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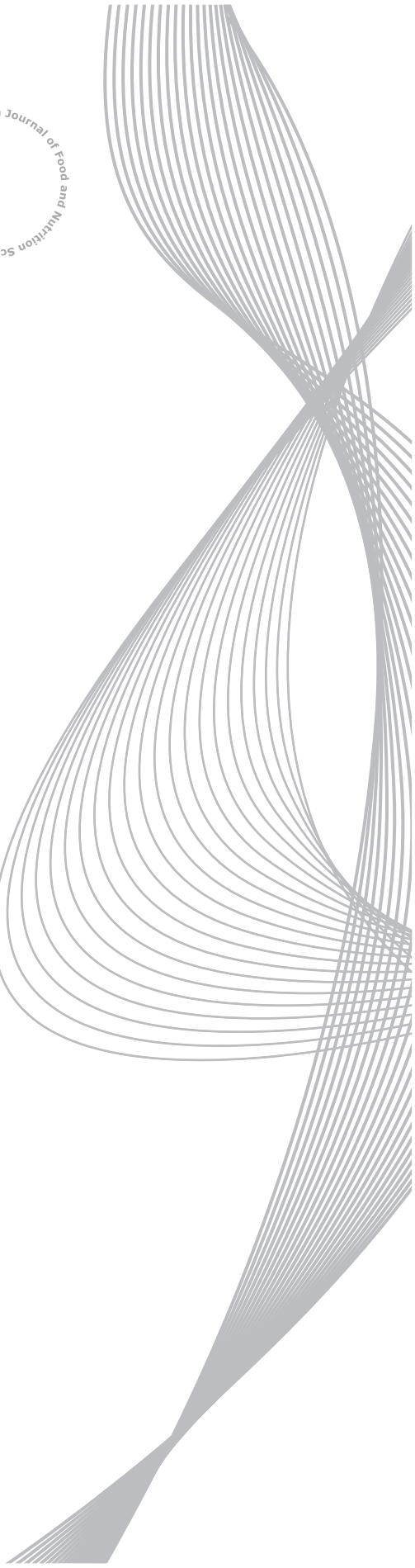

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Editorial
Red Beetroot Thematic Issue

Thematic Issue on *"Red Beetroot as a Source of Nutrients, Bioactive Compounds and Pigments"*

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Guest Editor of Thematic Issue

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This Thematic Issue of *Polish Journal of Food and Nutrition Sciences* deals with the trans-disciplinary aspects of red beetroot as well as the red beetroot-derived products in the following topics: processing and storage – effect on the quality and functionality; bioactive constituents and nutritional value; profile, absorption, metabolism, and bioavailability of phytochemicals; potential health-promoting and microbiological activities; and new analytical methods for compounds study.

Due to the development of civilization diseases, increasing attention is paid not only to the nutritional value and sensory attractiveness of certain food-plants and their products but, above all, to their profile and content of bioactive compounds since these issues may have a positive impact on human health. The bioactive compounds of red beetroots that have been studied in the seven published articles of the *Thematic Issue* are of a varied nature and activity, ranging from betalains and saponins to phenolic acids and flavonoids. In this context, the report of Kumorkiewicz *et al.* [2020] presents for the first time the new bidecarboxylated betanins (which might have a strong bioactivities) in red beetroot extract as well as their generation from betanin/isobetanin and mono-decarboxylated betanins during the process of heating. On the other hand, the thermal stability of betalains present in by-products of red beetroot and their degradation along with raising temperature is manifested by the production of various betalain degradation products that have been determined in the work of Otálora *et al*. [2020]. Next, Czyżowska *et al*. [2020] investigated the effect of long-term cold storage of fermented beetroots and fermented beetroots juice on the content of biologically active compounds and microorganisms. In turn, Tumbas Šaponjac *et al.* [2020] studied the encapsulation of bioactive compounds from red beetroot juice with soybean proteins and determined the physicochemical characteristics of these encapsulates. Another interesting research article by Spórna-Kucab & Wybraniec [2020] focused on the analysis of saponins, being natural plant compounds exhibiting health benefits, in red beetroot for the first time using high-speed counter-current chromatography in a new solvent system consisting of *tert*-butyl-methyl ether-butanol-acetonitrile-water. Aspects related to the fate of red beetroot phytochemicals after intake, such as the change in the total phenolics content, total flavonoids content, contents of betacyanins and, betaxanthins, phenolic acids profile as well as the antioxidant activity after simulated gastrointestinal digestion of red beetroot juice have been described by Desseva *et al.* [2020]. Finally, the study of Płatosz *et al.* [2020] shows the profile of phenolic acids and flavonoids of red beetroot and its fermentation product and explains whether the long-term consumption of fermented red beetroot juice affects phenolic compounds profile in plasma and urine of volunteers.

All these articles are actually highlighting the great potential of red beetroot and its products along with their various natural compounds as well as show that this vegetable arouses the interest of scientists from various corners of the world. At the same time, it should be emphasized that a number of aspects regarding the transformation of red beetroot phytochemicals during technological processes and also after consumption during digestion, absorption, and metabolism processes have not yet been discovered.

I would like to thank the Authors for their important work in the field of red beetroot as a source of bioactive compounds and pigments.

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Original article
Red Beetroot Thematic Issue

Thermal Decarboxylation of Betacyanins in Red Beet Betalain-Rich Extract

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Key words: decarboxylation, red beet root, colorants, betanin, betalain-rich extract, decarboxy-betacyanins

Betalains are one of the most common groups of plant pigments found in nature, especially in red beetroot (*Beta vulgaris* L.) which is the main commercially exploited source of betalains produced in the form of concentrates or powders. This report presents results of thermal decarboxylation studies on betacyanins present in a specifically purified highly concentrated betalain-rich extract (BRE). The first tentative structures formed by decarboxylation of the main pigment present in BRE, betanin and its diastereomer, were established by means of liquid chromatography coupled to diode array detection and electrospray ionization tandem mass spectrometry (LC-DAD-ESI-MS/MS). In the extract, two new isomeric bidecarboxylated betanins were tentatively identified. A high rate of generation of 2-decarboxy-betanin/-isobetanin which are present in the BRE extract at very low level was observed, which was dependent on the starting concentration of the BRE substrate. The bidecarboxylated derivatives were generated at a higher rate mostly from 17-decarboxy-betanin/-isobetanin as well as 15-decarboxy-betanin by further decarboxylation at carbon C-2. Further studies will be performed to demonstrate if the decarboxylated betanins being products of heating *B. vulgaris* preparations can be used for various food applications with new health-promoting actions and colorant properties.

INTRODUCTION

Betalains are one of the most common groups of plant pigments found in nature, however, they are not as well studied as compared to other natural pigments such as anthocyanins, carotenoids, and chlorophylls [Stafford *et al.,* 1994]. Betalain pigments which are composed of red-violet betacyanins together with yellow-orange betaxanthins are mainly found in most families of the Caryophyllales order [Chhikara *et al.,* 2019]. In addition, both betacyanins and betaxanthins can occur in the same plant part, despite the difference in its coloration [Martins *et al.,* 2017]. These pigments are commercially recognizable as food colorants due to their non-toxic, non-carcinogenic, and non-poisonous nature [Esatbeyoglu *et al.,* 2015; Siervo *et al.,* 2013].

Betalains are stable at pH values ranging from 3 to 7 and suitable for dyeing low acidic and neutral foods. In addition, they may be stabilized by ascorbic acid. In contrast, anthocyanins are unsuitable for coloration of such foods as they are unstable at pH values over 3, in addition their degradation is facilitated by ascorbic acid. For that reason, utilization of betalain pigments instead of anthocyanins for coloring food with a high vitamin C content or of vitamin C-supplemented products seems to be more favorable. Due to their thermolability, betalains are also utilized to color low-temperature products [Azeredo *et al.,* 2008; Herbach *et al.,* 2007; Stintzing *et al.,* 2004].

Red beetroot (*Beta vulgaris* L.) is the main commercially exploited source of betalain pigments which are produced in the form of concentrates or powders [Ciriminna *et al.,* 2018]. The most abundant pigments present in red beet are betanin (red betacyanin) and vulgaxanthin I (yellow betaxanthin). Due to their satisfying nutritional value and disease-preventing effects, such extracts are regarded beneficial to human health and applied as food additives, colorants, and dietary supplements [Nemzer *et al.,* 2011]. They are also characterized by the best quality in terms of the color and its intensity. In addition, betanin is approved by the US FDA and European Union as a natural colorant used for coloring dairy products, cosmetics, and pharmaceuticals [Esatbeyoglu *et al.,* 2015]. Beetroot extracts are utilized to emphasize the redness of such products as tomato soups, sauces, pastes, desserts, jams, sweets, and jelly beans. They are also used to protect meat from discoloration and to extend its shelf-life [Chhikara *et al.,* 2019; Tang *et al.,* 2015].

Several studies have attributed a wide spectrum of bioactive properties to betalain pigments and betalain-rich extracts. They may serve as biologically active nutraceuticals

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with a wide variety of therapeutic, anti-carcinogenic, hepato-protective, and antitumor properties [Chhikara *et al.,* 2019; Vulić *et al.,* 2013]. It has also been shown that some of the betalain pigments exhibit even higher antioxidant activity in comparison to typical natural antioxidants such as ascorbic acid, rutin, and catechin [Cai *et al.,* 2006; Gandía-Herrero *et al.,* 2009]. The free radical-scavenging activity of betanin extracted from red beet measured in a TEAC assay at pH 7.4 and in a 1,1-diphenyl-2-picrylhydrazy (DPPH) assay is about 7.5- and 3.0-fold higher, respectively, than that of vitamin C, which is an effective natural antioxidant [Cai *et al.,* 2003; Gengatharan *et al.,* 2015]. Furthermore, studies with different cell lines have demonstrated that betalains exhibit a chemopreventive potential [Gandía-Herrero *et al.,* 2016]. *In vivo* anti-tumor formation activity in mouse skin has been demonstrated for *B. vulgaris* extracts. Results showed a significant decrease in the incidence and number of papillomas found in mice skin. In the same study, lung tumor formation was induced to mice, and inhibited by the oral administration of *B. vulgaris* extracts [Kapadia *et al.,* 2003]. Pure betanin was assayed, revealing a strong inhibition of the proliferation of melanoma cancer cells [Wu *et al.,* 2006]. Betanin from red beet also showed excellent growth inhibition of MCF-7 (breast), HCT-116 (colon), AGS (stomach), SF-268 (CNS), and NCI-H460 (lung) cancer cell lines with IC_{50} values of 162, 142, 158, 164, and 147 μg/mL, respectively [Reddy *et al.,* 2005]. Recently a novel betalain-rich extract/concentrate (BRE) was tested in a pilot clinical study that reported short-term treatment with BRE which improved the function and comfort of knee joints in individuals with knee distress [Pietrzkowski *et al.,* 2014]. The chemopreventive and strong antioxidant properties of betalains stimulate research on their new structures, derivatives, and especially their influence on health.

Recently, several detailed research have been published on new products of degradation of betacyanins present in preparations subjected to thermal processing, especially on decarboxylated derivatives as well as their influence on human health [Cai *et al.,* 2005; Stintzing *et al.,* 2004; Tesoriere *et al.,* 2005]. Such derivatives were obtained by heating the natural substrates, previously isolated in aqueous and alcoholic solutions [Wybraniec, 2005; Wybraniec & Mizrahi, 2005]. The research on betanidin decarboxylation in ethanolic solutions was performed by Dunkelblum *et al.* [1972] as well as by Minale & Piattelli [1965]. Additionally, thermal treatment of betanin in aqueous /alcoholic media was described by Altamirano *et al.* [1993] and by Simon *et al.* [1993] however, without structural studies. In the case of *B. vulgaris* L. juice, thermal treatment led to the formation of different mono- (17-decarboxy- and 2-decarboxy-), bi- (2,17-bidecarboxy-), and tri- (2,15,17-tridecarboxy-) decarboxylated betacyanins along with their diastereomers, and minor levels of 14,15-dehydrogenated (neo-) derivatives which were identified by LC-DAD-ESI-MS/MS [Herbach *et al.,* 2004, 2006; Wybraniec, 2005; Wybraniec & Mizrahi, 2005]. Due to lower polarity of decarboxylated derivatives, their retention times during HPLC analysis on the reversed phase are longer in contrast to their starting substrates. Furthermore, different mechanisms of decarboxylation influenced by the type of alcoholic or aqueous media were indicated based on different mono-decarboxylation products of betanin/isobetanin. Definitely faster degradation process, leading to the formation of double decarboxylation products, was observed in ethanolic solutions, which should be taken into account during analytical samples preparation [Wybraniec, 2005; Wybraniec & Mizrahi, 2005].

Degradation products of thermal processing of isomeric to betanin gomphrenin pigments present in *Basella alba* L. fruit juice were also identified and their tentative structures were established for the first time by LC-DAD-ESI-MS/MS and LCMS-IT-TOF. The research reports that the principal degradation products present in heated *B. alba* fruit juice were 2-, 17-, and 2,17-decarboxy-gomphrenins, their diastereomers, as well as minor levels of their 14,15-dehydrogenated derivatives (neo-derivatives). It was also noticed that the position of betanidin glucosylation at carbon C-5 or C-6 affected the chromatographic differences between betanin and gomphrenin derivatives. Due to various chromatographic properties as well as greater stability in relation to their corresponding betacyanins, the processed betacyanins arising in the process of thermal decarboxylation and dehydrogenation represent a very interesting research material suitable for wider applications [Kumorkiewicz *et al.,* 2017].

In this contribution, further studies on betacyanins thermal decarboxylation are reported, especially these searching for new structures formed by decarboxylation of the main pigments present in the BRE extract by means of liquid chromatography coupled to diode array detection and electrospray ionization tandem mass spectrometry (LC-DAD-ESI-MS/MS). Influence of different heating conditions on generation of decarboxylated betanins was investigated as well.

MATERIALS AND METHODS

Reagents

Formic acid, acetic acid, LC-MS grade methanol, and water were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Plant material

Betalain-rich extract (BRE) [Nemzer *et al.,* 2011] was obtained from FutureCeuticals, Inc. (Momence, IL, USA).

Heating experiments

BRE aqueous solutions (30 mL) were prepared at concentrations: 0.70, 0.50, 0.25, and 0.10 g/L (Table 1) and acidified with acetic acid (1.0 and 2.5 g/L). These samples were heated at 85°C in a water bath for 45 min. Aliquots (1 mL) of the heated samples were taken in one repetition for LC-DAD-ESI-MS/MS analysis every 15 min.

Chromatographic analysis in the LC-DAD-ESI-MS/MS system

An LCMS-8030 mass spectrometric system (Shimadzu, Kyoto, Japan) coupled to LC-20ADXR HPLC pumps controlled with LabSolutions software (Shimadzu) was used for the chromatographic and mass spectrometric analyses.

TABLE 1. Composition of aqueous solutions containing the betalain-rich extract (BRE) heated at 85°C.

Test No.	Sampling time (min)	Concentration of BRE (g/L)	Concentration of acetic acid (g/L)
H1	0, 15, 30, 45	0.70	2.5
H2	0, 15, 30, 45	0.50	2.5
H3	0, 15, 30, 45	0.25	2.5
H4	0, 15, 30, 45	0.10	2.5
H5	0, 15, 30, 45	0.70	1.0
H6	0, 15, 30, 45	0.50	1.0
H7	0, 15, 30, 45	0.25	1.0
H8	0, 15, 30, 45	0.10	1.0

The samples were eluted through a 150 mm × 4.6 mm i.d., 5.0 μm, Kinetex C18 chromatographic column preceded by a guard column of the same material (Phenomenex, Torrance, CA, USA). The injection volume was 20 μL, and the flow rate was 0.5 mL/min. The column was thermostated at 40°C. The analytes were separated using a gradient system as follows: 5% B in A (v/v) at 0 min; gradient to 70% B in A at 12 min, gradient to 20% B in A at 15 min, isocratic at 20% B in A till 19 min, with A – 2% aqueous formic acid (v/v), and B – methanol. Online UV/Vis spectra were acquired using the PDA (photodiode-array detection) mode.

The positive ion electrospray mass spectra (*m/z* range 100–2000) were recorded on the LC-MS system which was controlled with LabSolutions software (electrospray voltage 4.5 kV; capillary 250°C; sheath gas: N_2), recording total ion chromatograms, mass spectra and ion chromatograms in selected ion monitoring mode (SIM) as well as the fragmentation spectra. Argon was used as the collision gas for CID experiments. The relative collision energies for MS/MS analyses were set at -35 V.

RESULTS AND DISCUSSION

The LC-MS selected ion chromatograms present in Figure 1 depict a typical betacyanin and decarboxylated betacyanin profile in a betalain-rich extract/concentrate (BRE) before the heating experiments. The dominant presence of betanin (**1**) and its isoform (**1'**) with substantial participation of very well-separated 17-decarboxy-betanin/-isobetanin (**2/2'**) as well as 15-decarboxy-betanin (**3**) (Table 2) confirms results from the previous research [Nemzer *et al*., 2011]. Further inspection of chromatograms revealed small quantities of a slightly resolved pair of 2-decarboxy-betanin/-isobetanin (**5/5'**) (absorption maximum at λ_{max} 533 nm) and an unresolved pair of 2,17-bidecarboxy-betanin/-isobetanin (**6/6'**), similarly to the previous reports (λ_{max} 507 nm) [Nemzer *et al.*, 2011; Wybraniec, 2005]. For the identification, a series of already known decarboxylated betanin standards was used in the study [Wybraniec *et al.,* 2006].

Figure 2 presents the structures of the studied pigments (Table 2) and possible decarboxylation reactions starting from betanin.

Interestingly, except for very well-known betanin derivatives identified as 2,17-bidecarboxy-betanin/-isobetanin (**6/6'**), other two new isomeric bidecarboxylated betanins (peaks **4** and **7**) were detected in BRE which fitted to the reaction scheme (Figure 2). These compounds displayed absorption maxima at λ_{max} 494 and 532 nm, respectively, thus differing from the maximum for compound **6/6'** and pseudomolecular ions at *m/z* 463 (Table 2), clearly confirming a loss of two CO_2 moieties from the starting Bt/IBt (**1/1'**). Subsequent fragmentation to ions of *m/z* 301 confirmed the existence of a bidecarboxylated fragment of betanidin and suggested the formation of bidecarboxylated betanin /isobetanin. These compounds could presumably be assigned to 15,17-bidecarboxy-betanin (**4**) and 2,15-bidecarboxy-betanin (**7**) based on analogous retention differences between 17-dBt (**2**) and 2-dBt (**5**) (2-decarboxylated betacyanins are more retarded on the column than the 17-decarboxylated derivatives)

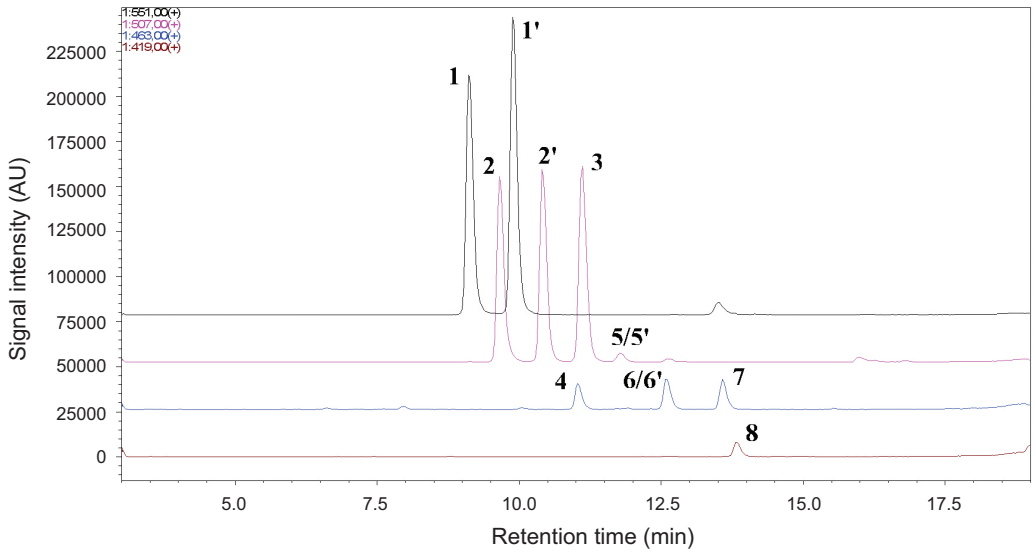

FIGURE 1. Chromatographic LC-MS traces of selected ions of betanin and its decarboxylated derivatives in the betalain-rich extract (BRE).

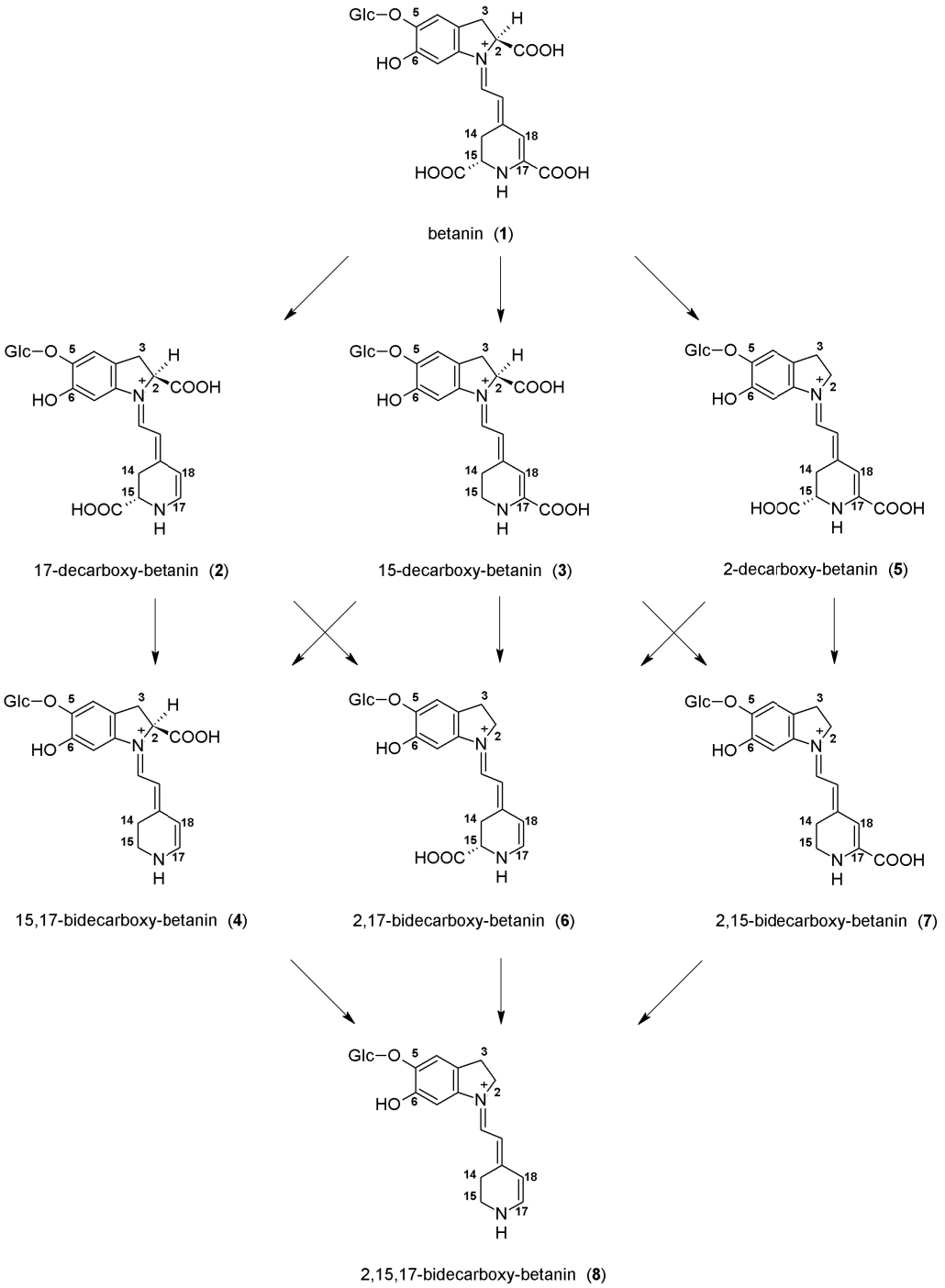

FIGURE 2. Chemical structures of detected betanin and its decarboxylated derivatives in the betalain-rich extract (BRE) before and after heating at 85°C for 15, 30 and 45 min. The reaction scheme of possible decarboxylation paths is also presented.

[Wybraniec, 2005]. The lack of the carboxyl moiety at carbon C-15 implicates the lack of the chirality at this position, therefore, only single forms of the pigments **4** and **7** are detected in the chromatograms, which supports their identification.

In contrast to the previous report [Nemzer *et al.*, 2011], the presence of 2,15,17-tridecarboxy-betanin (**8**) was acknowledged (Figure 2, Table 2). This compound displayed a pseudomolecular ion at *m/z* 419 during LC-MS analysis and an absorption maximum of λ_{max} 503 nm. Subsequent fragmentation experiments on the pseudomolecular ion at *m/z* 419 revealed fragmentation ions at *m/z* 257, which proved the existence of the tridecarboxylated fragment of betanidin. This conclusion was supported by the detection of only one chromatographic peak **8** in the HPLC system, which resulted from the loss of the chiral center at carbon C-15 of this compound. This confirmed the presence of a tridecarboxy-betanin for which the only possible structure can be predicted as 2,15,17-tridecarboxy-betanin similarly to the previous report based on the prolonged heating of *B. vulgaris* juice [Wybraniec, 2005].

The presence of some additional quantities of bi- and tridecarboxylated derivatives of betanin in the extract is pre-

TABLE 2. Chromatographic, spectrophotometric, and mass spectrometric data of the analyzed betanin-based decarboxylated betacyanins present in the betalain-rich extract (BRE) heated at 85°C for 15, 30, and 45 min.

No.	Compound name	Abbreviation	t_R	λ_{max}	*m/z*
			(min)	(nm)	$[M+H]^+$
1	betanin	Bt	9.2	536	551
2	17-decarboxy-betanin	17-dBt	9.7	505	507
1'	izobetanin	IBt	9.9	536	551
2'	17-decarboxy-isobetanin	17-dIBt	10.4	505	507
3	15-decarboxy-betanin	15-dBt	11.1	527	507
4	15,17-bidecarboxy-betanin	15,17-dBt	11.1	494	463
5/5'	2-decarboxy-betanin	2-dBt/-IBt	11.8	533	507
6/6'	2,17-bidecarboxy-betanin/isobetanin	2,17-dBt/-IBt	12.6	507	463
7	2,15-bidecarboxy-betanin	2,15-dBt	13.6	532	463
8	2,15,17-tridecarboxy-betanin	2,15,17-dBt	13.8	503	419

sumably a result of a deeper decarboxylation process which is inherent in the current preparation process of BRE. Nevertheless, further decarboxylation experiments performed in this study showed that the generation of these pigments can be due to the controlled thermal decarboxylation of Bt/IBt (**1/1'**) and especially mono-decarboxylated betanins (**2/2'**, **3** and **5/5'**).

The profiles of the main pigments in thermally-treated BRE were similar to the profiles of early heating products of *Beta vulgaris* L. root [Wybraniec, 2005]. The aqueous solutions acidified by acetic acid were heated for 45 min, and the temperature (85°C) of the heating process was high enough to enable monitoring changes in the compositions of the resulting mixtures within that time range. All the detected heating products (Figures 1–3) were less polar than their corresponding precursors. Similar experiments were performed also on betanin-based pigments, phyllocactin, and hylocerenin which released high quantities of mono-decarboxylated and especially bi-decarboxylated derivatives in aqueous or ethanolic solutions [Wybraniec & Mizrahi, 2005].

The experimental results (Table 3) obtained after 30 min of heating when betanin was almost completely degraded were presented graphically as a generation ratio (GR) which is the ratio between measured signals (chromatographic peak areas) for a selected compound after 30 min of heating and a reference before heating. Each reference was prepared for the defined concentration level (Table 1) of BRE (0.70, 0.50, 0.25, and 0.10 g/L). The GR index represents the tendency of a compound to be generated over being degraded, therefore, obviously only degradation was observed (very low GR values) for Bt/IBt (**1/1'**) (Table 3). Table 3 presents a full set of the results obtained for the sampling times of 15, 30, and 45 min.

In this study, the heating experiments performed with the higher concentration of acetic acid (2.5 g/L) in the dissolved extract sample revealed, first of all, a selective generation of 2-decarboxy-betanin/-isobetanin (**5/5'**) (Figure 3, Table 3) which are present in the BRE extract at a very low level. As a result, high levels of the GR index reaching the value of 20–40 after heating for 30 min (Figure 3) indicate mostly the generation of compounds **5/5'** and their low tendency to be degraded under the experimental conditions. This also means that the bidecarboxylated compounds (**6/6'** and **7**) generated at a higher rate (Table 3) according to the reaction scheme (Figure 2) are formed rather not from compounds **5/5'** but mostly from 17-dBt/-IBt (**2/2'**) as well as 15-dBt (**3**), respectively, by further decarboxylation at carbon C-2.

The diastereomeric pair of 2-decarboxy-betanin/-isobetanin (**5/5'**) can obviously be formed only from Bt/IBt (**1/1'**). The latter pigment was mostly degraded after 30 min of heating, especially at medium concentration levels (Figure 3, Table 3). Decreasing extract concentration from 0.7 to 0.1 g/L resulted in an increased signal ratio of compounds **5/5'** to **2/2'** or **5/5'** to **3**. This is also due to the further decarboxylation of 17-dBt/-IBt (**2/2'**) and 15-dBt (**3**) (Table 3) which are already present at high quantities in the BRE extract and are generated from betanin at a lower rate in these conditions. Similarly to betanin, pigments **2/2'** and **3** were degraded at the highest rate at BRE medium concentration levels (Table 3).

A previous report [Wybraniec, 2005] also presented data indicative of the preferential generation of 2-dBt/-IBt (**5/5'**) in aqueous acidic solutions of red beet extract in contrast to ethanolic solutions which enhanced the generation of 17-dBt/-IBt (**2/2'**). Our report more specifically defines conditions in which the formation of target derivatives occurs. According to the results, the most decisive is the concentration of the substrate (Bt/IBt (**1/1'**)). Another important factor is the concentration of acetic acid. Its lower concentration (1 g/L) increased the optimal substrate concentration (Table 3), which promoted the generation of 2-dBt/-IBt (**5/5'**).

