Application of ultrasound-assisted extraction method to recover betalains and polyphenols from red beetroot waste

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Application of ultrasound-assisted extraction method to recover betalains and polyphenols from red beetroot waste

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Abstract

Agriculture and food industries generate substantial quantities of waste material with huge potential for bioactive ingredients to be recovered and converted into high value chemicals. Red beetroot, known for its high content in betalains, natural red pigments, as well as polyphenols, fibre and nitrate, is experiencing increasing demand, in particular as juice, which is leaving behind large amounts of waste. The present study focused on the recovery of betalains and polyphenols from dried whole beetroot, wet and dried beet pulp waste from the juicing industry. As part of an ultrasound-assisted extraction, ethanol/water-based solvent mixtures were used as they were found to be more effective than single solvents. Enzyme-assisted extraction was initially examined in case of wet pulp, but was not able to retain betalains. Betalains appear to be more stable in dried pulp. Ultrasound-assisted extraction was found more suitable to effectively extract both betalains and polyphenols with high bioactive yield from dried pulp. The total betalain and polyphenol profiles as well as storage stability and antioxidant capacities were evaluated over a period of 4 weeks after extraction from the dried waste. During the 4 week storage, betalains quickly degraded at room temperature in contrast to -20 °C, whereas polyphenols and antioxidative activity were much less influenced by temperature. When compared, dried samples from the beetroot juicing industry demonstrate good betalain and polyphenol extractability, thus this data indicate that dried beet waste can serve as a good source of betalains for the color industry and other technological sectors.

Key words: Betalains, polyphenols, antioxidant capacity, storage, beetroot waste, ultrasound-assisted extraction, enzyme-assisted extraction

Introduction

The food industry is responsible for the generation of up to 60% of total food waste during their production, distribution and retail process[1]. In Europe, around 90 million tonnes of food waste are generated on a yearly basis, which corresponds to ca. 170 million tonnes of CO_2 equivalent emitted per year[2]. Of these, juice, canned and frozen fruits and vegetable industries approximately generate 11.5 million tonnes of waste annually excluding the waste from grape and wine industries[3]. This waste material has generally a high moisture content (~80% w/w) and is rich in sugars (~75% w/w dry matter)[4], which makes it prone to microbial spoilage. Their incineration has been proven to be unsustainable as it uses high temperatures, has a low energy yield, contributes to waste disposal in landfills, and downgrades organic material which could be used for other purposes. Other treatments such as composting and anaerobic digestion provide more stable final material from a microbiological perspective, but again downgrade the initial organic matter. The management of food waste becomes an increasingly relevant challenge to reduce pollution, increase the industry revenues and improve recycling. So far, most food waste is utilized for the production of biofuels, preparation of fiber and as animal feed[5]. However, there is good evidence that food waste could be more effectively used as a source of bioactive compounds with increased value and significance to human nutrition, target compounds being phenolics, pigments, vitamins, peptides, and aromatic compounds[3, 6]. For instance, it was reported that peels and seeds of citrus fruits, grapes, mangoes, avocados, and jackfruit contain over 15% more polyphenols than the edible parts[7]. As well, Choi, Kozukue, Kim and Friedman[8] reported that potato peels contain three times higher chlorogenic acids as compared to the cortex.

Beet (*Beta vulgaris* L.) is a popular crop grown around the world with some cultivars used for food as well as for sugar production. Sugar beet pulp, the main by-product of the sugar beet

industry is being extensively utilized[9], and is an excellent source for polyphenols[10]. In contrast to sugar beet, the waste from red beetroot processing has not been sufficiently considered for its alternative uses. The EU is the largest beetroot global producer (~70%), with the beetroot juice production in the UK alone generating waste corresponding to ~35-40% w/w of the initial biomass[11]. Red beetroot is a rich source of betalains, red pigments with strong tinctorial properties, which are receiving increasing popularity for different applications in the food and non-food industries[12]. The global beetroot market is expected to significantly increase in the next decade, with the global production of 690,000 tonnes of beet powder (in 2016) being projected to reach 11 million tonnes by 2027[13]. Apart from betalains, red beetroot also contains other bioactive compounds such as polyphenols, betaine, fiber, nitrate, ascorbic acid and carotenoids[14] and is considered as one of the top ten vegetables associated with superior health benefits[15]. In particular, the industrial production of beet juice, which is increasingly popular due to its blood pressure lowering properties, generates large amounts of pulp waste that are mostly ending up in landfill. In addition to peel and pomace, aerial parts of beet (leaves and stalks) are generally discarded after processing of beets[16]. Therefore, valorization of beet processing waste can contribute to reduction of waste generation and thereby support the concept of zero waste.

Extraction and maximum recovery of bioactive compounds are usually complex and require multistep techniques. The choice of solvent is extremely important for extraction of organic molecules from plant tissues such as betalains, polyphenols and other bioactives, with factors such as solubility of the target compounds, solvent polarity, solvent/target compound/waste matrix interaction, toxicity, cost and availability of solvents needing to be taken into account[17]. Commonly, organic solvents are used to extract bioactives and are combined with novel extraction approaches such as ultrasound-, microwave- and enzyme-assisted extraction methods[18, 19]. In the

present study, ultrasound- and enzyme-assisted extraction methods were selected as candidates to probe the feasibility of extracting betalains and polyphenols as they are considered more sustainable compared to conventional extraction, due to a reduced extraction time, solvent volume and energy consumption[17]. Betalains and polyphenols are located in vacuoles in the plant cells[20] and the acoustic cavitation caused by ultrasound facilitates the breakdown of cell walls and allows betalains as well as phenolic compounds to disseminate into the extraction solvent which can result in higher extraction yield compared to maceration. Further, ultrasound-assisted extraction uses a moderate temperature, which is favorable for extraction of heat-sensitive compounds and can easily be carried out in hybrid with other novel extraction techniques such as supercritical carbon dioxide extraction and microwave treatment[21]. In addition, ultrasound-assisted extraction has been applied to betalain extraction from different plant sources with better performance in comparison to conventional extraction methods such as maceration, magnetic agitation, orbital and metabolic shaking[22-24]. For example, Sivakumar, Anna, Vijayeeswarri and Swaminathan[22] demonstrated a 1.4 fold higher betalain yield when using ultrasound-assisted extraction (ultrasonication applied with probe) compared to maceration with magnetic stirring, while Righi Pessoa, Heloísa, Camila and Beatriz[25] and Ramli, Ismail and Rahmat[24] who used ultrasonic bath found 1.08 and 1.21 increase of extraction yield respectively.

Similarly, enzyme-assisted extraction is receiving an increasing interest and for highly effective extraction under comparatively mild extraction conditions (low temperature and short periods of time) with high recovery of bioactives as it facilitates to retrieve bound compounds[26]. Indeed, the wet waste pulp of red beet is a complex matrix and consists mainly of the plant cell wall polysaccharides (pectin, cellulose, hemicellulose), lignin, other small organic molecules (such as carbohydrates, betalains and polyphenols) and inorganic ions (such as Ca^{2+}, K^{+} and Na^{+}).

