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**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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*Authors*

*Ganwarige Sumali N. Fernando<sup>† ‡</sup>, Kelly Wood<sup>‡</sup>, Emmanouil H. Papaioannou<sup>#</sup>, Lisa J.*

*Marshall<sup>†</sup>, Natalia N. Sergeeva<sup>‡</sup>, and Christine Boesch<sup>† \*</sup>*

<sup>†</sup>School of Food Science and Nutrition, Faculty of Environment, University of Leeds, LS2 9JT, United Kingdom.

<sup>‡</sup>School of Chemistry, Faculty of Engineering and Physical Sciences, University of Leeds, LS2 9JT, United Kingdom.

<sup>#</sup>Department of Engineering, Faculty of Science and Technology, Lancaster University, LA1 4YW, Lancaster, United Kingdom.

\*Corresponding author

Tel: 0044-1133430268

Email: c.bosch@leeds.ac.uk

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6 17 **Abstract**  
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9 18 Agriculture and food industries generate substantial quantities of waste material with huge  
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11 19 potential for bioactive ingredients to be recovered and converted into high value chemicals. Red  
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13 20 beetroot, known for its high content in betalains, natural red pigments, as well as polyphenols,  
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15 21 fibre and nitrate, is experiencing increasing demand, in particular as juice, which is leaving behind  
16  
17 22 large amounts of waste. The present study focused on the recovery of betalains and polyphenols  
18  
19 23 from dried whole beetroot, wet and dried beet pulp waste from the juicing industry. As part of an  
20  
21 24 ultrasound-assisted extraction, ethanol/water-based solvent mixtures were used as they were found  
22  
23 25 to be more effective than single solvents. Enzyme-assisted extraction was initially examined in  
24  
25 26 case of wet pulp, but was not able to retain betalains. Betalains appear to be more stable in dried  
26  
27 27 pulp. Ultrasound-assisted extraction was found more suitable to effectively extract both betalains  
28  
29 28 and polyphenols with high bioactive yield from dried pulp. The total betalain and polyphenol  
30  
31 29 profiles as well as storage stability and antioxidant capacities were evaluated over a period of 4  
32  
33 30 weeks after extraction from the dried waste. During the 4 week storage, betalains quickly degraded  
34  
35 31 at room temperature in contrast to -20 °C, whereas polyphenols and antioxidative activity were  
36  
37 32 much less influenced by temperature. When compared, dried samples from the beetroot juicing  
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39 33 industry demonstrate good betalain and polyphenol extractability, thus this data indicate that dried  
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41 34 beet waste can serve as a good source of betalains for the color industry and other technological  
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43 35 sectors.  
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50 36 **Key words:** Betalains, polyphenols, antioxidant capacity, storage, beetroot waste, ultrasound-  
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52 37 assisted extraction, enzyme-assisted extraction  
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## 39 Introduction

40 The food industry is responsible for the generation of up to 60% of total food waste during their  
41 production, distribution and retail process<sup>1</sup>. In Europe, around 90 million tonnes of food waste are  
42 generated on a yearly basis, which corresponds to ca. 170 million tonnes of CO<sub>2</sub> equivalent emitted  
43 per year<sup>2</sup>. Of these, juice, canned and frozen fruits and vegetable industries approximately generate  
44 11.5 million tonnes of waste annually excluding the waste from grape and wine industries<sup>3</sup>. This  
45 waste material has generally a high moisture content (~80% w/w) and is rich in sugars (~75% w/w  
46 dry matter)<sup>4</sup>, which makes it prone to microbial spoilage. Their incineration has been proven to be  
47 unsustainable as it uses high temperatures, has a low energy yield, contributes to waste disposal in  
48 landfills, and downgrades organic material which could be used for other purposes. Other  
49 treatments such as composting and anaerobic digestion provide more stable final material from a  
50 microbiological perspective, but again downgrade the initial organic matter. The management of  
51 food waste becomes an increasingly relevant challenge to reduce pollution, increase the industry  
52 revenues and improve recycling. So far, most food waste is utilized for the production of biofuels,  
53 preparation of fiber and as animal feed<sup>5</sup>. However, there is good evidence that food waste could  
54 be more effectively used as a source of bioactive compounds with increased value and significance  
55 to human nutrition, target compounds being phenolics, pigments, vitamins, peptides, and aromatic  
56 compounds<sup>3,6</sup>. For instance, it was reported that peels and seeds of citrus fruits, grapes, mangoes,  
57 avocados, and jackfruit contain over 15% more polyphenols than the edible parts<sup>7</sup>. As well, Choi,  
58 Kozukue, Kim and Friedman<sup>8</sup> reported that potato peels contain three times higher chlorogenic  
59 acids as compared to the cortex.

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

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6 62 industry is being extensively utilized<sup>9</sup>, and is an excellent source for polyphenols<sup>10</sup>. In contrast to  
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8 63 sugar beet, the waste from red beetroot processing has not been sufficiently considered for its  
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10 64 alternative uses. The EU is the largest beetroot global producer (~70%), with the beetroot juice  
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12 65 production in the UK alone generating waste corresponding to ~35-40% w/w of the initial  
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14 66 biomass<sup>11</sup>. Red beetroot is a rich source of betalains, red pigments with strong tinctorial properties,  
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16 67 which are receiving increasing popularity for different applications in the food and non-food  
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18 68 industries<sup>12</sup>. The global beetroot market is expected to significantly increase in the next decade,  
19  
20 69 with the global production of 690,000 tonnes of beet powder (in 2016) being projected to reach 11  
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22 70 million tonnes by 2027<sup>13</sup>. Apart from betalains, red beetroot also contains other bioactive  
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24 71 compounds such as polyphenols, betaine, fiber, nitrate, ascorbic acid and carotenoids<sup>14</sup> and is  
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26 72 considered as one of the top ten vegetables associated with superior health benefits<sup>15</sup>. In particular,  
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28 73 the industrial production of beet juice, which is increasingly popular due to its blood pressure  
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30 74 lowering properties, generates large amounts of pulp waste that are mostly ending up in landfill.  
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32 75 In addition to peel and pomace, aerial parts of beet (leaves and stalks) are generally discarded after  
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34 76 processing of beets<sup>16</sup>. Therefore, valorization of beet processing waste can contribute to reduction  
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36 77 of waste generation and thereby support the concept of zero waste.  
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38 78 Extraction and maximum recovery of bioactive compounds are usually complex and require  
39  
40 79 multistep techniques. The choice of solvent is extremely important for extraction of organic  
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42 80 molecules from plant tissues such as betalains, polyphenols and other bioactives, with factors such  
43  
44 81 as solubility of the target compounds, solvent polarity, solvent/target compound/waste matrix  
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46 82 interaction, toxicity, cost and availability of solvents needing to be taken into account<sup>17</sup>.  
47  
48 83 Commonly, organic solvents are used to extract bioactives and are combined with novel extraction  
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50 84 approaches such as ultrasound-, microwave- and enzyme-assisted extraction methods<sup>18, 19</sup>. In the  
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6 85 present study, ultrasound- and enzyme-assisted extraction methods were selected as candidates to  
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8 86 probe the feasibility of extracting betalains and polyphenols as they are considered more  
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10 87 sustainable compared to conventional extraction, due to a reduced extraction time, solvent volume  
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12 88 and energy consumption<sup>17</sup>. Betalains and polyphenols are located in vacuoles in the plant cells<sup>20</sup>  
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14  
15 89 and the acoustic cavitation caused by ultrasound facilitates the breakdown of cell walls and allows  
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17 90 betalains as well as phenolic compounds to disseminate into the extraction solvent which can result  
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19 91 in higher extraction yield compared to maceration. Further, ultrasound-assisted extraction uses a  
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21 92 moderate temperature, which is favorable for extraction of heat-sensitive compounds and can  
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23 93 easily be carried out in hybrid with other novel extraction techniques such as supercritical carbon  
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25 94 dioxide extraction and microwave treatment<sup>21</sup>. In addition, ultrasound-assisted extraction has been  
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27 95 applied to betalain extraction from different plant sources with better performance in comparison  
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29 96 to conventional extraction methods such as maceration, magnetic agitation, orbital and metabolic  
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31 97 shaking<sup>22-24</sup>. For example, Sivakumar, Anna, Vijayeeswarri and Swaminathan<sup>22</sup> demonstrated a  
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33 98 1.4 fold higher betalain yield when using ultrasound-assisted extraction (ultrasonication applied  
34  
35 99 with probe) compared to maceration with magnetic stirring, while Righi Pessoa, Heloísa, Camila  
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39 100 and Beatriz<sup>25</sup> and Ramli, Ismail and Rahmat<sup>24</sup> who used ultrasonic bath found 1.08 and 1.21  
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41 101 increase of extraction yield respectively.