The profile of bidecarboxylated betanins (**4**, **6/6'** and **7**) generated during the heating experiments was also dependent on the starting concentration of the BRE substrate (Table 3). Concentration of bidecarboxylated betanin (**4**) decreased after

TABLE 3. Results of the 15, 30 and 45 min heating of the betalain-rich extract (BRE) expressed as the generation ratio (GR) between measured signals (chromatographic peak areas) after heating and a reference before heating for betanin as well as its generated decarboxylated derivatives.

		Generation ratio (GR)							
		2.5 g/L acetic acid				1.0 g/L acetic acid			
Sample code:		H1	H2	H3	H4	H5	H6	H7	H8
BRE conc. (g/L):		0.70	0.50	0.25	0.10	0.70	0.50	0.25	0.10
No.	Compound	15 min							
1	Bt	0.087	0.017	0.077	0.39	0.46	0.10	0.020	0.22
2	17-dBt	0.58	0.17	0.38	0.85	0.68	0.46	0.15	0.39
1'	IBt	0.070	0.014	0.059	0.34	0.43	0.086	0.023	0.21
2'	17-dIBt	0.51	0.13	0.36	0.81	0.64	0.40	0.11	0.33
3	15-dBt	0.66	0.30	0.34	0.52	0.89	0.64	0.23	0.59
4	15,17-dBt	0.41	0.12	0.32	0.71	0.60	0.29	0.029	0.30
5/5'	2-dBt/-IBt	27.2	19.1	22.2	22.5	42.4	21.9	5.7	4.0
6/6'	2,17-dBt/-IBt	9.7	6.1	1.8	1.1	3.3	3.6	1.0	0.34
7	2,15-dBt	4.2	3.5	1.6	1.6	2.4	2.9	0.93	0.001
8	2,15,17-dBt	1.2	1.0	0.44	0.41	0.83	0.72	0.33	0.46
No.	Compound	30 min							
1	Bt	0.049	0.002	0.002	0.059	0.20	0.025	0.002	0.11
2	17-dBt	0.42	0.095	0.24	0.56	0.68	0.34	0.031	0.046
1'	IBt	0.039	0.002	0.002	0.045	0.20	0.019	0.002	0.071
2'	17-dIBt	0.36	0.061	0.21	0.54	0.65	0.26	0.019	0.087
3	15-dBt	0.52	0.22	0.28	0.39	0.80	0.54	0.11	0.41
4	15,17-dBt	0.28	0.056	0.18	0.42	0.55	0.22	0.010	0.13
5/5'	2-dBt/-IBt	22.6	14.8	18.6	19.5	36.4	16.5	1.8	1.4
6/6'	2,17-dBt/-IBt	11.7	8.3	2.7	1.7	4.8	6.1	2.2	0.30
7	2,15-dBt	6.0	5.5	2.4	2.5	3.3	3.9	1.9	0.001
8	2,15,17-dBt	1.6	1.3	0.7	0.8	1.1	1.1	0.55	0.77
No.	Compound	45 min							
1	Bt	0.011	0.001	0.001	0.021	0.043	0.006	0.001	0.023
2	17-dBt	0.29	0.085	0.12	0.43	0.45	0.21	0.014	0.015
1'	IBt	0.015	0.001	0.001	0.017	0.041	0.008	0.003	0.018
2'	17-dIBt	0.25	0.042	0.10	0.37	0.42	0.16	0.006	0.026
3	15-dBt	0.37	0.16	0.19	0.25	0.58	0.37	0.077	0.28
4	15,17-dBt	0.10	0.035	0.07	0.27	0.43	0.13	0.003	0.040
5/5'	2-dBt/-IBt	17.4	14.6	15.0	15.0	20.3	8.9	1.4	0.63
6/6'	2,17-dBt/-IBt	14.0	9.4	3.4	2.3	6.0	6.3	3.2	2.0
7	2,15-dBt	7.3	7.0	3.1	3.2	4.9	5.0	2.6	1.6
8	2,15,17-dBt	1.8	1.7	0.96	1.1	1.4	1.2	0.46	0.68

the heating, therefore, this pigment was rather not meaningfully generated. It is possible that its presence results only from a chemical process taking place during production of the BRE extract, but it cannot be formed by heating. A high concentration of BRE enhances the formation of 2,17-bidecarboxy--betanin/-isobetanin (**6/6'**) over 2,15-bidecarboxy-betanin **7** (Table 3). During the heating experiment, contents of compounds **6/6'** and **7** successively increased at all conditions (Table 3), however, at the low concentration of BRE, the latter pigment signal outweighed and this effect was more pronounced at the higher concentration of acetic acid (2.5 g/L). These differences can, presumably, be attributed to the matrix effect.

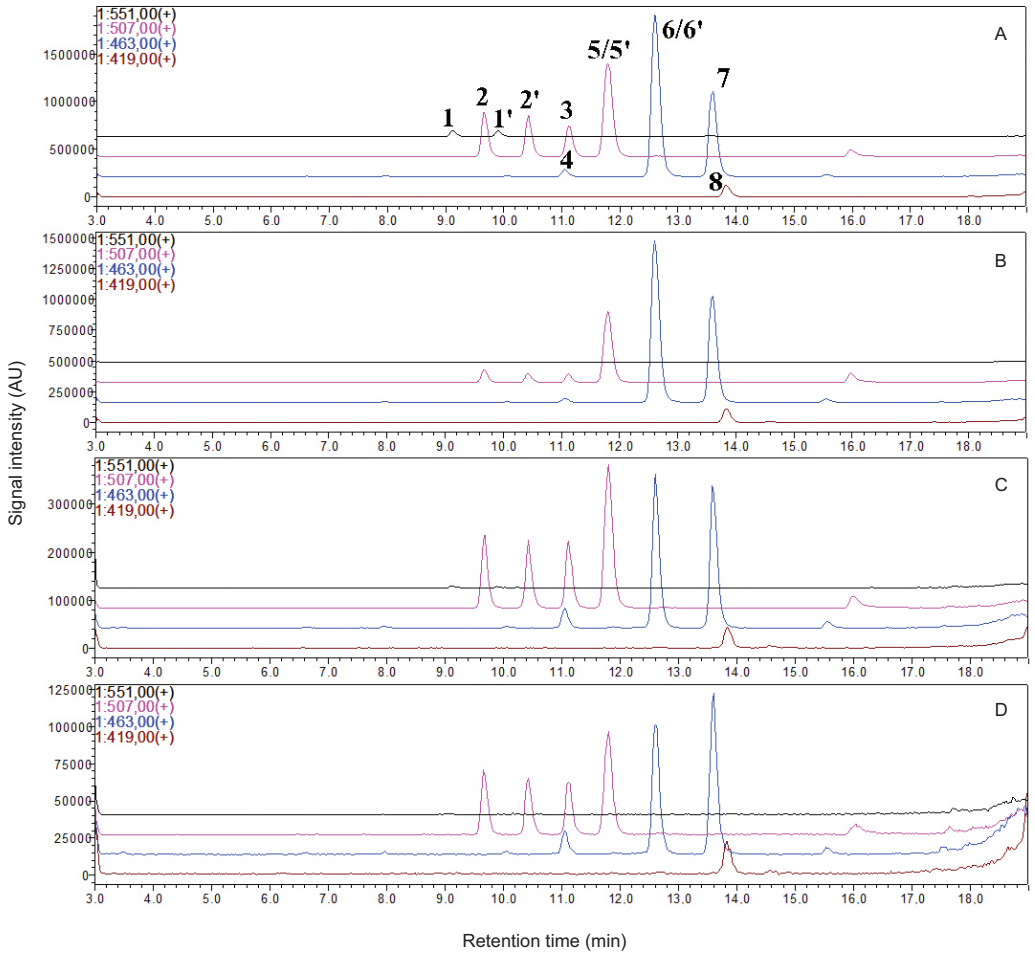

FIGURE 3. Chromatographic LC-MS traces of selected ions of betanin and its decarboxylated derivatives in the betalain-rich extract (BRE) after its heating at 85°C for 30 min. The experiments were performed for acetic acid concentration of 2.0 g/L and for BRE concentrations of 0.75 (A), 0.50 (B), 0.20 (C), and 0.10 g/L (D).

The presence of 2,15,17-tridecarboxy-betanin (**8**) in the reaction mixtures was strongly dependent on both the factors (Table 3) and acetic acid concentration increased enhanced the generation of pigment **8**, especially at the higher BRE concentration, however, during the heating experiment, the content of compound **8** increased successively at all conditions (Table 3).

CONCLUSION

This is the first report on the presence of new bidecarboxylated betanins in *B. vulgaris* extract as well as their generation by heating betanin/isobetanin and mono-decarboxylated betanins as the main ingredients of the extract mixture. Taking into account that especially 2,15-bidecarboxy-betanin can be present at higher quantities in the processed *B. vulgaris* juices and extracts, this compound – as an additional decarboxylated betanin – might have a strong influence on the bioactivities of *B. vulgaris* products, which was not considered before. Further studies will be performed to demonstrate if the decarboxylated betanins, which are degradation products of heated *B. vulgaris* preparations, can be used for various food applications with new health-promoting potentials and colorant properties.

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Original article
Red Beetroot Thematic Issue

Thermal Stability of Betalains in By-Products of the Blanching and Cutting of *Beta vulgaris* L. var *conditiva*

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Key words: red beetroot, blanching, cutting, by-products, betalains thermal stability

The objective of this study was to evaluate the thermal stability (5°C, 25°C, and 45°C) of betalains present in by-products of the blanching and cutting of *Beta vulgaris* L. var *conditiva*, to evaluate the possibility of taking advantage of them as a source of natural colorants to be incorporated in food products. The identification of the betalain compounds present in these by-products was also performed. Blanching waters showed pigment degradation at all the temperatures evaluated. The remnant tissues were freeze dried rendering beetroot powders whose pigments only presented thermal degradation at 45°C. Sixteen betalain compounds were identified in powders by chromatography and it was concluded that a thermal treatment at 45°C during six days affected the chemical stability of some of these compounds, producing a diversity of betalain degradation products. Results obtained allowed concluding that the red beetroot powder would have a better performance as a natural coloring additive than the blanching water at temperatures below 45°C. Probably, the low water activity of the powder and its lignin content ensured an effective protection of the pigments up to this temperature.

INTRODUCTION

The concern for healthy eating has driven the search for more natural ingredients and additives. As part of this reality, the use of natural pigments in food industrialization has been extended.

Natural pigments are extracted from plant tissues rich in such compounds as betalains, anthocyanins, carotenoids, and chlorophylls. Red beet (*Beta vulgaris* L. var *conditiva*) contains betacyanins and betaxanthins that are characterized by the presence of betalamic acid in their chemical structure [Polturak & Aharoni, 2018; Sakuta, 2014; Stintzing *et al.*, 2002]. The betacyanins (Bc) are a source of red-violet color with λ_{max} of absorption spectrum at 530 nm [Saenz *et al.*, 2012] whereas betaxanthins (Bx) provide food with yellow-orange color having a λ_{max} of absorption spectrum at 470 nm [Khan & Giridhar, 2015]. More than 80% of the red pigments in beet are betacyanins, mainly betanin (betanidin 5-*O*-β-glucoside) and its isomer isobetanin [Nemzer *et al.*, 2011; Sawicki *et al.*, 2016]. In addition, there are approximately 15 natural betaxanthins in red beet, with vulgaxanthin I and indicaxanthin being the main ones [Khan & Giridhar, 2015].

The stability of betalains is affected by different factors, such as temperature, pH, water activity, light, presence or absence of oxygen, and enzymatic action [Celli & Brooks, 2017; Herbach *et al.*, 2006; Wybraniec & Mizrahi, 2005]. Betacyanins in beet extracts have been noted as having pH stability in the range of 3–7 [Mikołajczyk-Bator & Czapski, 2017] and to be readily susceptible to thermal degradation [Gengatharan *et al.*, 2016]. Temperatures above 50°C are reported to produce the loss of color and antioxidant capacity. In the heat treatment, the betacyanins can be degraded by isomerization and/or decarboxylation [Kumorkiewicz & Wybraniec, 2017]. A slight hypsochromic and hypochromic change can occur displacing the maximum absorption in the spectrum, therefore imparting an orange-red color [Azeredo, 2009]. Also, betanin and isobetanin can be dehydrogenated and hydrolyzed causing the formation of neobetanin (4, 15-dehydrobetanin), which is bright yellow [Herbach *et al.*, 2006]. On the other hand, betaxanthins are also thermally sensitive and have a lower stability than betacyanins do [Pires Goncalves *et al.*, 2013].

In the food area, betalains application is accepted by the European Community [2008] and these pigments (named E162) are used in the production of jellies, jams, strawberry yogurt, ice cream, fruit cocktails, candies, and cookies [Esatbeyoglu *et al.*, 2015].

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Plant by-products are promising sources of high-value compounds with antioxidant and/or antimicrobial properties, such as fibers and polyphenols [O'Shea *et al.*, 2012]. Red beet processing generates large quantities of underutilized biomass [Fernandez *et al.*, 2017]. Bengardino *et al.* [2019] studied the extraction of bioactive compounds from beet leaves for the valorization of this by-product.

Red beet roots are consumed fresh, fermented, dried or after thermal processing. Polyphenol oxidase (PPO) and peroxidase (POX) are present in plant tissues and during preservation and storage of food products based on red beets, these enzymes are responsible for changes in color and nutritive value. For the reduction of enzymes activity, in general, a blanching treatment is applied in the frame of raw material industrialization [Latorre *et al.*, 2012]. It is also usual to subject these tissues to cutting and trimming to give them the desired geometrical characteristics [Jideani *et al.*, 2017]. Blanching and cutting give the origin to remnant water and solids that can give rise to additives and ingredients to be used in the food industry itself.

The objective of this study was to evaluate the thermal stability of betalains present in by-products of the blanching and cutting of *Beta vulgaris* roots, to evaluate the possibility of taking advantage of them as a source of natural colorants to be applied in food products. The identification of the betalain compounds present in these by-products was performed as well.

MATERIALS AND METHODS

Chemicals

Chemicals used were of analytical quality and provided by Sigma-Aldrich (Saint Louis, USA) or Merck Química (Buenos Aires, Argentina). The solvents for chromatography were of HPLC quality. Deionized water was used (Milli-Q™, Billerica, MA, USA).

Plant material

Samples of beet (*Beta vulgaris* L. var *conditiva*) roots were obtained from local markets in Buenos Aires city (Argentina).

Obtaining beet root by-products

Red beets were washed, peeled, and cut into slices 1 cm thick, 4.9 cm to 6.0 cm in diameter. Slices were subjected to a blanching treatment by immersion in water at 90°C for 7 min with a tissue/water ratio of 0.5 kg/L according to Latorre *et al.* [2010]. The remnant water was frozen (-18°C). The tissues were considered equivalent to those from the cutting operation in the industrialization and were also frozen at -18°C. Their water was sublimated in a Pennsalt freeze dryer (Pennsalt, Philadelphia, USA) at a chamber pressure of 100 mm and shelf temperature of 25°C. They were then milled in a domestic blade mill (DeLonghi, Buenos Aires, Argentina) and sieved to obtain powders with a particle size smaller than 105 μm.

The peroxidase and polyphenol oxidase activities in the powders were evaluated according to Latorre *et al.* [2010] and expressed as absorbance unit/(min × mg protein).

Chemical analysis of cell wall components in beetroot powders

Uronic acids, total (non-cellulosic) carbohydrates, cellulose, lignin, and protein contents were evaluated in red beet powders, according to Ng *et al.* [1998] by means of sulfuric acid hydrolysis. From the final residues, cellulose and lignin were determined gravimetrically, whilst the non-cellulosic carbohydrates, uronic acid as well as protein contents were determined in supernatants with the methods reported by Dubois *et al.* [1956], Filisetti-Cozzi *et al.* [1991], and Lowry *et al.* [1951], respectively.

Moisture content and water activity of beetroot powders

Moisture content of the powders was determined, in duplicate, by means of and infrared scale (Moisture Analyzer MB45 Ohaus Corporation, New Jersey, USA), using a ≈ 0.500 g sample.

Water activity was measured two times at 25°C in a Decagon AquaLab (Series 3 Water Activity Meter, Pullman, WA, USA), as explained by Basanta *et al.* [2016].

Evaluation of thermal stability of betalains in beetroot by-products

The powder was fractionated in amounts of ≈3.000 g in caramel glass flasks (volume 30 mL) and stored, for 6 days, at 5°C, 25°C, and 45°C. In the case of blanching water (pH=6.3), ≈ 2.00 mL were stored for 4 days in the same type of flasks, at the same temperatures. Storage was performed in duplicate.

After each storage day, the samples were characterized for their UV/Vis spectrum, total betalains content, and color parameters.

Betalain extraction and quantification

For the powder characterization, a quantity of ≈0.5000 g was extracted with 15 mL of Milli-Q™ water, stirred for 2 h, and centrifuged at 7700× *g* and 4°C for 15 min (Eppendorf 5804R, Hamburg, Germany). The supernatant was separated and used for measurement. In the case of blanching water, the measurement was performed directly on the sample.

According to Moβhammer *et al.* [2006], powder extracts obtained or blanching water were diluted in McIlvaine buffer (pH=6.5) to adjust the maximum absorption at 1.00±0.05 at wavelengths of 536 nm (betacyanins) or 476 nm (betaxanthins). The measurement was carried out in a UV-mini 1240UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). The content of betacyanins (Bc) and betaxanthins (Bx) was calculated as:

$$Bc \text{ or } Bx = [(A \times DF \times Mw \times 100/\varepsilon \times l)].$$

where: A is the absorption value of the betanin at its λ_{max} of 536 nm or vulgaraxanthin at its λ_{max} of 476 nm, corrected by the absorption at 600 nm; DF is the dilution factor and l is the pathlength (1 cm) of the cuvette; Mw is the molecular weight of betanin (550 g/mol) or vulgaraxanthin I (339 g/mol); and ε is the molar extinction coefficient of betanin (60,000 L/(mol × cm)) or vulgaraxanthin (48,000 L/(mol × cm)).

The contents of betacyanins and betaxanthins were expressed as mg/100 g for powder and as mg/L for blanching water.

Determinations were performed three times, and average and standard deviation (SD) are reported.

Absorption spectra

The spectra were determined for the extracts obtained from powders as described in the *Betalain extraction and quantification* section or directly on blanching water. Both were previously diluted with Milli-Q™ water to adjust the absorption maximum to 1.00±0.05. The whole visible spectrum (300–700 nm) was recorded at constant intervals ($\Delta\lambda$=2 nm) using a UV-mini 1240UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan) and 2 mm pathlength glass cells.

Color

Measurement of powders and water color was performed with a Minolta colorimeter (Minolta CM-600 Co. Ltd., Osaka, Japan) with natural daylight illuminant D65 and standard observer angle α: 10°. Each sample was placed on a white tile, registering the color through the chromatic coordinates of the CIELab space, L^* (ranging from 0, black, to 100, white), a^* (positive values for reddish colors and negative values for greenish ones), and b^* (positive for yellowish colors and negative for the bluish ones).

The average and SD for triplicate measurements are reported.

Chromatographic analysis of betalains in beetroot powders

To clarify the mechanism of betalains degradation, a chromatographic analysis was performed for beetroot powders at 45°C.

Extraction of betalains

A quantity of ≈1.0 g of powder was extracted twice with 10 mL of methanol/water (80:20, *v/v*) under continuous agitation, then centrifuged at 7700× *g* and 4°C for 15 min (Eppendorf 5804R, Hamburg, Germany). The supernatants were mixed and concentrated to a final volume of 2 mL, under reduced pressure at room temperature (25°C), then filtered through a 0.45 μm nylon filter and directly analyzed by HPLC [Swarna *et al.*, 2013].

HPLC-DAD analysis

HPLC-DAD analyses of the extracts were performed with a Waters HPLC 1525 system (Waters, Milford, USA), equipped with a binary pump system (model M0925P), a degasser (model M09DG2 455M), and with a photodiode array detector (model A10998). An Eclipse XDB-C18 column (150 mm x 4.6 mm, 5 μm particle size, Agilent, USA), with a mobile phase of formic acid (1 mL/100 mL water) (A) and acetonitrile (B) [Swarna *et al.*, 2013], at 0.3 mL/min flow rate and 20 μL injection volume was used in the study. A linear gradient was used, starting with 1% B and up till reaching 33% after 40 minutes. Chromatograms were recorded at 470 and 530 nm which correspond to the betaxanthins and betacyanins maximum absorption wavelengths.

HPLC-ESI-MS-MS analysis

HPLC-ESI-MS/MS analyses were performed with an Agilent 1200 HPLC system (Agilent Technologies, Wilmington, USA) provided with a binary pump (model G1312B), an automatic injector (model G1367D), a degasser (model G1379B), and a photodiode array detector (model G1315C) registering the chromatograms at 470 nm and 530 nm. The HPLC system was coupled with a high-resolution mass spectrometer Bruker micrOTOF-QII (Bruker Daltonics, Billerica, MA, USA) with an electrospray ionization source (ESI). The ionization conditions were 200°C and 4.5 kV for the capillary temperature and voltage, respectively. The nitrogen pressure as the nebulizer gas and its flow rate as the drying gas, was 3.0 bar and 6.0 L/min, respectively. The mass scan was performed between 50 and 950 m/z in positive mode. The acquisition and processing of data were done using the software Bruker Compass Data Analysis ver. 4.0 (Bruker Daltonics, Billerica, MA, USA). Peak identification was carried out by means of the UV spectra and mass spectra with identification of the $[M+H]^+$ ions of the individual compounds, as well as their fragmentation.

Statistical analysis

Results are reported as average and SD. The number of replicates is stated for each analysis. The comparison of the results was carried out by means of an analysis of variance, ANOVA, with the level of significance at α=0.05. The Tukey test was used "a posteriori" [Sokal & Rohlf, 2000].

Statistical analysis was performed using the Prism 5 utility (Statistical Software for Windows GraphPad, La Jolla, USA).

RESULTS AND DISCUSSION

Efficiency of blanching treatment

It is important to note that the results obtained in relation to the activity of POX and PPO (Figure 1) showed that the blanching method used was effective in inhibiting the activity of both enzymes. Therefore, the results reported below are not affected by them.

Chemical composition and physicochemical characteristics of the by-products

The powder was obtained with an average size <105 μm and a yield of 12.3 g/100 g tissue. The low moisture content (4.9 g/100 g powder) and a water activity of 0.28 guarantees the stability against deterioration during storage [Muggeridge & Clay, 2001].

Lightness (L^*) of the powder was low (23.1±0.1) and a^* (33.3±0.1) and b^* (3.9± 0.3) color coordinates were both above zero, with a^* being higher than b^*, which is coincident with the red color visually observed.

Non-cellulosic carbohydrates (hemicelluloses and pectins) were the main powder components (30.9 g/100 g) and, of them, 9.8 g/100 g were constituted by uronic acids. The rest of the components included proteins (8.5 g/100 g), lignin (1.7 g/100 g), and cellulose (7.0 g/100 g) (Table 1). It is worth noticing that, due to the technique used to obtain beetroot powders, water-soluble pectin and other residual water-soluble components belonging to the cytoplasmic medium (*i.e.*

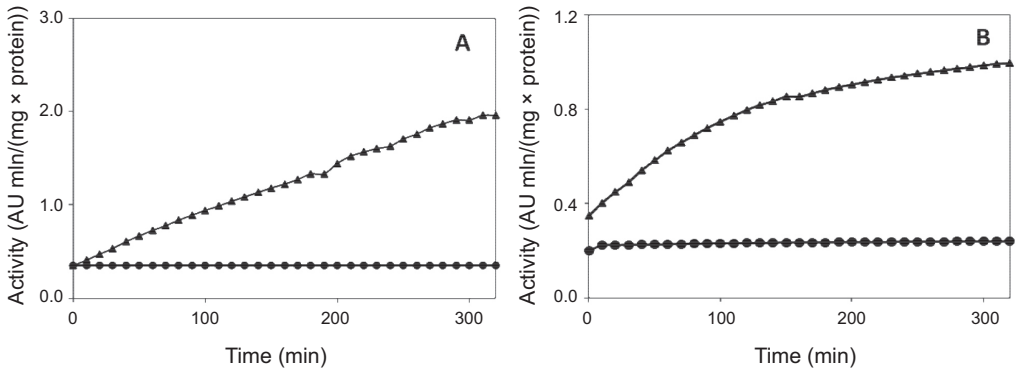

FIGURE 1. Efficiency of blanching procedure for enzymes inactivation.
(A) Peroxidase activity. (B) Polyphenol oxidase activity. AU: Absorbance unit. ▲ Non-blanched tissues. ● Blanched tissues.

TABLE 1. Chemical composition of the red beetroot powder prior to storage at different temperatures.

Component	Content
Moisture (g/100 g)	4.9±0.1
Cellulose (g/100 g)	7.0±0.1
Non-cellulosic carbohydrates (g/100 g)	30.9±0.8
Uronic acids (g/100 g)	9.8±0.8
Proteins (g/100 g)	8.5±0.4
Lignin (g/100 g)	1.7±0.1
Betacyanins (mg betanin/100 g)	0.47±0.01
Betaxanthins (mg vulgaxanthin/100 g)	0.26±0.01

Results are presented as mean ± SD (n=2). Values are reported per 100 g of powder.

globular proteins, amino acids, mono and di-saccharides) might have been lost during the procedure applied [Vincken *et al.*, 2003].

It can be observed in Table 1 that the powders had a higher content of betacyanins than betaxanthins (0.47 mg betanin/100 g *vs.* 0.26 mg vulgaxanthin/100 g), which is coincident with the trend observed for color parameters a^* and b^*. Betalains are found in the vacuoles of plant cells belonging to the order of Caryophyllales [Polturak & Aharoni, 2018]. According to Wiczkowski *et al.* [2018] and Sepúlveda-Jiménez *et al.* [2004], red beetroot accumulates mainly betacyanins in the form of betanin which is found at high concentration in the root (0.5 g/kg of betanin).

In the case of blanching water (pH 6.3), the L^* showed values of 25±1 and the a^* (50±1) and b^* (37±3) parameters were positive, with a^* being higher than b^*. The concentration was 5.57±0.56 mg betanin/L for betacyanins and 5.36±0.38 mg vulgaxanthin /L for betaxanthins.

Thermal stability of betalains in beetroot by-products

UV/Visible spectra

Figure 2 (A, B and C) shows the UV/Visible spectra, in a wavelength range between 300 nm and 700 nm, for beetroot powder extracts stored at 5°C, 25°C, and 45°C. The wavelengths where the peaks of maximum absorption occurred were 470 nm and 530 nm, corresponding to the absorption of betaxanthins and betacyanins, respectively. Similar spectrophotometric behavior was reported by other authors [Cejudo *et al.*, 2014; Cejudo-Bastante *et al.*, 2014; Stintzing & Carle, 2004]. All the extracts showed that the peak at 530 nm had a higher absorbance than the peak at 470 nm. The temperature exerted a null effect when the systems were stored at temperatures of 5°C and 25°C. Nevertheless, the absorbance values for the maximum wavelengths decreased throughout storage at 45°C, revealing the effect of this temperature on betalain stability.

In the case of blanching waters stored at the three temperatures, it can be observed (Figure 2D, E and F) that the peak at 470 nm showed greater absorbance than the peak at 530 nm. During storage at 5°C, it was observed that the peak at 470 nm decreased with storage time. At storage temperatures of 25°C and 45°C, it was observed a strong decay of both peaks with time and the decay increased with temperature. Khan & Giridhar [2014] stated that the degradation of betalains accelerated with increasing temperature and heating period.

Content of betalains

Figure 3 (A and B) shows the content of betacyanins and betaxanthins in red beetroot powders. For each storage time at different temperatures, the content of betacyanins was greater than the one of betaxanthins. The betalains were found to be much more stable at 5°C and 25°C with losses of less than 1% during storage time. Cai & Corke [2000] also studied the stability of betalains in amaranth powders, establishing that the stability of betalains increases as the dry matter increases and with a moisture content below 5%. Serris & Biliaderis [2001] reported that the stability of these pigments increases with lower water activity. In the present work, the red beetroot powders presented a moisture content lower than 4.9 g/100 g and a water activity of 0.28, facts that can justify the high stability of betacyanins and betaxanthins at 5°C and 25°C. It must be also considered that lignin was detected in the beetroot powder (Table 1), which can act as an antioxidant, protecting pigments from degradation [You *et al.*, 2019]. However, the content of betalains, varied during storage time at 45°C. A loss of 60% was observed for the be-

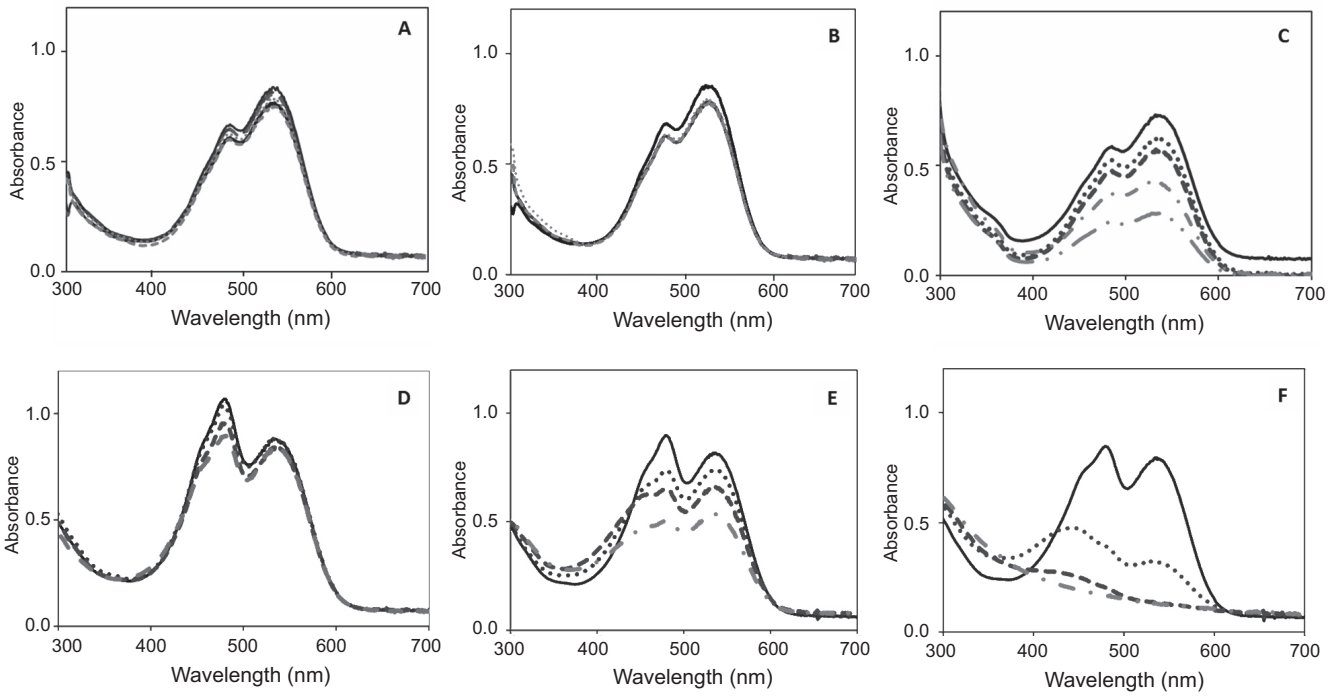

FIGURE 2. UV/Visible spectra at different storage temperatures.