Enzymatic pre-treatment of agri-food waste with appropriate hydrolyzing enzymes is an already established approach[27]. For instance, Papaioannou and Karabelas[28] studied lycopene recovery from tomato peel under mild conditions assisted by enzymatic pre-treatment and non-ionic surfactants, thereby allowing disruption of the cell wall structure for enhanced recovery of compounds from plant cell walls.

The aim of the present study was to establish an efficient and sustainable extraction method for betalain containing plant material. To this end, extraction was established in whole beet powder and applied to other betalain rich samples. samples. In addition to betalain yield, pattern and stability, polyphenol extraction and overall antioxidant activity was determined.

Experimental section

Materials

All chemicals and solvents were purchased from Sigma-Aldrich (Dorset, UK) and Fisher Scientific (Loughborough, UK). Betanin standard was obtained from Insight Biotechnology (Wembley, UK). Red beetroot powder (BP) and red beetroot juice powder (BJ) were purchased online from Whole Foods Ltd. (Ramsgate, UK). Food-grade beetroot waste powder (micronized, Beet waste (FD) and air-dried, Beet waste (AD)) were provided by Biopower (Milton Keynes, UK). The wet pulp was provided from James White Ltd. The enzymes Celluclast® 1.5L (cellulase enzyme) and Pectinex® Ultra Mash (pectinase enzyme) were provided by Novozymes A/S, Denmark.

Ultrasound- and enzyme-assisted extraction procedures

The ultrasound-assisted extraction of betalains was carried out using the method described by Righi Pessoa, Heloísa, Camila and Beatriz[25] with some modifications. A 1 g sample was mixed with 25 mL of extraction solvent (water and 20, 30, 50% v/v ethanol or methanol) for 2 min using

a vortex. The mixtures were then placed in an ultrasonic bath (XUBA3, Grant Instruments, UK) and sonicated at 44 kHz for 30 min at 30 °C. The ultrasonic bath has an inbuilt temperature control. The temperature was monitored before and during the treatment, which stayed within a 0.5 degrees difference to the target temperature. The nominal power used for the study was 35W and the energy input per unit volume (energy density (J/mL)) was calculated according to the following equation (eq 1) used by Arruda, Silva, Pereira, Angolini, Eberlin, Meireles and Pastore[29];

$$Energy\ density\ \left(\frac{J}{mL}\right) = \frac{Nominal\ ultrasonic\ power\ (W) \times Extraction\ time\ (s)}{Sample\ volume\ (mL)} \quad (1)$$

For the enzyme-assisted extraction, 17 mL of a 1:1 mixture of pectinase:cellulase enzymes with activity 200 Unit/mL each at pH 5.5 (acetate buffer), was added to 1 g of wet pulp sample and then placed on a controlled heating plate at temperatures 35, 45 and 55°C with magnetic agitation and left to hydrolyze for 2 h. The same procedure was followed with the pulp macerated in only 17 mL water and this was used as reference. Subsequently, ethanol was added to this mixture to achieve a final concentration of 30% (v/v) and, after an additional incubation for 2.5 h at 30°C, the resulting extracts were collected and analysed.

The samples of the above mentioned procedures were centrifuged (Centrifuge 5810 R, Eppendorf, Germany) for 10 min at 3500 × g at 4 °C and at each stage supernatants were collected separately and stored at -20ºC until analyzed. The residues were re-extracted as before with the same solvent that was used for the initial extraction stage (water and 20, 30, 50% v/v ethanol or methanol) for maximum pigment recovery. The supernatants were collected and filtered through a 45 µm pore

membrane. Aliquoted supernatants used for the stability study were stored at -20 °C and room temperature as indicated in the section below.

Quantification of total betalain and polyphenol content

The amount of betalains was determined using spectrophotometry (Specord 210 plus, Analytik Jena, Germany)[30] after appropriate dilution with distilled water into absorbance range (300 - 800 nm) and calculated using extinction coefficient values for 60,000 $cm^{-1}M^{-1}$ at λ_{max} 540 nm and 48,000 $cm^{-1}M^{-1}$ at λ_{max} 480 nm for betacyanin and betaxanthin, respectively. The total amount of betalain (in mg per g sample) was calculated by adding the values for betacyanin and betaxanthin. The total polyphenol content (TPC) in extracts from different solvents was analyzed using 96 well microplate format as recently described[31]. Gallic acid was used as the reference standard in the concentration range 0 – 250 µg/mL. For the assay, 10 µL of the sample or gallic acid standard was mixed with 40 µL of 10% Folin reagent (v/v) and 150 µL 4% sodium carbonate (w/v) incubated for 30 min at room temperature in the dark. Subsequently, absorbance was measured at 765 nm using a Tecan Spark™ 10M multimode microplate reader (TECAN, Männedorf, Switzerland). All samples and standards were analyzed in triplicate and the results were expressed as mg gallic acid equivalent (GAE)/ g sample.

Color measurement in beetroot extracts

The color of the different extracts was assessed using a portable Datacolor check 3 spectrophotometer (Datacolor, Lawrenceville, New Jersey, USA). The instrument was calibrated using a black trap and white tile before measuring the extracts. Extracts were placed in glass Petri dishes with lid and measurements were taken from three different random places of the petri dish.

The readings of L*C*h* were recorded and converted into the L*a*b* values using ColourMine conversion software. The color parameters were expressed as a mean of triplicate measurements.

Antioxidant capacity assays

The extracts were assayed for their potential to inhibit $ABTS^+$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical according to Re, Pellegrini, Proteggente, Pannala, Yang and Rice-Evans[32] with some modifications. Briefly, $ABTS^+$ stock solution (14 mM) was mixed with potassium peroxodisulfate (4.9 mM) at a ratio of 1:1 (v/v), and the mixture was allowed to stand in the dark to formation of radicals at room temperature for 12 – 24 hrs. $ABTS^+$ working solution was prepared by diluting the $ABTS^+$ stock solution with water to an absorbance of 0.700 ± 0.020 at 734 nm. A standard solution of Trolox was prepared to cover a range of 0 to 750 µM in ethanol: water (75:25 v/v). Then, 10 µL of sample or Trolox standard were mixed with 300 µL of $ABTS^+$ working solution and incubated for 60 min at room temperature in the dark. Subsequently, absorbance was measured at 734 nm using microplate reader.

