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Abstract

The aim of this study was to determine the impact of three different technological processes and in vivo digestion on the profile and content of betalains and inhibition of angiotensin I converting enzyme (ACE) in red beet. Betalains were analysed by the micro-HPLC-TOF-MS/MS method, while ACE inhibition was determined by an in vitro assay. In the tested samples, thirteen betalains were identified, constituting nine betacyanins, two betaxanthins and two betalain precursors. Among the betalains identified in the non-digested samples, betanin (betacyanin) and vulgaxanthin I (betaxanthin) were predominant. In the digested samples, 17-decarboxy-neobetanin (betacyanin) and cyclo-DOPA (betalain precursor) were the dominant compounds. The applied technological processes reduced the content of betalain by 31–64% in the obtained products. The contribution of betalains released from red beet products after in vitro digestion was detected within the range of 0.001–0.10%. The applied treatments lowered ACE inhibition of the non-digested red beet products, while in general, the in vitro digestion caused an increase in ACE inhibition in the digested red beet products.

Keywords	red beetroot, betalains, betalain precursors, ACE inhibitions, food processing, in vitro digestion

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Highlights

- Red beetroot samples contained thirteen different betalains.
- Applied technological methods reduce the content of betalains by 31-64%.
- *In vitro* digestion leads to the formation of betalain precursors.
- The ACE inhibitory activity differed significantly between tested samples.

The effect of processing and *in vivo* digestion on the betalains profile and ACE inhibition activity of red beetroot products

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The effect of processing and *in vivo* digestion on the betalains profile and ACE inhibition activity of red beetroot products

Abstract

The aim of this study was to determine the impact of three different technological processes and *in vivo* digestion on the profile and content of betalains and inhibition of angiotensin I converting enzyme (ACE) in red beet. Betalains were analysed by the micro-HPLC-TOF-MS/MS method, while ACE inhibition was determined by an *in vitro* assay. In the tested samples, thirteen betalains were identified, constituting nine betacyanins, two betaxanthins and two betalain precursors. Among the betalains identified in the non-digested samples, betanin (betacyanin) and vulgaxanthin I (betaxanthin) were predominant. In the digested samples, 17-decarboxy-neobetanin (betacyanin) and cyclo-DOPA (betalain precursor) were the dominant compounds. The applied technological processes reduced the content of betalain by 31–64% in the obtained products. The contribution of betalains released from red beet products after *in vitro* digestion was detected within the range of 0.001–0.10%. The applied treatments lowered ACE inhibition of the non-digested red beet products, while in general, the *in vitro* digestion caused an increase in ACE inhibition in the digested red beet products.

Keywords: red beetroot, betalains, betalain precursors, ACE inhibitions, food processing, in vitro digestion

1. Introduction

Previous studies indicate that food products can have nutritional values and additional beneficial effects on human health. These reports led to an increase in the production of so-called "functional foods". Consequently, an increased interest in root vegetables, which are rich in many of the ingredients with beneficial properties for consumers, has recently been observed. One of the root vegetables that is very popular among scientists is red beetroot (*Beta vulgaris* L.). Additionally, red beetroot is very popular among farmers and is grown in many countries. Extracts from red beet are often used in the food industry for food colouring known as E162 (Ninfali & Angelino, 2013; Georgiev et al., 2010). The red beetroot is a rich source of bioactive compounds, particularly betalains (Paciulli et al., 2016; Clifford et al., 2015).

Betalains are water-soluble nitrogen-containing pigments that can be divided into red–violet (betacyanins) and yellow–orange (betaxanthins) pigments (Ravichandran et al., 2013; Paciulli et al., 2016). Betalains are not widely dispersed in the plant world; however, as previously mentioned, due to their properties, they are commonly used in the food industry as a source of a natural red colouring (Azeredo, 2009). The highest content of betalains is found in red beetroot (Sawicki et al., 2016; Ravichandran et al., 2013). The concentrations of betacyanins and betaxanthins in the roots of red beet were between 400 and 2100 mg/kg fresh weight and between 200 and 1400 mg/kg fresh weight, respectively (Ninafli & Angelino, 2013). Moreover, research carried out by Sawicki et al. (2016) showed that the betalain content differs depending on the red beetroot variety. Betalains are very sensitive compounds that are degraded by heat, oxygen, light, pH and enzymes (Herbach et al., 2006). In addition, betalains have a high antioxidant capacity

(Kanner et al., 2001) and exhibit antibacterial, hepatoprotective, anticarcinogenic and anti-inflammatory actions (Račkauskiene et al., 2015; Liñero et al., 2017).

Vegetables, including red beetroots, are often eaten in processed form. Technological processes are mainly used to improve the taste or to improve the bioavailability of the ingredients contained in the food product (Murador et al. 2016). Thus, determining the effect of technological processing on bioactive compounds including betalains would facilitate the formulation of dietary recommendations. Scientific studies show different information regarding the impact of technological processes on the content and activity of biologically active food ingredients. Pellegrini et al. (2010) found a decrease in the total phenolic content of steamed broccoli, whereas Turkmen et al. (2005) showed an increase in the phenolic level of broccoli. Studies on the beneficial effects of red beetroot consumption on human health are increasing; in particular, the results indicate the beneficial effects of red beetroot on the prevention of hypertension. Hypertension is a primary risk factor for cardiovascular disease, and one billion people worldwide have this disease. The aim of this study was to investigate the effect of different technological processing (boiling, fermentation and microwave-vacuuming) and *in vivo* digestion of red beetroot products on the betalains content and angiotensin-converting enzyme (ACE) inhibition activity.

2. Materials and methods

2.1. Chemicals

Detergent-compatible (DC) protein assay reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Tripeptide Abz-Gly-Phe(NO_2)-Pro was purchased from Cymit Quimica

(Barcelona, Spain). ACE from rabbit lung was obtained from Sigma Aldrich (Madrid, Spain). Alpha-amylase, pepsin, pancreatin and bile salts were purchased from Sigma (St. Louis, MO, USA). MS-grade reagents, including acetonitrile, methanol, water and formic acid, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium was obtained from Fluka (Buchs, Switzerland).

2.2. Material

Fresh red beet (*Beta vulgaris* L. subsp. *vulgaris*) roots (25 kg) were obtained from a local market in Olsztyn, Poland. The roots were cleaned, mixed and then divided into five groups: 1) fresh whole roots, 2) roots for the fermentation process, 3) roots for boiling, 4) roots for fresh juice and 5) roots for red beet crunchy slices, which were kindly produced by the FPH PAULA company in Kalisz, Poland (www.crispy.pl). All red beet products were prepared from the same batch of fresh roots.

2.3. Processing

2.3.1. Fresh roots

The red beet roots assigned to the fresh group (5 kg) were equally divided into three subgroups: whole roots, peel and flesh separated from the roots. Then, after mixing within a subgroup, three samples (250 g) from each subgroup were taken to determine the betalain profile and content and to analyse the ACE inhibitory activity of the obtained material. After lyophilization, the samples were pulverized and stored at -80°C until analysis.