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44 102 Similarly, enzyme-assisted extraction is receiving an increasing interest and for highly effective  
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46 103 extraction under comparatively mild extraction conditions (low temperature and short periods of  
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48 104 time) with high recovery of bioactives as it facilitates to retrieve bound compounds<sup>26</sup>. Indeed, the  
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50 105 wet waste pulp of red beet is a complex matrix and consists mainly of the plant cell wall  
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52 106 polysaccharides (pectin, cellulose, hemicellulose), lignin, other small organic molecules (such as  
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54 107 carbohydrates, betalains and polyphenols) and inorganic ions (such as Ca<sup>2+</sup>, K<sup>+</sup> and Na<sup>+</sup>).

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6 108 Enzymatic pre-treatment of agri-food waste with appropriate hydrolyzing enzymes is an already  
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8 109 established approach<sup>27</sup>. For instance, Papaioannou and Karabelas<sup>28</sup> studied lycopene recovery  
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10 110 from tomato peel under mild conditions assisted by enzymatic pre-treatment and non-ionic  
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12 111 surfactants, thereby allowing disruption of the cell wall structure for enhanced recovery of  
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14 112 compounds from plant cell walls.

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17 113 The aim of the present study was to establish an efficient and sustainable extraction method for  
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19 114 betalain containing plant material. To this end, extraction was established in whole beet powder  
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21 115 and applied to other betalain rich samples. samples. In addition to betalain yield, pattern and  
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23 116 stability, polyphenol extraction and overall antioxidant activity was determined.

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## 27 118 **Experimental section**

### 28 119 **Materials**

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30 120 All chemicals and solvents were purchased from Sigma-Aldrich (Dorset, UK) and Fisher Scientific  
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32 121 (Loughborough, UK). Betanin standard was obtained from Insight Biotechnology (Wembley,  
33  
34 122 UK). Red beetroot powder (BP) and red beetroot juice powder (BJ) were purchased online from  
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36 123 Whole Foods Ltd. (Ramsgate, UK). Food-grade beetroot waste powder (micronized, Beet waste  
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38 124 (FD) and air-dried, Beet waste (AD)) were provided by Biopower (Milton Keynes, UK). The wet  
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40 125 pulp was provided from James White Ltd. The enzymes Celluclast® 1.5L (cellulase enzyme) and  
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42 126 Pectinex® Ultra Mash (pectinase enzyme) were provided by Novozymes A/S, Denmark.

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### 48 128 **Ultrasound- and enzyme-assisted extraction procedures**

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50 129 The ultrasound-assisted extraction of betalains was carried out using the method described by  
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52 130 Righi Pessoa, Heloísa, Camila and Beatriz<sup>25</sup> with some modifications. A 1 g sample was mixed  
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54 131 with 25 mL of extraction solvent (water and 20, 30, 50% v/v ethanol or methanol) for 2 min using



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6 132 a vortex. The mixtures were then placed in an ultrasonic bath (XUBA3, Grant Instruments, UK)  
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8 133 and sonicated at 44 kHz for 30 min at 30 °C. The ultrasonic bath has an inbuilt temperature control.  
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10 134 The temperature was monitored before and during the treatment, which stayed within a 0.5 degrees  
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12 135 difference to the target temperature. The nominal power used for the study was 35W and the energy  
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14 136 input per unit volume (energy density (J/mL)) was calculated according to the following equation  
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17 137 (eq 1) used by Arruda, Silva, Pereira, Angolini, Eberlin, Meireles and Pastore<sup>29</sup>;

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$$139 \quad \text{Energy density} \left( \frac{J}{mL} \right) = \frac{\text{Nominal ultrasonic power (W)} \times \text{Extraction time (s)}}{\text{Sample volume (mL)}}$$

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(1)

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

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

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6 153 membrane. Aliquoted supernatants used for the stability study were stored at -20 °C and room  
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8 154 temperature as indicated in the section below.  
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### 11 156 **Quantification of total betalain and polyphenol content**

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15 157 The amount of betalains was determined using spectrophotometry (Specord 210 plus, Analytik  
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17 158 Jena, Germany)<sup>30</sup> after appropriate dilution with distilled water into absorbance range (300 - 800  
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19 159 nm) and calculated using extinction coefficient values for 60,000 cm<sup>-1</sup>M<sup>-1</sup> at  $\lambda_{\max}$  540 nm and  
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21 160 48,000 cm<sup>-1</sup>M<sup>-1</sup> at  $\lambda_{\max}$  480 nm for betacyanin and betaxanthin, respectively. The total amount of  
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23 161 betalain (in mg per g sample) was calculated by adding the values for betacyanin and betaxanthin.  
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25 162 The total polyphenol content (TPC) in extracts from different solvents was analyzed using 96 well  
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27 163 microplate format as recently described<sup>31</sup>. Gallic acid was used as the reference standard in the  
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29 164 concentration range 0 – 250 µg/mL. For the assay, 10 µL of the sample or gallic acid standard was  
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31 165 mixed with 40 µL of 10% Folin reagent (v/v) and 150 µL 4% sodium carbonate (w/v) incubated  
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33 166 for 30 min at room temperature in the dark. Subsequently, absorbance was measured at 765 nm  
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35 167 using a Tecan Spark<sup>TM</sup> 10M multimode microplate reader (TECAN, Männedorf, Switzerland). All  
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37 168 samples and standards were analyzed in triplicate and the results were expressed as mg gallic acid  
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39 169 equivalent (GAE)/ g sample.  
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### 45 171 **Color measurement in beetroot extracts**

