

Moisture

Total moisture content of the samples was determined in a moisture analyzer balance (Radwag PMC 50/NH, Poland). The sample was placed in a dish and dried to constant mass at 105°C.

Total titratable acidity

The titratable acidity was measured by titration with a 0.1 M NaOH. The results are expressed as g citric acid in 100 mL juice as follows:

$$\text{TA, g/100 mL} = N_{NaOH} \times V_{NaOH} \times M_{eq}(\text{citric acid}) \times DF \times 100/1000/V_{sample}$$

where: N_{NaOH} is the normal concentration of NaOH, mol/L; V_{NaOH} is the volume of NaOH required to reach the equivalent point, mL, M_{eq}(citric acid) is the equivalent weight of citric acid (64.04 g/eq), DF is the dilution factor, and V_{sample} is the volume of BJ, mL.

Oxalic acid content determination

Oxalic acid content was determined as described by Wruss *et al*. [2015] without modifications using the permanganese reduction method. Beetroot juice was diluted (1:10, *v/v*) with H_2O, and 2 mL of the diluted sample was mixed with 6 mL of H_2O and 1 mL of 1 M H_2SO_4. The sample was heated to 50–60°C and titrated with a 0.02 M $KMnO_4$ solution until persisting pale pink appeared. The concentration of oxalic acid was determined using a reference curve generated

by pure oxalic acid (5–50 mg/mL, R^2=0.9987) and expressed as mg per g of dry weight (dw) of BJ (mg/g dw). All samples were measured in triplicate.

Determination of total phenolics content

The total phenolics content (TPC) was analyzed using the method of Singleton & Rossi [1965] with some modifications. Native or digested BJ (0.1 mL) was mixed with 0.5 mL of the Folin-Ciocalteu reagent and 0.4 mL of 7.5% Na_2CO_3. The mixture was vortexed and left at 50°C for 5 min. After incubation, the absorbance was measured at 765 nm. The TPC was expressed as mg gallic acid equivalents (GAE) per g of native or digested BJ dw (mg GAE/g dw). The linear range for gallic acid standard was 5–100 mg/L (R^2=0.9965)

Determination of total flavonoids content

The total flavonoids content (TFC) was evaluated according to a modified method described by Park *et al.* [1997]. An aliquot of 0.5 mL of the native or digested BJ was added to 0.1 mL of 10% $Al(NO_3)_3$, 0.1 mL of 1 M CH_3COOK, and 3.8 mL of ethanol. After incubation at ambient temperature for 40 min, the absorbance was measured at 415 nm. Quercetin was used as a standard in the linear range of 5–80 μg/mL (R^2=0.9972) and the results were expressed as μg quercetin equivalents (QE) per g of dw of sample (μg QE/g dw).

Spectrophotometric quantification of betalains

Betalains quantification was performed as described by Stintzing *et al*. [2003]. Samples of native or digested BJ were diluted with McIlvaine buffer (pH 6.5) to obtain absorption values of $0.9 \leq A \geq 1.0$ at their respective absorption maxima. The betalain contents (BC), separately for betacyanins and betaxanthins, were calculated as follows:

$$BC[mg/g\ dw] = A \times DF \times MW/(\varepsilon x l x g)$$

where: A is the absorption value at the absorption maximum corrected by the absorption at 650 nm, DF is the dilution factor, l is the path length (1 cm) of the cuvette, and g is the dry weight in 1 mL of sample. For quantification of betacyanins and betaxanthins, the molecular weights (MW) and molar extinction coefficients (ε) of betacyanins (MW=550 g/mol; ε=60,000 L/(mol/cm) in H_2O; λ=536 nm) and betaxanthins (MW=339 g/mol; ε=48,000 L/(mol/cm) in H_2O; λ=485 nm) were applied. All measurements were performed in triplicate.

Determination of antioxidant activity

DPPH• scavenging activity

The ability of the sample to donate an electron and scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams *et al*. [1995]. Freshly prepared 4×10^{-4} M solution of DPPH radicals was mixed with native or digested BJ in a ratio of 2:0.5 (*v/v*). The light absorption was measured at 517 nm after 30-min incubation. The DPPH radical scavenging activity of native or digested BJ was presented as Trolox equivalents (TE) in the linear range of the standard of 50–500 μmol/L (R^2=0.9985) and expressed as μmol TE per g dw of sample (μmol TE/g dw).