2.3.2. Fermentation and red beetroot juice production

Beet roots (5 kg) were peeled and then cut into slices 2-3 mm thick. After mixing, shredded roots were equally divided into three traditional stoneware pots flooded with marinade (1.5 L) containing salt (12 g) and sugar (12 g). Next, the obtained materials were thoroughly mixed, and three independent fermentations were started. During the spontaneous fermentation process, the pots containing red beetroots were kept in a dark room at a temperature of 23°C. Fermentation was carried out until the pH was stabilized. The pH was measured once a day (Radiometr PHM85, Denmark), and the pH values obtained, ranging from 7.06 ± 0.01 (fresh juice) to 3.59 ± 0.02 (after a 7-day fermentation process), clearly showed that the process was conducted properly. After 7 days, the fermentation process ended, and the fermented red beetroots (250 g) and juice obtained during the fermentation process were collected from each stoneware pot and frozen. The fermented beet root samples were pulverized and stored at -80°C until analysis. Fresh juice was obtained from 5 kg of roots using a commercial juice extractor and then centrifuged (Centrifuge 5415R, Eppendorf, Niemcy) for 20 min (13,200× *g* at 4°C). The supernatant was collected and stored at -80°C until analysis.

2.3.3. Boiling

The boiling group consisted of 5 kg of red beet roots. Roots were placed into a stainless steel pot with boiling distilled water (2.5 L) and covered with a lid. When the water reached the boiling point, the red beet roots were boiled for 45 min. Subsequently, boiled roots were immediately frozen with liquid nitrogen and then lyophilized. The process was carried out in triplicate, and the freeze-dried materials collected were pulverized and stored at -80°C until analysis.

2.3.4. Microwave-rotating-vacuum power

The crunchy slices were produced under industrial conditions at the FPH PAULA company by means of microwave-rotating-vacuum power (MIRVAC technology). The products obtained with this technology retain all taste, odour and nutritious characteristics of their fresh equivalents. It is important to emphasize two other attributes of MIRVAC products: microbiological purity, the lack of which posed great difficulties in the past, and their relatively low price (Zieliński et al., 2012).

2.4. In vitro digestion

The *in vitro* digestion was conducted using a previously described method (Delgado-Andrade, 2010). Briefly, 0.5 g of powder or 0.5 mL of juice of red beet was weighed, combined with 10 mL of distilled water and 250 µL of an alpha-amylase solution (32.5 mg of alpha-amylase dissolved in 25 mL of 1 mM $CaCl_2$ pH 7.0) per gram of sample and then incubated at 37°C for 30 min. Next, the pH was adjusted to 2 using 6 M $NaHCO_3$, and pepsin solution (0.4 g of pepsin dissolved in 2.5 mL 0.1 M HCl) was added. The mixture was then incubated at 37°C for 2 h. After this step, the pH was adjusted to 6 using 1 M HCl, and pancreatin and bile salts (0.1 g of pancreatin and 0.625 g of bile salts dissolved in 25 mL of 0.1 M $NaHCO_3$) were added to the mixture. Then, the pH was adjusted to 7.5, and the mixture was incubated at 37°C for 2 h. After the incubation, enzyme inactivation was conducted by raising the temperature to 100°C for 4 min. Next, the mixture was centrifuged (Centrifuge MPW-350R, MPW MED. INSTRUMENTS, Poland) for 1 h (3200× *g*, 4°C), and the supernatant was collected in a 15 mL flask and stored at -80°C until analysis.

2.5. Extraction & chromatographic analysis

The extraction and analysis of betalains in red beetroot products were carried out as described previously by Sawicki et al. (2016). Approximately 0.05 g of each dried red beet root sample was extracted by vortexing for 30 s in a 1 mL mixture of 15% methanol with 0.05% formic acid. Then, the mixture was sonicated for 30 s (VC 750, Sonics & Materials, Newtown, CT, USA), vortexed and sonicated again, and centrifuged (Centrifuge 5415R, Eppendorf, Wesseling, Germany) for 10 min (13,200 x *g* at 4°C). The supernatant was collected in a 5 mL flask. This step was repeated 5 times. Finally, before the analysis, the extract was centrifuged (20 min, 13,000× *g*). Extracts of red beetroot products before and after *in vivo* digestion were identified by a micro-HPLC system (LC 200, Eksigent, Vaughan, ON, Canada) coupled with a TripleTOF 5600^+ mass spectrometer (AB SCIEX, Vaughan, ON, Canada). The quantities of betacyanins and betaxanthins were calculated from the micro-HPLC-TOF peak area against betanin and vulgaxanthin I, respectively, as external standards (Sawicki et al., 2016).

2.6. Determination of ACE inhibitory activity

ACE inhibitory activity was assessed in non-digested and digested samples according to the method described by Martinez-Villaluenga et al. (2012). Briefly, 50 µL of ACE working solution was added to each microtiter plate well and then adjusted to 100 µL by adding either distilled water to the control or samples in the inhibition studies. To determine the ACE inhibitory activity, the samples were diluted in the range from two- to tenfold. The enzyme reaction was initiated by the addition of 200 µL of 0.45 mM Abz-Gly-Phe(NO_2)-Pro dissolved in 150 mM Tris-base buffer pH 8.3, containing 1.125 M NaCl, immediately mixed and incubated at 37°C. The generated fluorescence was measured at 1 min intervals for 30 min using a Multiscan microplate fluorometer (Biotek, Winooski, VT, USA) at λ_{exc}=355 nm and λ_{emi}=405 nm. All measurements were conducted three times.

2.7. Statistical analysis

The results are given as the mean values and standard deviation (SD) of three independent measurements. The results were subjected to one-way analysis of variance (ANOVA) supported by Duncan's multiple range test, and significant differences ($P < 0.05$) were calculated. Statistical analyses were performed using Statistica (Stat Soft, Tulsa, OK, USA).

3. Results and discussion

3.1. *The profile and content of betalains in red beet products*

The profile and content of betalains before and after *in vitro* digestion of red beet products were analysed using micro-HPLC-TOF-MS. The obtained MS data for red beet betalains are presented in Table 1. As mentioned above, betalains were identified by means of a comparison of their retention time in MS/MS spectra with previously published data (Sawicki et al., 2016; Wybraniec et al., 2016).

In the non-digested red beetroot products analysed, eleven betalain compounds were detected (Table 1). Among the identified betalains, nine compounds belong to the betacyanins group (betanin, isobetanin, betanidin, 17-decarboxy-neobetanin, neobetanin, isobetanidin, 2-decarboxy-neobetanin, 2,17-bidecarboxy-betanidin and 6´-*O*-feruloyl-betanin), while two compounds belong to the betaxanthin group (vulgaxanthin I and dopamine-betaxanthin). Among the identified betalain compounds, in addition to betanin and isobetanin, aglycones of these compounds (betanidin and isobetanidin, respectively) and a dehydrogenated form of betanin (neobetanin) were detected. Apart from betanin derivatives characterized by the loss of glucose molecules or hydrogen, compounds with additional structures were also detected. This compound consisted of an additional feruloyl residue and was identified as 6´-*O*-feruloyl-betanin. Other betalains identified in the red beet products analysed were decarboxylated forms of betanidin and neobetanin (2,17-bidecarboxy-betanidin, 17-decarboxy-neobetanin and 2-decarboxy-neobetanin). Previous studies have also shown the presence of decarboxylated derivatives of betanidin and neobetanin in red beetroots (Sawicki & Wiczkowski, 2018; Slatnar et al., 2015).