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48 172 The color of the different extracts was assessed using a portable Datacolor check 3  
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50 173 spectrophotometer (Datacolor, Lawrenceville, New Jersey, USA). The instrument was calibrated  
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52 174 using a black trap and white tile before measuring the extracts. Extracts were placed in glass Petri  
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54 175 dishes with lid and measurements were taken from three different random places of the petri dish.  
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6 176 The readings of  $L^*C^*h^*$  were recorded and converted into the  $L^*a^*b^*$  values using ColourMine  
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8 177 conversion software. The color parameters were expressed as a mean of triplicate measurements.  
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### 11 12 13 179 **Antioxidant capacity assays**

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15 180 The extracts were assayed for their potential to inhibit  $ABTS^+$  [2,2'-azino-bis(3-  
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17 181 ethylbenzothiazoline-6-sulfonic acid)] radical according to Re, Pellegrini, Proteggente, Pannala,  
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19 182 Yang and Rice-Evans<sup>32</sup> with some modifications. Briefly,  $ABTS^+$  stock solution (14 mM) was  
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21 183 mixed with potassium peroxodisulfate (4.9 mM) at a ratio of 1:1 (v/v), and the mixture was allowed  
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23 184 to stand in the dark to formation of radicals at room temperature for 12 – 24 hrs.  $ABTS^+$  working  
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25 185 solution was prepared by diluting the  $ABTS^+$  stock solution with water to an absorbance of 0.700  
26  
27 186  $\pm$  0.020 at 734 nm. A standard solution of Trolox was prepared to cover a range of 0 to 750  $\mu$ M in  
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29 187 ethanol: water (75:25 v/v). Then, 10  $\mu$ L of sample or Trolox standard were mixed with 300  $\mu$ L of  
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31 188  $ABTS^+$  working solution and incubated for 60 min at room temperature in the dark. Subsequently,  
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33 189 absorbance was measured at 734 nm using microplate reader.  
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37 190 The ferric reducing antioxidant power assay (FRAP) was performed according to Lotito and Frei<sup>33</sup>  
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39 191 with some modifications. Acetate buffer (300 mM, pH 3.6) was mixed with 10 mM TPTZ and 20  
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41 192 mM  $FeCl_3$  at 10:1:1 (v/v) to prepare the FRAP reagent. Trolox was used as the standard and  
42  
43 193 prepared to cover a concentration range of 0 to 1000  $\mu$ M in ethanol: water (75:25 v/v). Briefly, 10  
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45 194  $\mu$ L of the sample or Trolox standard were mixed with 300  $\mu$ L of FRAP reagent and incubated for  
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47 195 15 min at 37 °C. Subsequently, absorbance was measured at 593 nm using the microplate reader.  
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50 196 For both assays, samples and standards were run in triplicate and the results were expressed as  
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52 197 mean  $\pm$  standard deviation in  $\mu$ M Trolox equivalent (TE)/ g of sample.  
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6 199 **Identification of betalains and polyphenols**  
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8 200 Identification of betalains in the extracts was performed using the Shimadzu application note<sup>34</sup> for  
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10 201 betalain analysis with some modifications and polyphenols were analyzed using the method  
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12 202 described by Ifie, Marshall, Ho and Williamson<sup>35</sup>. HPLC (LC-2010 HT) coupled with a 2020  
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14 203 quadrupole mass spectrophotometer (Shimadzu, Kyoto, Japan) fitted with an electrospray  
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16 204 ionization source (ESI-MS) with a reverse phase Phenomenex Gemini C<sub>18</sub> column (4.6 mm × 250  
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18 205 mm, 5 μm) was used for both analyses. Both Single Ion Monitoring (SIM) and scan were used in  
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20 206 positive mode for betalains and negative mode for polyphenols. The chromatographic conditions  
21  
22 207 for betalain analysis were defined as follows; mobile phase A 2% (v/v) formic acid in water and  
23  
24 208 mobile phase B pure methanol, flow rate 0.95 mL/ min. Betalains were separated using gradient  
25  
26 209 elution mode started with 5-25% B for 15 min, 25-70% B for 4 min, and 70-5% B for last 7.10  
27  
28 210 min. The temperature of the column oven was set for 40 °C and the injection volume was 10 μL.  
29  
30 211 Betacyanins and betaxanthins were monitored at 536 nm and 486 nm, respectively. The  
31  
32 212 chromatographic conditions for polyphenol analysis were as follows: mobile phase A 0.5% (v/v)  
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34 213 formic acid in water and mobile phase B mixture of acetonitrile, water, and formic acid  
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36 214 (50:49.5:0.5, v/v), flow rate 0.5 mL/ min. The gradient conditions were as follows; the initial  
37  
38 215 condition started with 8% B and was increased to 18% B at 5.32 min, 32% B at 27.36 min, 60%  
39  
40 216 B at 42.56 min reaching 100% B at 49.04 min, held at 100% B for 6.08 min and returning to initial  
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42 217 conditions for 4.52 min. Identification of different polyphenols present in the extracts was  
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44 218 performed using the *m/z* values taken from the literature<sup>30, 36</sup>.  
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## 222 **Data analysis**

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

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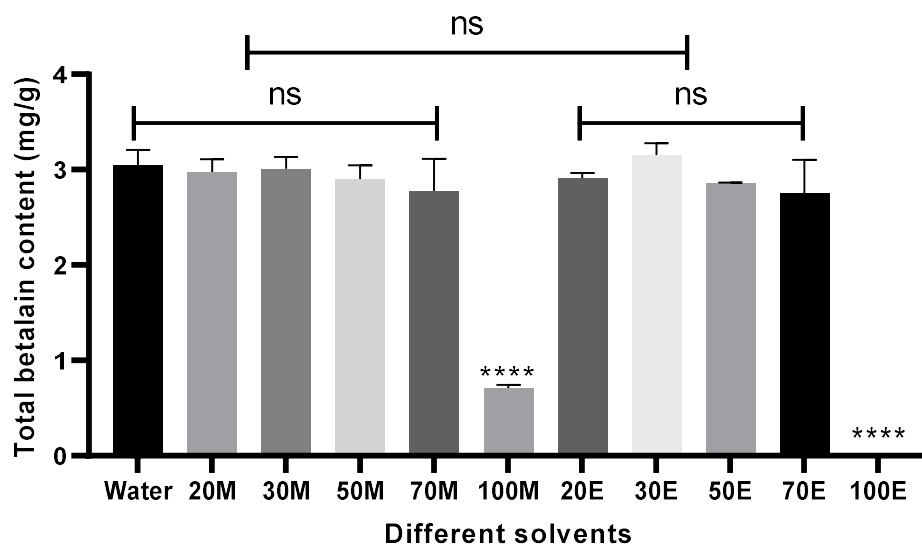
## 229 **Results and discussion**

### 230 **Effect of different solvents on extraction of betalains using ultrasound**

231 In the present study, different solvents and solvent-water mixtures were initially tested to optimize  
232 extraction conditions for betalains from red beetroot samples using ultrasound-assisted extraction  
233 and to assess the stability of betalains and polyphenols at different storage temperatures (RT, -20  
234 °C, for 4 weeks). The principle of ultrasound-assisted extraction involves the acoustic cavitation  
235 which is resulted in microjetting<sup>37</sup>. The microjetting generates the effects such as surface peeling  
236 and particle breakdown which can promote higher extraction yield<sup>38</sup>. Use of high nominal power  
237 (power provided by the device) creates greater extent of shear force and results in high extraction  
238 yield<sup>37</sup>. However, there is energy loss in the device during the conversion of mechanical energy  
239 into the cavitation<sup>39</sup>. The nominal power and the energy density during the extraction process of  
240 present study were 35W and 252 J/mL respectively.