$ABTS^{\bullet+}$ scavenging activity

The scavenging activity of the native or digested BJ against 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical action ($ABTS^{\bullet+}$) was estimated according to Re *et al.* [1999]. Briefly, $ABTS^{\bullet+}$ was produced by reacting ABTS stock solution (7 mM) with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 14 h before use. Afterward, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7±0.02 at 734 nm and equilibrated at 30°C. After the addition of 1.0 mL of diluted $ABTS^{\bullet+}$ solution to 0.01 mL of native or digested BJ, the absorbance reading was taken at 30°C after 6 min. The results were expressed as Trolox equivalent antioxidant capacity (TEAC, μmol TE/g dw) in the linear range of the standard of 500–2000 μmol/L (R^2=0.9966).

Ferric-reducing antioxidant power

The ferric-reducing antioxidant power (FRAP) assay was carried out according to the procedure of Benzie & Strain [1999] with slight modification. The FRAP reagent was prepared fresh daily and was warmed to 37°C prior to use. One hundred and fifty microliters of the native or digested BJ were allowed to react with 2850 μL of the FRAP reagent at 37°C for 4 min. The absorbance was recorded at 593 nm and the results were expressed as Trolox equivalents (μmol TE/g dw) in the linear range of the standard of 50–500 μmol/L (R^2=0.9970).

Cupric ion reducing antioxidant capacity (CUPRAC) assay

The CUPRAC assay was carried out according to the procedure of Apak *et al*. [2004]. One mL of $CuCl_2$ solution (1.0×10^{-2} M) was mixed with 1 mL of neocuproine methanolic solution (7.5×10^{-3} M), 1 mL NH_4Ac buffer solution (pH 7.0), and 0.1 mL of the native or digested BJ followed by addition of 1 mL water (total volume = 4.1 mL), and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. Trolox was used as a standard in the linear range of 200–2000 μmol/L (R^2=0.9929) and the results were expressed as μmol TE/g dw.

Acetylcholinesterase (AChE) inhibitory assay

The experimental conditions of the AChE assay were based on the method described by Lobbens *et al*. [2017] with slight modifications. The acetylcholinesterase inhibitory assay was carried out in a 96-welled microplate. Each well contained 30 μL of AChE (final concentration of 0.05 U/mL), 125 μL of 1.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) dissolved in phosphate-buffered saline (PBS) pH 7.5, 45 μL of PBS pH 7.5, and 25 μL of test solution or 25 μL negative control (water). A blank sample was prepared by adding buffer instead of enzyme. The microplate was shaken for 10 s and made to 30°C for 5 min. Hereafter, 30 μL of 7.5 mM acetylthiocholine (ATCI) dissolved in water were added to each well and the absorbance was measured every 30 s for 1 min at 412 nm. The blank corrected data were plotted

against time and the reaction rate (the slope of the plot) was calculated. Last, the inhibition was calculated by comparing the reaction rate in the test solution compared to the negative control. The experiment was performed in triplicate. The inhibition express in percentages as follows:

$$\%\text{inhibition} = 100\text{-}(\text{Slope}_{\text{sample}}/\ \text{Slope}_{\text{negative control}}) \times 100$$

HPLC analysis of phenolic acids

HPLC separation of the BJ phenolic acids was performed on Supelco Discovery HS C18 column (5 μm, 250 × 4.6 mm, Sigma-Aldrich, St. Louis, USA), operated at 30°C under gradient conditions with mobile phase consisting of 2% (*v/v*) acetic acid (solvent A) and acetonitrile (solvent B) as reported by Mihaylova *et al*. [2019]. The samples were filtered thought 0.45 μm syringe filter (polytetrafluoroethylene filter) and 20 μL were injected into the system. The gradient program used was: 0–1 min – 95% A and 5% B; 1–40 min: 50% A and 50% B; 40–45 min: 100% B; 46–50 min: 95% A and 5% B. The detection of chlorogenic, caffeic, *p*-coumaric, and sinapic acids was carried out at 320 nm in the linear range of 10–100 μg/mL for all the standards. The corresponding correlation coefficients were 0.9986, 0.9983, 0.9900, and 1.0000, respectively. The identification was done by comparing the retention time of the compound and those of the corresponding standard. The flow rate was 0.8 mL/min. The results were expressed in μg/g dw.