In the literature, only scarce information is available regarding the profile and content of betalains in red beetroot products. The results of a study by Paciulli et al. (2016) showed the presence of only one betalain compound (betanin) after blanching treatment. In comparison, in fresh red beetroot and after six different processes (boiling, drying, pickling, jam processing, juice processing, and puree processing), Guldiken et al. (2016) identified only two betalain compounds (betanin and isobetanin). Additionally, Ravichandran et al. (2013) found a small number of betalains (betanin, isobetanin and betanidin) after processing, such as boiling, roasting and microwaving. However, in a previous study, fifteen compounds from the betacyanin group, three compounds from the betaxanthin group and two precursors of betalains in nine solid red beet products were identified (Sawicki & Wiczkowski, 2018). In the above study, the red beet products contained nine more compounds (2´-*O*-glucosyl-betanin/ isobetanin, prebetanin, isoprebetanin, 17-decarboxy-betanin, 2,17-bidecarboxy-neobetanin, 2,17-bidecarboxy-betanin/ isobetanin, 2-decarboxy-betanin, 6´-*O*-feruloyl-isobetanin and threonine-betaxanthin) than those in our study. Interestingly, in our study, the tissues of red beet products contained one compound (2,17-bidecarboxy-betanidin) that was not identified in the abovementioned study. On the other hand, after lactic acid fermentation by *Lactobacillus* sp., six betalains were detected in the obtained juices (Czyżowska at al., 2006). Furthermore, in another study, eight betalain compounds were found in the obtained juice during spontaneous fermentation (Sawicki et al., 2017). In the same study, five betalains were detected in fresh juice; of these, four compounds belong to the betacyanins group, and one compound belongs to the betaxanthins group (Sawicki et al., 2017). The different numbers of identified betalains may be due to the use of different varieties of red beet. Additionally, vegetation season conditions (light, temperature, level of precipitation), climatic parameters and cultivation conditions influence the differences in the profiles of bioactive compounds, as demonstrated previously (Wiczkowski et al., 2014). However, previous studies have shown that thermal treatment contributes to the conversion of betalains to its decarboxylated and dehydrogenated derivatives (Celli & Brooks, 2017; Wybraniec, 2005; Wybraniec & Mizrahi 2005).

As shown in Table 2, the total concentration of betalains was found within the range of 2.58 ± 0.04–10.76 ± 0.13 mg/g dm (dry matter) for the solid samples. In the case of betacyanins, the total content of these red–violet compounds in our study varied between 2.40 ± 0.02–9.43 ± 0.11 mg/g dm. Similar to the total betalain level, the highest content of total betacyanins in the red beet products was observed in the peel (9.43 ± 0.11 mg/g dm), while the lowest was in fermented red beet (2.40 ± mg/g dm). The total betaxanthin content for the obtained products oscillated between 0.01 ± 0.00 mg/g dm for the beetroot crunchy slices and 2.19 ± 0.02 mg/g dm for the whole root. The obtained data indicate that the red beetroot is a good source of betalain compounds, allowing us to examine the association between the profile and content of these natural pigments and the applied technological process. In our study, the fate of betalains was investigated after boiling, fermentation and microwave-vacuum treatment because these methods are the most popular types of industrial treatments for red beet. Our study showed that the processing application decreased the total betalain content in the analysed red beet products. The highest content of betalain compounds in the solid samples was characterized by the peel of red beet (10.76 ± 0.13 mg/g dm), which agrees with previous studies (Slatner et al., 2015; Kujala et al., 2002). In our work, the total betalain content in the peel was approximately 33% and 30% higher than the total betalain content of these compounds in the whole roots or flesh, respectively. Previous studies (Slatnar et al., 2015; Kujala et al., 2002) also showed that the peel was characterized by the largest betalain content. On the other hand, the lowest betalain concentration was detected in the fermented red beet (2.58 ± 0.04 mg/g dm), showing that the betalains were leached by using marinade or that these compounds were degraded by the bacteria that appear during the fermentation process. The order of total betalains content for the solid red beet products was as follows: fermented roots < boiled roots < red beet crunchy slices < whole fresh roots < flesh of red beet < peel of red beet.

The main compound among the betacyanins group in the analysed products was betanin (ranging from 55.2% to 78.9% of total betacyanins), while the predominant compound from the betaxanthins group was vulgaxanthin I (between 66.7–100.0% of total betaxanthins). Previous studies also demonstrated that betanin and vulgaxanthin I were the dominant compounds in fresh (Slatnar et al., 2015; Kujala et al., 2002) and processed red beet (Sawicki & Wiczkowski, 2018). The highest percentage of decarboxylated derivatives was found in the peel. This phenomenon could be a result of exposing red beetroots to light and temperature, causing the transformation of betacyanins into decarboxylated forms (Mikołajczyk-Bator & Pawlak, 2016; Wybraniec, 2005). Moreover, in the peel, flesh, and fermented and boiled roots, neobetanin was the second most dominant compound. However, isobetanin and 2,17-bidecarboxy-betanidin were the second most predominant compounds in the whole roots and beetroot crunchy slices, respectively. Moreover, a much higher percentage of betanidin was detected in the fermented roots compared to that in other red beet products. However, isobetanidin was found only in the fermented roots. This finding indicates that betanin and/or isobetanin undergoes hydrolysis to betanidin and/or isobetanidin by lactic acid bacteria during the fermentation process. A higher percentage of 6′-*O*-feruloyl-betanin was detected in the peel than in the flesh and whole roots, which may indicate the accumulation of the feruloyl derivative of betanin in the older part of the root. Dopamine-betaxanthin, identified in the whole roots and flesh, was not found in the peel. As suggested by previous research, betaxanthin derivatives may degrade with the ageing of red beet root tissues (Sawicki & Wiczkowski, 2018).

The 45-min boiling process of whole roots led to a reduction in the total betalain content of approximately 46% (Table 2), while the total betacyanin and betaxanthin contents were degraded by approximately 28% and 86%, respectively. Previously published data also showed decreased levels of betacyanins and betaxanthins after boiling. Ravichandran et al. (2013) found decreases in betacyanin levels of 6%, 22%, and 51% and in betaxanthin levels of 18%, 23% and 33% after heating for 60, 120 and 180 s at 80°C, respectively. Jiratanan & Liu (2004) also demonstrated decreases in the content of betacyanins in red beetroots of 24%, 62% and 81% and in betaxanthins of 13%, 60% and 73% after treatment for 30 mins at 105°C, 115°C and 125°C, respectively. In a study by Sawicki & Wiczkowski (2018), the process of boiling whole roots for 60 min led to a decrease in the total betalain content of approximately 54%. The results clearly showed that the time and temperature of the treatment are responsible for the stability of betalains.