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

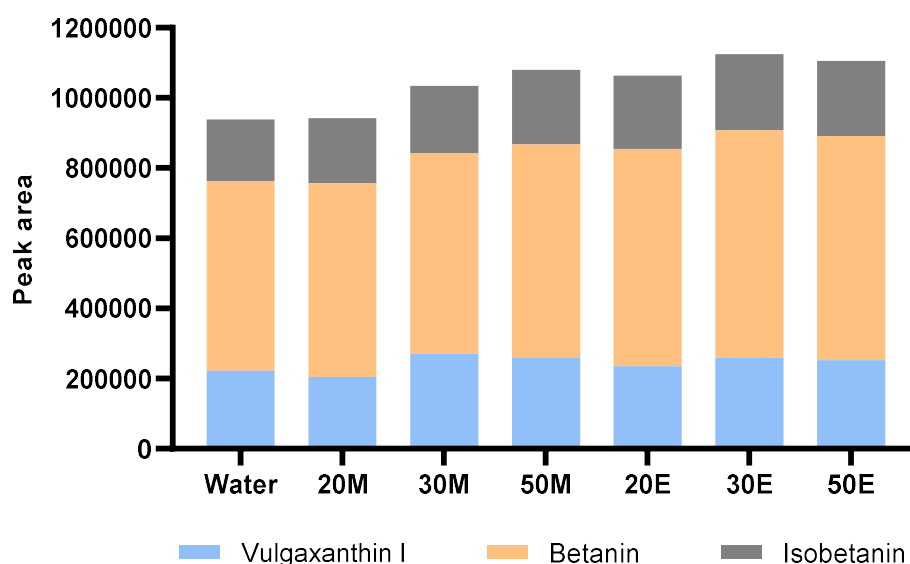
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6 245 of the target compounds. Betalains are hydrophilic pigments; therefore mixing of organic solvents  
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8 246 with water increases the extraction yield when compared to pure organic solvents such as alcohols.  
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10 247 Although, pure water can improve the betalain yield, it has caused severe difficulties during the  
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12 248 solute separation by filtration due to co-extraction of mucilaginous compounds such as pectin<sup>42</sup>.  
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15 249 The results are in agreement with the findings of Righi Pessoa, Heloísa, Camila and Beatriz<sup>25</sup> who  
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17 250 demonstrated total betalain contents in red beetroot ranging from 0.13 mg/g to 6.97 mg/g using  
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19 251 different combinations of water with organic solvents. Interestingly, whilst there was no change in  
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21 252 betalain yield when extracted with water in comparison to solvent mixtures, some studies  
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23 253 suggested that the use of aqueous ethanol or methanol is required to achieve efficient extraction of  
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25 254 betalains<sup>15, 42, 43</sup>. Compared to methanol, ethanol proved to be a better choice as an extraction  
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27 255 solvent due to being considered non-toxic; it can also be bio-produced from renewable resources  
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29 256 and is thus “greener” in environmental assessments, with the added benefit that it can be readily  
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31 257 used in the food industry<sup>44</sup>. According to the literature ethanol can reduce the co-extraction of  
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33 258 pectin, some soluble fiber and proteins<sup>45</sup> while increasing the extraction of compounds of lower  
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35 259 molecular weight<sup>46</sup>, thereby enhancing the overall extraction of bioactives such as polyphenols  
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37 260 and betalains. Indeed, the preliminary experiments showed 18.3% lower total values of  
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39 261 polyphenols when extraction was performed using water in comparison to 30% ethanol (data not  
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44 262 shown).  
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263  
 264 Figure 1. Effect of solvents on total betalain content in dried red beetroot powder extracts. Data  
 265 are mean with SD of three independent extractions. (M = Methanol v/v%, E = Ethanol v/v%). \*\*\*\*  
 266 indicates significant difference ( $p < 0.05$ ), Tukey's multiple comparison test.

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

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6 278 dominant contributors to solvation of polar molecules e.g. betalains are charge-dipole, dipole-  
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8 279 dipole, H-bonding, which favor polar solvents. On the other hand, weaker electrostatic interactions  
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10 280 e.g. ion- $\pi$ ,  $\pi$ - $\pi$  interactions involving neutral or less polar fragments present in betalains will favor  
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12 281 extraction solvents of lower polarity. This indicates ethanol to be a better choice to embrace both  
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14 282 types of molecular interactions when combined with polar solvent such water. The results  
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16 283 demonstrate a preference for 30% v/v ethanol for extraction, which was selected for further  
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18 284 experiments.  
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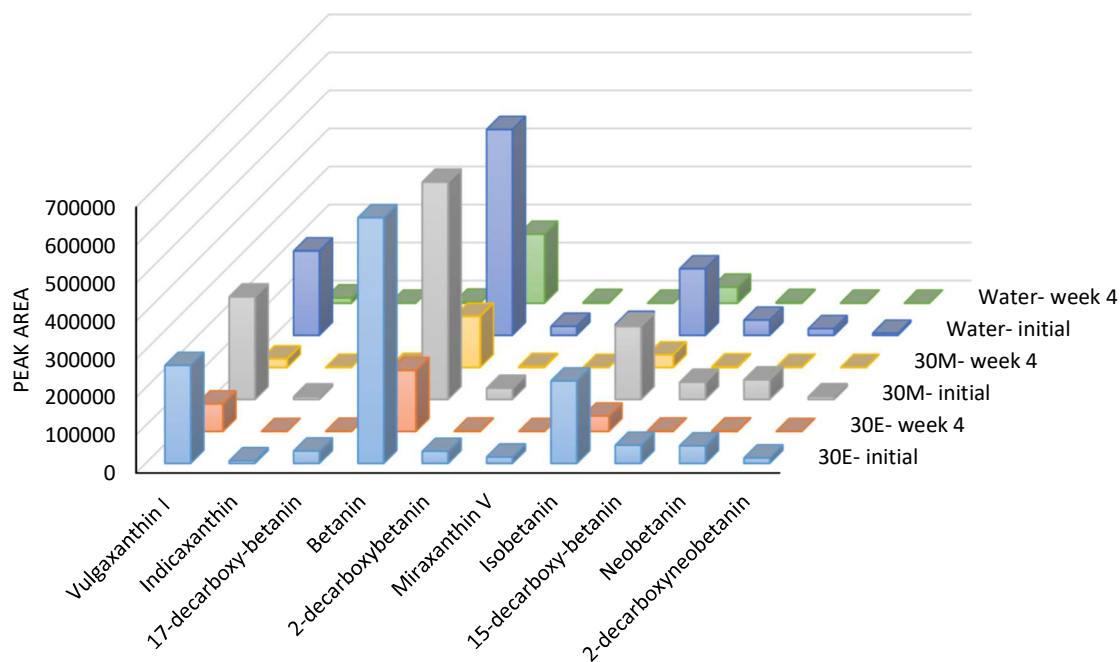
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286 Figure 2. Peak areas of main betalains under different solvent extraction conditions. Data are from  
287 HPLC with vulgaxanthin I and betanin/isobetainin monitored at 486 nm and 536 nm, respectively.  
288 The same solvent mixtures were used to evaluate the extraction of betalains and polyphenols from  
289 the wet pulp ( $81.5 \pm 0.67$  % w/w moisture content) under the temperatures of 35, 45 and 55°C.  
290 Similar results were obtained in this case with the 30% v/v aqueous ethanol lead to the recovery  
291 of  $6.86 \pm 0.23$  mg/g dry weight polyphenols after three repeating extraction steps, whereas the  
292 increase of ethanol in the mixture didn't improve further the polyphenols extraction (data not