HPLC analysis of sugars

Chromatographic separations and determination of BJ sugars were performed on an Elite Chrome Hitachi HPLC system, coupled with a Chromaster 5450 refractive index detector (RID). The separation was done on a Shodex® Sugar SP0810 (7 μm , 300 × 8.0 mm, Tokyo, Japan) and a guard column Shodex SP – G (5 μm, 50 × 6 mm) operating at 85°C, mobile phase H_2O with flow rate 1.0 mL/min and the injection volume of 20 μL as described by Petkova *et al.* [2014]. The detection of sucrose, glucose, and fructose was performed in the linear range of 0.5–10 mg/mL and the corresponding correlation coefficients were 0.9988, 0.9985, and 0.9995, respectively. The results were calculated as g/100 g dw, the identification was done by comparing the retention time of the compound and those of the corresponding standard.

Statistical analysis

All tests were carried out in triplicate and the results were presented as mean ± standard deviation (SD) using Microsoft Excel 2010.

RESULTS AND DISCUSSION

Analysis prior to digestion

Freshly cold pressed red beetroot juice was subjected to several analyses prior to digestion. The results for moisture content, pH, and titratable acidity of the juice are presented in Table 1. These parameters provide interrelated information about food quality. Oxalic acid content was also determined, because beetroot was considered as naturally rich in oxalic acid – 400––600 mg/100 g fresh weight (fw) [Duke, 2000]. This compound is a strong metal ion chelator leading however to the formation of kidney stones [Holmes & Assimos, 2004]. In our study, oxalic acid concentration was 224.8 mg/g dw of BJ.

TABLE 1. Chemical composition, pH value, and total titratable acidity of beetroot juice prior to digestion.

Parameter	Value
Moisture(g/100 mL)	92.86±0.12
pH	6.35±0.01
Total titratable acidity (g/100 mL)	0.24±0.03
Oxalic acid (mg/g dw)	224.8±1.2
TPC (mg GAE/g dw)	30.81±2.96
TFC (μg QE/g dw)	6.72±0.16
Betacyanins (mg/g dw)	2.81±0.10
Betaxanthins (mg/g dw	1.27±0.00
Sucrose (g/100 g dw)	7.20±0.15
Glucose (g/100 g dw)	3.14±0.22
Fructose (g/100 g dw)	4.06±0.25

TPC, total phenolics content; TFC, total flavonoids content; GAE, gallic acid equivalent; QE, quercetin equivalent.

To red beetroots are attributed numerous health benefits, associated to their wide-ranging array of bioactive molecules. The presence of phytochemicals has most often been explored. Although recommendations for the daily intake of phenolics and other antioxidants have not yet been well defined, it is considered that their consumption is beneficial for human health [Karam *et al*., 2018; Mihaylova *et al*., 2018]. The BJ tested in this study showed a significant TPC – 30.81 mg GAE/g dw (Table 1). Vasconcellos *et al*. [2016] obtained 3.67 mg GAE/g dw, which is approximately 8 times lower than reported here. In other studies, TPC in BJ was reported to be 0.52 mg GAE/mL [Porto *et al*., 2017] and 0.98 mg GAE/mL [Wootton-Beard & Ryan, 2011], which is respectively 4 and 2 times lower compared to our results (data not shown).

Flavonoids, which are part of the phenolic compound family, have an important contribution to the overall antioxidant activity of a given simple. That is why their content is also evaluated. In the present study, significantly lower TFC of BJ was measured, *i.e.* 6.72 μg QE/g dw (Table 1). In other studies, 83.34 mg QE/g and 0.42 mg QE/g total flavonoid contents of fresh beetroot juice were reported [da Silva *et al*., 2016; Olumese & Oboh, 2016]. This difference is likely due to the various origins of the beetroot material, including the various climatic and agricultural growth conditions.