Taking into account the fate of individual betalain compounds during the boiling process, this method of treatment leads to both a decrease and increase in the percent contribution of individual betalains. Betanin was the main compound from the betacyanins group in the boiled roots. The contribution of betanin in the boiled roots (62.5%) was close to the level in the fresh roots (61.4%). Furthermore, the highest increase in the dehydrogenated form of betanin was observed in the boiled roots, as the contribution of neobetanin increased almost threefold. A previous study showed that betanin may convert to isobetanin, and both of these compounds can change into neobetanin (Stintzing et al., 2005). Furthermore, Mikołajczyk-Bator & Czapski (2017) demonstrated an increase in neobetanin content during heating. Decreases were observed in the other betacyanins detected (isobetanin, 17-decarboxy-neobetanin, 2-decarboxy-neobetanin, 2,17-bidecarboxy-betanidin and 6´-*O*-feruloyl-betanin) in the boiled roots, and the highest decrease was observed for isobetanin. The concentration of isobetanin was over two and a half times lower in boiled roots than in the fresh roots. The change in the betacyanin profile can be associated with the compounds in the red–violet group having the same main core, and the applied heating method led to a transformation of one compound to another (Wybraniec, 2005). In the yellow–orange group, only two compounds in this group of betalains (vulgaxanthin I and dopamine-betaxanthin) were identified. Moreover, vulgaxanthin I was the predominant compound in the boiled roots, similar to the fresh roots; however, the contribution of this compound was decreased from 97.7% to 66.7% of the total betaxanthins content. This phenomenon may have been caused by vulgaxanthin I being the only betaxanthin in the peel, and the exposure of the outer parts of the roots to high temperature activity led to the degradation of this compound. Moreover, previous studies have shown that temperature is the main factor in the degradation of betaxanthins (Wendel et al., 2015).

Spontaneous fermentation was the treatment that caused the highest degradation of betalains in red beet roots among all the processing methods applied in this study. The fermentation process of whole roots for 7 days led to a reduction in the total betalain concentration of 64%, compared to the total betalain content of fresh roots (Table 2). In contrast, in a study by Sawicki & Wiczkowski (2018), spontaneous fermentation of red beetroots conducted for 7 and 14 days caused a decline in betalains content in the roots by 61% and 88%, respectively. The lower content of betalain in fermented roots may be related to the degradation or release of these pigments from the roots into the marinade, which seems more likely after a high concentration of these compounds were found in the obtained juice.

The fermented roots were found to have the richest profile of betalain compounds among all red beet products investigated in this study. In the fermented roots, eleven betalains were detected (Table 2). The main compound in this red beet product was betanin, similar to the fresh and boiled roots. However, a higher increase was observed for betanidin and isobetanidin. The increased contribution of betanidin and isobetanidin in the fermented roots can be associated with the hydrolysis of betanin and/or isobetanin to these compounds. However, isobetanin is characterized by the highest degradation (approximately fivefold), which agrees with the above statement that during the fermentation process, isobetanin undergoes transformation to an aglycone. In addition to isobetanin, the contributions of betanin, 17-decarboxy-neobetanin, 2-decarboxy-neobetanin and 2,17-bidecarboxy-betanidin were decreased. On the other hand, the contributions of neobetanin and 6´-*O*-feruloyl-betanin in the fermented roots were similar to those in the fresh roots. The obtained data suggest that as a result of the fermentation process, betacyanin compounds may undergo dehydrogenation, decarboxylation and deglucosylation. In the case of the betaxanthins, the predominant compound in the fermented roots and in other red beet products was vulgaxanthin I, with a 77.8% contribution to the total yellow–orange pigments. In the fermented roots, an increase in the share of the second compound from the betaxanthin group (dopamine-betaxanthin) was observed. The increase in dopamine-betaxanthin of approximately tenfold may be associated with the softening of beet tissue by using marinade and as a consequence, better extractability of this compound.

The level of betalains in red beet crunchy slices decreased by approximately 31% compared to the fresh roots and was the smallest loss of these pigments among tested solid red beet products. Furthermore, the betacyanin content in red beet crunchy slices (4.92 ± 0.04 mg/g dm) was almost the same as that in the fresh roots (4.98 ± 0.04 mg/g dm), which suggests that the applied method did not cause a loss of compounds from the betacyanin group (Table 2). A previous study reported that the production of red beet crunchy slices by MIRVAC technology is effective with regard to the final betacyanin content in obtained products (Wiczkowski et al., 2018). On the other hand, the total betaxanthin content was the smallest among the obtained red beet products (0.01 ± 0.00 mg/g dm), and the degradation of yellow–orange pigments increased to 99.5%. In comparison, a study by Ravichandran et al. (2013), which examined the influence of non-thermal vacuum-dried treatment on the degradation of betalains, showed increases in betacyanins of 20% and in betaxanthins of 12% compared to the respective levels in the control.

The red beet crunchy slices contained seven compounds, i.e., six from the betacyanins group and one from the betaxanthin group (Table 2). Among the betalains detected, betanin was the predominant betacyanin (78.9%), and vulgaxanthin I (100%) was the main betaxanthin in the crunchy slices. At lower levels, 17-decarboxy-neobetanin, neobetanin, isobetanin, 2-decarboxy-neobetanin and 2,17-bidecarboxy-betanidin were detected (ranging from 2.0% to 5.9%). In the case of betanin, its contribution was 21% higher in the red beet crunchy slices than in the fresh roots. However, in a study by Wiczkowski et al. (2018), only two compounds (betanin and isobetanin) from to the betacyanins were found in the red beet crunchy slices. In the study cited, a different profile of betacyanins was detected in the crunchy slices: betanin constituted 60%, while isobetanin constituted 40% of the total betacyanins content. This difference in the number of compounds and the different profile in red beet may result from the use of different varieties in the study (Slatnar et al., 2015; Lee et al., 2014).

Among the liquid products examined, fresh red beet juice had a higher concentration of betalains (approximately 21%) than fermented juice (Table 2). The reverse was the case for the betacyanin content, where the content of red–violet pigments in fermented juice was approximately 13% higher than in fresh juice. This phenomenon may have resulted from three facts: first, betacyanins were effectively leached from the red beet tissues by the marinade used (Sawicki & Wiczkowski, 2018); second, acidification of the obtained juice (pH 3.59 ± 0.02) during the fermentation process may prevent the degradation of these compounds (Fernandez-Lopez et al., 2007; Strack et al., 2003); and/or third, the fermentation process avoids oxidation by endogenous enzymes such as polyphenol oxidases and peroxidases (Strack et al., 2003). However, betaxanthins were found only in fresh juice. This finding confirms that the compounds in the betaxanthin group are unstable (Rodriguez-Sanchez et al., 2017; Wendel et al., 2015) and can also be degraded during the fermentation process. In comparison, fermented red beet juice obtained by spontaneous fermentation for 7 days had a higher content of total betalains, betacyanins and betaxanthins (102.33 ± 0.32 mg/L, 92.01 ± 0.17 mg/L and 10.32 ± 0.20 mg/L, respectively) (Sawicki & Wiczkowski, 2018) compared to the juice in our research. A study by Klewicka & Czyżowska (2011) examining the influence of fermentation by two bacteria (*Lactobacillus brevis* and *Lactobacillus paracasei*) for 48 h at 30°C, showed that the level of betalains was 960 mg/L. On the other hand, as shown in Table 2, the total concentration of betalains in fresh juice was 82.49 ± 2.14 mg/L. Interestingly, the previously published data showed a higher concentration of betalains in fresh red beet juices. Klewicka & Czyżowska (2011) found approximately 1270 mg/L betalains in fresh juice obtained from the "Chrobry" beetroot variety (Poland), while Bazaria & Kumar (2016) observed 405.28 mg/L betalains in fresh juice prepared from red beet cultivated in the northern part of India. Similar results were reported by Jagannath et al. (2015) for fresh juice obtained from red beet cultivated in the southern part of India. With regard to the first group of betalains, the content of betacyanins in our research was 56.26 ± 1.56 mg/L. In comparison to our results and those of Bazaria & Kumar (2016), fresh juice obtained from red beet harvested in India had a higher content of red–violet pigments, i.e. 256.86 mg/L. On the other hand, the total concentration of betaxanthins in red beet cultivated in India was fivefold higher than in Poland (Bazaria & Kumar, 2016).