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6 293 shown). The amount of polyphenols extracted by 30% v/v ethanol was 19.3% and 71% higher than  
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8 294 these of the pure water and pure ethanol, respectively. This is indicative of the extracted  
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10 295 polyphenols mixture higher affinity to lower ethanol concentrations (~30% v/v). Apart from  
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12 296 solvent-based extractions, enzyme-assisted extraction, which is considered as a highly effective  
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14 297 and sustainable extraction option to achieve high product yields, reduced by-product formation  
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16 298 under avoidance of harsh conditions<sup>26</sup> was employed in this study. The enzymes used, cellulose  
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18 299 and pectinase, are able to hydrolyze cell wall components and release bioactives that are associated  
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20 300 with these, therefore, allowing an overall more efficient extraction of bioactives. In the current  
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22 301 study, however, pre-treatment with cellulase and pectinase enzymes was unsuccessful to increase  
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24 302 total bioactive recovery, especially betalains from wet pulp. Enzyme treatments were performed  
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26 303 at three temperatures, 35, 45 and 55 °C, prior to extraction. There was an enhancement in recovered  
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28 304 polyphenols (10.06±0.21 mg/g dry weight) at 45 °C with a net recovery of 3.2 mg/g dry weight as  
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30 305 determined by Folin assay compared to extracted polyphenols by maceration. Betalains were not  
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32 306 detectable in the macerated wet and enzyme treated samples. Given the absence of the targeted  
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34 307 betalains, from these waste material, enzyme-assisted extraction was not further pursued. Betalain  
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36 308 absence in the case of wet pulp can be explained by the fact that there are enzymes present which  
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38 309 could lead to betalain degradation, whereas in the dry samples these enzymes are not active.  
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40 310 Further, high water activity induces the aldimine bond cleavage and promotes the betalain  
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42 311 degradation<sup>19</sup>.  
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51 313 **Effect of extraction solvent and temperature on betalains, total polyphenols and antioxidant**  
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53 314 **activity during four weeks of storage**  
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6 315 There are many internal and external factors such as pH, light, temperature, oxygen and water  
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8 316 activity that may influence the stability of betalain pigments during storage<sup>15</sup>. Storage temperature  
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10 317 in particular can be considered as one of the crucial factors that determine betalain stability<sup>50</sup>.  
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12 318 Following extraction with different solvents, the present study sought to establish betalain content  
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14 319 and pattern, total polyphenol content and antioxidant activity as well as color measurement as  
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16 320 potential indicators of sample deterioration during storage at -20 °C and RT. Betalain content  
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18 321 displayed a fast and marked decrease when stored at RT whilst extracts stored at -20 °C remained  
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20 322 at the same level (Figure S2 A and B). This results, covering a period of 4 weeks, are in line with  
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22 323 others indicating that temperatures below 10 °C are required to preserve betalains from  
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24 324 degradation<sup>20, 48, 51</sup>. Only Castellar, Obón, Alacid and Fernández-López<sup>52</sup> observed that betalains  
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26 325 in extracts from *Opuntia* varieties were preserved for 19 days at 25 °C. Sapers and Hornstein<sup>53</sup>  
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28 326 reported that the degradation of betalains during storage was mainly depending on the pH and light  
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30 327 exposure, and directly proportional to the initial concentration of betalains in the samples. In this  
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32 328 study, the storage temperature also had an impact on individual betalains; as shown in Figure 3,  
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34 329 betalain pattern during storage indicate that vulgaxanthin I was around 20% less prone to  
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36 330 degradation in 30% ethanol as compared to methanol at the same percentage of solvent or  
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38 331 compared to water. Other betalains displayed much less of a compositional change among the  
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40 332 different extracts.  
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333  
 334 Figure 3. Changes in betalain pattern during 4-week storage of extracts at room temperature.  
 335 Presented are the peak areas of samples extracted with water, 30% methanol and 30% ethanol,  
 336 initially and at the end of the storage period.

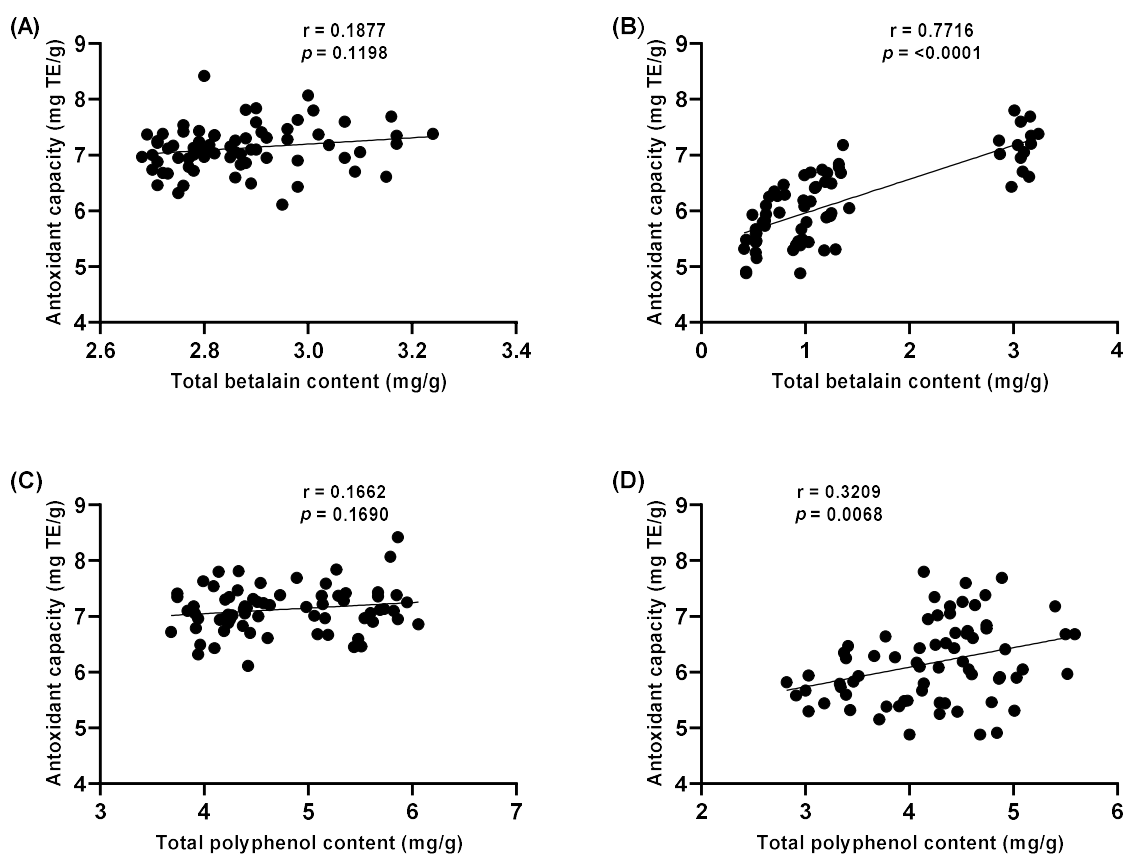
337 In contrast to betalains, the TPC of the beetroot extracts showed a different pattern. Whilst the  
 338 initial values of total polyphenols did not differ between the samples (Figure S3), around 20% of  
 339 increase was observed up to the second week and then a gradual decline until the end of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