Powder stored at 5°C (A), 25°C (B), 45°C (C). Blanching water stored at 5°C (D), 25°C (E), 45°C (F). Time: ——— day 0, ····· day 1, – – – day 2, ·–·–· day 4, ·–··–·· day 6.

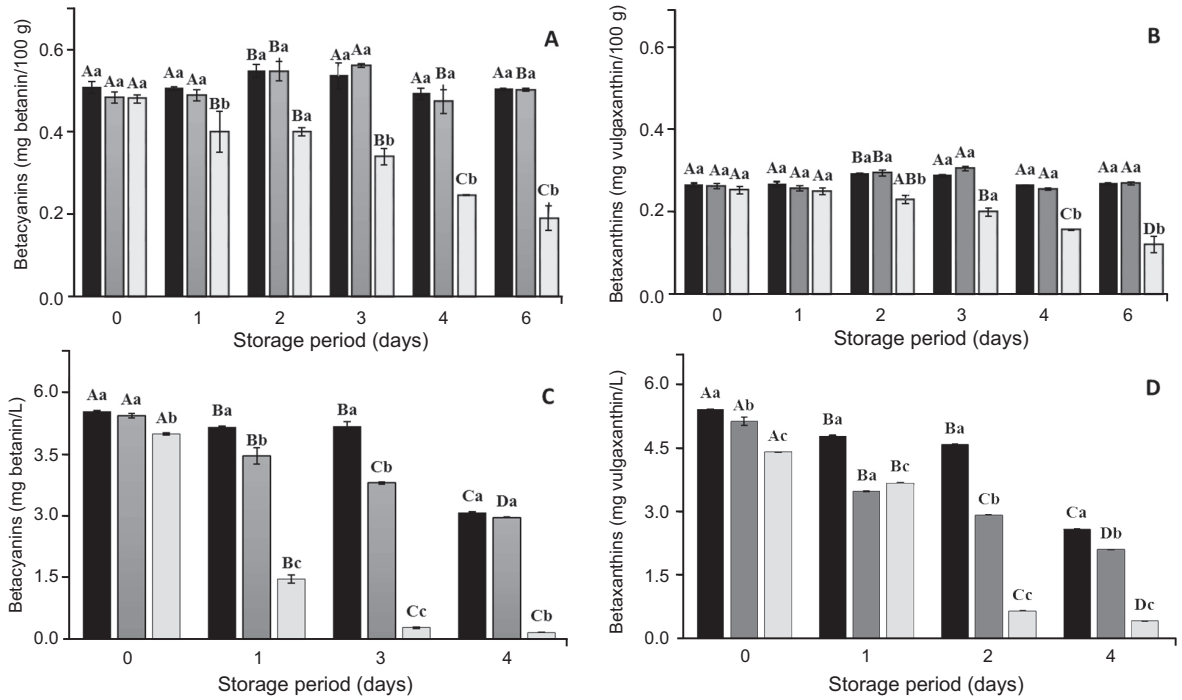

FIGURE 3. Betalain content at different storage temperatures.

Betacyanins in powder (A). Betaxanthins in powder (B). Betacyanins in blanching water (C). Betaxanthins in blanching water (D). Storage temperatures: ■ 5°C; ■ 25°C; ■ 45°C. Results are presented as mean ± SD (n=3). Different lower case letters indicate significant differences ($p<0.05$) between storage temperatures. Different capital letters indicate significant differences ($p<0.05$) between storage days.

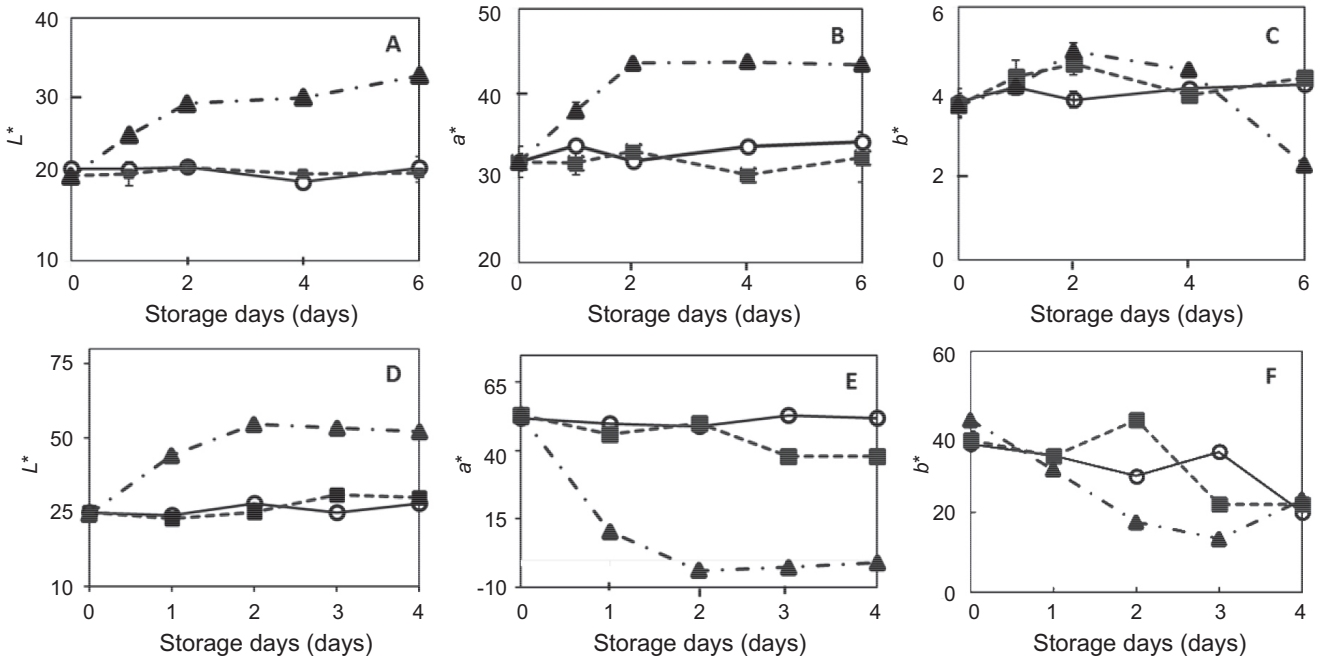

FIGURE 4. Color parameters at different storage temperatures.
Powder: L^* (A), a^* (B), b^* (C). Blanching water: L^* (D), a^* (E), b^* (F). Storage temperatures: ○ 5°C; ■ 25°C; ▲ 45°C.

tacyanins and 53% for betaxanthins, showing that these compounds were unstable at this higher temperature.

In the case of blanching water, the betalains concentration was affected by all the three temperatures studied, as can be observed in Figure 3 (C and D). The losses observed were 44% (betacyanins) and 52% (betaxanthins) at 5°C, 45% (betacyanins) and 59% (betaxanthins) at 25°C as well as 97% (betacyanins) and 90% (betaxanthins) at 45°C. Cai *et al.* [1998] reported a higher stability of pigments in amaranth powders compared to aqueous solutions, attributing this trend to the lower water activity of the powders.

Color parameters

The CIELab color parameters (L^*, a^*, b^*) are reported in Figure 4. The powders stored at 5°C, 25°C, and 45°C (Figure 4 A, B and C) showed high values for the a^* parameter, which is expressed as a strongly red color of the powders. At 5ºC and 25ºC there was practically no variation in the color parameters with storage time. During storage at 45°C, an increase for L^* and a^* was observed, in contrast with a decrease for b^* parameter. It can be concluded that the color stability of the powders was not affected during storage at 5°C and 25°C but was in fact affected when the powders were stored at 45ºC. This trend matches the ones observed for spectra and betacyanins and betaxanthins content which varied significantly during storage time at 45°C.

In the case of blanching water (Figure 4 D, E and F), only the parameter b^* showed a clear trend to a decrease at 5ºC. At 25°C, both a^* and b^* decreased and, at 45°C, L^* increased while both a^* and b^* decreased showing negative values for parameter a^* at the higher storage times which means a change from red to green color.

It can be concluded from the studies of spectra, pigment concentration and color parameters, that the changes suffered by blanching waters with storage time at 5°C, 25°C and 45°C are greater than those occurring in beetroot powders. While blanching waters showed changes at all temperatures tested, the powders only suffered changes at 45°C. This can be ascribed to their low water activity and to the presence of lignin of the cell wall material which can diminish, by means of its antioxidant activity, the pigments oxidation at 5°C and 25°C. As a consequence, among the products evaluated, the powders would have a better performance as a natural coloring additive than the blanching waters at temperatures below 45°C.

Therefore, it is interesting to clarify the changes suffered by betalains present in powders during storage at 45°C to have a more complete picture of the mechanisms involved in their decay as well as of their potential as coloring additives.

HPLC-DAD-ESI-MS/MS analysis of the betalains in beetroot powders

The betalain compounds of beetroot powders non stored (I) and stored at 45°C for 6 days (II), were evaluated by HPLC-DAD and HPLC-MS/MS, to analyze the effect of temperature on these pigments contained in a solid matrix. The results are shown in Figure 5. The compounds were identified by comparing their retention times (tr) and MS/MS spectra with bibliographic information [Herbach *et al.*, 2004; Nemzer *et al.*, 2011; Sawicki *et al.*, 2016].

From the analysis of the HPLC-DAD chromatograms registered at 470 and 530 nm wavelength, it was confirmed that these powders contained betacyanins, with a maximum absorbance in the visible spectrum at 530 nm being responsible for the intense red color, and compounds of the family of betaxanthins, with a maximum absorbance at 470 nm.

Within the family of betaxanthins (Figure 5 and Table 2, I-470 nm), vulgaxanthin I (glutamine bx) corresponding to

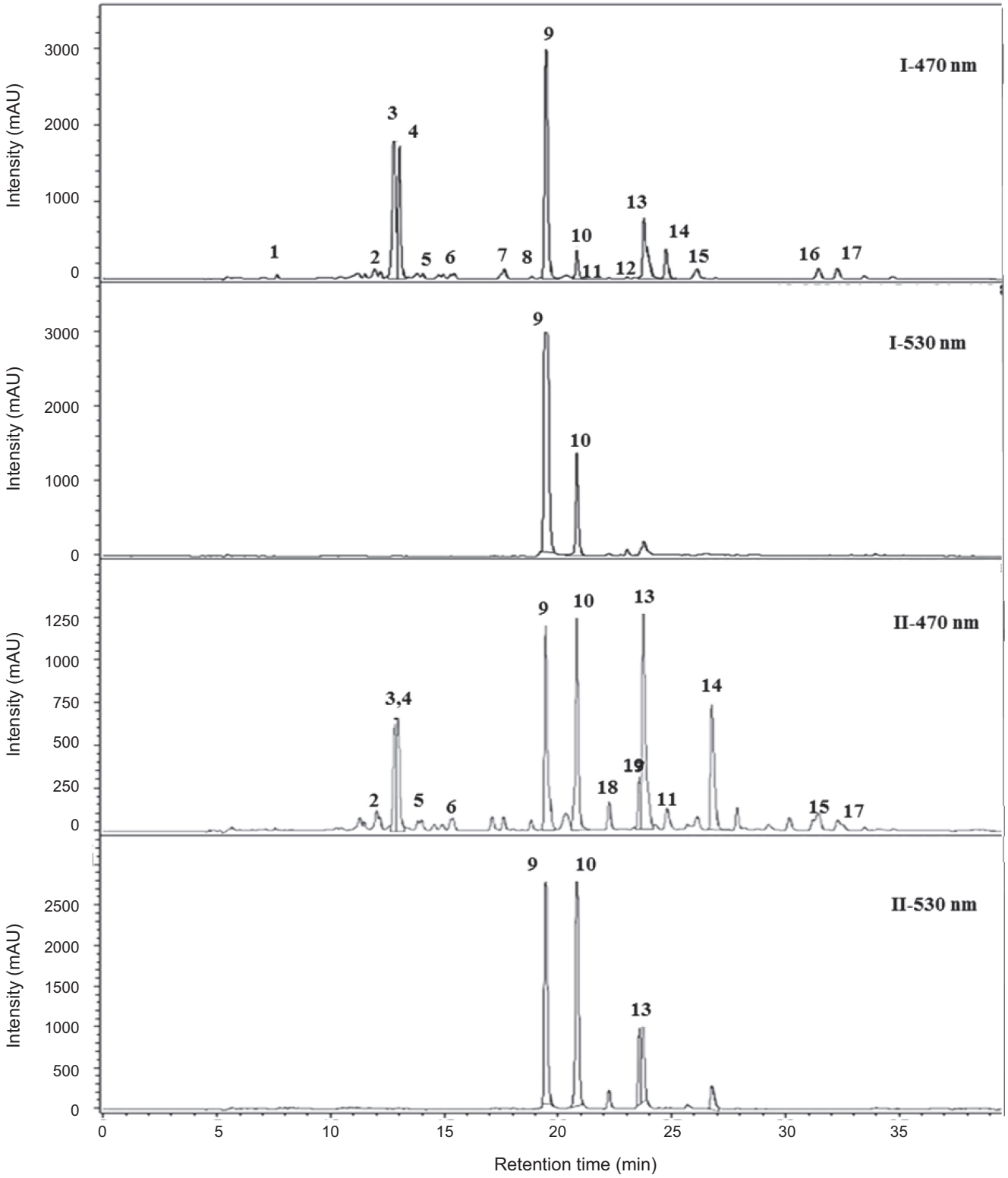

FIGURE 5. HPLC chromatograms of the betalain compounds present in powders.
Non-heat-treated (I) and 45°C heat-treated powders (II). The detection was performed at 470 nm and 530 nm. mAU: one-thousandth of an absorbance unit.

peak 3 and 4 was found through the pseudomolecular ions $[M+H]^+$ at retention times of 12.8 and 13 min both with *m/z* 340.11 ($C_{14}H_{18}N_3O_7$). The MS/MS spectra showed two characteristic fragments, one at *m/z* 323.09 $[M+H-17]^+$ that could be formed by the elimination of hydroxyl group, and another at *m/z* 277.08 $[323-46]^+$ originated by decarboxylation and di-deprotonation [Sawicki *et al.*, 2016]. It can be inferred that these two compounds (peaks 3 and 4) that present the same λ_{max}, and MS/MS spectra, are the two possible diasteroisomers of vulgaxanthin I due to the different configurations that C11 can adopt. According to the literature [Kujala *et al.*, 2002; Mégard, 1993; Singer & von Elbe,

TABLE 2. Analysis of the betalamic compounds by HPLC-DAD and HPLC-ESI- MS/MS of the non-heat-treated (I) and 45°C heat-treated powders (II).

Peak no.		Betalain name	Chemical formula	Retention time (min)		λ_{max} (nm)	*m/z* MS[M+H]$^+$	*m/z* MS/MS of [M+H]$^+$
I	**II**			**I**	**II**			
1	**-**	Asparagine bx (Vulgaxantin III)	$C_{13}H_{16}N_3O_7$	7.8	-	471	326.10	-
2	**2**	Serine bx	$C_{12}H_{15}N_2O_7$	11.9	11.8	470	299.09	-
3,4	**3**	Glutamine bx (Vulgaxanthin I)	$C_{14}H_{18}N_3O_7$	12.8/13	12.8	470	340.11	323.09 277.08
5	**5**	2,17-Bidecarboxy-2,3--dehydro-neobetanin	$C_{22}H_{23}N_2O_9$	14	14	463	459.16	330.11
6	**6**	Glutamic acid bx (Vulgaxanthin II)	$C_{14}H_{17}N_2O_8$	14.9	15	471	341.10	194.04 150.06
7	**-**	γ-Aminobutyric acid bx	$C_{13}H_{17}N_2O_6$	17.5	-	463	297.11	-
8	**-**	Proline bx (Indicaxanthin)	$C_{14}H_{17}N_2O_6$	18.7	-	477	309.11	-
9,10	**9,10**	Betanin /Isobetanin	$C_{24}H_{27}N_2O_{13}$	19.5/20.8	19.5/20.8	536	551.15	389.09
11	**11**	Dopamine bx (Miraxanthin V)	$C_{17}H_{19}N_2O_6$	21.8	24.8	456	347.12	303.13 211.07 137.06
12	**-**	Tyrosine bx (Portulacaxanthin II)	$C_{18}H_{19}N_2O_7$	22.8	-	472	375.12	211.08 178.07
13	**13**	4,15-Dehydro- betanin (Neobetanin)	$C_{24}H_{25}N_2O_{13}$	23.8	23.9	464	549.13	387.07
14	**14**	Valine bx	$C_{14}H_{18}N_2O_6$	24.6	26.8	466	311.12	267.13 221.13 193.13 150.05
15	**15**	Leucine bx (Vulgaxanthin IV)	$C_{15}H_{21}N_2O_6$	26.2	31.9	470	325.14	281.15
16	**-**	Phenylalanine bx	$C_{18}H_{19}N_2O_6$	31.2	-	470	359.12	-
17	**17**	Tryptophan bx	$C_{20}H_{20}N_3O_6$	32.1	32.2	472	398.13	-
-	**18**	17-Decarboxy-betanin	$C_{23}H_{27}N_2O_{11}$	-	20.8	515	507.16	345.11
-	**19**	2-Decarboxy-neobetanin	$C_{22}H_{25}N_2O_{11}$	-	21.9	[a]	505.15	343.09

a: λ_{max} was not determined due to co-elution with another compound or to low concentration. PI: Peak number corresponding to the chromatogram I-470/530 nm (non-heat-treated). PII: Peak number corresponding to the chromatogram II-470/530 nm (45°C heat-treated powders).

1980], the predominant yellow pigment in red beetroot juice is vulgaxanthin I. There could also be identified, serina bx, vulgaxanthin II, III and IV, isoindicaxanthin and miraxanthin V, but in a lower proportion. This family of compounds presented fragmentations that correspond to the loss of amino acids, desamination, and decarboxylation.

Betacyanins (Figure 5 and Table 2, I-530 nm) were detected at retention times of 19.5 and 20.8 min (peaks 9 and 10) and were identified as betanin and isobetanin [Sawicki *et al.*, 2016; Wybraniec, 2007], showing the pseudomolecular ion [M+H]$^+$ at *m/z* 551.15 ($C_{24}H_{27}N_2O_{13}$) and in the MS/MS spectrum, a characteristic fragment at *m/z* 389.09 [M+H – 162]$^+$ which was formed by the loss of the glucose molecule resulting in the presence of aglycones [betanidin+H]$^+$ or [isobetanidin+H]$^+$. Neobetanin was also found (I-470 nm, peak 13), with a λ_{max} of 464 nm because it has an extra double bond, which changes the resonance, generating a λ_{max} shift. In the mass spectrum, it gave an origin to the [M+H]$^+$ ion at *m/z* 549.13 and in the MS/MS spectrum to a fragment at *m/z* 387.07, also formed by the loss of the glucose molecule, characteristic rupture of these compounds [Sawicki *et al.*, 2016].

Comparing the information for the non-heat-treated (I) and the heat-treated (II) powders (Figure 5 and Table 2), it can be observed that peaks 1, 7, 8, 12, and 16 corresponding to asparagine bx, γ-aminobutyric acid bx, proline/Iso bx, tirosine bx, and phenylalanine bx, respectively, did not appear in the heat-treated samples, showing that some betaxanthins are highly sensitive to temperature. In addition, two new compounds derived from betacyanins appeared (peaks 18 and 19). Peak 18 corresponded to 17-decarboxy-betanin giving the pseudomolecular ion [M+H]$^+$ at *m/z* 507.16 and an MS/MS fragment at *m/z* 345.11 (507–162) and λ_{max} of 515 nm. Peak 19 corresponded to 2-decarboxy-neobetanin, with [M+H]$^+$ at *m/z* 505.15 and an MS/MS fragment at *m/z* 343.09 (505–162) and resulted from decarboxylation of neobetanin (549–44). 17-Decarboxy-betanin was the product of the decarboxylation of betanin (551–44). Decarboxylation is induced by the thermal effect as it was reported by Herbach *et al.* [2004]. The displacement of the maximum absorbance

of 17-decarboxy-betanin (λ_{max} of 515 nm) with respect to the one of betanin/isobetanin (λ_{max} of 536 nm) was produced by the delocalization of electrons π due to the decarboxylation of the C17 [Minale *et al.*, 1965; Stintzing *et al.*, 2004]. Previous studies on red beetroot showed that decarboxylations can occur in the C2, C15, and C17 although these positions differ in their susceptibility [Herbach *et al.*, 2004; Wybraniec, 2007; Wybraniec & Mizrahi, 2005].

Analyzing the ratio of the isobetanin/betanin and neobetanin/betanin areas for the non-heat-treated and the heat--treated powders (Figure 5), it could be observed an increase in the isobetanin/betanin ratio after the heat treatment (from 0.25 to 1) due to isomerization of betanin to isobetanin by the thermal effect [Herbach *et al.*, 2004]. On the other hand, for the neobetanin/betanin ratio, its increase was found to be non-significant (from 0.21 to 0.25).

CONCLUSIONS

The thermal stability at 5, 25, and 45° C of betalains present in by-products of the blanching and cutting of *Beta vulgaris* tissues has been studied.

Blanching waters showed pigment degradation at all the temperatures evaluated. The red beetroot powders suffered pigments thermal degradation only at 45°C. This can be ascribed to their low water activity and to the presence of lignin which can protect pigments from degradation, through its antioxidant activity, allowing to use these powders as a coloring additive up to 45°C.

Chromatographic studies showed that storage at 45ºC for six days, affected the chemical stability of betalains. Degradation reactions might impair the use of these powders as natural pigments in foods that are heat treated at temperatures higher than 45°C after pigment inclusion in the formulation.

It is expected that the results of this research will contribute to the addition of value to the *Beta vulgaris* tissues, thus contributing to its integral use as well as to the development of both sustainable processing technologies and healthy foods.

RESEARCH FUNDING

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Original article
Red Beetroot Thematic Issue

Optimisation of Beetroot Juice Encapsulation by Freeze-Drying

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Sensitivity of natural antioxidants could be improved using encapsulation technologies. In this study, encapsulation of beetroot juice (BJ) with soy proteins has been optimized in terms of the wall:core ratio, BJ dilution and mixing time of both components, maximizing DPPH radical scavenging activity (SA) and the encapsulation efficiency (EE) based on the total phenolics content. Multi response optimization has indicated that the optimal encapsulation is accomplished when soy protein is mixed with undiluted BJ at a wall:core ratio of 50 g/L for 9.8 min. Applying these conditions, the optimal encapsulate has been produced, where the contents of total phenolics, flavonoids and betalains were 150.71 mg GAE/100 g, 9.31 mg RE/100 g, and 521.28 mg/100 g, respectively, while EE and SA were in accordance with the values obtained by optimization, *i.e.* 92.48% and 1.01 mmol TE/100 g, respectively, confirming the validity of the optimization process. The resulting encapsulates have favorable physicochemical and functional characteristics and can be potentially applied as natural color additives.

LIST OF ABBREVIATIONS

a_w – water activity; BE – betanin equivalents; BJ – beetroot juice; CPC – core phenolic compounds; DW – dry weight; EE – encapsulation efficiency; GAE – gallic acid equivalents; OE – optimal encapsulate; RE – rutin equivalents; RP – reducing power; RSM – response surface methodology; SA – DPPH radical scavenging activity; SPC – surface phenolic compounds; SPI – soy protein isolate; TE – Trolox equivalents; VE – vulgaxanthin-I equivalents.

INTRODUCTION

Human body produces reactive oxygen and nitrogen species which, when imbalanced with antioxidants as protective mechanisms, can contribute to cellular aging and endanger health [Poljsak *et al.,* 2013; Valko *et al.,* 2007]. Overproduction of these reactive species results in oxidative stress, which is known to cause damage to biological molecules such as lipids, proteins and nucleic acids [McCord, 2000]. Antioxidants in cells scavenge excessively produced damaging species.

Fruit and vegetables are a rich source of antioxidant compounds such as ascorbic acid, carotenoids, flavonoids, and other phenolics [Rouanet *et al.,* 2010]. Research has shown that many chronic diseases are diet-induced [López-Varela *et al.,* 2002]. It has been suggested that a diet rich in fruit and vegetables might strengthen the antioxidant defense network endogenously, and contribute to protection from oxidative damage.

Beetroot (*Beta vulgaris* L.) is a vegetable cultivated for its roots rich in betalains, water-soluble nitrogenous pigments which include betacyanins colored from purple to violet and betaxanthins with colors from yellow to orange formed in the root [Chevallier, 1996] Betalain profile in a plant depends on its maturity degree, variety, and climatic conditions [Ampleatil *et al.,* 2015]. Betalains are commonly used as natural food dye (E162), but also have potential health benefits due to their antioxidant and anti-inflammatory activities [Georgiev *et al.,* 2010; Zielinska-Przyjemska *et al.,* 2009]. Beetroot contains also phenolic acids such as *p*-coumaric, protocatechuic, ferulic, vanillic, *p*-hydroxybenzoic, and syringic [Kujala *et al.,* 2000].

The sensitivity of natural compounds, such as betalains and phenolics, to environmental or technological process conditions (temperature, pH, oxygen content, water activity, light, radiation, presence of metal ions and redox enzymes) could be improved by microencapsulation [Krajka-Kuzniak *et al.,* 2012; Ravichandran *et al.,* 2013]. Pitalua *et al.* [2010] have found that betalain content, color, antioxidant activity, and redox potential of beetroot juice encapsulated in gum Arabic obtained were stable during storage for 44 days at a_w<0.521. Also, betalains from *Lampranthus productus* have been stable during six months of storage when spray dried with maltodextrin or chitosan [Gandía-Herrero *et al.,* 2013]. This technology presents packing target molecules in capsules which release the content at controlled rates and conditions, protecting them from deterioration [Shahidi & Han,

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1993]. One of the most widely used techniques of microencapsulation is freeze-drying which is based on the phenomenon of sublimation. Its main advantage is that the most of the favorable properties of raw material, *e.g.* shape, dimensions, appearance, taste, color, flavor, texture, and bioactivity, remain preserved [Ceballos *et al.,* 2012]. Saikia *et al.* [2015] found a higher efficiency of the encapsulation process and retention of phenolics in the freeze-dried samples compared to the spray-dried ones, and explained that by the large surfaces area exposed to air and high temperatures used in the spray drying technique.

The materials used as the wall of an encapsulate in food usually include protein isolates, gum Arabic, pectin, skim milk powder, non-fat dry milk solids, soy, modified starch, maltodextrin and sugars, which are food-grade and biodegradable [de Vos *et al.,* 2010; Nedovic *et al.,* 2011]. A soy protein isolate (SPI) is a well-known food carrier, due to its high purity, low cost, availability, high nutritional value, and functional properties such as solubility; the ability to absorb water and oil, the ability to stabilize emulsions; the ability to form gels, foams and films; as well as its fine organoleptic properties [Franzen & Kinsella, 1976; Maltais *et al.,* 2009; 2010].

This study was designed to investigate the optimal conditions for beetroot juice (BJ) encapsulation using soybean proteins, using the freeze-drying method. The optimal conditions for obtaining the encapsulates with the highest encapsulation efficiency (based on the total phenolic compound content) and the highest antioxidant activity against the stable DPPH radicals were defined with the response surface methodology (RSM). The optimal encapsulate (OE) was characterized regarding its physicochemical characteristics; total phenolics, flavonoid and betalain contents; antioxidant activity against DPPH radicals; and reducing power.

MATERIAL AND METHODS

Reagents, chemicals and instruments

The Folin-Ciocalteau reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and trichloroacetic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA); ferric chloride was obtained from J.T. Baker (Deventer, Holland); and sodium nitrite from LACH-NER (Brno, Czech Republic). Other chemicals and solvents used were of the highest analytical grade. Distilled water was produced using the DESA 0081 Water Still destilator water purification system (POBEL, Madrid, Spain). Soy protein isolate was purchased from "Macrobiotic Prom" company (Belgrade, Serbia). Absorbances in spectrophotometrical assays were measured using a Multiskan GO microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) and a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan).

Beetroot as plant material

Beetroot (*Beta vulgaris* L.) was obtained at a local supermarket. Roots of the beet were washed, cut into pieces, and blended in a laboratory blender (model Neo SK-400, TCL King Electrical Appliances Co., Ltd., Huizhou, China). Beetroot juice (BJ) was separated from pomace by vacuum filtration and stored at -20°C until analysis.

Encapsulation procedure

The BJ (initial pH 5.45) was encapsulated *via* freeze-drying. BJ (core) was mixed with a soy protein isolate (wall) according to the experimental design (Table 1). The samples were frozen at -40°C for 2 h in a Martin Crist Alpha 2–4 (Osterode, Germany) freeze-drier. The main drying process was performed at pressure of 0.01 mbar and temperatures from -40 to 20°C for 59.5 h. The final drying lasted 5.5 h at pressure of 0.005 mbar and temperatures from 20 to 30°C. The collected freeze-dried samples were stored at –20°C until further use. Freeze-drying experiments were executed in a laboratory freeze-drier (model Alpha 2–4 LSC, Martin Christ, Osterode, Germany), under the conditions described above.

Optimization of encapsulation

Parameters of encapsulation were optimized using the response surface methodology (RSM). The adopted experimental model was a Box-Behnken design for three variables at three levels. Three independent variables (parameters of encapsulation) were: the ratio of wall:core (X_1), dilution of BJ (X_2), and mixing time (X_3). The coded values of the independent variables were –1, 0, and 1. The actual values, selected from the preliminary study, based on literature survey [Cheng *et al.,* 2012; Ezhilarasi *et al.,* 2013; Ramírez *et al.,* 2015; Roopchand *et al.,* 2013; Saikia *et al.,* 2015; Tumbas Šaponjac *et al.,* 2016] by the corresponding coded values of three independent variables are given in Table 1. The complete model consisted of 15 experiments with three replicates.

For process optimization, encapsulation efficiencies (EE) and DPPH radical scavenging activity (SA) of encapsulates were chosen as the responses. Single response as well as multi response optimization were performed to enable the selection of optimal parameters for the production of the optimal encapsulate (OE), which will be further examined.