In the juices, ten betalain compounds were identified (Table 2). In the fermented juice, nine compounds were detected, and all identified substances belonged to the betacyanins group. However, in the fresh juices, eight compounds were detected: seven betacyanins and one betaxanthins. Among the betacyanins in fresh red beet juice, two compounds (betanidin and isobetanidin) that were present in fermented juices were not detected. These two compounds were the aglycones of betanin and/or isobetanin, which formed during the fermentation process, as shown in the case of fermented roots. Both in the fresh and fermented juices, betanin was the dominant compound; however, this compound had a higher contribution in the fresh juice (almost twofold). This situation may be related to the conversion of betanin to betanidin and isobetanidin, which were the second and third most predominant compounds in fermented juice. In the case of fresh juice, the second and third most dominant compounds were decarboxylated derivatives of neobetanin (17-decarboxy-neobetanin and 2-decarboxy-neobetanin). In addition to betanin, betanidin and isobetanidin in fermented juice, a higher contribution to total betacyanin content was also detected from isobetanin, neobetanin and 6´-O-feruloyl-betanin compared to that in the fresh juice. However, in addition to betanin, 17-decarboxy-neobetanin and 2-decarboxy-neobetanin, 2,17-bidecarboxy-betanidin constituted a higher contribution to the total betacyanin concentration in fresh juice than in the fermented juice. In comparison, the largest number of betalain compounds in the fermented juice obtained during spontaneous fermentation was detected by Sawicki & Wiczkowski (2018) and Czyżowska et al., 2006. On the other hand, only five compounds from the betacyanin group were detected in the fermented red beet juice obtained after a 24-h fermentation of red beet at 30°C by three probiotic bacteria and three infant intestinal microbiota of the Lactobacillus sp. (Czyżowska et al., 2006). Similar results were reported by Klewicka & Czyżowska (2011) for fermented red beet juice obtained by lactic fermentation conducted for 48 h at 30°C.

3.2. The profile and content of betalains in red beet products after in vitro digestion

In vitro digestion was determined by simulating the digestion conditions, which included oral, gastric and intestinal phases. The profile and content of the betalains in the digestion phases resulting from *in vitro* gastrointestinal digestion of red beet products were determined (Table 3). The present study offers the opportunity to track changes in the profile and content of betalain compounds after *in vitro* digestion and potential *in vitro* bioaccessibility determination of these compounds released from the various red beet product matrices.

To the best of our knowledge, the literature available does not provide sufficient data regarding the profile and content of betalains in the digestion phases obtained from the *in vitro* digestion of red beet products. Tesoriere et al. (2008) presented only three compounds (betanin, isobetanin and vulgaxanthin I) in the phases obtained after simulated gastrointestinal digestion of four red beet products (raw red beet, steamed red beet, red beet jam and juice). In comparison, nine betalains were identified in our research in all digestion phases of red beet products. Apart from the seven betalains present in non-digested red beet products (betanin, isobetanin, betanidin, 17-decarboxy-neobetanin, 2-decarboxy-neobetanin, 2,17-bidecarboxy-betanidin and 6´-*O*-feruloyl-betanin), two additional precursors of these compounds (betalamic acid and *cyclo*-DOPA) were found in the digestion phase. However, in the digestion phases, neobetanin and isobetanidin, which were present in the non-digested samples, were not detected. Notably, in the digested samples, compounds from the betaxanthins group were not found. Among the nine compounds detected in the digestion phases (two betalain precursors and seven betacyanins), *cyclo*-DOPA (52.4%) had the highest average contribution to the total betalain precursor concentration, while 17-decarboxy-neobetanin (32.2%) had the highest total betacyanins content. In addition, only three compounds in all tested digested samples were detected; two of them belong to the precursors of betalains (betalamic acid and *cyclo*-DOPA), and one compound belongs to the betacyanins group (6´-*O*-feruloyl-betanin). The betalain precursors constitute the main structure of betalain compounds (Azeredo, 2009) and appear in the digestion phases as a result of the decomposition of betalains during *in vitro* digestion. However, 6´-*O*-feruloyl-betanin was probably liberated from the red beet matrix products by *in vitro* digestion. The other compounds (betanin, isobetanin, betanidin, 17-decarboxy-neobetanin, 2-decarboxy-neobetanin and 2,17-bidecarboxy-betanidin) were found in one or a few digestion phases. The betanin, isobetanin and 2,17-bidecarboxy-betanidin were present only in the phases obtained after digestion of the peel. Betanidin was detected after digestion of two red beet products (crunchy slices and fermented juices) in the obtained phases. Importantly, this compound was not present in the non-digested crunchy slices. However, in crunchy slices, the main compound was betanin, which can be converted to betanidin by losing one molecule of glucose (Sawicki et al., 2016). Factors that may have caused the conversion of betanin to betanidin were higher temperature, pH changes and the enzymes used (Wiczkowski et al., 2018) during the *in vitro* digestion. The 2-decarboxy-neobetanin was detected in the phases after digesting four red beet products, such as peel, boiled roots, fermented and fresh juices. The other decarboxylated derivative (17-decarboxy-betanin) was present in five digested phases. This compound was detected after *in vitro* digestion of fresh roots, flesh, fermented roots, boiled roots, and crunchy slices.