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6 346 biosynthesis of polyphenols, during storage which induced the de novo synthesis of secondary  
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8 347 metabolites and may therefore contribute to increased phenolic content. Klimczak, Małecka,  
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10 348 Szlachta and Gliszczyńska-Świgło<sup>54</sup> observed an increase of free p-coumaric and ferulic acids in  
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12 349 orange juice during storage at different temperatures due to the release of free acids from their  
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14 350 bound form (at 18, 28 and 38 °C) which could be a further reason for changes of TPC content.  
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16 351 Folin reagent itself is lacking specificity, and some other reducing compounds such as phenolic  
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18 352 amino acids and ascorbic acid are known to react with Folin, thereby increasing the TPC values  
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20 353 independently of polyphenols<sup>57</sup>. In addition, the high polyphenol content during the storage period  
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22 354 could be linked to the preferential oxidation of betalains that prevents the degradation of  
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24 355 polyphenols present in the samples. In studies involving ABTS+, betanin was 1.5 to 2 times more  
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26 356 efficient as a free radical scavenger than anthocyanins at neutral or basic pH<sup>58</sup>. It was also observed  
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28 357 that among betacyanins such as betanidin, betanin, and phyllocactin, betanidin was the most potent  
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30 358 antioxidant against peroxy radical and nitric oxide indicating that glycosylation decreases the  
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32 359 radical scavenging activity of betacyanins<sup>59, 60</sup>.

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34 360 Antioxidant activities were determined using TEAC assay, commonly used method to assess  
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36 361 ABTS+ radical scavenging properties. This assay, as well as others such as FRAP, ORAC, DPPH,  
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38 362 superoxide radical scavenging, have been shown to correlate with betalain content as demonstrated  
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40 363 in several studies<sup>30, 61, 62</sup>. As shown in [Figure S2 E and F](#), antioxidant capacity was similar among  
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42 364 samples after extraction and remained largely unaffected during storage at -20 °C. In the case of  
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44 365 stored samples at RT, there was a successive decline in antioxidant capacity over the four-week  
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46 366 period to around 22%. This loss of antioxidant activity was highly correlated with the betalain  
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48 367 decline during RT storage ( $r=0.7716$ ,  $p < 0.0001$ ), but no correlation at -20 °C was evident  
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50 368 ( $r=0.1877$ ,  $p = 0.1198$ ) (Figure 4 A and B). Similarly, the TPC and antioxidant capacity showed a  
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369 significant correlation at RT ( $r=0.3209$ ,  $p = 0.0068$ ), but not at  $-20\text{ }^{\circ}\text{C}$  storage ( $r=0.1662$ ,  $p =$   
370  $0.1690$ ) (Figure 4 C and D).

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373 Figure 4. Correlation of betalain content and polyphenol content with antioxidant activity (TEAC  
374 -Trolox equivalent antioxidant capacity) of the red beetroot extracts stored in  $-20\text{ }^{\circ}\text{C}$  (A and C) and  
375 room temperature (B and D).

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

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6 380 known antioxidants such as ascorbic acid, tocopherols and rutin<sup>58,63</sup>. Moreover, there is evidence  
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8 381 indicating that the degradation products of betalains, such as neobetanin, have even higher  
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10 382 antioxidant activity than the betalains themselves<sup>64,65</sup>. This appears irrelevant for this study as the  
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12 383 neobetanin concentrations were lower after 4 weeks compared to the initial data (Figure 3). In  
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14 384 addition, other compounds are potentially present in extracts such as betains, carotenoids and  
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16 385 dietary nitrate and nitrite and contributing to overall antioxidant activity<sup>14, 66</sup>. The data are  
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18 386 demonstrating a substantial decline (80%) of betalains at RT storage, but lower loss of antioxidant  
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20 387 activity (22%) emphasizing possible synergetic effects of polyphenols, betalains and their  
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22 388 metabolites as well as other compounds present in the extracts in radical scavenging and iron  
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24 389 reducing capabilities<sup>62</sup>.

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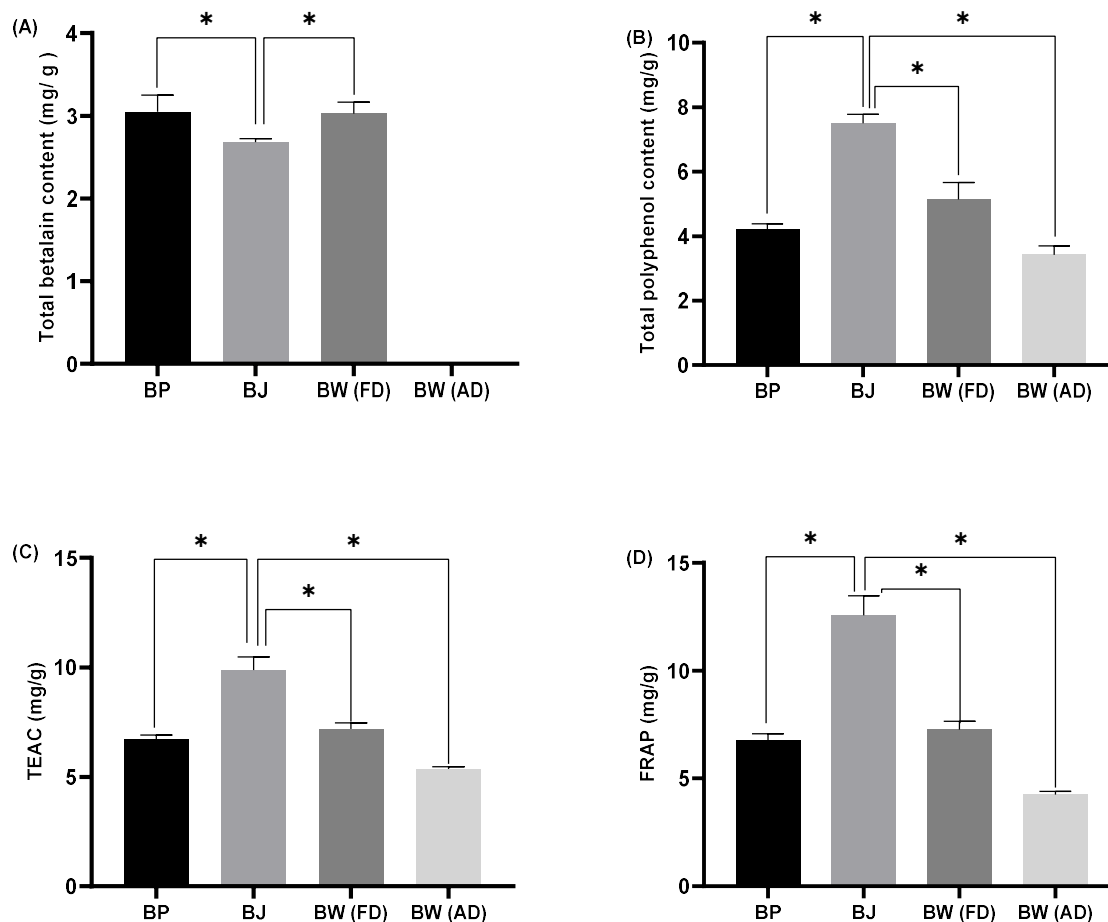
### 391 **Color measurements as indicators for pigment degradation during storage**