Spectrophotometric determination of the total phenolics content of beetroot juice and its encapsulates

The content of total phenolics in BJ, in the core of encapsulates (CPC), and in the surface of encapsulates (SPC) was determined by the method with the Folin-Ciocalteu reagent [González-Molina *et al.,* 2008]. Briefly, the reaction mixture was prepared in a 96-well microplate by mixing 170 μL of distilled water, 15 μL of BJ/encapsulate extract, 12 μL of the Folin-Ciocalteu's reagent, and 30 μL of 20% sodium carbonate. The mixture was incubated in the dark at room temperature for 1 h. After incubation, the absorbance was read at 750 nm. The corrections for interfering substances originating from beetroot have been made by simultaneous preparation of control samples with a matching concentration of BJ/encapsulate extract in the same way. Results were expressed as gallic acid equivalents (GAE) per 100 mL of BJ or per 100 g of encapsulate.

To determine the encapsulation efficiency, the total contents of phenolic compounds in the core (CPC) and surface (SPC) were evaluated using the encapsulate extraction procedure described by Vergara *et al.* [2014]. For CPC, 100 mg

of the sample were suspended in 1 mL of an ethanol:acetic acid:water mixture (50:8:42, *v/v/v*), vortexed for 1 min, centrifuged for 2 min at 14,000×*g* (model Rotilabo-mini-centrifuge, Carl Roth, Karlsruhe, Germany), and then the supernatant was separated. For SPC, 100 mg of the sample were suspended in 1 mL of an ethanol:methanol mixture (1:1, *v/v*). The mixture was vortexed for 1 min, centrifuged at 3018.6×*g* for 2 min, and then supernatant was separated. CPC and SPC were determined with the Folin-Ciocalteu method [González--Molina *et al.,* 2008]. The encapsulation efficiency (EE) was determined by using the equation:

$$EE\ (\%) = ((CPC\text{-}SPC)/CPC) \times 100 \quad \text{(Eq. 1)}$$

Spectrophotometric determination of the total flavonoid contents in beetroot juice and its optimal encapsulate

The total content of flavonoids in BJ and OE was determined spectrophotometrically with a modified method described by Markham [1989]. The reaction mixture was prepared in a 96-well microplate by mixing 125 μL of BJ/OE extract obtained using ethanol:acetic acid:water (50:8:42, v/v/v), 25 μL of distilled water, and 62.50 μL of $AlCl_3$. Blank sample was prepared in the same manner replacing $AlCl_3$ with water in the reaction mixture. The absorbance was measured immediately at 430 nm. Absorbance of the experimental samples was corrected for the absorbance of control samples with the appropriate concentration of BJ or extract encapsulated without the reagent. The total content of flavonoids was expressed as rutin equivalents (RE) per 100 mL of BJ or per 100 g of OE.

Determination of betalain content in beetroot juice and its optimal encapsulate

Contents of betalain pigments (betacyanins and betaxanthin) in OE and BJ were determined as described by von Elbe [2003] in a 96-well microplate by mixing 240 μL or 250 μL of a phosphate buffer (0.05 M, pH 6.5) and BJ/ethanol:aceticacid:water (50:8:42, v/v/v) OE. Phosphate buffer was used as a blank. Wavelengths of 545 nm and 476 nm were used for the analysis of betacyanins and betaxanthin, while the wavelength of 600 nm was used for correction. Total betalains content was calculated as the sum of betacyanin and betaxanthin contents. Content of betacyanins was expressed as mg betanin equivalents (BE) per 100 mL of juice or per 100 g of encapsulate, while the content of betaxanthins was expressed as mg vulgaxanthin-I equivalents (VE) per 100 mL of BJ or per 100 g of OE.

Determination of radical scavenging activity against DPPH radical

The ability of BJ and OE to scavenge DPPH radicals was determined using the spectrophotometric method which is based on monitoring changes in solution color from purple-colored stable nitrogen DPPH radicals to yellow-colored reduced non-radical form DPPH-H [Brand-Williams *et al.,* 1995]. Samples of the encapsulate were prepared in the same way as for determination of CPC. Briefly, 250 μL of a DPPH solution in methanol was mixed with 10 μL of the sample (BJ or encapsulate extract) in the microplate well and left in dark at room temperature for 50 min. Afterwards, absorbance was read at 515 nm. The samples containing corresponding concentrations of BJ and encapsulate extracts without DPPH radicals were analyzed in parallel. Their absorbance was used to correct the absorbance of experimental samples. The following equation was used to calculate the DPPH radical scavenging activity:

$$SA\ (\%) = (A_C - A_S) / A_C \times 100 \quad \text{(Eq. 2)}$$

where: A_C is the absorbance of the control (without antioxidant) and A_S is the absorbance in the presence of the sample (BJ or encapsulate extract) after correction. The calibration curve was made with Trolox and results were expressed as mmol of Trolox equivalents (TE) per 100 mL of BJ or per 100 g of encapsulate.

Determination of the reducing power of beetroot juice and its optimal encapsulate

The method of Oyaizu [1986] was used to determine the reducing power (RP) of BJ and OE. OE was prepared in the same way as for determination of CPC: 25 μL of the sample (BJ/OE extract), or 25 μL water as a blank, 25 μL of sodium phosphate buffer (pH 6.6), and 25 μL of 1% potassium ferricyanide, were mixed and incubated in a water bath for 20 min at 50°C. When the solution was cooled, 25 μL of 10% trichloroacetic acid was added and solutions were centrifuged at 3018.6×*g* for 10 min and afterwards 50 μL of the supernatant with 50 μL of distilled water and 10 μL of 0.1% ferric chloride were mixed in a microplate well. Absorbance was measured immediately at 700 nm. The control samples with matching concentration of BJ and encapsulate extracts were prepared in the same way. The calibration curve for this test was made with Trolox and results were expressed as mmol of Trolox equivalents (TE) per 100 mL of BJ or per 100 g of OE.

Water activity

Water activity (a_w) was determined by placing approximately 3 g of OE in a sample holder of a LabSwift a_w-meter "Novasina" (Lachen, Switzerland) at 25°C. The a_w values were recorded after equilibration.

Moisture content

The moisture content of OE was determined according to the procedure described by Şahin Nadeem *et al.* [2011] by drying in an oven at 70°C until constant weight.

Particle size distribution

Particle size distribution of OE was determined using the Mastersizer 2000 laser diffraction particle size analyzer (Malvern Instruments, Malvern, England). The Scirocco dispersion unit was used for dispersing encapsulate in the air. The sample was added at ambient temperature until an adequate obscuration was obtained (5–10%). The results were quantified as the volume-based particle size distribution by means of the Mastersizer 2000 software.

Statistical analysis

All experiments were performed in triplicate and the results are presented as means ± standard deviation (±SD, n= 3). Data were analyzed by one-way analysis of variance (ANOVA) and *t*-test, where applicable, and the least significant difference (LSD) test ($p<0.05$). Statistical analysis was performed using Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA). Optimization of experiments was conducted using Design-Expert® Version 7.0.0 (Stat-Ease, Inc., Minneapolis, MN, USA, 2005).

RESULTS AND DISCUSSION

Beetroot encapsulation

Optimization of the BJ encapsulation parameters (wall:core ratio, BJ dilution and mixing time), in terms of encapsulation efficiency (EE) and DPPH radical scavenging activity (SA), was conducted using RSM with the Box-Behnken design (Table 1). The experimental model included 15 different combinations of encapsulation parameters for the measurements of EE and SA as responses. The actual values were chosen from the preliminary studies. Encapsulation efficiency of BJ with soy protein based on total phenolics content ranged from 47.79% (experiment 13) to 92.47% (experiment 2), while the experimental values for SA were in the range from 0.03 mmol TE/ 100 g (experiment 12) to 1.05 mmol TE/100 g (experiment 1). Microencapsulation of phenolics highly depends on the carrier and the technique employed. Figure 1 shows the influence of independent variables (X_1, X_2, X_3) on EE and SA of encapsulates obtained in experiments 1–15. According to the response surfaces, it can be concluded that increasing the wall:core ratio caused a slight decrease in SA and EE values. Mixing time had the same effect on the responses, while increasing dilution decreased SA and increased EE of the encapsulates.

The results of single and multi-response optimization are reported in the Table 2. When using single response optimization it was found that a high wall:core ratio (149.50 g/L), low extract dilution (0.2), and medium mixing time (18.9 min) are needed to ensure the maximal encapsulation efficiency (93.44%). The single response optimization was used to optimize the response SA as well. In this case, the optimal sample, with maximum SA (1.01 mmol TE/100 g) may be obtained by using relatively low wall:core ratio (50.0 g/L), no BJ dilution (0.0) and medium mixing time (12.9 min). The conditions for producing the optimal encapsulate sample (OE) were found by using a multi response optimization, where the EE and SA values are considered at the same time, and represent the optimal conditions that provide maximum values of both responses (Table 2).

TABLE 1. Experimental design, encapsulation efficiency (EE), and DPPH radical scavenging activity (SA) of beetroot juice encapsulates.

Exp	X_1 (g/L)	X_2	X_3 (min)	EE (%)[a]	SA (mmolTE/100g)[a]
1	50 (-1)	0 (-1)	15 (0)	89.07±0.40	1.05±0.04
2	150 (+1)	0 (-1)	15 (0)	92.47±1.67	0.99±0.05
3	50 (-1)	4 (+1)	15 (0)	91.87±0.53	0.21±0.02
4	150 (+1)	4 (+1)	15 (0)	87.40±3.27	0.14±0.00
5	50 (-1)	2 (0)	5 (-1)	91.07±0.17	0.34±0.07
6	150 (+1)	2 (0)	5 (-1)	89.97±3.34	0.10±0.00
7	50 (-1)	2 (0)	25 (+1)	84.59±4.41	0.22±0.00
8	150 (+1)	2 (0)	25 (+1)	90.70±2.57	0.07±0.02
9	100 (0)	0 (-1)	5 (-1)	86.29±3.55	0.58±0.03
10	100 (0)	4 (+1)	5 (-1)	79.73±12.51	0.08±0.01
11	100 (0)	0 (-1)	25 (+1)	89.57±2.07	0.56±0.03
12	100 (0)	4 (+1)	25 (+1)	91.25±6.18	0.03±0.00
13	100 (0)	2 (0)	15 (0)	47.79±3.03	0.08±0.01
14	100 (0)	2 (0)	15 (0)	58.02±0.28	0.11±0.00
15	100 (0)	2 (0)	15 (0)	58.83±3.44	0.10±0.00

[a]Results are presented as mean values of three replications ± SD. The ratio of wall:core (X_1), dilution of beetroot juice (X_2) and mixing time (X_3); TE – Trolox equivalent.

Ramírez *et al.* [2015] optimized the encapsulation of model fruit juice in gum Arabic and/or maltodextrin using spray- or freeze-drying, following the concentration of gallic acid as a response. RSM optimization showed that the freeze-dried encapsulates achieved with a wall blend ratio close to 100% gum Arabic and core concentration from 10 to 20% had higher contents of gallic acid. Maltodextrin concentration of 15% was found to be the optimal for encapsulation of beetroot juice using spray-drying [Bazaria & Kumar, 2018]. The wall concentration in these studies was much higher than in our study (5%), although the wall material in our study was protein, not polysaccharide. Robert *et al.* [2010] optimized the encapsulation of polyphenols and anthocyanins from pomegranate using maltodextrin and soy protein isolate by spray drying. Encapsulation efficiency was significantly better upon the use

TABLE 2. Single (EE and SA) and multi response (EE+SA) optimization of encapsulation parameters using response surface methodology.

Optimization	Variable codes			Variable values			Optimal responses	
	X_1	X_2	X_3	X_1	X_2	X_3	EE (%)	SA (mmol TE/100 g)
EE	0.99	-0.90	0.39	149.50	0.20	18.9	93.44	–
SA	-1	-1	-0.21	50.00	0.0	12.9	–	1.01
EE+SA	-1	-1	-0.52	50.00	0.0	9.80	92.48	1.01

The ratio of wall:core (X_1), dilution of BJ (X_2) and mixing time (X_3);EE –encapsulation efficiency; SA – DPPH radical scavenging activity.

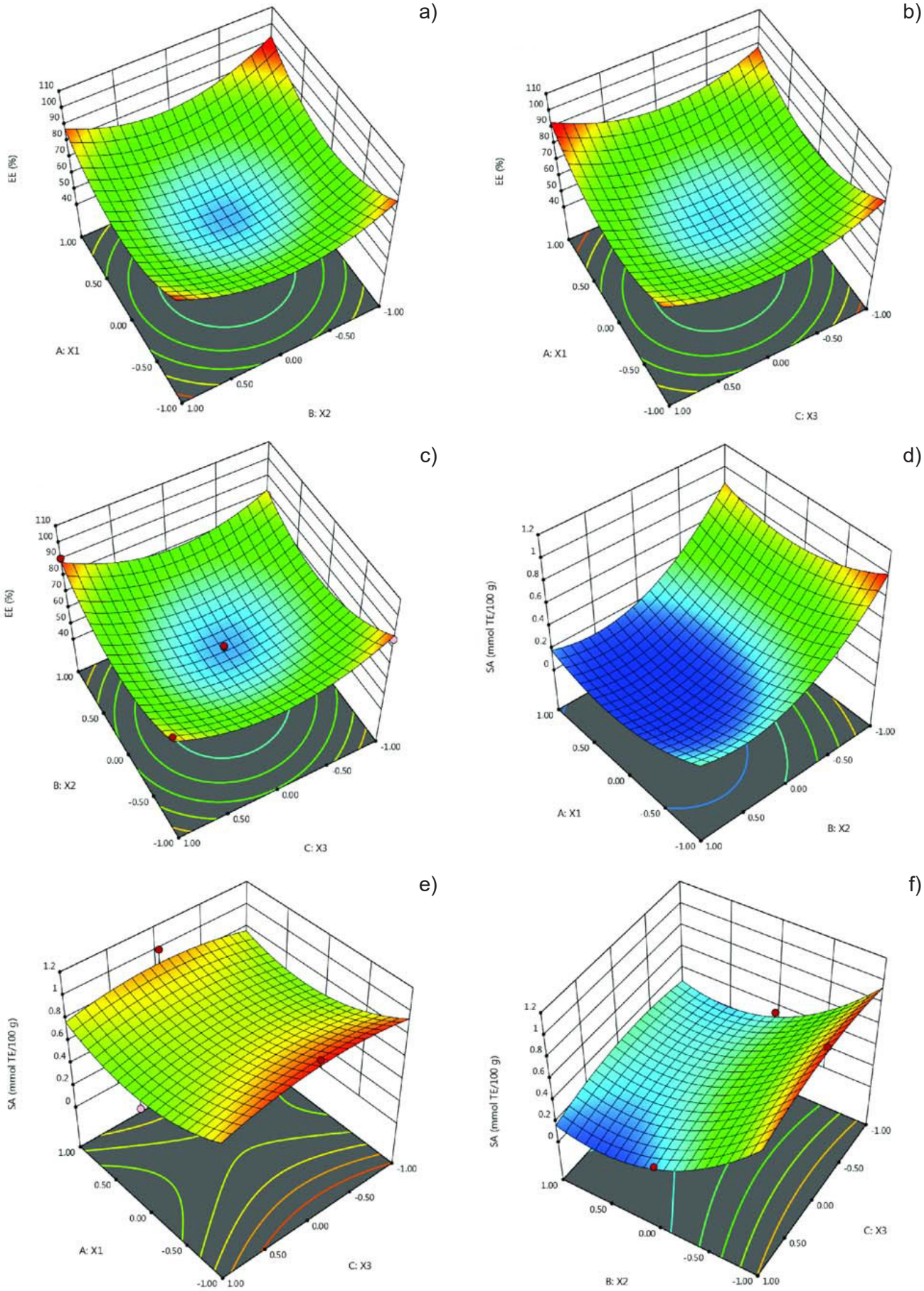

FIGURE 1. The influence of wall:core ratio (X_1), juice dilution (X_2), and mixing time (X_3) on encapsulation efficiency (EE) of beetroot juice with soy protein based on total phenolics content (a, b and c) and DPPH radical scavenging activity (SA) of encapsulates (d, e and f).

of the soy protein isolate (36.60–81.50%). However, the encapsulation efficiency using soy protein isolate in that study was lower than in our study (47.79–92.47%). Storage capacity of soy protein isolate was also better than that of maltodextrin, where polyphenol and anthocyanin retention increased during storage, while in the maltodextrin-based microcapsules the retention of these compounds diminished. Spray- and freeze-drying techniques require different temperature regimes and can lead to different physicochemical characteristics of encapsulates. Many factors such as light, temperature, and oxygen are considered to influence their quality.

Functional characteristics of beetroot and its optimal encapsulate

Janiszewska [2014] reported that the drying of beetroot juice is a method to obtain a pure and easy to use pigment in powder form. However, it is not possible to dry the beetroot juice without addition of carriers, because of its low glass transition temperature (measured Tg of juice 43± 4°C). In this study, we decided to use freeze-drying due to its favorable mild process parameters (low temperature) to avoid degradation of bioactive compounds from BJ. Before drying, BJ was characterized in terms of contents of total phenolics, total flavonoids, total betacyanins, and total betaxanthins, as well as free radical scavenging activity against DPPH$^{\bullet}$ (SA) and reducing power (RP) (Table 3).

Beetroots have a high content of betalains (betacyanins and betaxanthins) that serve as color pigments [Delgado-Vargas *et al*., 2000]. From Table 3 it can be observed that betalains were the dominant bioactive compounds in BJ with their concentration reaching 236.51 mg/100 mL, which is higher than the value of 767–1309 mg/L reported by Wruss *et al.* [2015]. The ratio between betacyanins and betaxanthins was from 1.75 to 1, which is close to the value obtained in this study (0.99). However, the total phenolics content obtained in this study was much lower compared to the report of Wruss *et al.* [2015] (0.85–1.29 g/L). Wootton-Beard & Ryan [2011] determined also a higher total content of phenolic compounds in the beetroot juice (101.50 mg GAE/70 mL) than that presented in this study. Flavonoids had a small share in total phenolics content found in the BJ. Kujala *et al.* [2002] reported the presence of betagarin, betavulgarin, cochliophilin A, and dihydroisorhamnetin, in extracts of beetroot peel. The differences in the physicochemical characteristics of beetroot could be due to the environmental characteristics, period of harvesting, cultivar variability, and maturity stage.

Free radical scavenging activity of BJ against DPPH$^{\bullet}$ was determined to be 1.93 mmol TE/100 mL and reducing power to be 107.80 mmol TE/100 mL. Wootton-Beard & Ryan [2011] analyzed 23 vegetable juices and found that beetroot juices displayed the highest ferric reducing antioxidant power (FRAP) values (8355–9500 μmol/L) and antioxidant capacity (100% DPPH$^{\bullet}$ inhibition and 92.1–92.3% ABTS$^{+\bullet}$ inhibition) compared to the other juices analyzed (tomato, carrot, mixed vegetable, mixed fruit and vegetable). Wruss *et al.* [2015] found that the oxygen radical absorbance capacity (ORAC) and FRAP of beetroot juices ranged from 19.70 to 37.90 mmol TE/L, and from 17.40 to 37.10 mmol TE/L, respectively.

TABLE 3. Physicochemical characteristics of beetroot juice (BJ) and its optimal encapsulate (OE).

Determination	BJ	OE
Total phenolics content[a]	56.65±1.00	150.71±3.07
Total flavonoids content[b]	7.33±8.52	9.31±0.64
Total belatains content	236.51±10.95	521.28±25.52
Betacyanins[c]	117.61±10.16	259.73±2.03
Betaxanthins[d]	118.90±10.27	261.55±2.05
SA[e]	1.93±0.04	1.02±0.08
RP[e]	107.80±5.12	1.81±0.04
Moisture content (%)	–	2.61±0.12
a_w	–	0.028±0.001
Particle size (μm)	–	174.57±8.51

Data represent mean value of three replicates±SD.
Results are expressed as: [a] mg GAE/100 mL BJ or 100 g OE, [b] mg RE/100 mL BJ or 100 g OE, [c] mg BE/100 mL BJ or 100 g OE, [d] mg VE/100 mL BJ or 100 g OE, [e] mmol TE/100 mL BJ or 100 g OE; GAE – gallic acid equivalent; RE – rutin equivalent; BE – betanin equivalent; VE – vulgaxanthin equivalent; TE –Trolox equivalent; SA – DPPH radical scavenging activity; RP – reducig power; a_w– water activity.

The OE was produced using the encapsulation parameters from multi response optimization (Table 2). Water is an important basic element in food. OE had a low moisture content (2.61%), which corresponds to the powdered product of good stability, effective packaging and storage [do Carmo *et al*., 2018; Sinija *et al.,* 2007]. Higher moisture content in products enables microbial growth and caking [do Carmo *et al.,* 2018]. In the study of do Carmo *et al.* [2018], beetroot extract encapsulated in whey protein isolate alone and mixtures with maltodextrin and inulin presented higher moisture values (3.81–4.24%) than the powders obtained with maltodextrin and/or inulin (3.33–3.58%) because of the greater ability of proteins to maintain moisture trapped in the particles [Jayasundera *et al*., 2009].

The water activity (a_w) measurement, often used as a critical control point for dry and dehydrated products, provides important information about the quality of a product such as the possibility of microbiological growth on the surface and sample stability and shelf-life. The control of the water activity allows preserving its structure, texture, stability, density, and the possibility of reconstitution. Water activity of the freeze-dried OE sample was found to be 0.028, which is more than acceptable to ensure microbiological, chemical, and physical stability of the powders. Jafari *et al.* [2016] reported that saffron petals extract encapsulated by freeze-drying with different combinations of maltodextrin, Arabic gum, and seed cress gum had their a_w values in the range from 0.07 to 0.29 and moisture content from 1.88 to 3.13%. Red wine encapsulated with maltodextrin and gum Arabic, employing freeze-drying as well, had a_w value of 0.11 [Rocha-Parra *et al.,* 2016]. The results of this study indicate that water activity was a key factor affecting phenolics stability during storage. Do Carmo *et al.* [2018] reported color

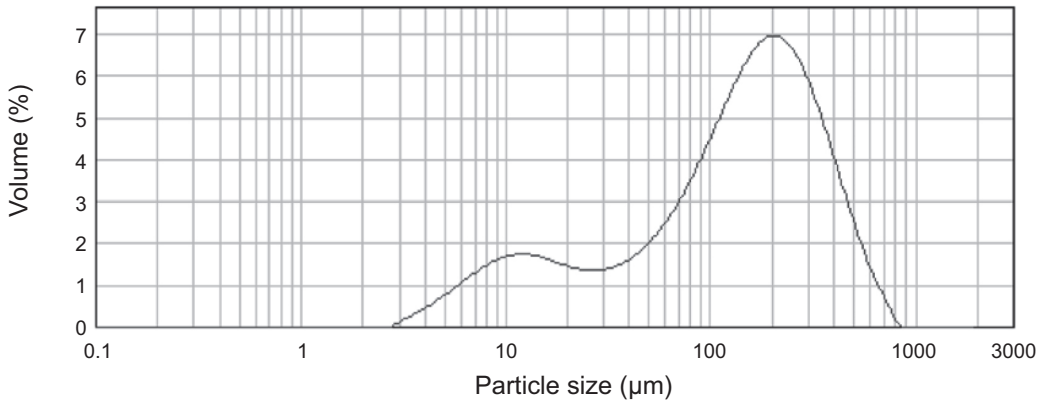

FIGURE 2. Particle size distribution of optimal encapsulate of beetroot juice.

change of the beetroot juice powders at high water activity (a_w=0.843), resulting from high moisture adsorption which may have facilitated betalains hydrolysis to yellow colored betalamic acid [Herbach *et al.,* 2006]. Pitalua *et al.* [2010] also concluded that the stability of betalains in microcapsules and scavenging activity depend on the a_w value. They found that when a_w values were in the range of 0.11–0.52 there were no significant differences in betalains content, color, scavenging activity and reducing power, during 45 days of storage. However, during storage of encapsulates with a_w values of 0.75 and 0.90, the concentration of betalains decreased, while scavenging activity increased significantly. It is speculated that the degradation of betalains occurs through hydrolytic reaction, due to the increase of moisture in the dry product, which leads to the diffusion of oxygen within encapsulated material, causing oxidation.

The mean particle size of the OE powder was determined to be 174.57 μm (Table 3, Figure 2), which is not in the range of fine powders (<5 μm), as reported by Medina-Torres *et al.* [2013] From the particle size distribution of OE presented in Figure 2 it is evident that there are two distinct peaks representing predominant sizes, the largest volume of the OE being of the particles with diameters ranging from 100 to 300 μm. Kuck & Noreňa [2016] obtained similar sizes (104.30–684.90 μm) of particles of a grape skin phenolic extract encapsulated by freeze-drying. Ezhilarasi *et al.* [2013] reported the particle size of *Garcinia* fruit extract encapsulated with different wall materials, employing freeze-drying, in the range of 15–100 μm. Janiszewska [2014] determined that the average diameter of spray-dried beetroot juice encapsulated in maltodextrin and gum Arabic and their mixture was in the range of 7.60–12.80 μm. Man *et al.* [1999] reported that the particle size of spray-dried powders is usually in the range of 1–15 μm, while freeze-dried powder particles can reach 300 μm. According to Chen *et al.* [2012], the larger size of the particles obtained by freeze-drying could be caused by low temperatures used in this process and lack of forces for breaking up the frozen liquid into droplets or to substantially alter their surface topology during the drying process. Ramírez *et al.* [2015] reported that in freeze-drying the product's structure is mainly developed during the previous freezing step, where fast freezing rates generate small ice crystals and slow cooling rate generates large crystals. Kuck & Noreňa [2016] highlighted that due to the larger particle size freeze-dried powders have lower hygroscopicity compared to the spray-dried powders, because larger particles have smaller exposed surface area and lower water absorption rate [Tonon *et al.,* 2010].

The content of bioactive compounds (total phenolic compounds, total flavonoids, and total betalains), and biological activity (reducing power and DPPH radical scavenging activity) were determined to characterize the OE (Table 3). According to the results obtained, flavonoids had a small contribution (6.18%) in total phenolics content of the encapsulate as determined by the Folin-Ciocalteu assay. The content of betaxanthins (261.55 mg VE/100 g) did not differ significantly ($p>0.05$) from the content of betacyanins (259.73 mg BE/100 g). It is evident that the content of betalains in BJ is high, which indicates a good potential of the carrier to trap betalains. Encapsulation efficiency of OE, based on the total phenolics contet, was 75.91%, which is lower than the result obtained in the multi response optimization (Table 2). On the other hand, predicted SA values (Table 2) were in accordance with the values obtained for OE as well, without any significant difference ($p>0.05$).

Bazaria & Kumar [2018] optimized the spray-drying of beetroot juice with whey protein concentrate. Total phenolics content of the obtained powders was in the range of 16.69–25.89 mg GAE/100 g, which is significantly lower than the value obtained in our study. It was suggested that lower inlet temperatures as well as higher addition of whey protein (30%) could contribute to the higher preservation of phenolics during spray-drying [Bhusari *et al.,* 2014]. Janiszewska [2014] investigated the most effective carrier that would preserve the stability of beetroot pigments. They have examined microencapsulation process using the spray--drying method and gum Arabic, maltodextrin and mixtures thereof as wall materials. The results showed that the highest yield of purple but the lowest yield of yellow pigments was obtained using gum Arabic as a carrier (42.60 mg/100 g DW and 35.10 mg/100 g DW, respectively). The content of betacyanins (109–129 mg/100 g) and betaxanthins (34–61 mg/100 g) in this study was much lower than in our study (259.73 and 261.55 mg/100 g). The results reported by do Carmo *et al.* [2018] demonstrate slightly lower contents for betaxanthins and betacyanins (136.86–155.37 and

211.93–230.10 mg/100 g) in spray-dried beetroot extract powders as well. This could be attributed to the high temperatures used in spray-drying, different carriers and wall:core ratios used in the experiments as well as the differences in beetroot juice composition. Otálora *et al.* [2015] improved betalain content (49.7 mg/100 g) as well as the a_w value (0.18) and moisture content (2.90%) in spray-dried cactus fruit juice by adding cactus cladode mucilage to maltodextrin as encapsulating agents. Pitalua *et al.* [2010] encapsulated beetroot juice using gum Arabic as a carrier and spray-drying as a method. Total betalain content in these encapsulates was even lower (11.98 mg/100 g), which is due to a much lower wall:core ratio (1:3) than we used in our study (50 g/L, or 1:20), apart from above mentioned differences in experimental conditions. Ahmed *et al.* [2010] have reported that a higher core:wall ratio increases the content of biologically-active components in the encapsulated system.

It is generally known that the DPPH radical scavenging activity of food is closely related to the content of bioactive compounds, such as phenolics and betalains, which are present in OE. Spray-dried beetroot extract powders obtained with whey proteins and their mixtures with inulin or maltodextrin exhibited higher antioxidant activities than the inulin- and/or maltodextrin-based powders [do Carmo *et al.,* 2018]. Authors suggest that in the case of protein-based encapsulates, antioxidant activity could be attributed not only to the bioactive compounds from beetroot extract but also to Maillard reactions occurring between sugars from beetroot extract and whey proteins.

CONCLUSION

Beetroot juice is a potential source of bioactive compounds and thus it can be used in the development of functional food. RSM was used to find the optimal conditions for producing the encapsulates of beetroot juice with soy proteins using the freeze-drying process with the highest content of phenolic compounds and the highest antioxidant activity. The optimization process was confirmed by applying the optimal conditions resulting in the sample with bioactive characteristics similar ($p>0.05$) to the predicted one. Based on these results, the optimized conditions obtained could be used for the encapsulation of bioactive compounds from beetroot juice with soybean proteins. The resulting encapsulates, with favorable physicochemical characteristics, have a potential to be used in the food industry as food colorants and as components of functional foods.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Original article
Red Beetroot Thematic Issue

Bioactive Compounds and Microbial Quality of Stored Fermented Red Beetroots and Red Beetroot Juice

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Key words: beetroot fermentation, betalains, bioactive compounds, LC-MS

The aim of this study was to investigate the effect of long-term cold storage of fermented beetroots and fermented beetroot juice on the content of biologically active compounds and microorganisms. Contents of total phenolics, as well as red and yellow betalains were determined. Total phenolics content in fermented grated beetroot was 920 mg/kg after 7 months of cold storage, while in juice it was 810 mg/L within the same timespan. At the end of the experiment, after 10 months of storage, these values decreased to 570 mg/kg and 540 mg/L, respectively. Concentration of red betalains after 7 months of storage was determined at 116 mg/kg for grated beetroot and at 69 mg/L for juice. At the same time, the content of yellow betalains was 14 mg/kg and 19 mg/L for grated beetroot and juice, respectively. In the case of fermented beets and juice, about 3-fold decrease of red pigments was observed during storage. Bioactive compounds were identified using LC-MS. Betanidin was shown to be the major compound among grated beetroot pigments at the beginning of the investigation, the beetroot juice was predominated by isobetanidin and betanidin. At the end of the study, the proportion of pigments changed slightly. Lactic acid bacteria predominated among the bacterial microbiota in the products tested. *Enterobacteriaceae* were not detected in fermented grated beetroots and investigated juices throughout storage time. To conclude, during long-term cold storage, the content of bioactive compounds decreases, however, remains at a high level.