As shown in Table 3, the total betalain content released from solid red beet products after *in vitro* digestion was detected within the range of 0.87–2.62 μg/g (corresponding to 0.012–0.10% of the betalains content in non-digested solid red beet products). Fermented roots of red beet were characterized by the highest betalains content released, while the flesh of red beet was characterized by the poorest release of these compounds (Table 3). This phenomenon may have resulted from the previous softening of the tissues of red beet by the fermentation process, which may result in a higher release of betalain compounds. On the other hand, the total concentration of released betalains from the fresh and fermented red beet juices in the digestion phases were found to be 1.12 ± 0.01 and 1.43 ± 0.01 mg/L, respectively (corresponding to 0.001% and 0.002% of the betalains content in non-digested juices, respectively). The order of released betalains from red beet products after *in vivo* digestion was fresh juice < fermented juice < flesh < peel < whole roots < crunchy slices < boiled roots < fermented roots. In the case of betacyanin, the total concentration of these compounds in our study varied between 0.50–1.49 μg/g (corresponding to 0.008–0.016% of the betacyanin content in non-digested solid red beet products). However, the highest contribution of released betacyanins in the digestion phases of fermented roots was noted to be similar to that of the total betalains. The fermented and fresh red beet juices were characterized by a total betacyanins concentration in the digested phases of 1.00 ± 0.00 and 0.61 ± 0.00 mg/L, respectively (corresponding to 0.0015% and 0.0010% of the betacyanins content in non-digested juices, respectively). With regard to the second group of betalains detected in the digested samples, the total content of betalain precursors in our study was found to be within the range of 0.37–2.00 μg/g. The highest content of total betalain precursors was observed after the digestion of fermented roots (2.00 ± 0.03 μg/g), while the lowest was in the digestion phase of the flesh (0.37 ± 0.00 μg/g). However, no significant differences were found in the total betalain precursor content among the peel, flesh and crunchy slices. The concentration of total betalain precursors in fresh juices was approximately 16% higher than in fermented juice. The presented data showed that the betalains released from fermented red beet products may be more bioaccessible, which may result in potential health benefits when consuming these red beet products.

3.3. ACE inhibitory activity of red beet products

ACE inhibitory activity is now one of the major therapies for treating hypertension (Hall et al., 2018). The available data concerning ACE inhibitory activity focus on the action of phenolic compounds (Sakulnarmrat et al., 2014; Afonso et al., 2013; López de Lacey et al., 2014) and mostly bioactive peptides (Hall et al., 2018; Garcia-Mora et al., 2015). In our study, betalains derived from eight red beet products were assessed for the potential to lower blood pressure by inhibiting ACE (Fig. 1). Furthermore, the ACE inhibitory activity of these products was evaluated after simulated gastrointestinal digestion (Fig. 2). The results were expressed as the percentage (%) of ACE inhibited by extracts or digestion phases of red beetroot products (Fig. 1 & 2).

The value of ACE inhibition for non-digested solid samples ranged from 4.72 to 86.97%. All red beet products demonstrated a strong potential to inhibit ACE activity, although their ACE inhibition differed significantly across the red beet products studied ($P < 0.05$). Among non-digested samples, fresh red beet juice was found to have the highest ACE inhibitory activity (86.97 ± 0.40%), while boiled beetroot was estimated to have the lowest (4.72 ± 0.92%). The reason for the low activity of the boiled red beetroot products was the heating treatment, which causes degradation of betalains with a simultaneous decrease in their activity (Pedreño & Escribano, 2001). Applied technological processes resulted in significant decreases in ACE inhibitory activity ($p < 0.05$) compared to that of fresh samples. Heating, fermentation and microwave-rotation-power vacuum treatment resulted in decreases in ACE inhibitory activity of 84.1%, 31.0% and 20.5%, respectively. In contrast, the fermented red beet juice was characterized by approximately 8.5% lower ACE inhibition compared to the fresh juice. The order of decreasing ACE inhibitory activity was as follows: fresh juice > fermented juice > peel > whole fresh roots > red beet crunchy slices ≥ fermented roots > flesh > boiled roots. In addition, the ACE inhibitory values were highly correlated with the total betalain ($r = 0.836$) and betacyanin ($r = 0.829$) contents.

However, for the digested samples, the values of ACE inhibition were between 9.53 and 87.99% (Figure 2). This finding indicates the high activity of digested red beet products against ACE. This phenomenon may result from the fact that red beets are good sources of phenolic acids (Guldiken et al. 2016; Ravichandran et al., 2012). Furthermore, phenolic acids mainly occur in a bound form, while thermal processing, fermentation, freezing (Dewanto et al., 2002) and hydrolysis (Garcia-Mora et al., 2015) can contribute to the release of phenolic acids from the cell wall. Garcia-Mora et al. (2015) observed that phenolic compounds can contribute to the ACE inhibitory activity of bean hydrolysates. The case may be similar for red beetroot.

The peel was found to have the highest value of ACE inhibition among the digestion phases (87.99%), while the smallest value of ACE inhibitory activity was observed for fermented red beetroot juice (9.53%) (Figure 2). Furthermore, only the digested sample of fermented red beetroot juice showed a decrease in the activity of ACE inhibition compared to non-digested fermented juice. The phenolic acids present in the fermented juices were liberated from the red beet matrix by the fermentation process, which can be demonstrated by the high ACE inhibition value of the non-digested fermented juice. However, the next step of this study most likely caused the degradation of these compounds as a result of *in vitro* digestion. The other samples demonstrated higher ACE inhibitory activity in relation to non-digested red beetroot products. In addition, the fresh products of red beets (whole roots, peel and flesh) showed greater inhibitory activity against ACE. The order of ACE inhibition for the digested red beet products was as follows: peel > fresh whole roots > flesh > boiled roots > fermented roots > red beet crunchy slices > fresh juices > fermented juices.

4. Conclusions

This study is the first to demonstrate changes in the content and profile of the betalain compounds in the eight red beet products before and after *in vitro* digestion. The research clearly showed that each of the red beet products possessed its own unique profile of betalain compounds and that the total content of betalains differed significantly between individual red beet products.

Moreover, during the *in vitro* digestion, the betalain content decreased and the number of compounds identified in the digestion phases was reduced. In addition, each red beet product and digestion phase of these products were characterized by a specific and unique ACE inhibitory activity. Most likely, in the case of non-digested red beet products, the betalain compounds were responsible for ACE inhibition, while in the case of digested samples, phenolic acids were responsible. Further studies are now needed to discover the biological properties of betalains and to determine how betalains from different red beet products behave after consumption.