Many fruits and vegetables have been reported to possess acetylcholinesterase inhibitory activity, making them useful for consumption by Alzheimer's patients [Szwajgier & Borowiec, 2012]. In the accessible literature there is a lack of information about the presence or not of this activity in beetroot. Solely, Murthy & Manchali [2013] stated in their review that red beetroot possesses anti-acetylcholinesterase activity. In our study no inhibition in the beetroot juice was detected.

TABLE 2. Antioxidant and acetylcholinesterase (AchE) inhibitory activities of beetroot juice.

Activity	Value
DPPH$^{\bullet}$ scavenging activity (μmol TE/g dw)	56.71±1.66
ABTS$^{\bullet+}$ scavenging activity (μmol TE/g dw)	97.04±1.35
FRAP (μmol TE/g dw)	184.74±2.62
CUPRAC (μmol TE/g dw)	222.84±2.35
AChE inhibitory activity (%)	n.d.

DPPH$^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical, ABTS$^{\bullet+}$, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical action; FRAP, ferric-reducing antioxidant power; CUPRAC, cupric ion reducing antioxidant capacity; TE, Trolox equivalent, n.d., not detected.

The antioxidant capacity of BJ is strongly linked to the content of red pigments. Based on their results, Czapski *et al.* [2009] considered that primarily betalains as responsible for the antioxidant capacity of red beets, therefore, they are probably the most studied beetroot phytochemicals. In this study, contents of betacyanins and betaxanthins were determined spectrophotometrically (Table 1). Content of betacyanins, the red pigments, was 2.2 times higher than that of betaxanthins, the yellow pigments, resulting to a ratio of 1:0.45. The betalain content is thought to be influenced by many factors, such as the size of roots, cultivar, climatic and agricultural conditions [Bazaria & Kumar, 2016]. Moreover, the extraction method is critical for their determination. Carrillo *et al*. [2017] demonstrated that betalains seemed to be largely responsible for the total antioxidant capacity found in the insoluble fraction, which could explain the lower values in this study. Kujala *et al*. [2002] reported slightly higher pigment contents in flesh of different red beet cultivars (4.4 to 9.2 mg/g dw) that are still comparable to the betalains content reported in our study (4.08 mg/g dw).

Beetroot has relatively sweet taste, so the determination of its sugars content is of particular interest. In this regard, contents of individual sugars (sucrose, glucose, and fructose) of BJ were evaluated and data are presented in Table 1. Although red beetroot is poorer in sugars than sugar beet, sucrose is still the main sugar followed by glucose and fructose [Bavec *et al*., 2010]. The BJ sucrose content found in this study was 7.20 g/100 g dw, which is in agreement with a previous study reported by Wruss *et al*. [2015]. The same author however indicated much lower glucose and fructose content (0.41% in total) than these cited here (3.14 and 4.06 g/100 g dw, respectively).

The antioxidant activity of plant materials is related to their natural self-defense mechanisms. Different *in vitro* methods are used in order to evaluate the strength of this activity. They are designed to imitate the antioxidant action that phytochemicals exercise *in vivo*. The most commonly used assays for the reducing potential evaluation are FRAP and CUPRAC while the anti-radical scavenging activity is studied according to DPPH and ABTS assays [Haida & Hakiman, 2019]. Red beet belongs to the group of 10 vegetables of the highest antioxidant potential [Wettasinghe *et al*., 2002]. The results of the antioxidant potential assay of red beetroot juice are presented in Table 2. Granato *et al*. [2015] reported 6363 μmol TE/L according to DPPH assay which is 0.64 times higher than that obtained in this study (4048±120 μmol TE/L). The same author reported average activity towards CUPRAC of 17664 μmol TE/L, which is comparable to our result by the same method (data not shown). The antioxidant potential of the juice toward the ABTS$^{\bullet+}$ was evaluated to be 97.04 μmol TE/g dw. The reducing potential according to FRAP assay was measured to be 184.74 μmol TE/g dw compared to Ou *et al*. [2002] that reported values from 12 to 120 μmol TE/g dw of beetroots of different varieties.