INTRODUCTION

The preservation of plant products by fermentation was known in ancient times, mainly in the Far East. This method was widely used in countries engaged in seasonal plant cultivation. It also made it possible to secure the food in the event of natural disasters. Fermentation is one of the oldest technologies used until today, which prolongs the shelf life of food products and increases their nutritional and organoleptic values [Kavitake *et al*., 2018]. The tradition of natural food preservation is related to the geographical area, climate, and the type of ingredients used. Fermented food is widely used in East Asia; fermenting vegetables and fruits is very important in the northern European countries, while the production of fermented olives and fermented sausages dominates in the southern Europe. Fermentation of cucumbers, cabbage, and olives plays also the most important economic role. Fermented: red beetroots, cauliflower, carrot, celery, onion, pepper, and tomatoes are less known.

The fermentation process affords the opportunity to obtain a large range of new products that will differ in their organoleptic characteristics from the primary raw material. The process of fermentation and the beneficial effect of fermented products on our organism [Swain *et al.*, 2014] causes their popularity and increases a group of supporters. Lactic acid from kimchi may prevent fat accumulation and obesity-induced heart diseases [Park *et al.*, 2008]. Kimchi has been reported to have anti-obesity effects [Jung *et al.*, 2014; Kim *et al.*, 2011; Park *et al.,* 2012]. Some studies have documented the protective effects of sauerkraut or sauerkraut juice against breast [Ju *et al.*, 2000; Licznerska *et al.*, 2013; Szaefer *et al.*, 2015] and colon [Kusznierewicz *et al.,* 2010] cancer. Microorganisms involved in the fermentation process (lactic acid bacteria like: *Lactobacillus*, *Leuconostoc*, and *Streptococcus* genera) impart the products a characteristic sour taste, form aromatic compounds [McFeeters, 2004], change the structure of fermented plant material [Parada & Aguilera, 2007], increase the digestibility of the plant mass [Swain *et al.*, 2014] as well as increase the content of vitamins (mainly B2 and PP ones) [Capozzi *et al.,* 2012; Thapa & Tamang, 2015]. Other functions of microorganisms include elimination of undesirable substances that can be found in plant raw materials (cyanides, gas-forming substances, hemagglutinins, thioglycosides) and bioconservation, *i.e.* protection against the development of undesirable microbiota [Septembre-Malaterre *et al.,* 2018].

Harmful microorganisms that may occur in improperly prepared fermented products include: putrefactive bacteria – aerobic and anaerobic ones, having proteolytic properties responsible for food spoilage; butyric acid bacteria, cellulo-

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lytic and pectinolytic bacteria – violating the structure of plant tissues [Zieliński *et al*., 2017], which results in "smeariness"; and bacteria of false lactic fermentation, producing carbon dioxide and unwanted products having the nature of organic acids [Franco *et al.*, 2012]. A significant threat can also be yeasts and molds, the development of which is harmful to products of fermentation [Perez Diaz *et al.*, 2014]. In addition, molds can produce toxic substances, breakdown plant tissues [Maruvada & McFeeters, 2009], and can also cause secondary acidification of the environment leading to the development of putrefactive bacteria [Franco *et al.*, 2012]. On the other hand, a small amount of yeast is favorable, as it stabilizes the microbiota [Arroyo-Lopez *et al*., 2012]. It is the ethanol produced by yeast that acts as a preservative. In addition, yeast produces vitamins and growth substances that favorably affect lactic acid bacteria [Arroyo-Lopez *et al.,* 2012]. However, when excessively growing in the fermented product, yeast become the prevailing microbiota, competing with lactic acid bacteria [Arroyo-Lopez *et al.*, 2012].

In recent years, the root of the *Beta vulgaris* L. plant has attracted considerable attention as a functional health promoting food [Chhikara *et al.*, 2019]. It is rich in valuable active compounds such as: betalains [Gengatharan *et al.*, 2015; Khan & Giridhar 2015; Sawicki *et al.*, 2016, 2017], phenolics [Kujala *et al.*, 2002; Ninfali *et al.*, 2017; Preczenhak *et al.*, 2019; Ravichandran *et al.*, 2012], saponins [Mikołajczyk-Bator *et al.*, 2016], and fiber [Lundberg *et al.,* 2008]. Beet juice contains a high level of biologically available antioxidants and many trace elements such as potassium, magnesium, zinc, iron, calcium, phosphorus, sodium, as well as vitamins: niacin and biotin [Wootton-Beard & Ryan, 2011].

Red beetroots and their bioactive compounds can inhibit lipid peroxidation, increase resistance to low density lipoprotein oxidation, and exert a cancer-preventing effect [Lechner & Stoner, 2019; Ninfali *et al.*, 2017].

Betalains are present in the tuberous part of the plant, giving its red-purple coloration. They can be divided into two groups: betacyanins (red-violet) and betaxanthins (yellow-orange). Betanin is the most abundant betacyanin in red beetroot, isobetanin is the second major one. Betalain profile in thirteen red beetroot varieties was investigated by Sawicki *et al.* [2016], who identified thirty betalains, including 18 betacyanins and 12 betaxanthins. Betanin and isobetanin prevailed among the betacyanins, whereas vulgaxanthin I or miraxanthin II among the betaxanthins, depending on the variety.

The first mention of fermented beetroot in Poland appeared in the Herbarium of Syreniusz, and one of the first studies on the processes taking place during borscht making were conducted by Panek [1905]. There is little research on biologically active compounds in fermented beet products. Our previous studies addressed changes of red beet juice composition during spontaneous and controlled fermentation [Czyżowska *et al.,* 2006] and changes of fermented juice composition during 6 months of cold storage [Klewicka & Czyzowska, 2011]. Studies of Sawicki & Wiczkowski [2018] were concentrated on the effect of boiling and fermentation of red beetroot products on betalain profiles and antioxidant capacities. These authors investigated betalain profile and content in fermented shredded beetroots at 7th and 14th day of fermentation. They identified fifteen betacyanins and their derivatives, and also two betaxanthins. They also controlled juices of fermented red beetroots during fermentation and observed 11 betacyanins and their derivatives as well as 4 betaxanthins. Fermentation reduced the content of betalains by 61–88%, however this decline was lesser in unpeeled beetroots.

To the best of our knowledge, there is no literature addressing changes in fermented beetroot products during long-term cold storage. Therefore, the aim of our study was to investigate the effect of long-term cold storage on the content of biologically active compounds (betalains and phenolics) and microorganisms in fermented beetroots and fermented beetroot juice.

MATERIALS AND METHODS

Material and its preparation

The beetroots of Wodan variety originated from Agricultural Farm Michał Sznajder/ Gospodarstwo Rolne Michał Sznajder (Karnice, Poland; 51°28'57"N, 16°50'27"E). Beetroots were washed and peeled only from hard parts. Next, they were grated using a Solia G450 vegetable cutter (AlexanderSolia GmbH, Remscheid, Germany).

Fermentation and storage conditions

Grated beetroots were fermented with 7% salt addition in December 2016 and stored in 200-L barrels made of certified PP5 polypropylene. For a month, they were fermented at a temperature of about 15–18^{o}C, for another month at a temperature of about 10–13^{o}C, and then transported to a cold room to be stored at 5^{o}C.

To obtain juice, fermented grated beetroots were pressed on a hydraulic fruit press and bottled. All these processes were carried out at the Agricultural Farm Michał Sznajder/ Gospodarstwo Rolne Michał Sznajder.

Three samples from 3 barrels were examined each time, giving the total number of 9 samples per one analysis per one product. Juice samples were centrifuged, diluted if necessary, and filtered before HPLC/LC-MS analysis, while grated beet samples were extracted with water in a 1 to 9 ratio at room temperature for 30 min.

Determination of total phenolics content

Total phenolics content (TPC) was determined using the Folin-Ciocalteu (F-C) reagent [Waterhouse, 2001]. The exact conditions of analysis were given in Czyzowska *et al.* [2015]. The results were expressed as gallic acid equivalent and calculated based on the weight/volume of samples collected for analysis.

Spectrophotometric betalain quantification

Red and yellow pigment contents were analyzed using the Nilsson's spectrophotometric method [Nilsson, 1970]. Samples were mixed with a phosphate buffer (pH 6.5), to ensure the absorbance between 0.3 and 0.8 at 538 nm. Absorbance was measured at 476, 538, and 600 nm using a Cecil CE2041 spectrophotometer (Cecil Instruments Limited, Cambridge, UK). The results were expressed as betanin or vulgaxanthin equivalents for red and yellow pigments, respectively.

Betalain profile determination by HPLC-DAD

A Finnigan Surveyor liquid chromatograph equipped with an autosampler, a diode array detector (Finnigan Surveyor--PDA Plus), and ChromQuest 5.0 chromatography software (Thermo Fisher Scientific Inc, Waltham, MA, USA) was used. Separation was performed on a Spherisorb ODS2 column (250 × 4.6 mm, particle size 5 μm, Waters, Milford, MA, USA) protected with a guard column of the same material. All samples were filtered through a 0.45 μm filter prior to chromatography. The HPLC method described by Czyżowska *et al*. [2006] was employed. Eluent A consisted of 0.2% TFA and 10% HCOOH (65:35, v/v), and eluent B was prepared by mixing 100% acetonitrile and 10% HCOOH (80:20, v/v). Complete separation of betalains was achieved within 80 min at room temperature and at a flow rate of 0.9 mL/min. The first 15 min were performed isocratically with 100% A, followed by linear gradient from 0 to 20% B in 65 min. Betalains were monitored at 470 and 538 nm for betaxanthins and betacyanins, respectively. Results were expressed as peak area (PA).

LC-MS^n analysis of bioactive compounds

The HPLC was coupled on-line with an MS LTQ Velos mass spectrometer (ThermoScientific, Waltham, MA, USA). Chromatographic separation was performed using a Hypersil Gold column (150 × 2.1mm, particle size 1.9 μm, ThermoScientific, Waltham, MA, USA). The mobile phase consisted of solvent A (1 mL formic acid in 1 L of deionized water) and solvent B (95% (v/v) acetonitrile). The analysis conditions were similar to those described by Nowak *et al*. [2016]. Elution began with 96% to 85% A for 8 min, from 85% to 82% A for 12 min, from 82% to 60% A for 40 min, from 60% to 50% A for 4 min, followed by washing and re-equilibration of the column. The injection volume was 10 μL. The flow rate was set to 220 μL/min. Electrospray ionization mass spectrometry was performed, in both the positive and negative ionization mode. Mass spectra were checked over the *m/z* range of 100–1000.

Determination of organic acids and ethanol contents

Organic acids were analyzed using a Finnigan Surveyor HPLC system (Thermo Fisher Scientific Inc, Waltham, MA, USA) equipped with an autosampler, a refractive index detector (Finnigan Surveyor-RI Plus), a diode array detector (Finnigan Surveyor-PDA Plus), and ChromQuest 5.0 chromatography software. Separation was performed on an Aminex HPX 87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) protected by a guard column. The analysis conditions were as given in Czyżowska *et al.* [2017]. The elution conditions were as follows: flow rate – 0.6 mL/min, oven temperature – 60°C, and solvent – 5 mM sulphuric acid.

Determination of microbial population count

Samples of the fermented grated beetroots and juice were prepared according to ISO 6887 [2010]. Total mesophilic count (TMC) was determined on a plate count agar (PCA) following incubation at 30°C for 96 h. Lactic acid bacteria (LAB) were quantified on De Man, Rogosa and Sharpe agar (MRS) following incubation at 30°C for 72 h under anaerobic conditions (Gas-Pack System, BBL, Becton–Dickinson, Franklin Lakes, NJ, USA). *Enterobacteriaceae* were determined on Violet Red Bile Dextrose agar (VRBD). Dichloran Rose Bengal Chloramphenicol agar (DRBC) was used for determination of yeast and mold counts. The lowest detection limit of these enumeration techniques was 10 CFU/g (CFU – colony forming units). Five samples of each product were analyzed. The results were expressed as CFU/g of beetroot or CFU/mL of juice.

Statistical analysis

All measurements were performed in nine independent replicates and the results are presented as mean values ± standard deviations (SD). The standard deviation was determined using STATISTICA 10 PL software (StatSoft, Krakow, Poland). The results were compared by one-way analysis of variance (ANOVA), whereas Tukey's test was carried out to test any significant differences among the mean values. Differences among mean values at 5% level ($P<0.05$) were considered statistically significant.

RESULTS AND DISCUSSION

Our previous investigations involved a six-month cold storage of fermented beetroot juices [Klewicka & Czyzowska, 2011]. There are no literature data about changes of fermented beetroot products after this time of storage. Therefore, we have decided to study changes of these products since the 7th month until the end of shelf-life.

Total phenolics content in fermented beetroot products

Total phenolics content in grated beetroot was approximately 900 mg/kg at the beginning of the experiment, while in juice it was approx. 800 mg/L at the same time (Table 1).

TABLE 1. Contents of total phenolics, and red and yellow pigments in fermented beetroot products during long-term cold storage.

Fermented product	Total phenolics content*	Red pigment**	Yellow pigment***
Grated beetroot (mg/kg)			
GB7M	920±120^a	116±11^a	14±1^b
GB8M	795±7^a	83±12^b	17±3^{ab}
GB9M	605±10^b	52±13^c	19±1^a
GB10M	570±5^c	38±2^c	13±2^b
Beetroot juice (mg/L)			
BJ7M	810±20^a	69±11^a	19±2^a
BJ8M	793±5^a	52±9^a	14±2^b
BJ9M	595±8^b	33±8^b	6±1^c
BJ10M	540±96^b	24±9^b	8±1^c

GB – grated beetroot; BJ – beetroot juice; 7M-10M time of storage in a cold room (5°C) in months. *Calculated as gallic acid equivalent. **Calculated as betanin equivalent. ***Calculated as vulgaxanthin equivalent. Data are expressed as mean±standard deviation, n=9. Different letters a-c for each product type indicate statistically significant differences ($p\leq0.05$).

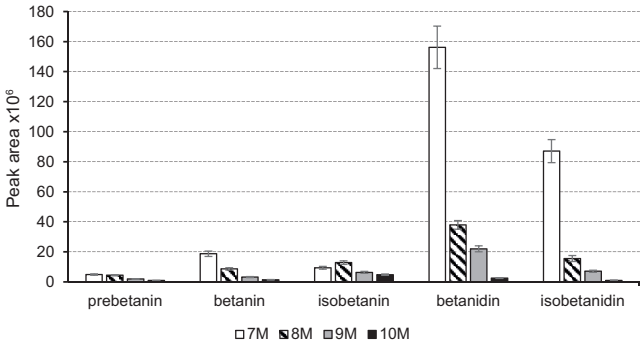

FIGURE 1. Quantitative composition of red betalains (betacyanins) in fermented grated beetroot during long-term storage at 5°C.

Content of compounds expressed as HPLC peak area. 7M-10M – time of storage in months.

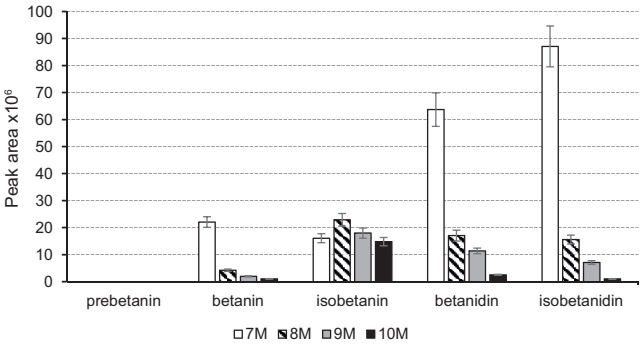

FIGURE 2. Quantitative composition of red betalains (betacyanins) in fermented beetroot juice during long-term storage at 5°C.

Content of compounds expressed as HPLC peak area. 7M-10M – time of storage in months.

The total phenolics content in the samples analyzed by Kavalcová *et al*. [2015] ranged from 820 to 1281 mg/kg in different varieties of red beetroot, and in the samples analyzed by Wootton-Beard & Ryan [2011] the content of phenolics was from about 620 to 1450 mg/kg, whereas their concentration in juices made of seven beet varieties ranged from 0.85 to 1.29 g/L [Wruss *et al.*, 2015]. As it can be seen, the concentration of this group of compounds in the investigated fermented grated beetroots and juices was comparable with the concentration of phenolics in fresh beets and in beet juices.

A downward trend was observed in phenolics content during storage (Table 1). The largest decrease was noticeable at the turn of the 8th and the 9th month of storage. At the end of the experiment, the content of phenolics was approx. 570 mg/kg in grated beetroot and 540 mg/L in beetroot juices. This could be due to the enzymatic oxidation of phenolic compounds in fragmented plant tissues and the activity of lactic acid bacteria enzymes, as well as oxygen access during the collection of subsequent batches of the products.

Betalain content

The concentration of betalains determined with the Nilson's spectrophotometric method [1970] was 116 mg/kg and 69 mg/L for red pigments and 14 mg/kg and 19 mg/L for yellow ones, for grated beetroot and juice, respectively. Study results indicate that red pigments predominated in the analyzed products. Their content in grated beetroots was about 8 times higher compared to the yellow ones (betaxanthins); while in the case of beetroot juice this difference was smaller (Table 1).

Both in the case of fermented beets and juice, about 3-fold decrease of red pigments content was observed during storage. Taking into account yellow pigments, their content did not change in grated beetroot, and their about 2-fold loss was observed in juices. These observations are consistent with literature data stating that betalains degrade during storage or processing which cause color changes [Esquivel, 2016].

As we stated before [Czyżowska *et al.*, 2006], for product color, not only the total content of the pigments is important but also their composition. The content of the main compounds (red betalains) found in fermented grated beetroots and fermented juice was also determined by HPLC. The results expressed as peak area (PA×10^6) are presented in Figures 1 and 2, respectively. Five main compounds from the betacyanin group were found in fermented grated beetroot: prebetanin, betanin, isobetanin, betanidin, and also isobetanidin. Prebetanin concentration in fermented juices was below the limit of quantitation.

Betanidin was shown to be the major compound among grated beetroot pigments at the beginning of the investigation, its content was above 50% of the total content of these pigments. The beetroot juice was predominated by isobetanidin (46%) and betanidin (34%). Their high concentrations can be effected by β-glucosidase activity of lactic acid bacteria (LAB) involved in the fermentation process. The activity of this enzyme is widespread among LAB typical of plant materials. β-Glucosidases release plant secondary metabolites from their β-*D*-glucosylated precursors [Michlmayr & Kneifel, 2014].

In most cases, the content of pigments decreased during storage, which could be influenced by many factors such as: matrix pH and water activity, enzymes activities, heat treatment, and exposure of product to oxygen and light during storage, as well as storage temperature [Belhadj Slimen *et al.*, 2017; Herbach *et al.*, 2006].

Minor increase of isobetanin content was observed between the 7th and the 8th storage month. Betacyanins, due to the C15 chiral center, exist in two epimeric forms, *e.g.* betanin and isobetanin. Epimerization can occur in an acidic medium and lead to an increased isobetanin concentration [Belhadj Slimen *et al.*, 2017; Herbach *et al.*, 2006].

Towards the end of the study, the proportion of pigments changed slightly, betanidin accounted for 45% and 30% of total betalains in grated beet and beet juice, respectively.

Compared to our previous research [Klewicka & Czyzowska, 2011] into the storage of fermented beet juice, the content of betanidin and isobetanidin is much higher, while the concentration of betanin and isobetanin is lower. This might be due to a longer storage time (up to 10 months), other fermentation conditions (addition of starter cultures in the case of previous studies), and scale of the research (laboratory and larger farm).

Considering our other research into the effect of supporting the fermentation of beet juice by the addition of a starter

TABLE 2. Tentative LC-MS^n identification of betalains in fermented products between the 7th and the 10th month of storage.

RT (min)	λ_{max} (nm)	$[M+H]^+$ *m/z*	MS^2 *m/z*	$[M-H]^-$ *m/z*	MS^2 *m/z*	Tentative identification	GB*	BJ*
2.28	263, 274, 460	273		271		portulaxanthin	+	+
3.36	475	459	295, 413, 251	457		unknown bidecarboxy-xanneobetanin isomer	+	+
3.62	284, 462	347		345		dopamine-bx (miraxanthin V)	+	+
4.32	280, 308	317		315	152, 162, 108	dopamine	+	+
4.70	280, 479	345		343	162, 180, 136	unknown decarboxy--betanidin isomer	+	+
5.06	483, 275	309		307		indicaxanthin	+	+
5.24	276, 479	391		389		dopaxanthin	+	-
5.78	536	505	356, 194			unknown decarboxy--dehydrobetanin isomer	+	+
5.93	537	551	389	549		betanin	+	+
6.05	535	713		711		2'-*O*-glucosyl-isobetanin	+	+
6.11	537	631		629		prebetanin	+	+
6.35		505	356, 194	503		unknown decarboxy--dehydrobetanin isomer	-	+
6.92	536	551		549		isobetanin	+	+
7.13	547	527				ni	-	+
7.23	542	389	343, 345, 150, 194, 258, 301, 178	387	299, 194, 343	betanidin	+	+
7.46		326		324		asparagine-betaxanthin (vulgaxanthin III)	+	-
7.63		311		309		valine-betaxanthin	-	+
8.35	542	389		387		isobetanidin	+	+
8.65	467	295	166, 120, 278	293	128, 275	unknown betanidin derivative	+	+
8.70	532	549		547	459, 297, 503, 415	neobetanin	+	+
9.07	280, 450	505				unknown betanidin derivative	+	+
9.80	280, 504	380		378	272, 306, 288, 254, 360, 179	ni	+	+
10.00	277, 527	231	214, 158, 188			ni	+	+
10.10	280, 482	325				vulgaxanthin IV	-	+
10.48	274, 456	461		459		2,17-bidecarboxy--neobetanin	+	+
10.35	277, 482	597				ni	+	-
10.58	279, 327, 482	323		321	128, 171, 303, 215	ni	+	+
12.25	465	331		329		tyramine-betaxanthin (miraxanthin III)	+	+
14.12	507	463		461		2,17-bidecarboxy--betanin/isobetanin	+	-
14.31	459	297		295		γ-aminobutyric acid--betaxanthin	+	+
14.52	532	727	551			6-*O*-feruloyl-betanin	+	-
21.32	534	637	593, 551, 389			isobetanidin 5-*O*-(6-*O*--malonyl)-β-glucoside	+	+

RT – retention time; GB – fermented grated beetroot, BJ – fermented beetroot juice, ni – not identified; * – presence of compounds at each time of storage (7M, 8M, 9M, 10M).

TABLE 3. Tentative LC-MS^n identification of other bioactive compounds in fermented products between the 7th and the 10th month of storage.

RT (min)	λ_{max} (nm)	$[M+H]^+$ *m/z*	MS^2 *m/z*	$[M-H]^-$ *m/z*	MS^2 *m/z*	Tentative identification	GB	BJ
2.18		329		327		betagarin	+	-
2.89	257, 470	259	163, 259	257		caffeic acid derivative	+	+
3.03	279, 320	181		179	135, 119, 92	caffeic acid	+	+
3.09	223, 280	155	137	153		protocatechuic acid	+	-
3.79	223, 276	139		137		*p*-hydroxybenzoic acid	+	-
4.32	280, 308	317		315	152, 162, 108	dopamine	+	+
4.77		166		164		ni	+	+
4.85		339		337		*p*-coumaroylquinic acid	+	+
5.86	364	321				2-*O*-(3,4-dihydroxy benzyl)-2,4,6- -trihydroxyphenylacetic acid	-	+
6.03				289	128, 271, 215	(epi)catechin	+	-
6.68	272, 310	293				ni	+	-
7.06		611		609		quercetin-glucoside	+	-
7.13	547	527				ni	-	+
7.81	271	345		343	192	theogallin	+	-
8.19	316	194		192	148, 174	ni	+	+
9.45	280, 491	571		569	371, 327, 389, 197	ni	+	+
9.80	280, 504	380		378	272, 306, 288, 254, 360, 179	ni	+	+
10.00	277, 527	231	214, 158, 188			ni	+	+
10.35	277, 482	597				ni	+	-
10.58	279, 327, 482	323		321	128, 171, 303, 215	ni	+	+
11.65	279			575	443, 425, 267	(epi)catechin-(epi) catechin (A type)	+	+
12.19	328	309		307		ni	+	+
12.85	225, 280	187	170, 158, 144			ni	+	-
13.06	280, 229			195	136, 151, 177	ni	+	+
17.03				301		quercetin	+	+
18.29	333			307	261, 97	ni	+	-
19.02				197	153, 180, 171, 136, 182, 93, 198	syringic acid	+	-

RT – retention time; GB – fermented grated beetroot, BJ – fermented beetroot juice, ni – not identified; * – presence of compounds at each time of storage (7M, 8M, 9M, 10M).

culture [Czyżowska *et al.*, 2006], it was noted that the addition of *Lactobacillus plantarum*, a plant-derived strain, caused enhanced synthesis of betanidin, compared to other bacterial strains. The influence of the variety on the proportions of the compounds tested was noted as well.

LC-MS identification of bioactive compounds in fermented beetroot products

To the best of our knowledge, there is no literature available regarding the betalain profile in fermented juices and grated beets during long-term cold storage. Available data relate to changes in this group of compounds during fermentation [Sawicki & Wiczkowski, 2018; Sawicki *et al.*, 2019].

The profile of bioactive compounds in fermented products (between 7 and 10 months of storage) was analyzed using the LC-MS^n technique. Compounds were identified by comparison of mass spectra, λ_{max} and retention times of available standards (phenolic acids and flavonols) or previously published data [Nemzer *et al*., 2011; Sawicki *et al.*, 2016; Sawicki & Wiczkowski, 2018; Slatnar *et al.*, 2015].

TABLE 4. The pH and the concentration of organic acids and ethanol in the fermented products.

Fermented product	pH	Lactic acid	Acetic acid	Propionic acid	Ethanol
Grated beetroot (g/kg)					
GB7M	3.71±0.32^{a}	6.03±1.04^{b}	4.77±1.02^{ab}	1.02±0.32^{b}	8.98±0.67^{a}
GB8M	3.56±0.13^{a}	6.63±0.20^{b}	5.54±1.30^{ab}	1.73±0.12^{a}	7.42±0.35^{b}
GB9M	3.32±0.25^{a}	8.68±0.92^{a}	3.54±1.03^{b}	1.04±0.94^{ab}	7.78±0.45^{b}
GB10M	3.61±0.08^{a}	6.40±0.15^{b}	6.30±0.65^{a}	1.59±0.36^{ab}	4.15±0.25^{c}
Beetroot juice (g/L)					
BJ7M	3.92±0.31^{a}	5.18±0.42^{a}	4.10±0.82^{a}	0.78±0.48^{b}	6.04±3.98^{ab}
BJ8M	3.83±0.25^{a}	5.03±0.36^{a}	4.28±1.08^{a}	0.56±0.14^{b}	3.85±1.05^{b}
BJ9M	3.81±0.48^{a}	5.01±0.76^{a}	4.90±0.62^{a}	2.26±1.02^{a}	9.06±3.06^{a}
BJ10M	4.12±0.37^{a}	4.76±0.64^{a}	3.86±0.58^{a}	0.32±0.10^{b}	3.28±0.62^{b}

GB – grated beetroot; BJ – beetroot juice; 7M-10M time of storage in a cold room (5°C) in month. Data are expressed as mean±standard deviation n=9. Different letters a-c for each product type indicate statistically significant differences ($p \leq 0.05$)

As expected, the majority of compounds belonged to the betalain family (Table 2). In addition to the basic betalains (prebetanin, betanin, isobetanin, betanidin, and isobetanidin) characterized by HPLC, additional 22 compounds were identified including, among others, neobetanin and its derivatives like 2,17-bidecarboxy-neobetanin and bidecarboxy-xanneobetanin isomer.

Studies of Wybraniec & Michałowski [2011] confirmed the highest enzymatic oxidating activity of neobetanin at pH 3, which is similar to the conditions prevailing in our products, and other studies of Starzak *et al.* [2016] have shown that one of the main products of enzymatic oxidation was 2,17-bidecarboxy-xanneobetanin.

Nine of these compounds were found in fermented roots and juices investigated by Sawicki *et al.* [2019] and fifteen in the research by Sawicki & Wiczkowski [2018].

Taking into account betaxanthins, 8 compounds from this group were found in fermented grated beetroots and juices. Four of them (miraxanthin V, indicaxanthin, miraxanthin III, and γ-aminobutyric acid-betaxanthin) were detected in both investigated products. One of these compounds – miraxanthin V – was found in fermented beetroot juices obtained by Sawicki & Wiczkowski [2018]. However, these authors identified three other betaxanthins not occurring in our juices. The newest studies of the aforementioned authors [Sawicki *et al.*, 2019] indicated the presence of two betaxanthins (vulgaxanthin I and miraxanthin V) in fermented beet roots. These authors did not identify any traces of these compounds in juices obtained during the fermentation process of beetroots. We did not find any traces of vulgaxanthin I in products studied, which is not consistent with our previous data concerning fermented juices [Czyżowska *et al.*, 2006] as well as data obtained by Sawicki *et al.* [2018]. But according to other literature data [Sawicki & Wiczkowski, 2018], this compound disappeared after the 5th day of fermentation of red beet juice. However, it was present in the fermented beets examined by the authors. The lack of this compound in our fermented products may be due to long storage or the variety used.

Considering phenolic compounds (Table 3), some acids were detected, including: *p*-hydroxybenzoic, protocatechuic, syringic, caffeic, *p*-coumaroylquinic, and galloylquinic (theogallin) acids. We did not find ferulic acid, that was detected in beet juices investigated by Wruss *et al.* [2015], but we identified its derivative with betanin – 6-*O*-feruloyl-betanin, a compound previously identified by Slatnar *et al*. [2015] in peel and petiole of red beet, by Sawicki *et al.* [2016] in 13 red beet varieties, by Sawicki *et al*. [2018] in fermented red beet juice, as well as by Sawicki & Wiczkowski [2018] and Sawicki *et al.* [2019] in fermented beetroot and juices. Flavonoids identified were: (epi)catechin, A type dimer of (epi)catechin-(epi) catechin, quercetin and its derivative quercetin-glucoside, and betagarin – a compound identified by Kujala *et al.* [2002].