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

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6 403 by the increasing values of  $b^*$  of the room temperature stored samples compared to the initial  
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8 404 values which indicates the development of yellow color in the samples. However, both  $a^*$  and  $b^*$   
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10 405 values remained unchanged with the samples stored at  $-20\text{ }^\circ\text{C}$  when compared to the initial color  
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12 406 values. The initial color results of this study were compatible with data from Prieto-Santiago,  
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14 407 Cavia, Alonso-Torre and Carrillo<sup>69</sup> on the relationship between the color and the thermal  
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16 408 degradation of beetroot betalain pigments. Pearson correlation coefficients ( $r$ ) between color  
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18 409 measurements ( $L^*$ ,  $c^*$ ,  $h^*$ ,  $a^*$  and  $b^*$ ) with TBC are shown in Table S1. The  $L^*$   $h^*$  and  $b^*$  values  
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20 410 were negatively correlated ( $p < 0.0001$ ) with TBC ( $r = -0.9074$ ,  $r = -0.9256$  and  $r = -0.8807$   
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22 411 respectively) while  $c^*$  and  $a^*$  values showed positive correlation ( $p < 0.0001$ ) with TBC ( $r = 0.5903$   
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24 412 and  $r = 0.8967$  respectively). Other studies<sup>69-71</sup> have reported that pigment content can be  
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26 413 correlated better with the combined color parameters than the single color measurements.  
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28 414 Therefore, the different combinations of color numeric values were calculated and shown in Table  
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30 415 S1. The  $L^*a^*b^*$  data is a good indicator of visual color assessment of the samples<sup>69</sup> and there was  
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32 416 a strong positive correlation between  $L^*a^*b^*$  data and total betalain content ( $r = 0.9820$ ,  $p$   
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34 417  $< 0.0001$ ). Additionally, the  $a/b$  ratio can be used as a convenient parameter for assess the color  
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36 418 degradation accurately as well as quantitatively<sup>71</sup>. Therefore, these correlations indicate that the  
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38 419 color measurement could be used as indirect assessment to determine the betalain pigments as easy  
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40 420 and inexpensive method.  
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6 426 Following extraction using 30% v/v ethanol, betalain values in the four samples ranged from 0-  
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8 427 3.06 mg/g as shown in Figure 5A. As indicated in the earlier section, the data of the present study  
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10 428 are in the range of others, some authors have shown a higher total betalain content in red beetroot  
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12 429 cultivars ranging from 4.43 – 9.60 mg/g dry matter<sup>47</sup>, and 7.42 – 8.56 mg/g dry matter<sup>72</sup>. In  
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14 430 contrast, Lee, An, Nguyen, Patil, Kim and Yoo<sup>66</sup> observed relatively low concentrations of  
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16 431 betalains (0.65 – 0.80 mg/g fresh weight) in red beetroot cultivars from USA. Variations of results  
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18 432 could, apart from extraction and extraction conditions (temperature, pH), be due to differences in  
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20 433 beet varieties and growth conditions<sup>73</sup>. The ratio of betacyanin to betaxanthin was 1.12, 1.35 and  
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22 434 1 for the BP, BJ and beet waste (FD) samples respectively, demonstrating that betalain  
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24 435 composition of the samples was varied. A similar ratio of betacyanin to betaxanthin has been  
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26 436 reported previously for different beetroot sources<sup>47, 74</sup>.  
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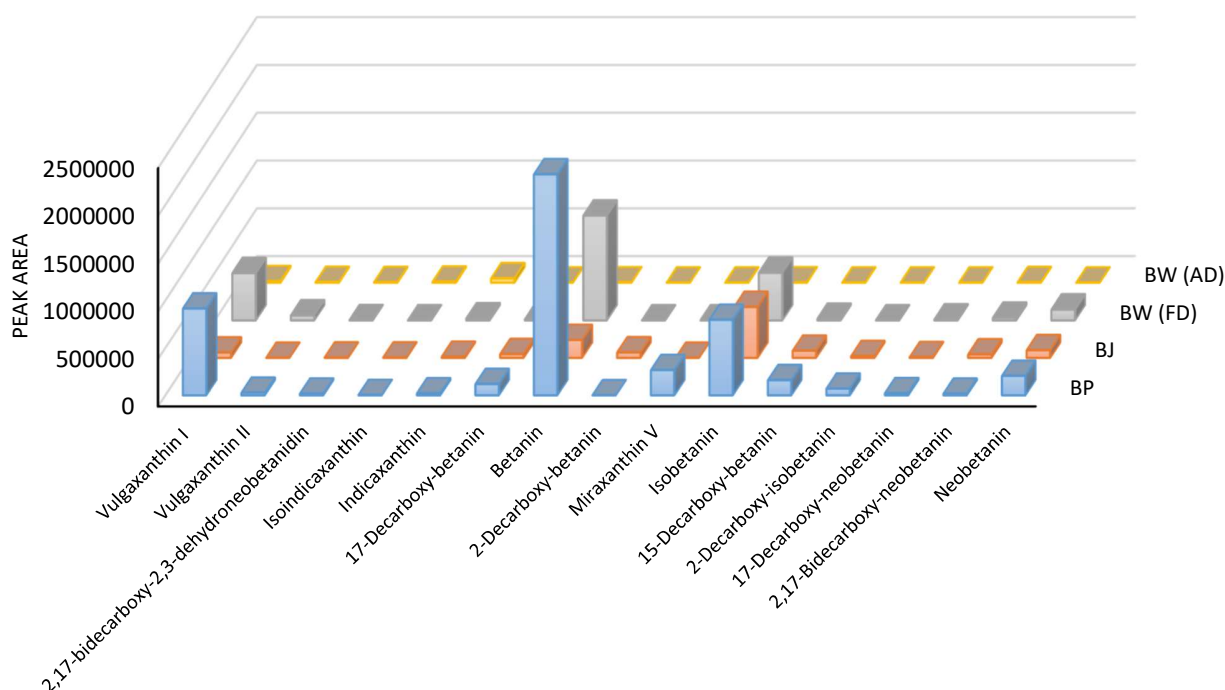


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438 Figure 5. Total betalains (A), total polyphenols (B) and antioxidant activities (C, D) in extracts of  
 439 different beetroot samples after ultrasound-assisted extraction. Data are mean with SD of three  
 440 independent extractions. \* indicates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