The ferric reducing antioxidant power assay (FRAP) was performed according to Lotito and Frei[33] with some modifications. Acetate buffer (300 mM, pH 3.6) was mixed with 10 mM TPTZ and 20 mM $FeCl_3$ at 10:1:1 (v/v) to prepare the FRAP reagent. Trolox was used as the standard and prepared to cover a concentration range of 0 to 1000 µM in ethanol: water (75:25 v/v). Briefly, 10 µL of the sample or Trolox standard were mixed with 300 µL of FRAP reagent and incubated for 15 min at 37 °C. Subsequently, absorbance was measured at 593 nm using the microplate reader. For both assays, samples and standards were run in triplicate and the results were expressed as mean ± standard deviation in µM Trolox equivalent (TE)/ g of sample.

Identification of betalains and polyphenols

Identification of betalains in the extracts was performed using the Shimadzu application note[34] for betalain analysis with some modifications and polyphenols were analyzed using the method described by Ifie, Marshall, Ho and Williamson[35]. HPLC (LC-2010 HT) coupled with a 2020 quadrupole mass spectrophotometer (Shimadzu, Kyoto, Japan) fitted with an electrospray ionization source (ESI-MS) with a reverse phase Phenomenex Gemini C_{18} column (4.6 mm × 250 mm, 5 µm) was used for both analyses. Both Single Ion Monitoring (SIM) and scan were used in positive mode for betalains and negative mode for polyphenols. The chromatographic conditions for betalain analysis were defined as follows; mobile phase A 2% (v/v) formic acid in water and mobile phase B pure methanol, flow rate 0.95 mL/ min. Betalains were separated using gradient elution mode started with 5-25% B for 15 min, 25-70% B for 4 min, and 70-5% B for last 7.10 min. The temperature of the column oven was set for 40 °C and the injection volume was 10 µL. Betacyanins and betaxanthins were monitored at 536 nm and 486 nm, respectively. The chromatographic conditions for polyphenol analysis were as follows: mobile phase A 0.5% (v/v) formic acid in water and mobile phase B mixture of acetonitrile, water, and formic acid (50:49.5:0.5, v/v), flow rate 0.5 mL/ min. The gradient conditions were as follows; the initial condition started with 8% B and was increased to 18% B at 5.32 min, 32% B at 27.36 min, 60% B at 42.56 min reaching 100% B at 49.04 min, held at 100% B for 6.08 min and returning to initial conditions for 4.52 min. Identification of different polyphenols present in the extracts was performed using the *m/z* values taken from the literature[30, 36].

Data analysis

The data are reported as mean ± standard deviation of three extractions measured in duplicate or triplicate and graphs were drawn using GraphPad Prism version 9.0 for Windows. One-way ANOVA was applied to determine the statistical significance among the extractions at $p < 0.05$ among the different groups. Pearson correlation coefficients were calculated using the GraphPad Prism version 9.0 for Windows.

Results and discussion

Effect of different solvents on extraction of betalains using ultrasound

In the present study, different solvents and solvent-water mixtures were initially tested to optimize extraction conditions for betalains from red beetroot samples using ultrasound-assisted extraction and to assess the stability of betalains and polyphenols at different storage temperatures (RT, -20 °C, for 4 weeks). The principle of ultrasound-assisted extraction involves the acoustic cavitation which is resulted in microjetting[37]. The microjetting generates the effects such as surface peeling and particle breakdown which can promote higher extraction yield[38]. Use of high nominal power (power provided by the device) creates greater extent of shear force and results in high extraction yield [37]. However, there is energy loss in the device during the conversion of mechanical energy into the cavitation[39] The nominal power and the energy density during the extraction process of present study were 35W and 252 J/mL respectively.

Previous studies have reported that aqueous mixtures of organic solvents are most effective for efficient extraction of water-soluble phytochemicals[40-42]. Indeed, different mixtures of solvents miscible with water (20, 30, 50%, v/v) showed superior performance in this study to extract betalains in comparison to pure methanol and ethanol (Figure 1). This is mainly due to the polarity

of the target compounds. Betalains are hydrophilic pigments; therefore mixing of organic solvents with water increases the extraction yield when compared to pure organic solvents such as alcohols. Although, pure water can improve the betalain yield, it has caused severe difficulties during the solute separation by filtration due to co-extraction of mucilaginous compounds such as pectin[42]. The results are in agreement with the findings of Righi Pessoa, Heloísa, Camila and Beatriz[25] who demonstrated total betalain contents in red beetroot ranging from 0.13 mg/g to 6.97 mg/g using different combinations of water with organic solvents. Interestingly, whilst there was no change in betalain yield when extracted with water in comparison to solvent mixtures, some studies suggested that the use of aqueous ethanol or methanol is required to achieve efficient extraction of betalains[15, 42, 43]. Compared to methanol, ethanol proved to be a better choice as an extraction solvent due to being considered non-toxic; it can also be bio-produced from renewable resources and is thus "greener" in environmental assessments, with the added benefit that it can be readily used in the food industry[44]. According to the literature ethanol can reduce the co-extraction of pectin, some soluble fiber and proteins[45] while increasing the extraction of compounds of lower molecular weight[46], thereby enhancing the overall extraction of bioactives such as polyphenols and betalains. Indeed, the preliminary experiments showed 18.3% lower total values of polyphenols when extraction was performed using water in comparison to 30% ethanol (data not shown).

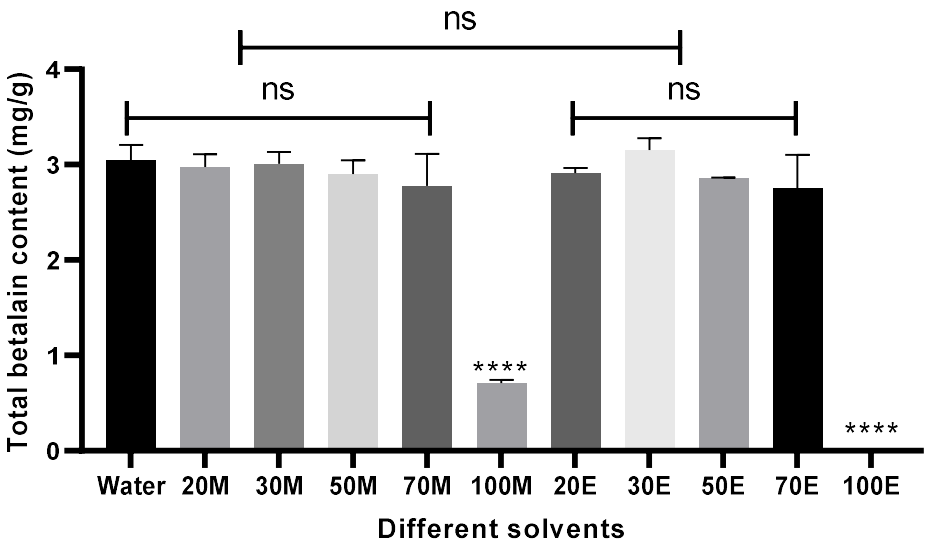

Figure 1. Effect of solvents on total betalain content in dried red beetroot powder extracts. Data are mean with SD of three independent extractions. (M = Methanol v/v%, E = Ethanol v/v%). **** indicates significant difference ($p < 0.05$), Tukey's multiple comparison test.