Organic acids and ethanol

Table 4 shows the concentration of organic acids and ethanol in fermented grated beetroot and juice after 7–10-month storage. On the basis of chromatographic separation, the presence of three acids was revealed in the tested samples. They were products of lactic acid fermentation. Of the acids, lactic acid showed the highest concentrations in the tested samples, *i.e*. 6.03–8.68 g/kg in fermented grated beetroots, and 4.76–5.18 g/L in juices, and in most cases did not differ significantly ($p>0.05$) during storage time. Acetic and propionic acids occurred at levels of 3.54–6.30, 3.86–4.90 g/kg and 1.02–1.73 and 0.32––2.26 g/L, in fermented grated beetroots, and juices respectively. About 50% ethanol loss between the 7th and the 10th storage month was observed in both products tested.

There are no literature data concerning organic acids content in fermented beetroot products. But taking into account other fermented products, like olives, the major organic acids were lactic acid as the main biochemical product of fermentation followed by acetic acid, the presence of which could be attributed to homo- or hetero-fermentative metabolism of LAB strains due to nutrient limitation, salt concentration, as well as to yeast metabolism [Arroyo-López *et al*., 2012; Blana *et al*., 2014].

TABLE 5. Microbiological quality of fermented beetroot products during long-term cold storage.

Fermented product	TMC	LAB	*Enterobacteriaceae*	Yeast and molds
Grated beetroot (CFU/g)				
GB7M	$(1.8\pm0.5)\times10^{5b}$	$(4.5\pm1.3)\times10^{5a}$	$<10^{a}$	93 ± 35^{a}
GB8M	$(5.2\pm3.6)\times10^{4c}$	$(4.5\pm1.9)\times10^{3c}$	$<10^{a}$	$<10^{b}$
GB9M	$(8.0\pm2.0)\times10^{5a}$	$(4.6\pm1.2)\times10^{5a}$	$<10^{a}$	$<10^{b}$
GB10M	$(7.0\pm2.5)\times10^{3d}$	$(5.6\pm2.5)\times10^{4b}$	$<10^{a}$	$<10^{b}$
Beetroot juice (CFU/mL)				
BJ7M	$(3.6\pm0.7)\times10^{4c}$	$(2.4\pm0.8)\times10^{5b}$	$<10^{a}$	$(4.0\pm0.4)\times10^{3a}$
BJ8M	$(2.4\pm0.7)\times10^{3d}$	$(8.3\pm1.8)\times10^{5a}$	$<10^{a}$	$(1.4\pm0.7)\times10^{3b}$
BJ9M	$(1.5\pm0.1)\times10^{6a}$	$(1.4\pm0.5)\times10^{6a}$	$<10^{a}$	$(1.3\pm0.3)\times10^{3b}$
BJ10M	$(2.8\pm0.2)\times10^{5b}$	$(2.2\pm0.3)\times10^{5b}$	$<10^{a}$	$(1.2\pm0.5)\times10^{3b}$

GB – grated beetroot; BJ – beetroot juice; 7M-10M time of storage in a cold room (5°C) in months; TMC – total mesophilic count, LAB – lactic acid bacteria. Data are expressed as mean±standard deviation, n=9. Different letters a-d for each product type indicate statistically significant differences ($p\leq0.05$).

Microbiology

Results of microbiological analyses of the investigated products during storage are presented in Table 5. The total number of mesophilic bacteria in the tested samples reached 10^3–10^5 CFU/g in grated beetroots and 10^3–10^6 CFU/mL in juices. In most cases, there were no significant differences ($p>0.05$) between the number of mesophilic bacteria and the number of LAB. In some cases (especially in juices between the 7th and the 8th month of storage), the number of LAB was slightly higher, which may indicate better recovery of these bacteria in the MRS broth. The results indicate that lactic acid bacteria predominated among the bacterial microbiota in the fermented products. Larger numbers of LAB were observed in the fermented juices samples.

The count of yeast was lower in the fermented grated beetroots: at the beginning of investigated period at the level of almost 10^2, and at the end of observation lower than 10 CFU/g. The higher count of yeast was found in the juices (10^3 CFU/mL). *Enterobacteriaceae* were not detected in the fermented grated beetroots and beetroot juices throughout the storage time. These results indicate a high quality of the investigated products, which is ensured by the manufacturer, and that they are safe for the consumers in terms of their microbiological quality. In the case of fermented grated beetroots at the end of the storage period, the counts of mesophiles and LAB decreased in a statistically significant manner ($p\leq0.05$) as compared to their initial counts. In the case of juices, the numbers of all microorganisms were almost at the same levels during the storage period.

The storage survival of lactic acid bacteria, including potentially probiotic strains, depends on the environment and the type of strain used. Peñas *et al*. [2010] observed a steady increase in the number of lactic acid bacteria, including *Lb. plantarum* and *Lb. mesenteroides* strains, used for fermentation of cabbage juice, during 3-month storage at 4°C. Gardner *et al*. [2001], found a decrease in the number of bacteria from 10^9 CFU/mL after fermentation to 10^6 CFU/mL on the 90th day of storage of cabbage, beet, and carrot juices at 4°C, fermented with a mixture of *Lactobacillus*, *Leuconostoc*, and *Pediococcus* strains. According to these authors, the viability of bacterial cultures may result from the specificity of the strain used and the type of the food matrix (vegetables from which the juice was obtained). In turn, Yoon *et al.* [2005] studied the survival of LAB strains in fermented (72 h, temp. 30°C) beetroot juice, stored at 4°C for 4 weeks. The survivability of the strains was observed at levels ranging from 10^6 to 10^8 CFU/mL, except for *Lb. acidophilus* LA39. Finally, a research by Klewicka & Czyżowska [2011] regarding the storage of fermented beet juices with the addition of starter cultures showed the survival of LAB at 10^6 CFU/mL after 180 days of storage at 4°C.

CONCLUSIONS

This is the first report on biologically active compounds (betalains and phenolics) composition in fermented grated beetroots and beetroot juices during long-term cold storage.

The total phenolics content decreased during the process, and at the end reached 570 mg/kg in grated beetroot and 540 mg/L in juices. Betalains and phenolics profiles in these products were characterized and it was found that there were both quantitative and qualitative differences between them. Lactic acid bacteria predominated among the bacterial microbiota of the fermented products.

The tested products were found to be a rich source of biologically active compounds, and their health-promoting potential was enhanced by the presence of metabolically active lactic acid bacteria. During long-term storage, the content of their bioactive compounds decreased, however, remained at a high level.

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Original article
Red Beetroot Thematic Issue

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

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Key words: red beetroot juice, antioxidant activity, bioaccessibility, *in vitro* digestion, phytonutrients, HPLC

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].

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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 50ºC 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$^\bullet$ 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

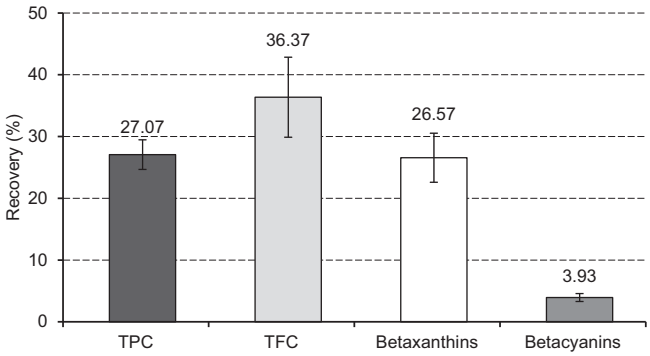

FIGURE 1. Recovery of total phenolics content (TPC), total flavonoids content (TFC), and contents of betaxanthins and betacyanins after *in vitro* simulated gastrointestinal digestion (SGD) of red beetroot juice.

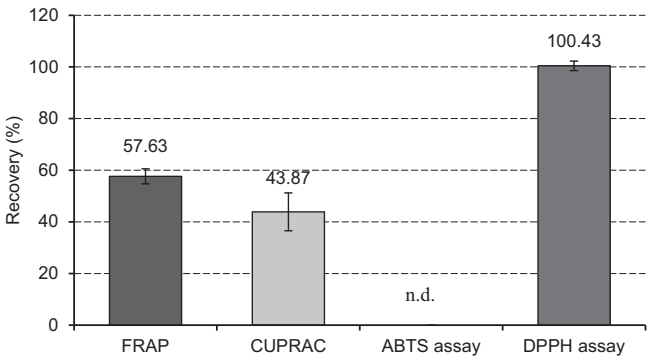

FIGURE 2. Recovery of antioxidant activity after *in vitro* simulating gastrointestinal digestion (SGD) of red beetroot juice.

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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Original article
Red Beetroot Thematic Issue

Profile of Phenolic Acids and Flavonoids of Red Beet and Its Fermentation Products. Does Long-Term Consumption of Fermented Beetroot Juice Affect Phenolics Profile in Human Blood Plasma and Urine?

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Key words: phenolic acids, flavonoids, red beet, fermentation, consumption, human body fluids profile

The aim of this study was to determine the profile of phenolic acids and flavonoids in red beetroot products and these compounds fate in the human body after long-term exposure to fermented red beet juice. Phenolic acids and flavonoids were analyzed by HPLC-MS/MS. The performed analysis revealed that beetroot and its products were the notable sources of phenolic acids and flavonoids, with isoferulic acid, protocatechuic acid, epicatechin, and apigenin predominating among the twenty compounds detected. However, the compounds analyzed appeared mainly in the bound form in the fresh material. The results obtained indicate that the fermentation process caused an increase in the content of free phenolic acids and reduced the content of conjugated phenolic acids. Contrarily to phenolic acids, the same process caused a reduction in the content of free flavonoids and an increase in the content of conjugated flavonoids. Moreover, the 14-day spontaneous fermentation process resulted in a significant reduction (by 45.18%) in the total content of phenolics (phenolic acids and flavonoids). However, it was observed that forty-two days of the regular intake of the fermented beetroot juice (200 mL/60 kg body weight) generally did not affect concentrations of phenolic acids and flavonoids in the volunteers' body fluids compared to their concentrations in fluids before fermented beetroot juice consumption.

INTRODUCTION

Red beetroot (*Beta vulgaris* L. subsp. *vulgaris*) is a great source of minerals (such as manganese, iron, potassium, magnesium, and cobalt), vitamins B and C as well as a number of bioactive substances such as polyphenols, carotenoids, nitrate, and betalains. Due to the high contents of these phytochemicals, beetroot is in the top of ten vegetables with the most powerful antioxidant influence [Carrillo *et al*., 2017; Raikos *et al.*, 2016]. Additionally, these features may determine the other biological aspects of beetroots. It has been suggested that red beet has anti-neurodegenerative, antitumor, anti-inflammatory, antibacterial, antiviral, cardioprotective, and lipid peroxidation inhibitory activities [Clifford *et al*., 2015]. For these reasons, consuming red beetroot might have beneficial effects on human and animal organisms.

Beetroot is eaten fresh and after cooking. In addition, this raw material is used in the processing industry for the production of drinking and concentrated juices, frozen foods, dried fruit, lyophilizate, and for the production of natural red dyes [Chhikara *et al*., 2019]. One of the technological processes used in the food industry is fermentation, which involves the use of transformations occurring under the influence of enzymes produced by yeast and bacteria. During the fermentation process, in addition to basic end products, additional nutrients are produced and toxins removed. What is more, lactic acid bacteria found in all non-heat treated fermented foods have a positive effect on digestive health [Hasan *et al*., 2014]. It is so important, therefore, to determine to what extent an applied technological process affects the profile and content of biologically active compounds of vegetables and fruits.

Phenolics compounds, which include flavonoids and phenolic acids, are very important secondary metabolites of plants and have highly varied structures and properties. In addition, numerous studies point to their strong antioxidant activities [Saxena *et al*., 2012]. Moreover, long-term consumption of diets rich in biologically active polyphenols provides protection against the development and progression of lifestyle diseases such as cardio- and cerebrovascular diseases, cancer, diabetes or neurodegenerative diseases [Pandey & Rizvi, 2009]. Previous studies have indicated the absorption of phenolic acids and flavonoids by animal and human organisms and the occurrence of native and conjugated forms in their body fluids [Wiczkowski *et al*., 2008]. It should be noted, however, that the consumption of bioactive foods is not synonymous with good bioavailability and the potentially protective effect on the human body. To verify whether bioactive compounds can have a positive effect on the human body,

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at first should be determined the bioavailability and structure of the compounds that occur in the body fluids after the consumption of foods rich in bioactive substances [Rein *et al*., 2013]. In this context, the aim of this study was to analyze the profile and content of red beetroot phenolics (phenolics acids and flavonoids) in red beetroot products (fresh roots, fermented roots, and commercial fermented red beet juice) and whether the long-term and regular consumption of red beetroot affects the profile and concentration of phenolics in blood plasma and urine of volunteers.

MATERIALS AND METHODS

Chemicals and reagents

Water, methanol, acetonitrile, formic acid, and diethyl ether were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Protocatechuic acid, *m*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, syringic acid, sinapic acid, ferulic acid, isoferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, *trans*-cinnamic acid, vitexin, rutin, epicatechin, luteolin, quercetin, apigenin, kaempferol, and orientin were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Extrasynthese (Genay, France).

Research materials and processes

Healthy red beetroots (3 kg), with no signs of mechanical damage, were acquired from the local market in Olsztyn (Poland). Next, the roots were cleaned, mixed, and divided into two groups: fresh roots and roots for the fermentation process. Commercially fermented red beetroot juice was obtained from Zakład Przetwórstwa Owocowo-Warzywnego VITAPOL Sp. J. M. T. Ciszkiewicz, Podkowa Leśna, Poland (Eng. Fruit and Vegetable Processing Plant VITAPOL Sp. J. M. T. Ciszkiewicz).

Fresh roots

The red beetroots assigned to the fresh group (1.5 kg) were cut into four parts, then two opposite quarters were frozen at -80°C, lyophilized, pulverized, and stored at -80°C until analysis of the profile and content of phenolic acids and flavonoids.

Fermentation process

The spontaneous fermentation process was conducted according to the method described by Sawicki & Wiczkowski [2018]. Briefly, the whole roots (1.5 kg) were cut into 2–3 mm thick slices. Then, the root slices were placed in a stoneware dish and submerged in a properly prepared marinade (1.5 L water, 12 g salt, and 12 g sugar). The materials obtained were thoroughly mixed, and three independent fermentations were started. The process was carried out over two weeks in the dark at a constant temperature of 23°C until the pH stabilized. The pH was measured once a day using a pH-meter PHM85 (Radiometer, Copenhagen, Denmark), and the pH values obtained, ranging from 7.02±0.02 (fresh juice) to 3.63±0.012 (after a 14-day fermentation process), clearly showed that the process was conducted properly. After 14 days, the spontaneous fermentation process ended, and the fermented red beetroots (200 g) were collected from each stoneware pot and frozen. Next, the roots were lyophilized, pulverized, and stored at -80°C until analysis.

Subjects and study design

Samples of blood plasma and urine were obtained from volunteers who participated in a 42-day non-clinical medical study with an experimental diet. The study was led in accordance with the ethical principles of the Declaration of Helsinki, and the study protocol was accepted by the Bioethical Committee at the Faculty of Medical Science of the University of Warmia and Mazury in Olsztyn (Poland, No. 7/2015). All volunteers were fully informed about the study and signed an informed consent form. Moreover, the study was carried out in the NZOZ Atarax Clinic in Olsztyn, Poland under medical supervision.

Men (5) and women (19) between 24 and 40 years of age participated in the experiment. Inclusion criteria were as follows: they had to be certified healthy at a medical interview, have a body mass index (BMI) under 30, and have no gastrointestinal disturbances, including gastric and duodenal ulcers; they could not participate in other clinical trials within 90 days prior to the survey, take drugs, abuse alcohol, be pregnant and breast-feeding, or take any medications or vitamin supplements.

During the 7 days before starting the experimental diet, the volunteers followed their everyday diet while excluding all products obtained from red beet (red beets, strawberry yogurt, strawberry ice cream, fruit juice, wine, and other products containing E162 dye) to eliminate the main compounds within their systems originating from this vegetable. Then, for 42 days, once a day, directly after breakfast, the volunteers drunk a dose of the fermented beetroot juice (200 mL/60 kg body weight). All volunteers included in the study visited NZOZ Atarax every 7 days to have their health status evaluated and to receive the juice of fermented beetroot for the next 7 days. Under fasting conditions, before consumption (sample zero) and after 7, 21, and 42 days of daily consumption of fermented beet juice, elbow or forearm vein blood samples were taken in heparinized vacutainers, and the blood obtained was centrifuged (500× *g*, 15 min, 1000× *g*, 10 min, 4°C, MPW-351R Centrifuge, MPW-Med. Instruments, Warsaw, Poland). In addition, according to the above sampling scheme (0, 7, 21, and 42 days), urine samples were collected from the volunteers. Thereafter, the separated plasma and collected urine were frozen and stored at -80°C until analysis.

Extraction of free forms of phenolic acids and flavonoids

The profile and content of phenolic acids and flavonoids were analyzed according to the method described by Wiczkowski *et al.* [2016], with some modifications. Extracts from fresh and fermented red beetroots were prepared. About 0.15 g of each solid sample were extracted with 1 mL of a mixture of methanol/water/formic acid (80/19.9/0.1; *v/v/v*) by stirring overnight (about 24 h) at 10°C using a ThermoMixer (Benchmark Scientific, Saryeville, NJ, USA). The solutions were centrifuged (13,200 × *g*, 20 min, 4°C, Centrifuge 5415R, Eppendorf, Hamburg, Germany) and the supernatants were combined into 1-mL volumetric flask. The volume was brought up to 1 mL with distilled water. The obtained extracts were stored at -80°C until analysis.

For HPLC-MS/MS analysis, extracts from fresh and fermented red roots as well as fermented red beetroot juices, blood plasma and urine were prepared according to the following procedure: samples of fresh (100 μL), fermented red roots (100 μL), commercial fermented juice (100 μL), blood plasma (200 μL), and urine (100 μL) were transferred to 15-mL glass test-tubes and next evaporated to dryness under a nitrogen atmosphere at 30°C. The sediments obtained were dissolved in 400 μL of distilled water, acidified to pH 2, were extracted by vortexing (60 s) and sonication (60 s). Next, the free forms of phenolic acids and flavonoids were extracted three times with 2 mL of diethyl ether by vortexing (60 s) and sonication (60 s) (VC 750, Sonics & Materials Inc., Newtown, CT, USA). After centrifugation for 10 min (3,000× *g*, 4°C, Centrifuge, Beckman, Indianapolis, IN, USA) the separated ether layer was collected and evaporated to dryness with a stream of nitrogen at 30°C. The extraction procedure with diethyl ether was repeated three times and the organic layers were combined. The dried samples were stored at -80°C until analysis. The remaining aqueous extracts were left for about 10 min under nitrogen to remove residual diethyl ether and then stored in sealed test tubes at 4°C until the extraction of phenolic acids and flavonoids released from soluble esters.

Extraction of phenolic acids and flavonoids released from soluble esters

Next, 1 mL of 4 M NaOH was added to the aqueous extracts remaining from the extraction of free forms of phenolic acids and flavonoids. The mixture was placed in a nitrogen atmosphere and hydrolyzed for 4 h at room temperature using a magnetic stirrer. The solutions were then adjusted to pH 2 with 6 M HCl. Liberated phenolic acids and flavonoids were extracted three times with 2 mL of diethyl ether by vortexing (60 s) and sonication (60 s), and then centrifuged (3,000× *g* at 4°C, 10 min). The organic layers were collected, combined, and evaporated to dryness with a stream of nitrogen at 30°C. The remaining aqueous extracts were left for about 10 min under nitrogen to remove residual diethyl ether and then were stored in closed tubes at 4°C until the extraction of phenolic acids and flavonoids released from soluble glycosides.

Extraction of phenolic acids and flavonoids released from soluble glycosides

After alkaline hydrolysis, acid hydrolysis was performed by adding 0.2 mL of 6 M HCl into the test tube and incubation in a Heat Block (Benchmark Scientific, Saryeville, NJ, USA) at 100°C for 60 min. Next, the samples were cooled, adjusted to pH 2 with 8 M NaOH, and handled in the same manner as after alkaline hydrolysis.

Chromatographic analysis

The dry residues containing free phenolic compounds, as well as the compounds released from ester and glycosidic bonds (fresh and fermented red roots, commercial fermented juice, blood plasma, and urine) were dissolved in 100 μL of 80% (*v/v*) methanol containing 0.95% (*v/v*) formic acid, centrifuged (13,200× *g* at 4°C, 20 min), and analyzed by using the HPLC system (LC-200, Eksigent, Vanghan, ON, Canada) coupled with a mass spectrometer (QTRAP 5500, AB Sciex, Vaughan, ON, Canada) consisting of a triple quadrupole, ion trap, and ion source of electrospray ionization (ESI). The chromatographic separation was conducted with an HALO C_{18} column (50 mm × 0.5 mm × 2.7 μm, Eksigent, Vaughan, ON, Canada) at 45°C, at the flow rate of 15 μL/min. The elution was conducted using a solvent gradient system consisting of solvent A (0.9% (*v/v*) formic acid aqueous solution) and solvent B (0.9% (*v/v*) formic acid acetonitrile solution). The gradient used was as follows: 0.5% B for 0.5 min, 0.5–90% B in 1.5 min, 90% B for 0.5 min, 90–0.5% B in 0.2 min, and 0.5% B for 0.3 min. An optimal identification of the analyzed phenolic acids and flavonoids was achieved

TABLE 1. Phenolic acids and flavonoids identified in fresh and fermented beetroot, commercial fermented red beet juice, as well as in blood plasma and urine of the volunteers.

No	Compounds	R_t (min)	$[M]^-$ (m/z)	MS/MS (m/z)	Sample
	Phenolic acids				
1	Protocatechuic acid	0.94	153	91/81	B, F, J, P, U
2	*m*-Hydroxybenzoic acid	0.99	137	93/65	B, F, J, P, U
3	Chlorogenic acid	1.00	353	191/179	B, F, J, P, U
4	Caffeic acid	1.04	179	135/107	B, F, J, P, U
5	Syringic acid	1.06	197	182/153	B, F, J, P, U
6	Sinapic acid	1.13	223	208/179/164	B, F, J, P, U
7	Ferulic acid	1.15	193	178/134	B, F, J, P, U
8	Isoferulic acid	1.20	193	178/134	B, F, J,
9	*p*-Coumaric acid	1.22	163	119/93	B, F, J, P, U
10	*p*-Hydroxybenzoic acid	1.28	137	98/93	B, F, J, P, U
11	3,4-Dihydroxyphenylacetic acid	1.30	169	125/109/95	B, F, J, P, U
12	*trans*-Cinnamic acid	1.35	147	119/109/103	B, F, J, P, U
	Flavonoids				
13	Vitexin	1.00	431	323/311/283	B, F, J
14	Rutin	1.02	609	463/301	B, F, J
15	Epicatechin	1.04	289	245/203/109	B, F, J, P, U
16	Luteolin	1.26	285	151/133	B, F, J, P, U
17	Quercetin	1.27	301	179/151	B, F, J, P, U
18	Apigenin	1.33	269	225/151/117	B, F, J, P, U
19	Kaempferol	1.35	285	185/93	B, F, J, P, U
20	Orientin	1.95	447	357/339/296	B, F, J

R_t – retention time; B – fresh beetroot; F – fermented beetroot; J – commercial fermented red beet juice; P – blood plasma; U – urine.

under the following ESI-MS/MS conditions: negative ionization, curtain gas: 20 L/min, collision gas: ion spray voltage: 5300 V, temperature: 350°C, 1 ion source gas: 35 L/min, 2 ion source gas: 30 L/min, declustering potential: 100 V, entrance potential: 10 V, collision energy: 40 eV, and collision cell exit potential: 20 V. Identification and quantitation of the phenolic acids and flavonoids were based on the comparison of their retention times and the presence of the respective parent and daughter ion pairs (Multiple Reaction Monitoring method, MRM) (Table 1) with data obtained after analysis of the authentic standards. The external standards (0.01––0.5 μg/mL) had linear calibration curves with a coefficient of determination of 0.997–0.999. The concentrations of individual phenolics were expressed as concentrations of their free and conjugated forms (a sum of phenolics released from ester and glycosidic bonds) of phenolic acids or flavonoids. The results were expressed in μg/g dry matter (dm) of fresh and fermented red beetroot, and in μg/mL of red beetroot juice, blood plasma, and urine. All analyses were performed in triplicate for each sample.

Statistical analyses

The data are presented as the mean±the standard deviation (SD). Statistical differences among the samples were tested using one-way ANOVA with the Tukey's test. Differences were considered significant at $P<0.05$. The statistical analysis was performed using the Statistica software (Stat Soft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Profile and content of phenolic acids and flavonoids in red beet products

In the fresh and fermented red beetroots as well as in the commercial fermented red beet juices, twelve phenolic acids were found (Table 1). Four of the identified phenolic acids were hydroxybenzoic acid derivatives (protocatechuic, *m*-hydroxybenzoic, syringic, and *p*-hydroxybenzoic acids), while seven phenolic acids belonged to the hydroxycinnamic acid derivative classification (chlorogenic, caffeic, sinapic, ferulic, isoferulic, *p*-coumaric, and *trans*-cinnamic acids), and one phenylacetic acid derivative (3,4-dihydroxyphenylacetic acid) was detected as well. Previously published data have also shown the presence of derivatives of hydroxybenzoic acid (protocatechuic acid, *p*-hydroxybenzoic acid, syringic acid, vanillic acid) and hydroxycinnamic acid (chlorogenic acid, caffeic acid, ferulic acid, coumaric acid, cinnamic acid) in red beetroot products [Mattila & Hellström, 2007; Georgiev *et al*., 2010; Ravichandran *et al*., 2012; Vulić *et al.,* 2014; Ben Haj Koubaier *et al*., 2014; Székely *et al*., 2014; Wruss *et al*., 2015; Değirmencioğlu *et al*., 2016]. To the best of our knowledge, our study is the first to show the full profile of phenolic acids in red beetroot products. Moreover, this is the first study that identifies phenylacetic acid derivative in these products.

The main compound among the phenolic acids in the fresh and fermented red beetroot analyzed was isoferulic acid (88.9% and 61.9% of the total phenolic acids, respectively) (Table 2). Furthermore, in the fresh roots, the second most dominant compound was syringic acid, followed by ferulic, *p*-coumaric, and sinapic acids. On the other hand, in fermented beetroot, the second major compound was protocatechuic acid, followed by sinapic, ferulic, caffeic, and *p*-coumaric acids. Interestingly, in contrast to the fresh and fermented red beetroot, the main compound detected in the commercial fermented red beetroot juice was protocatechuic acid. Its total content was at 0.979 μg/mL (30.7% of the total phenolic acids in the fermented red beetroot juice). In addition, *trans*-cinnamic acid was the second major compound, followed by isoferulic, *p*-hydroxybenzoic, *m*-hydroxybenzoic, syringic, and sinapic acids (Table 2). Interestingly, the results we obtained are significantly different from those available in the literature. The available data show that the major phenolic acids in solid red beetroot were *p*-hydroxybenzoic acid [Ravichandran *et al*., 2012], caffeic acid [Georgiev *et al*., 2010], and vanillic acid [Ben Haj Koubaier *et al*., 2014]. On the other hand, in liquid red beet products, the dominant compound was gallic acid (fresh juice) [Wruss *et al*., 2015], while in fermented juice, the main detected acid was vanillic acid [Değirmencioğlu *et al*., 2016].

The results of the study showed that the fermentation process resulted in a change in phenolic acid content. In our research, contents of the hydroxybenzoic acid derivatives (protocatechuic, *m*-hydroxybenzoic, syringic, and *p*-hydroxybenzoic acids) and hydroxycinnamic acid derivatives (chlorogenic, caffeic, sinapic, ferulic, isoferulic, *p*-coumaric, and *trans*-cinnamic acids) in the fresh roots were at 1.55±0.00 μg/g dm and 30.66±0.00 μg/g dm, respectively. However, the content of the detected phenylacetic acid derivative (3,4-dihydroxyphenylacetic acid) in fresh roots approximated the limit of detection. As in the case of fresh beetroot, in fermented red beetroot, the content of hydroxycinnamic acid derivatives was much higher (14.22±0.01 μg/g dm) than of the hydroxybenzoic acid derivatives (3.27±0.00 μg/g dm). In general, phenolic acids in fresh, as well as in fermented beetroot, appeared mostly in bound forms (31.79±0.01 μg/g dm and 16.40±0.01 μg/g dm) compared with the presence the free forms (0.42±0.00 μg/g dm and 1.11±0.00 μg/g dm, respectively). It is important to note that the fermentation process resulted in a 2.64-fold increase in the content of free phenolic acids, including: sinapic, isoferulic, *p*-coumaric, *p*-hydroxybenzoic, and *trans*-cinnamic acids, but caused no changes in the proportion of free protocatechuic, *m*-hydroxybenzoic, chlorogenic, syringic, and 3,4-dihydroxyphenylacetic acids. A decrease in the proportion of the free forms after the fermentation treatment was observed only in the case of ferulic acid. The fermentation of red beetroots affected also contents of the bound forms of phenolic acids. The process applied increased the proportion of the conjugated protocatechuic, *m*-hydroxybenzoic, chlorogenic, caffeic, sinapic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, and 3,4-dihydroxyphenylacetic acids (Table 2). Chlorogenic and 3,4-dihydroxyphenylacetic acids in fresh beetroot roots were at the limit of detection. In fermented roots, chlorogenic acid accounted for only 0.1% of total phenolic acids and was found only in the bound form (after alkaline and acid hydrolysis). The low level of chlorogenic acid and detection of this compound after hydrolysis may be due to the fact that chlorogenic acid occurs in the larger structures (polysaccharides, lignins) in the red

TABLE 2. Content and contribution of phenolic acids and flavonoids in red beet products.