In vitro gastrointestinal digestion of beetroot juice

Once entered into a human body food is subjected to digestion. This is a complex, multistage process that has its general rules but remains specific to each individual. That is why it is not easy to imitate digestion entirely in a laboratory setting; however, efforts are being made to determine the conditions closest to the human body [Minekus *et al*., 2014]. Although this process begins in the human mouth, in our study this step is omitted because of the liquid form of the sample, which is usually not chewed and passes directly into the stomach and small intestines afterwards. Food bioaccessibility

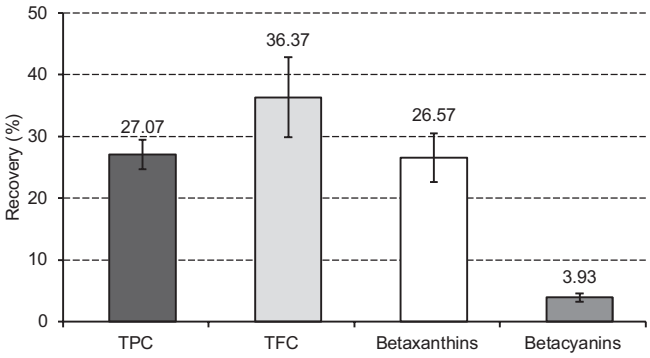

FIGURE 1. Recovery of total phenolics content (TPC), total flavonoids content (TFC), and contents of betaxanthins and betacyanins after *in vitro* simulated gastrointestinal digestion (SGD) of red beetroot juice.

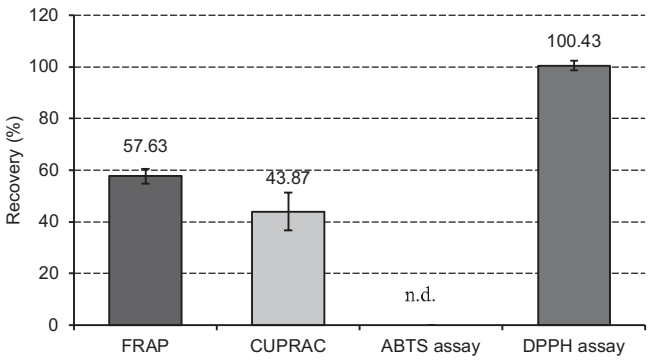

FIGURE 2. Recovery of antioxidant activity after *in vitro* simulating gastrointestinal digestion (SGD) of red beetroot juice.

n.d., not detected; FRAP, ferric-reducing antioxidant power; CUPRAC, cupric ion reducing antioxidant capacity; ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; DPPH – (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical.

TABLE 3. Contents of individual phenolic acids in beetroot juice before and after simulated gastrointestinal digestion (SGD) (μg/g dw).

Phenolic acid	Before SGD	After SGD
Chlorogenic acid	16.99±0.55	42.42±1.02
Caffeic acid	22.21±0.75	n.d.
p-Coumaric acid	52.69±0.98	n.d.
Sinapic acid	19.86±0.80	16.15±0.77

dw – dry weight, n.d. – not detected.

(the amount of released soluble food components which are ready for intestinal absorption) is among the most important factors determining the bioavailability [Parada & Aguilera, 2007].

Digestion of phytochemicals is a complex process, and their bioaccessibility depends on both the characteristics of the food matrix and the physiological conditions throughout the digestion [Alminger *et al*., 2014]. Since phenolic phytochemicals are very different in their chemical structure and properties, this leads to their different bioaccessibility. In the present study, the bioaccessibility was evaluated by determining the total phenolic and flavonoid contents, betaxanthin and betacyanin contents as well as the remaining antioxidant activity after digestion of BJ. Figure 1 presents the data for the recovery of contents of total phenolics, total flavonoids, and betalains after SGD. Loss of 72.93% of total phenolics and 63.63% of total flavonoids was reported. Much lower recovery was claimed by Guldiken *et al.* [2016], only 5.0% for TPC and 10.0% for TFC. Significant reduction of TPC was also reported by Bouayed *et al*. [2011] and Pavan *et al*. [2014] in the analysis of different apple varieties and araticum and papaya extracts submitted to SGD. Flavonoids loss during digestion of red chiltepin was established by Ovando-Martinez *et al*. [2018]. The same negative tendency was observed for betalains bioaccessibility. While nearly 27% of betaxanthins were present after the simulated digestion, almost all betacyanins (96.07%) have been lost. Our results are in agreement with the findings of Sawicki *et al*. [2019], who also noticed betalain content reduction during the *in vitro* digestion. Betalains are very sensitive compounds that are degraded by high temperature, oxygen, light, pH changes, and enzyme activity [Ravichandran *et al*., 2013]. Although, both betacyanins and betaxanthins have a broad pH stability in the pH range of 3.0–7.0, a loss of their content after digestion is reported [Tesoriere *et al*., 2008] probably due to isomerization, decarboxylation, and/or cleavage that may occur [Stitzing & Carle, 2004].