The further analysis into individual betalain composition of dried red beetroot powder extracts was conducted using HPLC demonstrating the presence of a range of betalains and metabolites in all samples (Figure S1). As expected, they were the main red pigments betanin and isobetanin as well as the predominant yellow pigment vulgaxanthin I, which is in accordance with the literature[47, 48]. A comparison of the peak areas of three main betalain pigments is presented in Figure 2, showing a similar pattern for all the samples. Based on peak area analysis, ethanol performed better with regards to extraction of betalains: the total betalain extractability with aqueous ethanol was 7.7 % and 19.9% higher in comparison to methanol (both at 30% v/v) and water, respectively (although not significant, $p>0.05$). The variation of the yield can be attributed to a different polarity of the extraction solvents e.g. relative polarity: water (1.000), methanol (0.762) and ethanol (0.654)[49]. Efficiency of the extraction process depends on the ability to solvate target molecules. The

dominant contributors to solvation of polar molecules e.g. betalains are charge-dipole, dipole-dipole, H-bonding, which favor polar solvents. On the other hand, weaker electrostatic interactions e.g. ion-π, π-π interactions involving neutral or less polar fragments present in betalains will favor extraction solvents of lower polarity. This indicates ethanol to be a better choice to embrace both types of molecular interactions when combined with polar solvent such water. The results demonstrate a preference for 30% v/v ethanol for extraction, which was selected for further experiments.

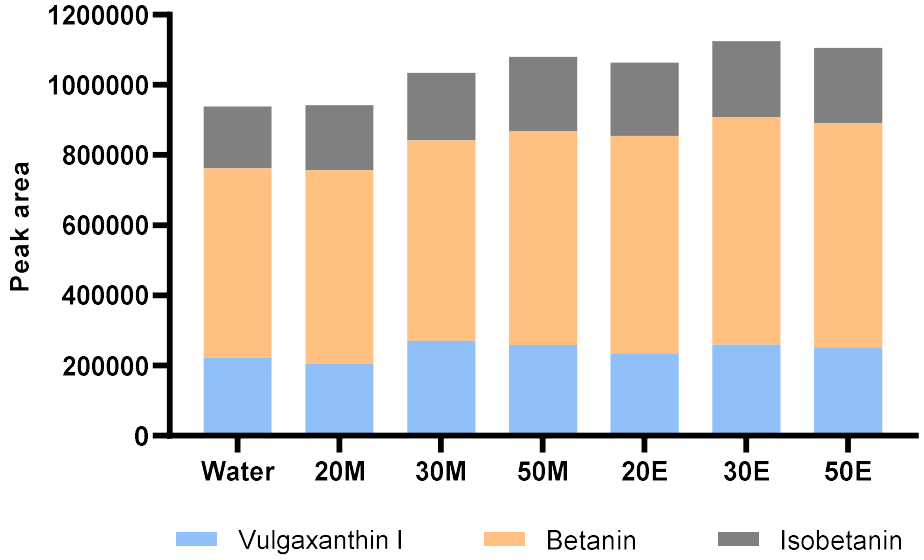

Figure 2. Peak areas of main betalains under different solvent extraction conditions. Data are from HPLC with vulgaxanthin I and betanin/isobetanin monitored at 486 nm and 536 nm, respectively.

The same solvent mixtures were used to evaluate the extraction of betalains and polyphenols from the wet pulp (81.5±0.67 % w/w moisture content) under the temperatures of 35, 45 and 55°C. Similar results were obtained in this case with the 30% v/v aqueous ethanol lead to the recovery of 6.86±0.23 mg/g dry weight polyphenols after three repeating extraction steps, whereas the increase of ethanol in the mixture didn't improve further the polyphenols extraction (data not

shown). The amount of polyphenols extracted by 30% v/v ethanol was 19.3% and 71% higher than these of the pure water and pure ethanol, respectively. This is indicative of the extracted polyphenols mixture higher affinity to lower ethanol concentrations (~30% v/v). Apart from solvent-based extractions, enzyme-assisted extraction, which is considered as a highly effective and sustainable extraction option to achieve high product yields, reduced by-product formation under avoidance of harsh conditions[26] was employed in this study. The enzymes used, cellulose and pectinase, are able to hydrolyze cell wall components and release bioactives that are associated with these, therefore, allowing an overall more efficient extraction of bioactives. In the current study, however, pre-treatment with cellulase and pectinase enzymes was unsuccessful to increase total bioactive recovery, especially betalains from wet pulp. Enzyme treatments were performed at three temperatures, 35, 45 and 55 °C, prior to extraction. There was an enhancement in recovered polyphenols (10.06±0.21 mg/g dry weight) at 45 °C with a net recovery of 3.2 mg/g dry weight as determined by Folin assay compared to extracted polyphenols by maceration. Betalains were not detectable in the macerated wet and enzyme treated samples. Given the absence of the targeted betalains, from these waste material, enzyme-assissted extraction was not further pursued. Betalain absence in the case of wet pulp can be explained by the fact that there are enzymes present which could lead to betalain degradation, whereas in the dry samples these enzymes are not active. Further, high water activity induces the aldimine bond cleavage and promotes the betalain degradation[19].

Effect of extraction solvent and temperature on betalains, total polyphenols and antioxidant activity during four weeks of storage

There are many internal and external factors such as pH, light, temperature, oxygen and water activity that may influence the stability of betalain pigments during storage[15]. Storage temperature in particular can be considered as one of the crucial factors that determine betalain stability[50]. Following extraction with different solvents, the present study sought to establish betalain content and pattern, total polyphenol content and antioxidant activity as well as color measurement as potential indicators of sample deterioration during storage at -20 °C and RT. Betalain content displayed a fast and marked decrease when stored at RT whilst extracts stored at -20 °C remained at the same level (Figure S2 A and B). This results, covering a period of 4 weeks, are in line with others indicating that temperatures below 10 °C are required to preserve betalains from degradation[20, 48, 51]. Only Castellar, Obón, Alacid and Fernández-López[52] observed that betalains in extracts from *Opuntia* varieties were preserved for 19 days at 25 °C. Sapers and Hornstein[53] reported that the degradation of betalains during storage was mainly depending on the pH and light exposure, and directly proportional to the initial concentration of betalains in the samples. In this study, the storage temperature also had an impact on individual betalains; as shown in Figure 3, betalain pattern during storage indicate that vulgaxanthin I was around 20% less prone to degradation in 30% ethanol as compared to methanol at the same percentage of solvent or compared to water. Other betalains displayed much less of a compositional change among the different extracts.