No	Compounds	Form	Red beet products					
			Fresh		Fermented		Commercial juice	
			μg/g dm	%	μg/g dm	%	μg/mL	%
			Phenolic acids					
1	Protocatechuic acid	F	0.000	0.0	0.000	0.0	0.016	0.5
		C	0.258	0.8	2.644	15.1	0.963	30.2
2	*m*-hydroxybenzoic acid	F	0.000	0.0	0.000	0.0	0.029	0.9
		C	0.097	0.3	0.070	0.4	0.144	4.5
3	Chlorogenic acid	F	0.000	0.0	0.000	0.0	0.003	0.1
		C	0.000	0.0	0.018	0.1	0.022	0.7
4	Caffeic acid	F	0.000	0.0	0.525	3.0	0.016	0.5
		C	0.258	0.8	0.263	1.5	0.185	5.8
5	Syringic acid	F	0.000	0.0	0.000	0.0	0.041	1.3
		C	0.934	2.9	0.263	1.5	0.102	3.2
6	Sinapic acid	F	0.000	0.0	0.018	0.1	0.003	0.1
		C	0.387	1.2	1.033	5.9	0.096	3.0
7	Ferulic acid	F	0.193	0.6	0.018	0.1	0.041	1.3
		C	0.612	1.9	1.016	5.8	0.045	1.4
8	Isoferulic acid	F	0.193	0.6	0.438	2.5	0.102	3.2
		C	28.441	88.3	10.401	59.4	0.360	11.3
9	*p*-coumaric acid	F	0.000	0.0	0.018	0.1	0.003	0.1
		C	0.483	1.5	0.368	2.1	0.035	1.1
10	*p*-hydroxybenzoic acid	F	0.032	0.1	0.035	0.2	0.070	2.2
		C	0.225	0.7	0.263	1.5	0.211	6.6
11	3,4-dihydroxyphenylacetic acid	F	0.000	0.0	0.000	0.0	0.000	0.0
		C	0.000	0.0	0.018	0.1	0.029	0.9
12	*trans*-cinnamic acid	F	0.000	0.0	0.053	0.3	0.086	2.7
		C	0.097	0.3	0.053	0.3	0.587	18.4
Total content			32.21±0.01[a]*	100%	17.51±0.01[b]*	100%	3.19±0.00*	100%
			Flavonoids					
13	Vitexin	F	0.001	0.1	0.001	0.2	0.002	0.3
		C	0.020	2.5	0.036	6.1	0.023	2.9
14	Rutin	F	0.001	0.1	0.001	0.1	0.002	0.3
		C	0.017	2.1	0.013	2.2	0.037	4.6
15	Epicatechin	F	0.001	0.1	0.001	0.1	0.005	0.6
		C	0.253	31.3	0.202	34.1	0.034	4.2
16	Luteolin	F	0.000	0.0	0.000	0.0	0.002	0.2
		C	0.015	1.9	0.004	0.7	0.138	17.1
17	Quercetin	F	0.023	2.9	0.002	0.4	0.009	1.1
		C	0.133	16.4	0.098	16.5	0.023	2.9
18	Apigenin	F	0.008	1.0	0.008	1.4	0.015	1.9
		C	0.121	14.9	0.153	25.8	0.135	16.8
19	Kaempferol	F	0.002	0.2	0.001	0.2	0.006	0.8
		C	0.192	23.7	0.036	6.0	0.161	20.0
20	Orientin	F	0.000	0.0	0.002	0.3	0.021	2.6
		C	0.022	2.7	0.035	5.9	0.191	23.7
Total content			0.81±0.00[a]*	100%	0.59±0.00[b]*	100%	0.81±0.00*	100%
Total phenolics content			33.02±0.01[a]*		18.10±0.01[b]*		4.00±0.00*	

*Data are expressed as mean±SD (n=3). Means in line related to a total content of phenolic acids, flavonoids and phenolics which characterized by the different letters are significantly different ($P<0.05$).
F – free forms of phenolic acids or flavonoids; C – conjugated forms of phenolic acids or flavonoids released from soluble esters and glycosides; dm – dry matter.

beetroot matrix or that the hydrolysis process might have not been sufficiently effective. In contrast, the fermentation process decreased the contribution of the conjugated forms of syringic and isoferulic acids by 48.28% and 32.73% of total phenolic acids, respectively. Moreover, no changes were observed in the conjugated form of *trans*-cinnamic acid. The proportion of the free phenolic acids in fermented red beet juice was 12.9% (0.41±0.00 μg/mL), while the proportion of the conjugated phenolic acids was 87.1% (2.78±0.00 μg/mL). In general, the obtained results indicate that the fermentation process caused an increase in the proportion of free phenolic acids and reduced the proportion of conjugated phenolic acids in the phenolic acids profile. The proportion of free and bound phenolic acids in the fresh red beetroot is consistent with the results obtained by Mattila & Hellström [2007]. In the cited study, phenolic acids in fresh and pickled red beetroot were found mainly in the bound form, however the pickling process caused an increase in the amount of free phenolic acids [Mattila & Hellström, 2007]. Furthermore, according to our results, lactic acid fermentation caused different changes in the contents of the free and bound forms of hydroxybenzoic, hydroxycinnamic, and phenylacetic acid derivatives. As presented above, the lactic acid fermentation caused not only an increase in the content of the free forms of hydroxybenzoic, hydroxycinnamic, and phenylacetic acid derivatives, but also a decrease or no changes in the case of some compounds in fermented red beetroot. Similar observations were made for the conjugated forms. The same process not only reduced the content of the conjugated form of certain compounds but also, in some cases, caused an increase or did not show any effect on the content of other compounds. The results obtained in our study are in line with those obtained by Manach *et al.* [2004]. The cited authors reported that hydroxycinnamic acids frequently occurred in the form of simple esters with quinic acid or glucose in fruits and vegetables. In contrast, hydroxybenzoic acid derivatives have been shown to occur mainly in the form of glucosides, which reduces their availability to organisms. Fermentation is one of the food processes that increases the release of the conjugated phenolics, which was observed in our study [Shrestha *et al*., 2010; Hur *et al*., 2014; Huynh *et al.,* 2014]. Research carried out by Değirmencioğlu *et al.* [2016] showed that microorganisms involved in the fermentation process had the ability to breakdown the food matrix, thereby releasing associated phytochemicals, which agrees with our data. However, the increased content of free phenolic acids may be related to β-glucosidase activity, an enzyme produced by microbial starter cultures that are used in the fermentation process. β-Glucosidase can release phenolic acids that are conjugated to sugars and glycosides by hydroxyl groups bound to cell-wall structural components. Breaking these bonds contributes to the release of conjugated phenolic acids by loosening the cell wall and thus increases the availability of phenolic acids in red beet products [Acosta-Estrada *et al*., 2014].

In the present study, the fresh roots were characterized by a statistically significantly ($P<0.05$) higher content of phenolic acids compared to the fermented roots (Table 2). The fermentation process of red beetroots led to a reduction in the total phenolic acids content by approximately 45.6%. Moreover, the total concentration of the phenolic acids in fermented beetroot juice was 3.19±0.00 μg/mL. There are few available data on the total content of phenolic acids in fresh and fermented red beetroot. The results obtained in our study are consistent with these reported by Guldiken *et al*. [2016], according to which, the content of total phenolic acids in pickled red beetroot was 25% lower than that of fresh beetroot. According to Svensson *et al.* [2010], the reduction in the total content of phenolic acids after the fermentation process can be attributed to the activity of the phenolic acids' decarboxylases and reductases. Interestingly, Mattila & Hellström [2007] observed that the pickling process did not seem to destroy phenolic acids in the product of red beetroots. However, in the case of fermented beetroot juice, the study carried out by Değirmencioğlu *et al*. [2016] showed that the total concentration of phenolic acids ranged from 34.35 to 59.91 μg/mL, depending on the strain used for fermentation.

Another group of phenolic compounds identified in the red beet products were flavonoids, including vitexin, rutin, epicatechin, luteolin, quercetin, apigenin, kaempferol, and orientin (Table 1). Previously published data have shown the presence of flavonoids in red beetroot products. Georgiev *et al*. [2010] identified two flavonoids (catechin hydrate and epicatechin) in extracts from red beetroots. In contrast, Ben Haj Koubaier *et al*. [2014] showed that the stems of red beet contained four flavonoids (myricetin, quercetin, kaempferol and rutin), while no rutin was identified in the roots of red beetroot. In contrast, Székely *et al*. [2014] did not identify any flavonoids in the roots of different red beet cultivars. However, Rembiałkowska *et al*. [2011] identified six flavonoids (rutin, quercetin glycoside, quercetin, kaempferol, myricetin, and luteolin) in pickled red beetroot. Moreover, in the case of fermented red beetroot juice, Değirmencioğlu *et al*. [2016] identified ten flavonoids (myricetin, quercetin, kaempferol, rutin hydrate, naringenin, hesperidine, catechin, epicatechin, and epigallocatechin). Interestingly, completely different research results were obtained by Wruss *et al*. [2015]. They did not detect any flavonoids in juices prepared from seven beetroot varieties. The authors explain the lack of identified flavonoids in red beetroot juices by the low concentration of these compounds in the juices obtained compared to their concentration found in extracts prepared from dry matter samples. The different numbers of detected phenolics may be due to the use of different parts of the roots and may also depend on the species, variety, degree of maturity of the raw material, harvest period and storage conditions, as well as the vegetation season conditions [Wiczkowski *et al*., 2014; Sawicki *et al.,* 2016].

In the samples of fresh and fermented red beets we tested, the dominant flavonoid was epicatechin (34.4% and 34.1% of the total flavonoids content, respectively) (Table 2). Its content in fresh red beetroot was 0.254 μg/g dm, while in fermented beetroot it was 0.203 μg/g dm. The second major compound among the flavonoids in the fresh roots analyzed was kaempferol (0.194 μg/g dm), which accounted for 23.9% of the total flavonoids. In the fresh red beetroot, we detected a much higher content of quercetin (0.156 μg/g dm) and apigenin (0.129 μg/g dm) than in the fermented beetroot, which accounted for 19.3% and 15.9% of total flavonoids, respective-

ly. The fresh red beetroot had also the lowest content of luteolin (0.015 μg/g dm). In addition, in the fermented red beetroot, apigenin (0.161 μg/g dm) was the second major compound, followed by quercetin (0.100 μg/g dm). A much lower content, compared to that of luteolin, apigenin, and quercetin in fermented red beetroot, was detected for vitexin (0.037 μg/g dm), kaempferol (0.037 μg/g dm), orientin (0.037 μg/g dm), and rutin (0.014 μg/g dm). Likewise in the fresh roots, luteolin also occurred in the lowest amount (0.004 μg/g dm) in the fermented roots. In contrast with the fresh and fermented red beetroots, the main flavonoid analyzed in the fermented red beetroot juice was orientin. The total concentration of orientin was 0.212 μg/mL (26.3% of the total flavonoids in the fermented red beetroot juice). In addition, kaempferol (0.167 μg/mL) was the second major compound, followed by apigenin (0.150 μg/mL) and luteolin (0.140 μg/mL). Lower concentrations of rutin (0.039 μg/mL), epicatechin (0.039 μg/mL), and quercetin (0.032 μg/mL) were determined in red beet juice, with the lowest concentration being that of vitexin (0.025 μg/mL). Our data differ from those reported in the literature. For instance, Georgiev *et al*. [2010] reported catechin hydrate to be the major flavonoid in extracts from red beetroot, whereas Ben Haj Koubaier *et al*. [2014] showed that kaempferol was the dominant flavonoid in the roots of red beet, while quercetin in the stems. Moreover, research carried out by Kazimierczak *et al*. [2014] showed that quercetin was the main flavonoid that occurred in fresh red beetroot. In the case of fermented beet juice, the results are also variable. Research carried out by Kazimierczak *et al*. [2014] showed quercetin-3-*O*-glucoside to be the major flavonoid of fermented beetroot juice, while Değirmencioğlu *et al*. [2016] found catechin and myricetin to be the dominant flavonoids in beetroot juice fermented by *Saccharomyces cerevisiae* and *Saccharomyces boulardii*.

The fermentation process caused a reduction in the total content of free form of flavonoids and an increase in their conjugated form. The total content of free flavonoids in fresh red beet was at 0.04±0.00 μg/g dm (4.4% of the total flavonoids content), while after the fermentation process it decreased to 0.02±0.00 μg/g dm (2.7% of the total flavonoids content). In contrast, the total content of conjugated flavonoids in fresh red beet was at 0.77±0.00 μg/g dm (95.6% of the total flavonoids content), while after the fermentation process it was 0.57±0.00 μg/g dm (97.3% of the total flavonoids content). However, the same process not only reduced the content of the free form of certain compounds but also, in some cases, caused a decrease or did not show any effect on the content of other compounds. As presented in Table 2, the application of the fermentation process caused a decrease in the content of the free form of quercetin, and an increase in the content of the free form of apigenin. In addition, no changes were observed in the contents of free epicatechin, luteolin, or kaempferol. It was also found that the fermentation process increased the concentration of vitexin and orientin while caused no changes in rutin concentration. For the conjugated forms of flavonoids, the fermentation process caused an increase in the content of rutin, epicatechin, and quercetin. At the same time, it decreased contents of the conjugated form of luteolin and kaempferol. In the case of the fermented red beetroot juice, the proportion of free flavonoids was 7.8% (0.06±0.00 μg/mL), while the proportion of conjugated flavonoids was 92.2% (0.75±0.00 μg/mL). The results obtained show that fresh and fermented beetroot can be a good source of flavonoids in an everyday diet. In contrast to phenolic acids, the application of the fermentation process decreased the amount of free flavonoids. Likewise for phenolic acids, the fermentation process may affect the ester and glycosidic bonds and thus can contribute to changes in the content of flavonoids by releasing them from being bound to the insoluble cell wall material [Svensson *et al*., 2010]. It may be surprising that the conjugated forms of rutin, vitexin, and orientin were found in fermented beet products, as they ought to be hydrolyzed in the fermentation process. This might be explained by the fact that C-glucosides of phenolics do not undergo hydrolysis during fermentation. In the case of rutin, similarly to chlorogenic acid, its presence in the analyzed material after hydrolysis may be associated with the loosening of the red beetroot matrix and releasing this compound from larger structures it was incorporated into. Changes in the profile of the flavonoids during fermentation are mainly due to the activity of enzymes produced by a huge variety of microflora, often uncharacterized. As a result of the activity of the cellulolytic, ligninolytic, and pectinolytic enzymes, the cell walls of the food matrix can be completely ruptured by the hydrolysis of ester bonds, resulting in oxidative degradation of the matrix and release of the bound flavonoids [Huynh *et al*., 2014]. However, it should be noted that the fermentation process does not always result in an increase in phytochemicals content due to the loosening of the food matrix, but can also cause a decrease in content of certain components. Phytochemicals could be metabolized (*e.g*., deglycosylated) into other low molecular forms [Aura *et al*., 2002]. In general, the potential of lactic acid bacteria to metabolize secondary plant compounds remains unknown. Therefore, further research in this field is needed to elucidate the microbiological pathways for phytochemical conversion, metabolite identification, and biological activity determination.

As in the case of phenolic acids, the richest source of flavonoids turned out to be the fresh roots, which contained 0.81±0.00 μg flavonoids/g dm. A lower by approximately 27.2% total flavonoids content was observed in the fermented red beetroot. However, the commercial fermented beetroot juice contained 0.81±0.00 μg flavonoids/mL. As with the content of phenolic acids, our data for flavonoids differed from those available in the literature, which may be related to the fact that the authors of the publications have expressed the results per beetroot dry extracts [Georgiev *et al*., 2010; Čanadanović-Brunet *et al*., 2011; Ben Haj Koubaier *et al*., 2014]. On the other hand, Kazimierczak *et al.* [2014] compared the profile of flavonoids in fermented beetroot juices from organic *versus* conventional production. Interestingly, they did not notice any significant differences in the content of flavonoids between the studied materials. As mentioned for phenolic acids, the differences in the total content of flavonoid compounds between results obtained in this study and the results from the cited papers may have been due to the use of different beetroot varieties. The impact of the variety, as well as the growing conditions, growing season, and the method

and length of storage on the level of various phytochemicals in vegetables was demonstrated in earlier works [Wiczkowski & Piskuła, 2004; Sawicki & Wiczkowski, 2018]. Moreover, the type of microorganisms used in the fermentation process as well as its conditions and duration could play a key role in the total content of flavonoids [Shrestha *et al.,* 2010].

Total contents of phenolic acids and flavonoids in red beetroot products

Based on the results of HPLC-MS/MS analysis, the fresh roots were shown to be the richest source of phenolics. The total phenolics content in fresh red beet was 33.02±0.01 μg/g dm, while in the fermented beetroot, it was 18.10±0.01 μg/g dm. In contrast, in commercial red beet juice it was as low as 4.00±0.00 μg/mL. Our data differ from those available in the literature [Vulić *et al*., 2014; Ben Haj Koubaier *et al*., 2014; Wruss *et al*., 2015; Guiné *et al*., 2018]. It is important to note, however, that the most common method for determining total phenolics content is the Folin-Ciocalteau method rather than the HPLC-MS method, which is not an absolute measurement of the amount of phenolics in a tested sample. Some substances contained in the sample studied, including organic acids, residual sugars, amino acids, proteins and other hydrophilic compounds, interfere with this test. Thus, the results achieved for the samples tested can differ significantly, which was also the case in the studies presented above. Therefore, detailed identification and quantification of phenolic compounds are essential.

Moreover, it should be noted that the 14-day spontaneous fermentation process resulted in a significant reduction of the total content of phenolics by 45.18%. Results of our research are consistent with the findings reported by Hunaefi *et al.* [2013], who observed that the total phenolics content increased slightly until day seven of the fermentation process and successively decreased for a longer time of fermentation. Hunaefi *et al*. [2013] and Rodríguez *et al.* [2008] explain that the reduction in total phenolics content may be due to an increase in the amounts of lactic acid bacteria. There is evidence that lactic acid bacteria are involved in the metabolism of dietary phenolics, *i.e.* their cause their degradation and thus positively affect the final product, imparting it a characteristic taste, aroma, and texture.

In the case of fermented beet juice, it is important to pay attention to the food processing, which usually consists of the pre-treatment, including cleaning, rinsing and slicing, and the basic treatment involving thermal food processing. Treatments applied in the food processing are aimed at eliminating unwanted impurities, microbes, non-nutrients, inactivating enzymes; and also at increasing the digestibility and availability of nutrients; at improving the structure and consistency of a food product; and finally at ensuring its appropriate organoleptic characteristics. On the other hand, these treatments can have a destructive effect on health-promoting ingredients, causing their losses at every stage of processing [Ravichandran *et al*., 2013; Wiczkowski *et al.*, 2015].

Content of phenolic acids and flavonoids in blood plasma and urine of volunteers consuming fermented beetroot juice

To the best of our knowledge, this is the first study that has characterized the profile of phenolic acids and flavonoids in human body fluids after long-term and regular consumption of red beetroot juice. Sixteen compounds, including four hydroxybenzoic acid derivatives (protocatechuic, *m*-hydroxybenzoic, syringic, and *p*-hydroxybenzoic acids), six hydroxycinnamic acid derivatives (chlorogenic, caffeic, sinapic, ferulic, *p*-coumaric, and *trans*-cinnamic acids), one phenylacetic acid derivative (3,4-dihydroxyhydrophenylacetic acid), and five flavonoids (epicatechin, luteolin, quercetin, apigenin and kaempferol) were identified in the blood plasma and urine samples before and after the intake of fermented red beetroot juice (Table 1). All the identified compounds were also detected in the material tested (red beet juice). However, isoferulic acid, vitexin, rutin, and orientin, which were found in the consumed commercial fermented red beet juice, were not identified in the samples of human body fluids.

Before and after regular consumption of fermented beetroot juice, phenolic acids and flavonoids appeared in blood plasma and urine throughout the forty-two days of the experiment (Figure 1). The total concentration of phenolic acids in the volunteers' blood plasma before drinking fermented beetroot juice was 6.68±0.59 μg/mL and increased to 10.84±0.92 μg/mL after seven days of regular juice intake. Afterwards, it decreased slightly to 6.51±0.67 μg/mL after

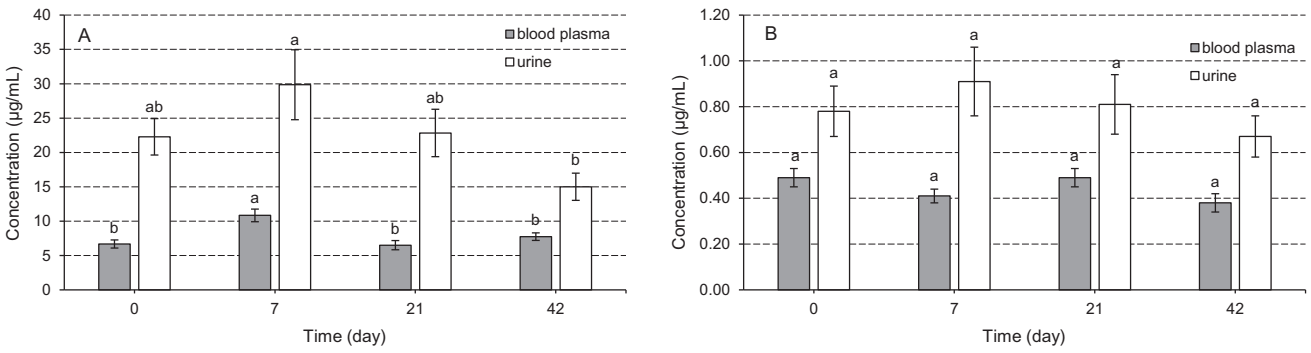

FIGURE 1. Concentration of phenolic acids (A) and flavonoids (B) in blood plasma and urine of volunteers after the intake of fermented beetroot juice for a period of 42 days.

Values are mean±SD (n=24). Means related to a total content of phenolic acids or flavonoids marked by different letter above bars are significantly different (P<0.05).

twenty-one days and then increased again to 7.75±0.55 μg/mL after forty-two days of regular intake of the juice. An increase in the total concentration of phenolic acids ($P<0.05$) was noted as soon as after seven days of regular consumption of fermented beetroot juice. Differences in the total concentration of flavonoids in blood plasma were not found to be statistically significant ($P\geq0.05$) before (0.49±0.04 μg/mL) and after seven (0.41±0.03 μg/mL), twenty-one (0.49±0.04 μg/mL), and forty-two (0.38±0.04 μg/mL) days of regular consumption of juice from red beetroot. Furthermore, urine analysis of the volunteers before the intake of fermented beetroot showed that the total concentration of phenolic acids was at 22.28±2.65 μg/mL. After forty-two days of consumption of fermented beetroot juice, as in the case of blood plasma, the total concentration of phenolic acids in the urine did not change statistically significantly ($P\geq0.05$). Regular intake of fermented beetroot juice for seven days has resulted in a slight increase in the total concentration of phenolic acid. Total urinary excretion of flavonoids has not changed statistically ($P\geq0.05$) throughout the entire forty-two day long period of fermented beetroot juice intake. Before starting the regular consumption of fermented beetroot juice, the total concentration of flavonoids in the urine of volunteers was at 0.78±0.11 μg/mL. After seven days, it increased to 0.91±0.15 μg/mL, and afterwards decreased to 0.81±0.13 μg/mL after twenty-one days and to 0.67±0.09 μg/mL after forty-two days. It was found that after forty-two days of consumption of fermented beetroot juice by the volunteers, the total plasma and urine concentrations of phenolic acids and flavonoids did not change compared to those determined in volunteers' fluids before starting the regular consumption of juice. To the best of our knowledge, limited data are available on the bioavailability of phenolics after long-term ingestion.

The factor affecting the bioavailability of phenolics is the nature of the food matrix itself. Phenolics can react with food ingredients, *e.g*., some proteins and carbohydrates, which can significantly affect their absorption from digestion. Other important factors are the intestinal environment, such as the pH or the presence of bile salts [Manach *et al.,* 2004]. Interindividual variability, and more specifically, differences in the intestinal microflora of volunteers, may affect the bioavailability of phenolic acids and flavonoids as well. Research conducted by Aura *et al*. [2002], van't Slot & Humpf [2009], and Gonthier *et al*. [2003] showed a successive postprandial appearance of microbial phenolic metabolites in plasma after 0.5–4 h. Moreover, a study conducted by Feliciano *et al.* [2016] showed ingestion of foods rich in polyphenols to result in an increase in the concentration of phenolic acids in plasma compared to that on the first day of the intervention. According to the aforementioned reports, flavonoids are degraded during digestive processes, resulting in phenolic acids formation and thus decreasing the concentration of flavonoids. Unfortunately, we did not notice this correlation in our study. Additionally, the bioavailability of phenolic compounds may be limited due to the interactions with their transporters. In addition, we cannot exclude the possibility of phenolic acids and flavonoids distribution and accumulation in other tissues. Another explanation for such an insignificant variation in plasma levels of phenolic acids and flavonoids is that although the diets were controlled, the subjects followed the dietary instructions well (exclusion of foods rich in betalains) but did not eat exactly the same amounts of polyphenols-rich fruits and vegetables. Interestingly, the research conducted by Moon *et al*. [2000] showed that the long-term consumption of foods rich in flavonoids did not cause the accumulation of significant amounts of flavonoids in the plasma. It should be noted, however, that despite a number of studies that have been carried out, there is not enough data on phenolic acids and flavonoids in human tissues. Previous studies have indicated that despite the low absorption of phenolic acids and flavonoids, these compounds are extensively metabolized in the gut and liver [Marín *et al*., 2015]. A number of chemical processes change the physical properties of phenolics, making them more soluble in water. Moreover, the observed insignificant variability of the urinary excretion of these compounds after forty-two days of consumption of fermented beetroot juice suggests that the majority of the phenolic acids and flavonoids supplied with juice have been metabolized by the intestinal microflora to smaller low molecular weight metabolites. Generally, phenolic acids and flavonoids are rapidly excreted with urine, indicating that the daily consumption of plant products is necessary to maintain high blood metabolite levels. All things considered, it should be noted that the bioavailability of plant phenolic acids and flavonoids may be affected by internal factors (including age, sex, ethnicity, disease and genetic polymorphisms) and external factors (including doses, food matrix, and eating habits).

Summarizing, our results showed that forty-two days of consumption of a commercial fermented beetroot juice did not affect the concentration of phenolic acids and flavonoids in plasma and urine of the volunteers. In turn, Sawicki *et al.* [2018] observed that the long-term and regular consumption of the red beet juice caused stabilization of both the profile and content of betalains in the physiological fluids of volunteers. Irregular changes in the phenolic acid and flavonoid profiles in human plasma and urine, in contrast to the profile and content of betalains, may be due to the low concentration of phenolic acids (3.19±0.00 μg/mL) and flavonoids (0.81±0.00 μg/mL) (Table 1) and a high concentration of betalains (0.21±0.00 g/mL) in beetroot juice [Sawicki *et al.,* 2018]. In addition to beetroot juice, volunteers consumed a number of foods that are rich sources of phenolic acids and flavonoids. Taking into consideration that the estimated daily intake of phenolic compounds by the Polish population was 1756.5±695.8 mg/day [Grosso *et al*., 2014], it seems difficult to assume that such a small contribution to the daily intake of phenolic compounds as 200 mL of beetroot juice (*i.e.* 0.8 mg, being not even 0.1% of daily intake) will significantly affect levels of metabolites in human fluids.

CONCLUSIONS

To the best of our knowledge, this is the first time when the effects of fermentation on the profile and content of red beet phenolic acids and flavonoids have been determined as well as when the impact of the long-term and regular consumption of this vegetable on the profile and content of phenolics in plasma and urine of volunteers has been established.

Our research has shown that red beet and its products are notable source of phenolic acids and flavonoids. The analyses performed have revealed that isoferulic acid, protocatechuic acid, epicatechin, and apigenin predominated among the twenty compounds detected in beetroot and its products. Furthermore, the total content of phenolic acids and flavonoids differed significantly between the red beet products studied. The fermentation process simultaneously increased contents of free phenolic acids and conjugated flavonoids as well as decreased contents of conjugated phenolic acids and free flavonoids. However, our results showed that the long-term and regular consumption of fermented beetroot juice generally did not affect the concentration of phenolics in plasma and urine of the volunteers. From the physiological and nutritional points of view, information on the full profile of red beet phenolic acids and flavonoids is very important because it may help predict their biological activity. Moreover, further studies are now needed to determine how red beetroot phytochemicals, including phenolic acids and flavonoids, behave under the conditions of other food processing treatments and upon consumption of other red beet products (influence of the food matrix).

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CONFLICT OF INTERESTS

All authors declare no conflict of interests.

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Original article
Red Beetroot Thematic Issue

High-Speed Counter-Current Chromatography in Separation and Identification of Saponins from *Beta vulgaris* L. Cultivar Red Sphere

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Key words: saponins, *Beta vulgaris* L., high-speed counter-current chromatography

Saponins, natural plant compounds exhibiting health benefits, were extracted from *B. vulgaris* L. cultivar (cv.) Red Sphere and separated by high-speed counter-current chromatography (HSCCC) in a new solvent system composed of: TBME-BuOH-ACN-H_2O (1:2:1:5, *v/v/v/v*). The system was used in the head-to-tail mode. The flow rate of the mobile phase was 3 mL/min and the column rotation speed was 860 rpm. The retention of the stationary phase was 47%. The LC-MS/MS analyses were performed for the identification of separated saponins in the crude extract as well as HSCCC fractions,.

Significantly different elution orders of the analytes were observed in the HSCCC and HPLC systems, indicating the complementarity of both the techniques in the fractionation of saponins. Moreover, during the HSCCC separation, acetal-type saponins were eluted faster than pentose/hexose-type saponins and then dioxolane-type saponins. The separation of saponins in the HSCCC solvent system enabled the fractionation and preconcentration of 13 saponins for further fragmentation experiments in the LC-MS/MS system. Nine saponins were detected for the first time ever in *B. vulgaris* L. cv. Red Sphere.

INTRODUCTION

The widely described health benefits of red beet root (*Beta vulgaris* L.) have led to increased scientific and consumer interest in this vegetable [Chhikara *et al*., 2019; Clifford *et al*., 2015; Nemzer *et al*., 2011]. Red beet is rich in several bioactive compounds which can be useful in the treatment of many diseases, such as hypertension, atherosclerosis, type 2 diabetes, and dementia [Clifford *et al*., 2015; Nemzer *et al*., 2011].

Beet root is a rich source of highly bioactive saponins which are associated with haemolytic [Voutquenne *et al*., 2003]; molluscicidal [Huang *et al*., 2003]; anti-inflammatory [da Silva *et al*., 2002]; antifungal, anti-yeast activity, and antibacterial [de Lucca *et al*., 2002]; as well as with antiparasitic activity [Traore *et al*., 2000]. They exhibit also cytotoxic, antitumoric [González *et al*., 2003], and antiviral effects [Gosse *et al*., 2002].