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

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6 446 previously reported betanin derivatives and betaxanthins could not be detected. Sawicki, Bączek  
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8 447 and Wiczowski<sup>73</sup> reported the presence of eighteen betacyanins with twelve betaxanthins in  
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10 448 thirteen Polish varieties of red beetroot. In comparison, only three betalains (betanin, isobetanin  
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12 449 and vulgaxanthin I) were identified in red beet cultivars grown in USA and Finland<sup>47, 66</sup>.  
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14 450 Differences in betalain content and pattern may be due to varietal diversity, local growth and  
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16 451 climate conditions as well as post-harvest conditions<sup>76</sup>. The most prominent peaks identified in the  
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18 452 current study were betanin, isobetanin, vulgaxanthin I and neobetain. Further, not all samples  
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20 453 contained all betalains that had been identified. The sample that had been originally air dried was  
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22 454 devoid of most peaks indicating large-scale degradation of betalains, likely UV and temperature  
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24 455 facilitated, whereas the peak areas in beetroot waste FD sample were more similar to the BP sample  
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26 456 which is derived from whole beet. In general, the betalain content (betacyanins and betaxanthins)  
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28 457 is highest in the peel of red beet in comparison to the inner rings<sup>47, 72, 73</sup>, which is also evident in  
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30 458 the present study. Peak areas of vulgaxanthin I and betanin are much lower in the beet juice sample  
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32 459 as compared to samples comprising the whole beet (BP) and pomace fraction (beet waste, FD)  
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34 460 (Figure 6). In summary, the results of betalain analysis demonstrate that the dried beetroot waste  
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36 461 from juicing industries can be a good source of betalain pigments, with regards to betalain yield  
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38 462 equivalent to whole beet and beet juice.  
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463  
 464 Figure 6. Comparison of betalain peaks present in different beetroot sources (BP –red beetroot  
 465 powder, BJ – Beetroot juice powder, BW (FD) – freeze dried red beetroot waste powder, BW (AD)  
 466 – air dried beetroot waste powder)

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

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6 475 most accurate quantification of betalains, by eliminating the aforementioned problems associated  
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8 476 with spectrophotometry. However, the standards have to be isolated from the plant materials in  
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10 477 case of quantification of betalains using HPLC due to lack of commercial availability<sup>79</sup>. Thus,  
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12 478 despite the relatively high discrepancy (~15%) in calculation between the two methods, HPLC and  
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14 479 UV-vis spectroscopy, the latter remains the most convenient and fastest method to quantify  
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16 480 betalains <sup>78</sup>. In the present study, peak areas were used as basis for comparing individual samples,  
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18 481 which has been applied by many other groups<sup>30, 72</sup>.

21 482 In contrast to betalains, there are detectable polyphenols in all samples, however, the total  
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23 483 polyphenol content is much higher in BJ compared to BP and Beet waste samples Figure 5.  
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25 484 Current TPC values ( $3.42 \pm 0.27 - 7.50 \pm 0.28$  mg/g) are in the range that others have reported  
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27 485 from  $0.51 \pm 0.07$  to  $15.5 \pm 0.1$  mg/g<sup>30, 80-83</sup> which include as main polyphenols gallic, syringic,  
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29 486 caffeic and ferulic acids<sup>30</sup>. In the present study twelve different polyphenols were identified of  
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31 487 which seven were hydroxycinnamic acid derivatives, four belonging to the flavonoids group and  
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33 488 one trihydroxybenzoic acid (  
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35 489 Table 1). Similar polyphenol composition was reported by the other studies which analyzed the  
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37 490 polyphenol composition of different varieties of beetroot including juice, roots and stem extracts<sup>47,</sup>  
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39 491 <sup>74, 84</sup>.

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

No	Compound	Retention time (min)	$\lambda_{\max}$	$[M-H]^-$	Beetroot sources			
					BP	BJ	BW (FD)	BW (AD)
1	Catechin	4.29	282	289	+	+	+	+
2	Cochliophilin A	4.98	283	281	+	+	+	nd
3	<i>p</i> -coumaric acid	5.33	282	163	+	+	+	+
4	Caffeic acid	5.67	265	179	+	+	+	+
5	<i>N-trans</i> -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	<i>N-trans</i> -feruloyltyramine	21.75	274	312	+	+	+	nd
11	Quercetin	52.40	361	301	+	+	+	+
12	Betavulgarin	56.42	279	311	+	+	+	nd

500 nd – not detected

501  
 502 In line with the polyphenol content, the antioxidant activity of BJ, determined as TEAC and FRAP,  
 503 was 32% and 46% higher compared with BP and 27% and 42% higher than beet waste (FD) and  
 504 45% and 66% higher than beet waste (AD), respectively (Figure 5C and D). A highly significant  
 505 correlation ( $p < 0.05$ ) was observed between the total polyphenol content with TEAC assay ( $r =$   
 506 0.9845) and FRAP assay ( $r = 0.9753$ ). Interestingly, the betalain content did not show any  
 507 significant correlation with TEAC ( $r = 0.2196$ ,  $p = 0.5314$ ) and FRAP ( $r = 0.2078$ ,  $p = 0.5442$ ) assays

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6 508 ( $p>0.05$ ). Several studies determined a strong relationship between radical scavenging activity and  
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8 509 betalains as well as polyphenols present in a range of fruits and vegetables<sup>63, 85, 86</sup>. For instance,  
9  
10 510 Čanadanović-Brunet, Savatović, Četković, Vulić, Djilas, Sinisa and Cvetković<sup>87</sup> observed a  
11  
12 511 significantly high linear correlation between hydroxyl ( $r > 0.81$ ) and superoxide ( $r > 0.92$ ) radical  
13  
14 512 scavenging activities with betacyanins and betaxanthins extracted from beetroot pomace.  
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## 18 19 514 **Conclusion**

20  
21 515 To conclude, effective combined extraction of betalains and polyphenols from red beet dried  
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23 516 powder has been demonstrated in an ultrasound-assisted approach establishing low ethanol  
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25 517 concentrations (30% ethanol) as the most suitable solvent combination compared to the enzyme-  
26  
27 518 assisted extraction method from wet pulp. The stability of betalains, in contrast to polyphenols,  
28  
29 519 was strongly affected by storage temperature leading to a rapid loss of betalains over the  
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31 520 observation period of four weeks at room temperature, irrespective of the solvent used, a finding  
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33 521 that is in good correlation with color measurements. The comparatively moderate loss of  
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35 522 antioxidant activity vs betalain content over time emphasizes the potential contribution of betalains  
36  
37 523 and polyphenols as well as their metabolites and/or degradation products to antioxidant activity.  
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39 524 These comparative extraction results indicate that the samples derived from the beetroot industry  
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41 525 can provide good pigment yield, after their initial drying, similarly to whole beet samples.  
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## 45 46 47 527 **Acknowledgments**

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51 529 is supported by a Commonwealth PhD scholarship.  
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6 531 **Supporting information**

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8 532 Supporting tables and figures are provided in a separate file. Experiment and characterization  
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10 533 details; HPLC chromatograms, variation of TBC, TPP and antioxidant activity during the storage  
11  
12 534 period, effects of different solvents on TBC, TPP and antioxidant activity of red beetroot extract,  
13  
14 535 correlation coefficients of color data, UV-Vis spectrum of beetroot samples and HPLC  
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16 536 chromatograms of red beetroot samples.  
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**827 Synopsis**

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