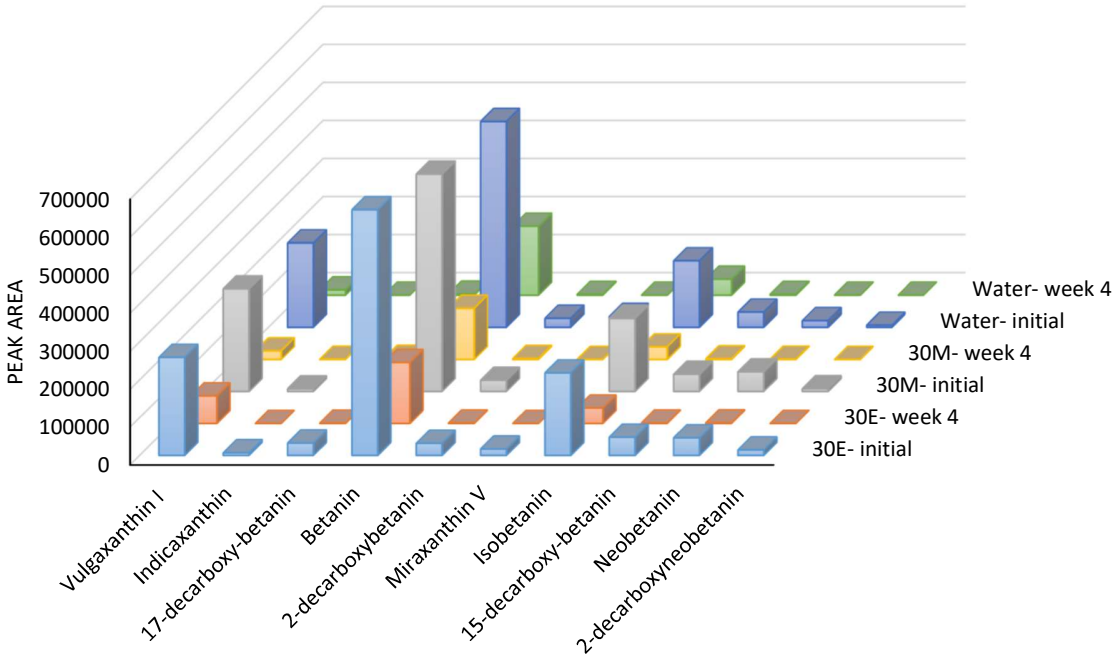

Figure 3. Changes in betalain pattern during 4-week storage of extracts at room temperature. Presented are the peak areas of samples extracted with water, 30% methanol and 30% ethanol, initially and at the end of the storage period.

In contrast to betalains, the TPC of the beetroot extracts showed a different pattern. Whilst the initial values of total polyphenols did not differ between the samples (Figure S3), around 20% of increase was observed up to the second week and then a gradual decline until the end of the storage period, irrespective of the storage conditions; however, this was much more pronounced in samples stored at -20 °C (Figure S2 C and D). The increased TPC is a phenomenon observed also by other relevant studies associating increases with the release of phenolic compounds bound to proteins or polysaccharides during storage[54], deglycosylation, new compound formation[55], and reactions occurring between (oxidized) polyphenols [56]. Indeed, Madiwale, Reddivari, Holm and Vanamala[55] demonstrated activation of phenylalanine ammonia-lyase (PAL), an enzyme which regulates the

biosynthesis of polyphenols, during storage which induced the de novo synthesis of secondary metabolites and may therefore contribute to increased phenolic content. Klimczak, Małecka, Szlachta and Gliszczyńska-Świgło[54] observed an increase of free p-coumaric and ferulic acids in orange juice during storage at different temperatures due to the release of free acids from their bound form (at 18, 28 and 38 ºC) which could be a further reason for changes of TPC content. Folin reagent itself is lacking specificity, and some other reducing compounds such as phenolic amino acids and ascorbic acid are known to react with Folin, thereby increasing the TPC values independently of polyphenols[57]. In addition, the high polyphenol content during the storage period could be linked to the preferential oxidation of betalains that prevents the degradation of polyphenols present in the samples. In studies involving ABTS+, betanin was 1.5 to 2 times more efficient as a free radical scavenger than anthocyanins at neutral or basic pH[58]. It was also observed that among betacyanins such as betanidin, betanin, and phyllocactin, betanidin was the most potent antioxidant against peroxyl radical and nitric oxide indicating that glycosylation decreases the radical scavenging activity of betacyanins[59, 60].

Antioxidant activities were determined using TEAC assay, commonly used method to assess ABTS+ radical scavenging properties. This assay, as well as others such as FRAP, ORAC, DPPH, superoxide radical scavenging, have been shown to correlate with betalain content as demonstrated in several studies[30, 61, 62]. As shown in Figure S2 E and F, antioxidant capacity was similar among samples after extraction and remained largely unaffected during storage at -20 °C. In the case of stored samples at RT, there was a successive decline in antioxidant capacity over the four-week period to around 22%. This loss of antioxidant activity was highly correlated with the betalain decline during RT storage ($r=0.7716$, $p < 0.0001$), but no correlation at -20 °C was evident ($r=0.1877$, $p = 0.1198$) (Figure 4 A and B). Similarly, the TPC and antioxidant capacity showed a

significant correlation at RT (r=0.3209, p = 0.0068), but not at -20 °C storage (r=0.1662, p = 0.1690) (Figure 4 C and D).