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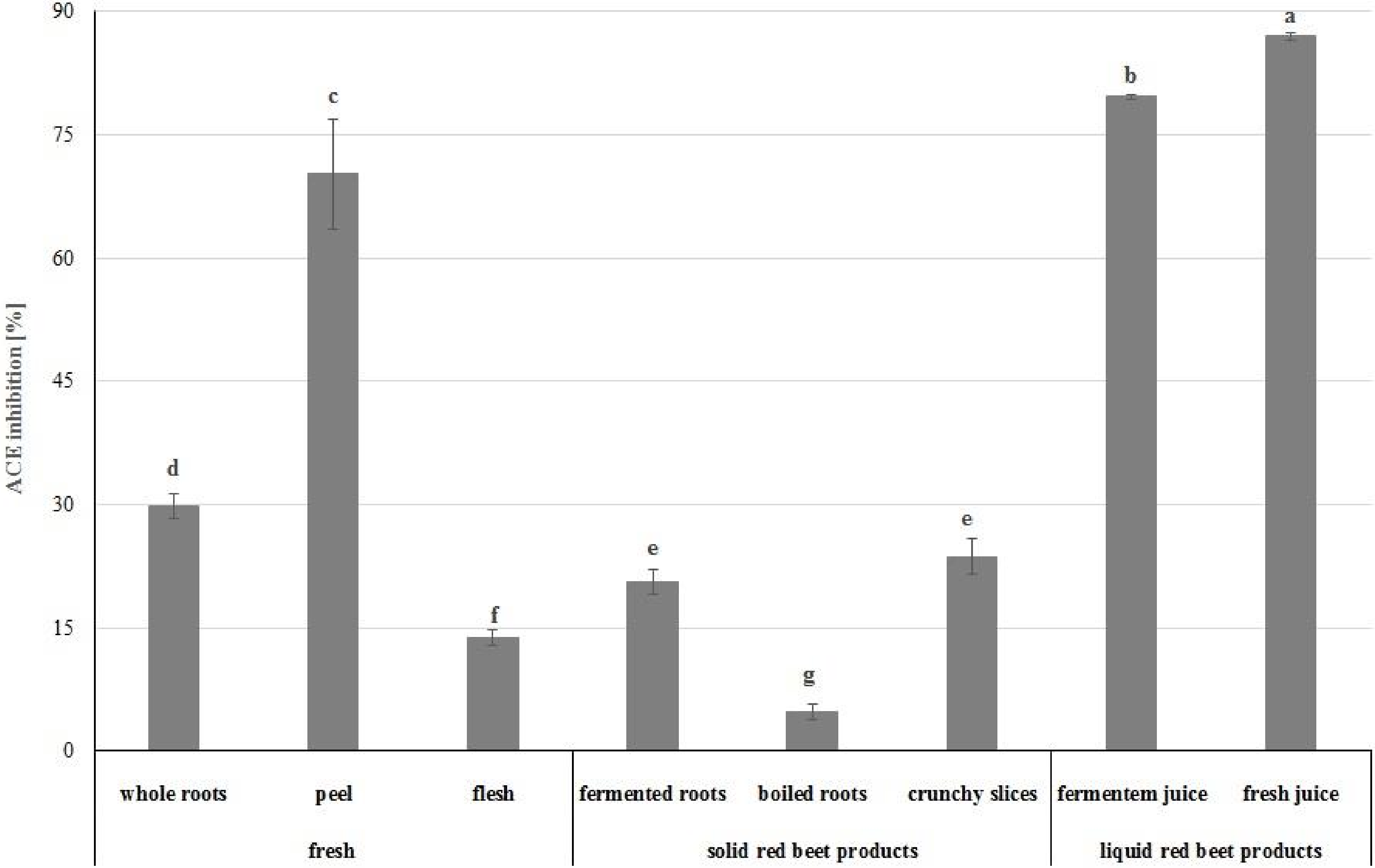
Figure caption

Fig. 1. ACE inhibitory activity of fresh and processed red beetroot

Fig. 2. ACE inhibitory activity of fresh and processed red beetroot after *in vivo* digestion



90
75
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45
30
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ACE inhibition [%]
d
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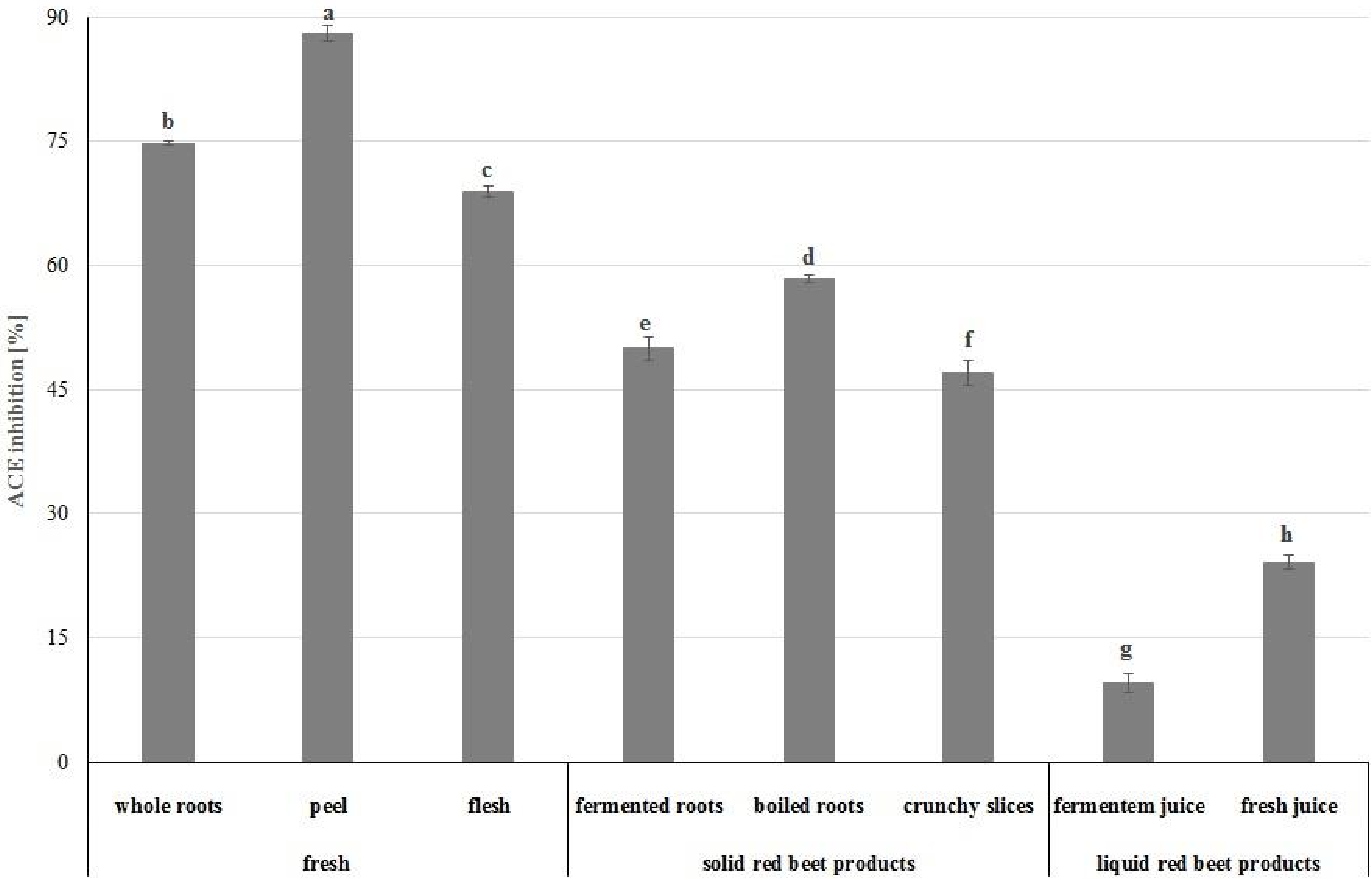
a
whole roots
peel
flesh
fermented roots
boiled roots
crunchy slices
fermentem juice
fresh juice
fresh
solid red beet products
liquid red beet products



ACE inhibition [%]
90
75
60
45
30
15
0
a
b
c
d
e
f
g
h
whole roots
peel
flesh
fermented roots
boiled roots
crunchy slices
fermentem juice
fresh juice
fresh
solid red beet products
liquid red beet products

Table 1. The MS data of found red beet betalains.