Saponins are divided into two groups: steroidal saponins and triterpenoid saponins. Some authors distinguish a third group called steroidal amines or steroidal alkaloids [Bruneton, 1995]. Triterpenoid saponins are a common group of saponins consisting of a triterpenoid aglycone containing 30 carbon atoms and comprising a pentacyclic structure [Sparg *et al*., 2004]. Triterpene saponins are glycosides containing one or two sugar chains connected *via* an ester to C-28 or ether to C-3. According to the literature, hexoses, pentoses, 6-deoxyhexoses, and uronic acids are primary sugar units in triterpene saponins. Moreover, the uronic acid moiety is bonded only to C-3 [Mikołajczyk-Bator *et al*., 2016; Yoshikawa *et al*., 1996, 1998]. So far, oleanolic as well as hederagenin, akebonoic, and gypsogenin aglycones were identified in red beet roots [Mikołajczyk-Bator *et al*., 2016].

The profile of triterpenoid saponins in the roots of red beet cv. Red Sphere (*Beta vulgaris* L.) was previously studied using reversed liquid chromatography and mass spectrometry by Mroczek *et al.* [2012, 2019] and 13 saponins have been described wherein 11 compounds contained oleanolic acids aglycone. Another research on saponins in red beet root cv. Nochowski was that by Mikołajczyk-Bator *et al.* [2016], wherein 44 saponins were characterized, including 22 compounds that were described for the first time ever. Moreover, 27 saponins, which contained oleanolic acids aglycone, were identified using the LC-MS/MS/MS technique.

The structural characterisation of triterpenoid saponins in the roots of red beet is very difficult and time-consuming due to the complexity of the matrix [Mikołajczyk-Bator *et al*., 2016; Mroczek *et al*., 2012, 2019], therefore, fractionation of the crude samples enables faster and more accurate identification of significantly preconcentrated saponins [Thakur *et al*., 2014]. High-speed counter-current chromatography is a very important technique in the separation of natural plant compounds [Jerz *et al*., 2008, 2010; Spórna-Kucab

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et al., 2019; Wybraniec *et al*., 2009, 2010] which enables subsequent identification of new structures of target compounds [Choi *et al*., 2015; Figueiredo *et al*., 2017]. Moreover, HSCCC is perceived as a rapid and convenient technique for the isolation of saponins [Thakur *et al*., 2014]. Additionally, it is worth noting that during HSCCC separations, no loss of target compounds arising from their irreversible adsorption onto the solid matrix is observed, often taking place in the conventional liquid chromatography, because mobile and stationary phases are liquids [Spórna-Kucab *et al*., 2013a, 2015; 2016; 2018a,b].

In this study, a crude saponin mixture from *Beta vulgaris* L. cv. Red Sphere was separated for the first time by semi-preparative HSCCC in a new solvent system. The HSCCC technique enabled the identification of new saponins in *Beta vulgaris* L. cv. Red Sphere.

MATERIALS AND METHODS

Plant material and reagents

Red beet roots (*Beta vulgaris* L.) of cv. Red Sphere were purchased from a local market in Cracow in June 2019. The roots were grounded and forthwith extracted.

For the HSCCC experiments and for analytes extraction, HPLC-grade butanol (BuOH), *tert*-butylmethylether (TBME), acetonitrile (ACN), and ethanol (EtOH) were obtained from Avantor Performance Materials Poland S.A. (Gliwice, Poland). LC-MS grade acetonitrile and formic acid (purity ≥98%) were obtained from Sigma-Aldrich (St. Louis, United States).

Crude pigment extracts

Fresh and grounded in a blender (thermomix, Vorwerk, Wuppertal, Germany) roots from *Beta vulgaris* L. cv. Red Sphere (1.5 kg) were extracted by maceration three times for 1 h, using 1 L of 80% ethanol each time. The extract obtained was filtered and pre-concentrated by a rotary evaporator (Heidolph, Schwabach, Germany) at 25°C to 100 mL under reduced pressure. Then, the extract was loaded into an RP-C_{18} cartridge pre-conditioned with ethanol and water. The cartridge was washed with water and then with 10%, 20%, 50%, and 100% ethanol. The eluates were pre-concentrated by a rotary evaporator at 25°C and freeze-dried. The eluates obtained were monitored by LC-DAD-ESI-MS/MS. Finally, 196.6 mg of saponins were eluted with 50% ethanol and 115.4 mg of saponins with 100% ethanol.

HSCCC separation

The separation of saponins (Table 1) was accomplished on a semi-preparative AECS QuikPrep HSCCC *J*-type hydrodynamic chromatograph (London, United Kingdom) with 121 mL capacity and 2.0 mm i.d..

A new biphasic system consisting of TBME-BuOH-ACN-H_2O (1:2:1:5, *v/v/v/v*) was prepared for the HSCCC run. The solvent system was prepared in a separating funnel. Required volumes of solvents were mixed in a separating funnel. Then, the two phases for the HSCCC separation were divided shortly before use and degassed by ultrasonication for 10 min. The upper organic phase was used as the stationary phase and the lower aqueous phase as the mobile phase in the 'head-to-tail' mode. Per definition, this mode of separation is named reversed-phase comparable to C_{18}-HPLC also using the aqueous phase as the eluting solvent phase [Spórna-Kucab *et al*., 2019; Wybraniec *et al*., 2010].

The HSCCC column was entirely filled with the upper phase (stationary phase). The rotation of the HSCCC instrument was started at 860 rpm. Then, the lower aqueous phase (mobile phase) was pumped at a flow rate of 3.0 mL/min (K-501 pump, Knauer, Berlin, Germany).

After column equilibration, the sample solution was injected by an injection valve. This solution was prepared by dissolving 300 mg of the extract in 4 mL of the lower phase. The effluent from the column was continuously monitored with a UV-Vis detector at 210 nm (Knauer). Twelve fractions were collected in a fraction collector (Foxy Jr., Knauer) at 2-min intervals and then analysed by LC-DAD-ESI-MS/MS (Figures 1–3). The HSCCC fractions were weighed after their pre-concentration on a rotary evaporator at 25°C and freeze-drying.

LC-DAD-ESI-MS/MS analysis

The eluates from the RP-C_{18} cartridge as well as a crude extract (Figure 1) and the HSCCC fractions (Figures 2 and 3) were analysed by LC-DAD-ESI-MS/MS using an LCMS-8030 mass spectrometric system coupled to an LC-20ADXR pump with a gradient elution mode at 40°C in the acetonitrile (A) and 2% aqueous formic acid (B) system: 5% A in B at 0 min, a gradient to 60% A in B at 12 min, then 80% A in B at 15 min. The injection volume was 5 μL and the flow rate was 0.5 mL/min. LC-DAD-ESI-MS/MS analyses were conducted on a 100 mm × 4.6 mm I.D., 5.0 μm Kinetex C_{18} chromatographic column from Phenomenex (Torrance, United States).

The LC-MS/MS system was controlled with LabSolutions software (Shimadzu, Japan), which was operated in a negative mode, at electrospray voltage of 4.5 kV, capillary temp. of 250°C, and N_2 used as the sheath gas. Scan range was from *m/z* 100 to 2000. Argon was used to improve trapping efficiency and as the collision gas for CID experiments. The collision energy for MS analyses was set at 50 V.

RESULTS AND DISCUSSION

MS/MS analysis of saponins

The first study by Mroczek *et al.* [2012, 2019] on *B. vulgaris* L. cv. Red Sphere reported thirteen saponins with pentose and hexose substituents. Another research on saponins in *B. vulgaris* L. cv. Nochowski conducted by Mikołajczyk-Bator *et al.* [2016] revealed 27 acetal-, dioxolane- as well as pentose/hexose-type saponins.

In this study, the MS/MS analysis of the crude extract of *Beta vulgaris* L. (Figure 1) revealed the presence of thirteen acetal-, dioxolane- as well as pentose/hexose-type saponins. All of the identified saponins and their MS/MS data are listed in Table 1.

The saponins detected in our research are derivatives of oleanolic acid. The MS/MS data show the presence of five acetal- and two dioxolane-type substituents, which have been never described in *B. vulgaris* L. cv. Red Sphere. These types

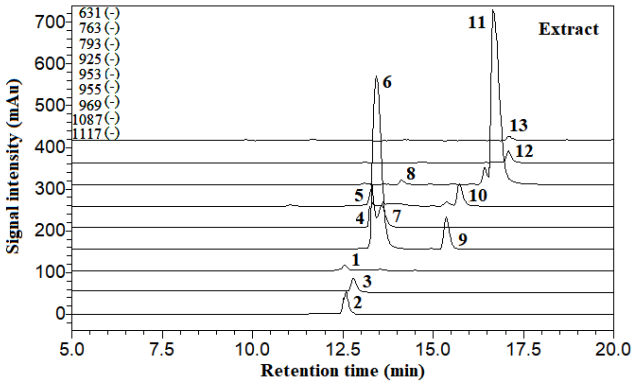

FIGURE 1. ESI-MS chromatogram of saponins from *B. vulgaris* L. cultivar Red Sphere extract.

of compounds were found by Mikołajczyk-Bator [2016] in *B. vulgaris* L. cv. Nochowski.

Fragmentation of saponins with oleanolic acid as the aglycone leads to yielding the daughter ion $[M-H]^-$ at *m/z* 631 which corresponds to hexuronic acid-oleanolic acid. The simplest saponin with the pseudomolecular ion $[M-H]^-$ at *m/z* 631 (peak **13** with t_R = 17.1 min) was identified in the studied *B. vulgaris* L. extract. As expected, this compound (**13,** HexUA-oleanolic acid) fragmented to *m/z* 455 which corresponded to oleanolic acid. HexUA-oleanolic acid was previously identified in the *B. vulgaris* L. cv. Red Sphere [Mroczek *et al*., 2012, 2019].

The peak **12** (t_R = 17.1 min) corresponded to the compound which exhibited the pseudomolecular ion $[M-H]^-$ at *m/z* 763. The fragmentation of this compound (**12)** yielded daughter ions at *m/z* 631 and 455 because of the losses of pentose (132 Da) and hexuronic acid (176 Da), and therefore compound **12** was identified as Pen-HexUA-oleanolic acid. Compound **12** was previously identified in the *B. vulgaris* L. cultivars Red Sphere and Nochowski [Mikołajczyk-Bator *et al*., 2016; Mroczek *et al*., 2012, 2019].

Peaks **8** (t_R = 14.1 min) and **11** (t_R = 16.7 min) corresponded to compounds with the identical pseudomolecular ions $[M-H]^-$ at *m/z* 793 and aglycone ions at *m/z* 455 which correspond to oleanolic acid. The fragmentation of the pseudomolecular ion $[M-H]^-$ at *m/z* 793 for compound **8** yielded daughter ions at *m/z* 631 and 455 because of the losses of 162 Da and 176 Da, confirming the presence of hexose and hexuronic acid. Finally, compound **8** was identified as Hex-HexUA-oleanolic acid. However, the fragmentation of compound **11** with the same pseudomolecular ion $[M-H]^-$ at *m/z* 793 resulted in the formation of daughter ions at *m/z* 673 and 631, indicating the loss of 120 Da and 162 Da characteristic for the acetal-type substituent; and this compound can be assigned to Act-HexUA-oleanolic acid. The research of saponins profile in the *B. vulgaris* L. cv. Red Sphere did not reveal the presence of compound **11** [Mroczek *et al*., 2012, 2019] but this compound was previously observed in the *B. vulgaris* L. cv. Nochowski [Mikołajczyk-Bator *et al*., 2016]. The structure of compound **11** was determined by Yoshikawa *et al*. [1996] who named this saponin betavulgaroside IV.

The identical pseudomolecular ions $[M-H]^-$ at *m/z* 925 were ascribed to peaks **5** (t_R = 13.3 min) and **10** (t_R = 15.8 min). These saponins consisted of the same aglycone ion at *m/z* 455, corresponding to oleanolic acid. Moreover, the daughter ions at *m/z* 631 and 793 (losses of pentose (132 Da) and hexose (162 Da), respectively) were observed for both saponins. The compounds **5** and **10** (Pen-Hex-HexUA-oleanolic acids) were previously identified in *B. vulgaris* L. cv. Red Sphere [Mroczek *et al*., 2012, 2019].

Peaks **4** (t_R = 13.3 min) and **7** (t_R = 13.6 min) corresponded to compounds which revealed pseudomolecular ion $[M-H]^-$

TABLE 1. Saponins tentatively identified by negative ion ESI-MS/MS in *B. vulgaris* L. cultivar Red Sphere.

No.	Saponin structure	t_R [min]	*m/z* $[M-H]^-$	*m/z* from MS/MS of $[M-H]^-$
1	HexUA-Hex-HexUA-oleanolic acid*	12.5	969	unknown
2	Act-Hex-Hex-HexUA-oleanolic acid	12.5	1117	997;955;835;793;631;455
3	Act-Hex-Pen-HexUA-oleanolic acid	12.8	1087	967;925;763;631;455
4	Diox-Hex-HexUA-oleanolic acid	13.3	953	909;793;631;455
5	Pen-Hex-HexUA-oleanolic acid	13.3	925	793;631;455
6	Act-Hex-HexUA-oleanolic acid	13.5	955	835;793;673;631;455
7	Diox-Hex-HexUA-oleanolic acid	13.6	953	909;793;631;455
8	Hex-HexUA-oleanolic acid	14.1	793	631;455
9	Act-Hex-HexUA-oleanolic acid	15.4	955	835;793;673;631;455
10	Pen-Hex-HexUA-oleanolic acid	15.8	925	793;631;455
11	Act-HexUA-oleanolic acid	16.7	793	673;631;455
12	Pen-HexUA-oleanolic acid	17.1	763	631;455
13	HexUA-oleanolic acid	17.1	631	455

*proposed structure Hex – hexose; Pen – pentose; HexUA – hexuronic acid; Act – acetal substituent; Diox – dioxolane substituent.

TABLE 2. Saponin distribution in the recovered HSCCC fractions obtained from *B. vulgaris* L. cultivar Red Sphere extract.

No.	*m/z* [M-H]$^-$	Relative content of pigments (%)*												Total HPLC peak area ($\times 10^{-5}$)
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
1	969								60.7	26.4	12.9			0.9
2	1117				80.3	19.7								37.8
3	1087			86.7	13.3									33.8
4	953						71.7	28.3						30.1
5	925							30.0	41.0	29.0				20.2
6	955			0.8	78.0	21.2								294.8
7	953						54.6	45.4						36.6
8	793										14.3	71.4	14.3	7.0
9	955	10.0	63.3	26.7										53.1
10	925				59.4	24.2	16.4							44.4
11	793	8.0	68.0	11.0	13.0									104.6
12	763		12.0	13.2	18.0	48.1	8.7							19.4
13	631					12.6	12.9	74.5						3.5
Fraction mass (mg)		30.3	94.2	55.2	31.2	30.9	6.0	6.6	3.0	1.5	0.9	0.9	0.6	

*analysed by LC-MS.

at *m/z* 953 and fragment ions at *m/z* 909, 793, 631, and 455. These compounds were characterised for the first time in *B. vulgaris* L. cv. Red Sphere. The fragmentation of the [M-H]$^-$ primarily yielded daughter ions at *m/z* 909 and 793 because of the loss of CO_2 and a dioxolane-type substituent, respectively. The MS/MS data exhibited additionally daughter ions at *m/z* 631 and 455, being characteristic for hexuronic acid-oleanolic acid and oleanolic acid. These two compounds were identified as Diox-Hex-HexUA-oleanolic acids. It is worth noting that saponins with dioxolane-type substituents were detected in *B. vulgaris* L. cv. Red Sphere for the first time ever, but this type of the structure was determined by Yoshikawa *et al*. [1996] who named it betavulgaroside I. The compounds **6** and **9** with t_R 13.5 and 15.4 min, respectively, exhibited identical [M-H]$^-$ ions at *m/z* 955, which corresponded to acetal-type substituent saponins because of the loss of 120 Da (daughter ion at *m/z* 835) and 162 Da (daughter ion at *m/z* 793) during fragmentation. The daughter ion at *m/z* 835 was not noticed during the fragmentation of compounds **6** and **9** identified by Mroczek *et al*. [2019] in *B. vulgaris* L. cv. Red Sphere. These acetal-saponins (compounds **6** and **9**) were also identified by Mikołajczyk-Bator *et al*. [2016] in *B. vulgaris* L. cv. Nochowski. Based on the MS/MS fragmentation of compound **6** and **9** as well as previous MS/MS data [Mikołajczyk-Bator *et al*., 2016], it can be deduced that these molecules consisted of aglycone ion at *m/z* 455 – oleanolic acid as well as acetal-type substituent, hexose and hexuronic acid. Taking into account the above elucidations, compounds **6** and **9** were designated as Act-Hex-HexUA-oleanolic acid.

Peak **1** (t_R = 12.5 min) corresponding to the compound revealing the pseudomolecular ion [M-H]$^-$ at *m/z* 969 was tentatively identified as hexuronic acid-hexose-hexuronic acid derivative of oleanolic acid (HexUA-Hex-HexUA-oleanolic acids). The concentration of compound **1** was not sufficient to identify daughter ions. Mikołajczyk-Bator *et al.* [2016] identified presumably identical saponin in *B. vulgaris* L. cv. Nochowski. The elution of compound **1** in further HSCCC fractions indicates that it is not an acetal-type saponin, because such saponins are eluted in early fractions.

The compound **3** (t_R = 12.8 min) was characterised by the pseudomolecular ion [M-H]$^-$ at *m/z* 1087 and fragment ions at *m/z* 967, 925, 763, 631, and 455. The fragmentation of compound **3** resulted in the formation of primary daughter ions at *m/z* 967 and 925 because of the loss of 120 Da and 162 Da (cleavage and loss of the acetyl-type substituent, respectively). The presence of the daughter ions at *m/z* 763 and 631 indicated the loss of the hexose and pen-

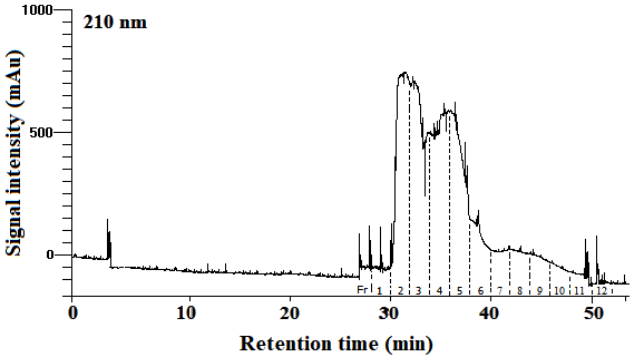

FIGURE 2. HSCCC chromatogram of *B. vulgaris* L. cultivar Red Sphere extract (300 mg) separated into 12 fractions in a solvent system: TBME-BuOH-ACN-H2O (1:2:1:5, *v/v/v/v*) at a flow rate of 3.0 mL/min in the head-to-tail mode; velocity 860 rpm; optical detection at a wavelength of 210 nm.

tose from the structure of compound **3**. Finally, this compound was identified as Act-Hex-Pen-HexUA-oleanolic acid. Compound **3**, named as betavulgaroside IX, was identified for the first time by Yoshikawa *et al*. [1996]. Betavulgaroside IX was only identified in Nochowski cv. by Mikołajczyk-Bator *et al*. [2016].

The MS spectra of the compound t_R = 12.5 min the pseudomolecular ion $[M-H]^-$ at *m/z* 1117 which fragmented into ions at *m/z* 997, 955, 835, 793, 631, and 455, indicated the presence of saponin with acetal-type substituent. The fragmentation of this compound (**2**) resulted in the formation of primary daughter ions at *m/z* 997 and 955 (losses of 120 and 162 Da) corresponding to the cleavage and loss of the acetal-type substituent, respectively. The presence of the daughter ions at *m/z* 793 and 631 indicated the loss of the two hexoses from the structure of compound **2**. The daughter ions at *m/z* 631 and 455 confirmed the presence of hexuronic acid--oleanolic acid and oleanolic acid, respectively. Taking into account the above elucidations, compound **2** was identified as Act-Hex-Hex-HexUA-oleanolic acid. The compound **2** has been never identified in *B. vulgaris* L. cv. Red Sphere but was identified in Nochowski cv. by Mikołajczyk-Bator *et al*. [2016]. Moreover, compound **2** was thoroughly described using NMR by Yoshikawa *et al*. [1996] who named it betavulgaroside V.

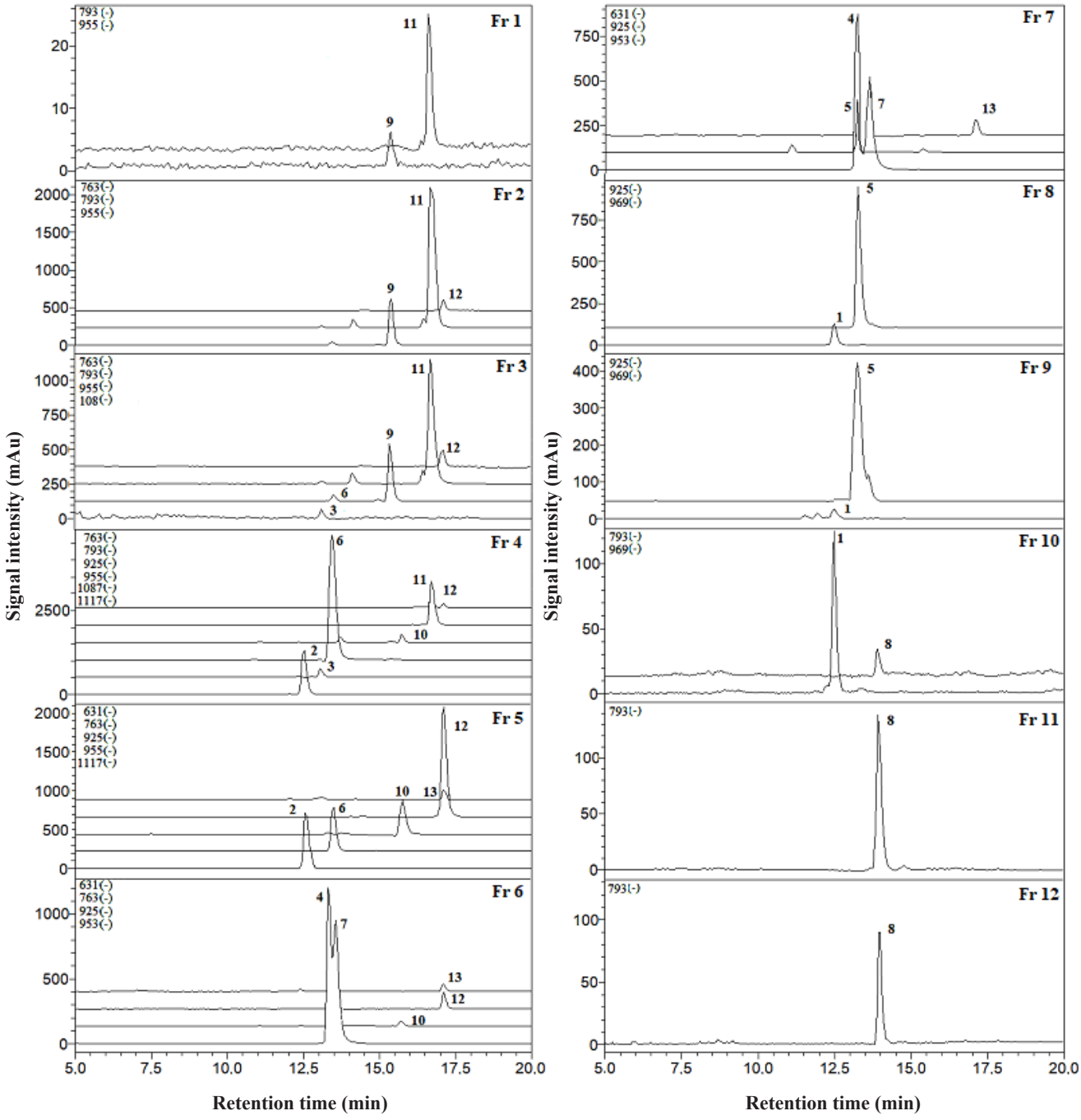

FIGURE 3. ESI-MS chromatograms of saponins analysed in the fractions (1–12) separated from the extract of *B. vulgaris* L. cultivar Red Sphere by HSCCC.

HSCCC separation of saponins

The crude extract (300 mg) was fractionated by the HSCCC technique and 12 fractions were collected and analysed by LC-DAD-ESI-MS/MS analysis (Table 2, Figures 1–3).

The HSCCC separation monitored at 210 nm (Figure 2) was realized in a new solvent system TBME-BuOH-ACN-H_2O (1:2:1:5, *v/v/v/v*) in a head-to-tail mode which corresponds to the reversed-phase mode in HPLC. Saponins were previously separated by HSCCC technique in a solvent system TBME-BuOH-ACN-H_2O (1:3:1:5, *v/v/v/v*) showing retention of the stationary phase amounting to 35% [Thakur *et al*., 2014], therefore, a new solvent system with higher retention was prepared. After the separation in the new solvent system, the retaining amount of stationary phase in the coil-system was calculated to be 47%. Elution order and separation effectiveness of saponins in the HSCCC and the C_{18} reversed--phase HPLC were compared in order to demonstrate the complementarity of these techniques.

The most polar HexUA-Hex-HexUA-oleanolic acid (**1**) with the pseudomolecular ion [M-H]- at m/z 969 was eluted in fractions 8–10 and it was completely separated from Act-Hex--Hex-HexUA-oleanolic acid (**2)** with the pseudomolecular ion at *m/z* 1117. Separation of compounds **1** and **2** was not possible in the HPLC system applied due to their identical retention times, therefore, HSCCC allowed for their complete separation.

Likewise, compounds Act-Hex-Hex-HexUA-oleanolic acid (**2**) and Act-Hex-Pen-HexUA-oleanolic acid (**3**) differed by their elution profiles in the HPLC and HSCCC technique. Compound **2** ([M-H]$^-$ at *m/z* 1117) and compound **3** ([M-H]$^-$ at *m/z* 1087) differed by one sugar moiety. Compound **2** with hexose in the structure as eluted later in the HSCCC than compound **3** with pentose. Different elution profiles of compounds **2** and **3** in the HPLC and the HSCCC mad their complete separation feasible.

Similar structures for Diox-Hex-HexUA-oleanolic acid (**4**) ([M-H]- at m/z 953) and Pen-Hex-HexUA-oleanolic acid (**5**) ([M-H]$^-$ at *m/z* 925) presumably contributed to their co-elution in the HPLC system. Compound **4** consisted of dioxolane-type substituent, whereas compound **5** consisted of a pentose. The elution order of compounds **4** and **5** was identical in the HPLC and the HSCCC but the HSCCC technique enabled their quite effective separation.

A complete separation of Pen-Hex-HexUA-oleanolic acid (**5**) ([M-H]$^-$ at *m/z* 925) and Act-Hex-HexUA-oleanolic acid (**6**) ([M-H]$^-$ at *m/z* 955) was observed in spite of their similar chromatographic properties indicated in the C_{18}-HPLC system. The difference in the structure consists in the presence of the acetal-type substituent in compound **6** instead of pentose in compound **5**. The presence of the acetal-type substituent presumably contributed to the faster elution of compound **6** in comparison to compound **5** in the HSCCC system. Similarly, the separation of the compounds **5** and **6** by HSCCC and HPLC also proves the complementarity of the two separation systems.

The principal compound present in the extract, Act-Hex--HexUA-oleanolic acid (**6**) ([M-H]$^-$ at *m/z* 955), differed from Diox-Hex-HexUA-oleanolic acid (**4**) and Diox-Hex-HexUA-oleanolic acid (**7**) ([M-H]$^-$ at *m/z* 953) by the presence of acetal-type substituent in compound **6** instead of dioxolane-type substituent in compound **4** and **7**. The differences in the structures translated into their properties during separation by the HSCCC technique. Acetal-type saponin (Act-Hex-HexUA-oleanolic acid, compound **6**) was eluted faster than Diox-Hex-HexUA-oleanolic acid (compounds **4** and **7**).

Separation of Hex-HexUA-oleanolic acid (**8**) ([M-H]$^-$ at *m/z* 793) which was present mainly in the fractions 11 and 12 was very effective. This saponin only partially co-eluted with HexUA-Hex-HexUA-oleanolic acid in fraction 10 in the HSCCC.

Act-Hex-HexUA-oleanolic acid (**9**) ([M-H]$^-$ at *m/z* 955) and Pen-Hex-HexUA-oleanolic acid (**10**) ([M-H]$^-$ at *m/z* 925), similarly to compounds **5** and **6**, were completely separated by the HSCCC according to differences in their structures (acetal-type substituent and pentose, respectively). Similarities can also be seen in the elution order.

Furthermore, comparison of the elution order of Act-HexUA-oleanolic acid (**11**) ([M-H]$^-$ at *m/z* 793) and Pen-HexUA-oleanolic acid (**12**) ([M-H]$^-$ at *m/z* 763) confirmed that the acetal-type saponins are eluted faster in the HSCCC system than the pentose-type saponins. Because compounds **11** and **12** are closely related, their complete separation by HSCCC was impossible. The separation of these saponins could be feasible in highly polar solvent systems with salts [Spórna-Kucab *et al*., 2013a].

HexUA-oleanolic acid (**13**) ([M-H]$^-$ at *m/z* 631), eluted as the last saponin in the HPLC, was not completely separated by the HSCCC technique but its different elution profiles in the HPLC and the HSCCC systems afford the possibility for its recovery using both techniques.

CONCLUSION

In this study, separation of saponins from *B. vulgaris* L., cultivar Red Sphere, has been realized for the first time using high-speed counter-current chromatography in a new solvent system consisting of *tert*-butyl-methyl ether-butanol-acetonitrile-water. The previously described [Thakur *et al*., 2014] solvent system for the separation of saponins by the HSCCC had much lower retention of the stationary phase, therefore, the new solvent system had been prepared. Separation and concentration of the compounds during the HSCCC process enabled tentative identification of 13 saponins by MS/MS technique. Nine saponins were detected for the first time in *B. vulgaris* L. cv. Red Sphere. Additionally, saponin with the pseudomolecular ion [M-H]$^-$ at *m/z* 969 has been tentatively identified for the first time and its possible structure has been proposed.

Analysis of the saponin elution order showed some tendencies. Acetal-type saponins were eluted faster than pentose/hexose-type saponins as well as dioxolane-type saponins. Moreover, the saponins differed in the elution order in the HPLC and HSCCC systems, therefore, their elution in HSCCC is rather dependent on the steric conditions than on polarity. The combination of the HPLC and HSCCC results in complementary elution orders and makes them a very versatile tool for the isolation of saponins which may open up the possibility of utilizing these compounds commercially.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Original article
Red Beetroot Thematic Issue

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