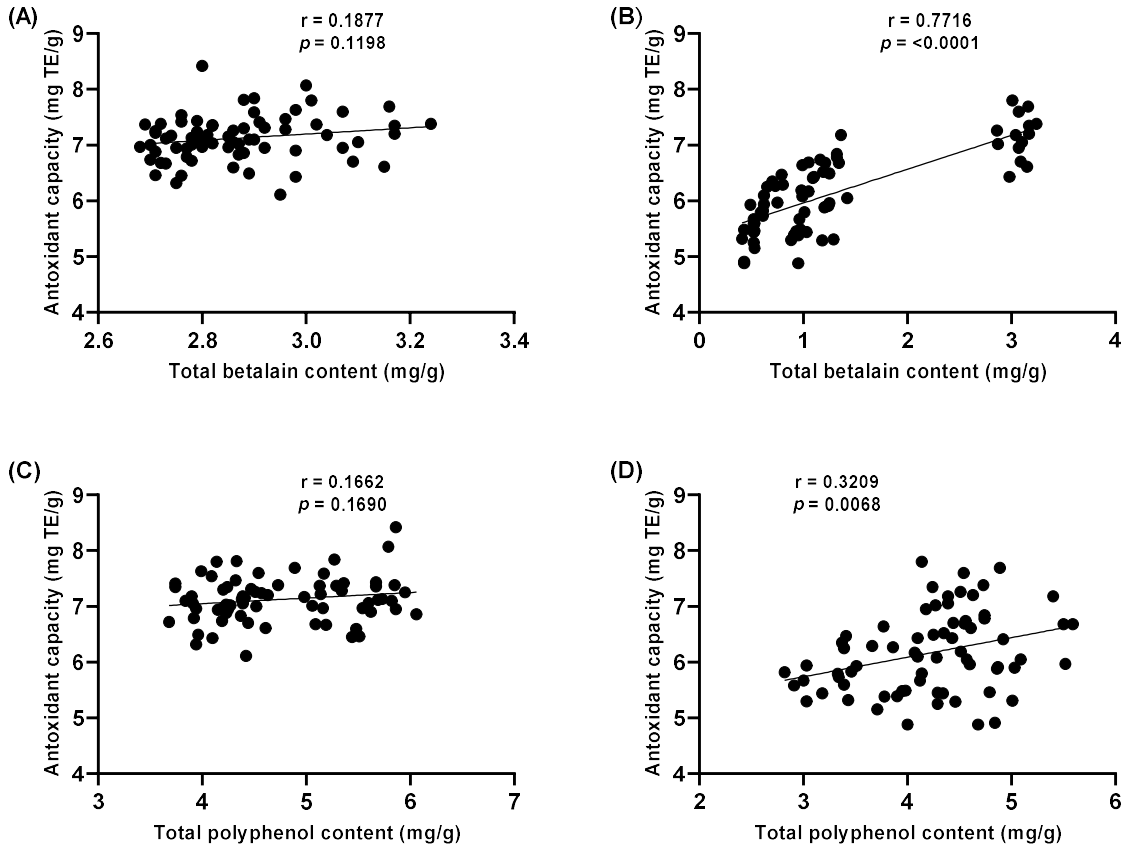

Figure 4. Correlation of betalain content and polyphenol content with antioxidant activity (TEAC -Trolox equivalent antioxidant capacity) of the red beetroot extracts stored in -20 ºC (A and C) and room temperature (B and D).

Results derived from antioxidant capacity measurements are a reflection of overall radical scavenging or reducing capabilities of a sample, which is, similarly to TPC, depending on the composition as well as individual structural features of bioactives in the mixture. Apart from polyphenols, betalains have demonstrated strong radical scavenging activities as compared to

known antioxidants such as ascorbic acid, tocopherols and rutin[58, 63]. Moreover, there is evidence indicating that the degradation products of betalains, such as neobetanin, have even higher antioxidant activity than the betalains themselves[64, 65]. This appears irrelevant for this study as the neobetanin concentrations were lower after 4 weeks compared to the initial data (Figure 3). In addition, other compounds are potentially present in extracts such as betains, carotenoids and dietary nitrate and nitrite and contributing to overall antioxidant activity[14, 66]. The data are demonstrating a substantial decline (80%) of betalains at RT storage, but lower loss of antioxidant activity (22%) emphasizing possible synergetic effects of polyphenols, betalains and their metabolites as well as other compounds present in the extracts in radical scavenging and iron reducing capabilities[62].

Color measurements as indicators for pigment degradation during storage

Betalains are sensitive to oxidation during storage in solutions, which affects their color stability as a result of structure changes. Color stability is a highly important factor when using betalains as natural colorants. Therefore, it is important to measure the color parameters of extracts as it gives indirect indication on the pigment concentration over time. Considering the color measurements, the L* value represent the lightness and darkness of the sample whereas the a* and b* values represent the color direction from red to green and yellow to blue of the samples, respectively[67]. The initial chromatic properties of the extracts did not show any significant difference ($p > 0.05$). There was a marked reduction of a* values (reduction of red color) of the RT stored samples during storage compared to the initial values which indicates the degradation of betalains in the extract, likely due to the decarboxylation of betacyanin and formation of degradation products leading to changes of the red color to yellow/orange[68]. This was confirmed

by the increasing values of b* of the room temperature stored samples compared to the initial values which indicates the development of yellow color in the samples. However, both a* and b* values remained unchanged with the samples stored at -20 °C when compared to the initial color values. The initial color results of this study were compatible with data from Prieto-Santiago, Cavia, Alonso-Torre and Carrillo[69] on the relationship between the color and the thermal degradation of beetroot betalain pigments. Pearson correlation coefficients (r) between color measurements (L*, c*, h*, a* and b*) with TBC are shown in Table S1. The L* h* and b* values were negatively correlated ($p < 0.0001$) with TBC ($r = -0.9074$, $r = -0.9256$ and $r = -0.8807$ respectively) while c* and a*values showed positive correlation ($p < 0.0001$) with TBC ($r = 0.5903$ and $r = 0.8967$ respectively). Other studies[69-71] have reported that pigment content can be correlated better with the combined color parameters than the single color measurements. Therefore, the different combinations of color numeric values were calculated and shown in Table S1. The L*a*b* data is a good indicator of visual color assessment of the samples[69] and there was a strong positive correlation between L*a*b* data and total betalain content ($r = 0.9820$, $p < 0.0001$). Additionally, the a/b ratio can be used as a convenient parameter for assess the color degradation accurately as well as quantitatively[71]. Therefore, these correlations indicate that the color measurement could be used as indirect assessment to determine the betalain pigments as easy and inexpensive method.

Application of selected extraction conditions to characterize different red beetroot samples

A further aim of this study was to apply the selected extraction conditions to different beet derived samples, which were, apart from whole beet, beet juice, beet pulp waste from juicing industries as air dried and freeze dried products.

Following extraction using 30% v/v ethanol, betalain values in the four samples ranged from 0-3.06 mg/g as shown in Figure 5A. As indicated in the earlier section, the data of the present study are in the range of others, some authors have shown a higher total betalain content in red beetroot cultivars ranging from 4.43 – 9.60 mg/g dry matter[47], and 7.42 – 8.56 mg/g dry matter[72]. In contrast, Lee, An, Nguyen, Patil, Kim and Yoo[66] observed relatively low concentrations of betalains (0.65 – 0.80 mg/g fresh weight) in red beetroot cultivars from USA. Variations of results could, apart from extraction and extraction conditions (temperature, pH), be due to differences in beet varieties and growth conditions[73]. The ratio of betacyanin to betaxanthin was 1.12, 1.35 and 1 for the BP, BJ and beet waste (FD) samples respectively, demonstrating that betalain composition of the samples was varied. A similar ratio of betacyanin to betaxanthin has been reported previously for different beetroot sources[47, 74].