Compound	MS [m/z]	MS/MS [m/z]	Retention time [min]
	betalain precursors		
betalamic acid	212.2	166.0	1.30
cyclo-DOPA	358.1	196.0/150.1	1.31
	betacyanins		
betanin	551.1	389.1	1.29
isobetanin	551.1	389.1	1.34
betanidin	389.1	343.1	1.35
17-decarboxy-neobetanin	505.1	343.1/297.1	1.39
neobetanin	549.1	387.1	1.45
isobetanidin	389.1	343.1	1.46
2-decarboxy-neobetanin	505.1	343.0/297.1	1.47
2,17-bidecarboxy-betanidin	301.1	257.1	1.56
6'-*O*-feruloyl-betanin	727.1	551.1/389.0	1.70
	betaxanthins		
vulgaxanthin I	340.1	323.0	0.67
dopamine-betaxanthin	347.1	303.1	1.50

Table 2. The content and % contribution of betalains in red beetroot products.

compounds	fresh			solid red beet products			liquid red beet products	
	whole root	whole root divided into		fermented roots	boiled roots	crunchy slices	fermented juice	fresh juice
		peel	flesh					
				betacyanins				
betanin	61.4	55.2	68.8	58.8	62.5	78.9	30.2	57.0
isobetanin	13.3	5.1	4.7	5.8	6.7	5.1	8.7	5.0
betanidin	0.4	0.4	0.2	6.3	0.0	0.0	25.0	0.0
17-decarboxy-neobetanin	6.6	9.1	4.2	4.6	3.9	2.0	3.3	15.4
neobetanin	4.0	9.1	10.9	7.5	16.2	2.8	5.1	2.4
isobetanidin	0.0	0.0	0.0	1.3	0.0	0.0	18.7	0.0
2-decarboxy-neobetanin	7.0	7.0	4.7	4.6	4.5	5.3	3.3	11.4
2,17-bidecarboxy-betanidin	4.6	4.5	4.4	6.3	3.4	5.9	3.3	6.9
6'-*O*-feruloyl-betanin	2.6	9.5	2.2	5.0	2.8	0.0	2.5	1.9
Total[1]	**4.98±0.04[c]**	**9.43±0.11[a]**	**6.42±0.04[b]**	**2.40±0.02[e]**	**3.57±0.07[d]**	**4.92±0.04[c]**	**64.84±1.26[A*]**	**56.26±1.56[B*]**
				betaxanthins				
vulgaxanthin I	97.7	100.0	84.2	77.8	66.7	100.0	0.0	100.0
dopamine-betaxanthin	2.3	0.0	15.8	22.2	33.3	0.0	0.0	0.0
Total[2]	**2.19±0.02[a]**	**1.33±0.02[b]**	**1.14±0.01[c]**	**0.18±0.01[e]**	**0.30±0.00[d]**	**0.01±0.00[f]**	**0.0**	**26.23±0.58[A#]**
Total betalains[3]	**7.17±0.05[c]**	**10.76±0.13[a]**	**7.56±0.04[b]**	**2.58±0.04[f]**	**3.87±0.08[e]**	**4.93±0.05[d]**	**64.84±1.26[B*]**	**82.49±2.14[A*]**

[1] All values were expressed as milligrams of betanin per gram dry matter of red beetroot / * milligram of betanin per liter. Data are expressed as mean ± SD (n=3). Means in line related to a total content of betacyanins for each red beetroot products by the different letters are significantly different (P<0.05).
[2] Values were expressed as milligrams of vulgaxanthin I per gram dry matter of red beetroot / # milligram of vulgaxanthin I per liter. Data are expressed as mean ± SD (n=3). Means in line related to a total content of betaxanthins for each red beetroot products followed by the different letters are significantly different (P<0.05).
[3] Values were expressed as milligrams of betanin per gram dry matter of red beetroot / * milligram of betanin per liter. Data are expressed as mean ± SD (n=3). Means in line related to a total content of betalains for each red beetroot products followed by the different letters are significantly different (P<0.05).

Table 3. The content and % contribution of betalains in red beetroot *in vitro* digested products.

compounds	fresh			solid red beet products			liquid red beet products	
	whole root	whole root divided into		fermented roots	boiled roots	beetroot chips	fermented juice	fresh juice
		peel	flesh					
betalain precursors								
betalamic acid	37.2	46.2	44.3	90.4	23.9	45.4	46.9	46.8
cyclo-DOPA	62.8	53.8	55.7	9.6	76.1	54.6	53.1	53.2
Total[1]	**0.48±0.00^{c}**	**0.41±0.00^{d}**	**0.37±0.00^{d}**	**2.00±0.03^{a}**	**0.74±0.05^{b}**	**0.38±0.01^{d}**	**0.43±0.00^{B*}**	**0.51±0.00^{A*}**
betacyanins								
betanin	0.0	17.9	0.0	0.0	0.0	0.0	0.0	0.0
isobetanin	0.0	20.6	0.0	0.0	0.0	0.0	0.0	0.0
betanidin	0.0	0.0	0.0	0.0	0.0	51.5	23.7	0.0
17-decarboxy-neobetanin	74.9	0.0	57.1	59.9	40.0	25.9	0.0	0.0
2-decarboxy-neobetanin	0.0	27.2	0.0	0.0	28.1	0.0	52.0	64.7
2,17-bidecarboxy-betanidin	0.0	18.6	0.0	0.0	0.0	0.0	0.0	0.0
6'-*O*-feruloyl-betanin	25.1	15.7	42.9	40.1	32.0	22.6	24.3	35.3
Total[2]	**0.70±0.02^{c}**	**1.49±0.01^{a}**	**0.50±0.00^{e}**	**0.63±0.01^{d}**	**0.72±0.01^{c}**	**1.09±0.04^{b}**	**1.00±0.00^{A*}**	**0.61±0.01^{B*}**
Total betalains[2]	**1.18±0.02^{d}**	**1.90±0.01^{b}**	**0.87±0.00^{e}**	**2.62±0.04^{a}**	**1.46±0.06^{c}**	**1.48±0.05^{c}**	**1.43±0.01^{A*}**	**1.12±0.01^{B*}**

[1] All values were expressed as micrograms of betanin per gram dry matter of red beetroot / * milligram of betanin per liter. Data are expressed as mean ± SD (n=3). Means in line related to a total content of betalain precursors for each red beetroot products by the different letters are significantly different (P<0.05).

[2] Values were expressed as micrograms of betanin per gram dry matter of red beetroot / * milligram of betanin per liter. Data are expressed as mean ± SD (n=3). Means in line related to a total content of betacyanins for each red beetroot products followed by the different letters are significantly different (P<0.05).

[3] Values were expressed as micrograms of betanin per gram dry matter of red beetroot / * milligram of betanin per liter. Data are expressed as mean ± SD (n=3). Means in line related to a total content of betalains for each red beetroot products followed by the different letters are significantly different (P<0.05).