Figure 2 presents the data for antioxidant activity recovery after *in vitro* SGD. The resulted antioxidant activity of digested juice varied across the four methods. No activity was detected according to the ABTS assay while full recovery was measured by the DPPH assay. Following the CUPRAC and FRAP, approximately half of the initial activity remained after digestion. Guldiken *et al.* [2016] also reported varied results about the antioxidant activity of digested red beetroot juice. They claimed no remaining activity based on the DPPH assay, with 0.4%, 8%, and 24% recovery determined with CUPRAC, FRAP, and ABTS assays, respectively. Although all four are electron transfer-based assays [Apak *et al*., 2007], some factors like: radical formation and stability, sample solubility in reaction media, affinity against the sample components *etc*., could make the difference in the results. In addition, it should be taken into account that despite the both radicals are synthetic; the reaction temperature and light conditions are completely different. DPPH radical is stabilized by itself, while ABTS cation radical has to be formed initially. During digestion, many reactions occurs leading to the inactivation of some active molecules or liberation of others from the matrix [Pavan *et al*., 2014]. The presence of bile acids should also be taken into account. That is why variations are expected in the remaining antioxidant activity.

The HPLC analysis of phenolic acids of native red beetroot juice revealed the presence of chlorogenic, caffeic, *p*-coumaric, and sinapic acids at contents of 16.99, 22.21, 52.69, and 19.86 μg/g dw, respectively (Table 3). Caffeic and *p*-coumaric acids were no longer detected after the digestion process. Interestingly, 2.5 times higher concentration of chlorogenic acid was measured in the digested sample. Sinapic acid's bioaccessibility remained at 16.15 μg/g dw, which represented 81% recovery.

Various research papers reported changes in the phenolic acids content after the digestion process. Loss in the sinapic acid of *H. cannabinus* L. was reported by Wong *et al*. [2014]. Decrease of chlorogenic acid content after SGD of various fruits was previously claimed as well [Bouayed *et al*., 2012], however reports on the stability of this acid during digestion also exist [Correa-Betanzo *et al*., 2014]. Moreover, Bermúdez-Soto *et al*. [2007] also reported 28% increase of chlorogenic acid content after digestion of chokeberry (*Aronia melanocarpa*) probably due to isomerisation reactions of neochlorogenic acid, whereas Ovando-Martínez *et al*. [2018] reported low bioaccessibility values of caffeic and *p*-coumaric acids in red chiltepin attributed to the gastrointestinal conditions. Therefore, further investigations on interactions between food components during digestion are needed.

CONCLUSIONS

Beta vulgaris (red beetroot) is consumed worldwide due to its high nutritive and medicinal values. Many studies have been focused on pre-treated beet products but data on the bioavailability of fresh juice phytochemicals are still limited. In this regard, in the present study the phytochemicals content and the antioxidant activity of freshly prepared beetroot juice were evaluated. It was demonstrated that this vegetable juice could be assumed as a valuable source of biologically active compounds such as phenolics (including flavonoids and phenolic acids) and betalains. Furthermore, the bioaccessibility of the phytochemicals was determined to assess the potential benefits of juice consumption. In conclusion, the SGD process resulted in lower recovery of total phenolics, total flavonoids, and betalains. The remaining antioxidant activity measured by four *in vitro* methods was variable. Digestion process led to a higher content of chlorogenic acid but decreased concentrations of caffeic, *p*-coumaric, and sinapic

acids. In this regards, further studies on different component interactions during digestion process are needed in order to better understand the potential health benefits of food.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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