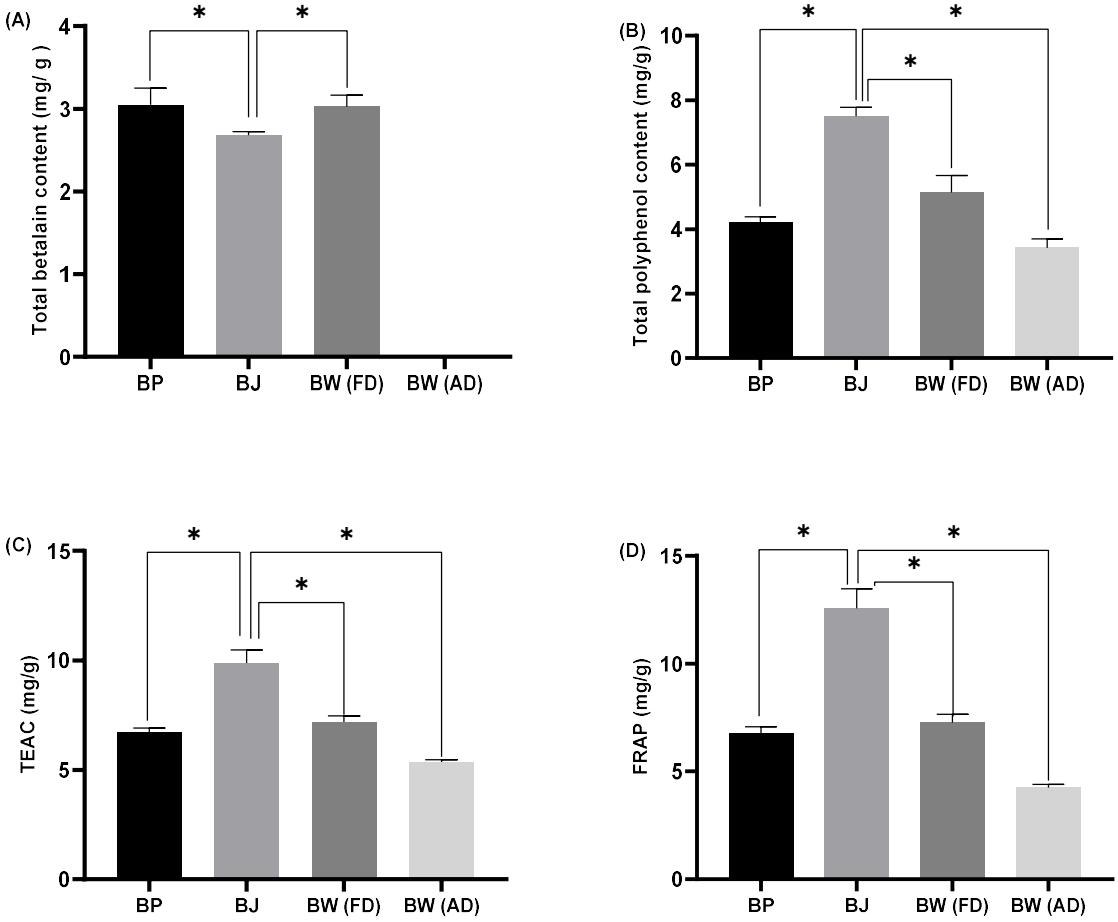

Figure 5. Total betalains (A), total polyphenols (B) and antioxidant activities (C, D) in extracts of different beetroot samples after ultrasound-assisted extraction. Data are mean with SD of three independent extractions. * indicates significant difference ($p < 0.05$), Tukey's multiple comparison test.

Betalain peaks were identified using individual retention times, interpretation of MS fragmentation spectrum (m/z values) and λ_{max} values compared with previously published data[75]. The red beetroot sources examined in the present study contained sixteen different betalain compounds with eleven of them belonging to the betacyanin group and five to betaxanthins Figure 6. However, some

previously reported betanin derivatives and betaxanthins could not be detected. Sawicki, Bączek and Wiczkowski[73] reported the presence of eighteen betacyanins with twelve betaxanthins in thirteen Polish varieties of red beetroot. In comparison, only three betalains (betanin, isobetanin and vulgaxanthin I) were identified in red beet cultivars grown in USA and Finland[47, 66]. Differences in betalain content and pattern may be due to varietal diversity, local growth and climate conditions as well as post-harvest conditions[76]. The most prominent peaks identified in the current study were betanin, isobetanin, vulgaxanthin I and neobetanin. Further, not all samples contained all betalains that had been identified. The sample that had been originally air dried was devoid of most peaks indicating large-scale degradation of betalains, likely UV and temperature facilitated, whereas the peak areas in beetroot waste FD sample were more similar to the BP sample which is derived from whole beet. In general, the betalain content (betacyanins and betaxanthins) is highest in the peel of red beet in comparison to the inner rings[47, 72, 73], which is also evident in the present study. Peak areas of vulgaxanthin I and betanin are much lower in the beet juice sample as compared to samples comprising the whole beet (BP) and pomace fraction (beet waste, FD) (Figure 6). In summary, the results of betalain analysis demonstrate that the dried beetroot waste from juicing industries can be a good source of betalain pigments, with regards to betalain yield equivalent to whole beet and beet juice.

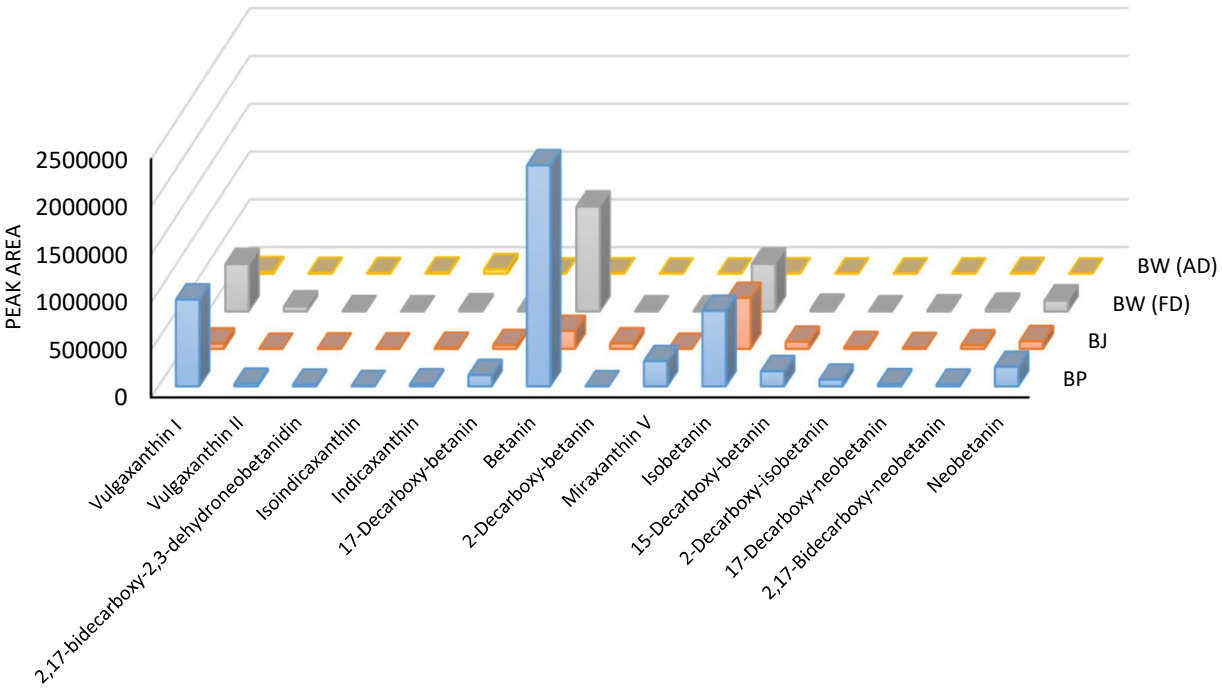

Figure 6. Comparison of betalain peaks present in different beetroot sources (BP –red beetroot powder, BJ – Beetroot juice powder, BW (FD) – freeze dried red beetroot waste powder, BW (AD) – air dried beetroot waste powder)

Generally, betalains are quantified using the spectrophotometric method based on the absorption at a single wavelength and the molar extinction coefficient of the prominent betacyanin and betaxanthin present in the extracts. However, the problems arising in spectrophotometric analysis of such complex mixtures have been highlighted in the literature and attributed mainly to overlapping peaks of betacyanins and betaxanthins, and absorption by the other interfering substances present in the extract[77, 78]. In the present study, air dried beet waste (AD) did not show any peaks around 486 nm or 536 nm in UV-vis spectrum (Figure S4), but some betalains were observed in the HPLC chromatogram (Figure S5). Therefore, HPLC is the method of choice for

most accurate quantification of betalains, by eliminating the aforementioned problems associated with spectrophotometry. However, the standards have to be isolated from the plant materials in case of quantification of betalains using HPLC due to lack of commercial availability[79]. Thus, despite the relatively high discrepancy (~15%) in calculation between the two methods, HPLC and UV-vis spectroscopy, the latter remains the most convenient and fastest method to quantify betalains [78]. In the present study, peak areas were used as basis for comparing individual samples, which has been applied by many other groups[30, 72].

In contrast to betalains, there are detectable polyphenols in all samples, however, the total polyphenol content is much higher in BJ compared to BP and Beet waste samples Figure 5. Current TPC values (3.42 ± 0.27 – 7.50 ± 0.28 mg/g) are in the range that others have reported from 0.51 ± 0.07 to 15.5 ± 0.1 mg/g[30, 80-83] which include as main polyphenols gallic, syringic, caffeic and ferulic acids[30]. In the present study twelve different polyphenols were identified of which seven were hydroxycinnamic acid derivatives, four belonging to the flavonoids group and one trihydroxybenzoic acid (

Table 1). Similar polyphenol composition was reported by the other studies which analyzed the polyphenol composition of different varieties of beetroot including juice, roots and stem extracts[47, 74, 84].

Table 1. HPLC-MS data (negative ionization mode) for identification of polyphenols present in different beetroot sources

No	Compound	Retention time (min)	λ_{max}	$[M-H]^-$	Beetroot sources			
					BP	BJ	BW (FD)	BW (AD)
1	Catechin	4.29	282	289	+	+	+	+
2	Cochliophilin A	4.98	283	281	+	+	+	nd
3	*p*-coumaric acid	5.33	282	163	+	+	+	+
4	Caffeic acid	5.67	265	179	+	+	+	+
5	*N-trans*-feruloylmethoxytyramine	6.13	278	342	+	+	+	+
6	Ferulic acid	6.39	274	193	+	+	+	+
7	Chlorogenic acid	7.77	281	353	+	+	+	nd
8	Gallic acid	9.25	282	169	+	+	+	+
9	Rosmarinic acid	16.65	265	359	+	+	+	nd
10	*N-trans*-feruloyltyramine	21.75	274	312	+	+	+	nd
11	Quercetin	52.40	361	301	+	+	+	+
12	Betavulgarin	56.42	279	311	+	+	+	nd

nd – not detected

In line with the polyphenol content, the antioxidant activity of BJ, determined as TEAC and FRAP, was 32% and 46% higher compared with BP and 27% and 42% higher than beet waste (FD) and 45% and 66% higher than beet waste (AD), respectively (Figure 5C and D). A highly significant correlation ($p<0.05$) was observed between the total polyphenol content with TEAC assay (r= 0.9845) and FRAP assay (r= 0.9753). Interestingly, the betalain content did not show any significant correlation with TEAC (r= 2196, p= 0.5314) and FRAP (r=0.2078, p=0.5442) assays

(p>0.05). Several studies determined a strong relationship between radical scavenging activity and betalains as well as polyphenols present in a range of fruits and vegetables[63, 85, 86]. For instance, Čanadanović-Brunet, Savatović, Ćetković, Vulić, Djilas, Sinisa and Cvetković[87] observed a significantly high linear correlation between hydroxyl ($r > 0.81$) and superoxide ($r > 0.92$) radical scavenging activities with betacyanins and betaxanthins extracted from beetroot pomace.

Conclusion

To conclude, effective combined extraction of betalains and polyphenols from red beet dried powder has been demonstrated in an ultrasound-assisted approach establishing low ethanol concentrations (30% ethanol) as the most suitable solvent combination compared to the enzyme-assisted extraction method from wet pulp. The stability of betalains, in contrast to polyphenols, was strongly affected by storage temperature leading to a rapid loss of betalains over the observation period of four weeks at room temperature, irrespective of the solvent used, a finding that is in good correlation with color measurements. The comparatively moderate loss of antioxidant activity *vs* betalain content over time emphasizes the potential contribution of betalains and polyphenols as well as their metabolites and/or degradation products to antioxidant activity. These comparative extraction results indicate that the samples derived from the beetroot industry can provide good pigment yield, after their initial drying, similarly to whole beet samples.

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Supporting information

Supporting tables and figures are provided in a separate file. Experiment and characterization details; HPLC chromatograms, variation of TBC, TPP and antioxidant activity during the storage period, effects of differet solvents on TBC, TPP and antixodant activity of red beetroot extract, correlation coefficients of color data, UV-Vis spectrum of beetroot samples and HPLC chromatograms of red beetroot samples.

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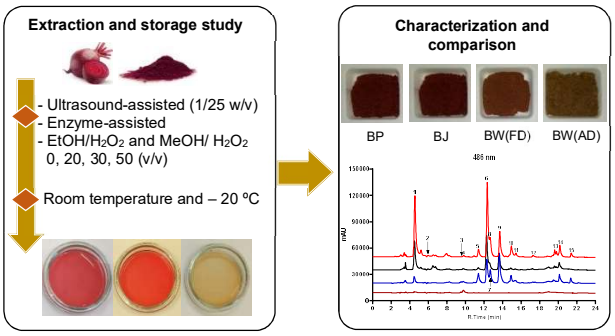

Synopsis

Extraction of betalains from beetroot waste using ultrasound-assisted extraction method for their valorization as a value-added natural colouring additive with antioxidant properties.