

# Comparative evaluation of different methods for determining phytochemicals and antioxidant activity in products containing betalains – verification of beetroot samples

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## ABSTRACT

This study presents methods that can be used to assess the health quality of products containing betalains. The paper compares and verifies data on the phytochemical composition of three different pigmented beetroot cultivars using spectrophotometric, HPLC-DAD, HPTLC and LC-Q-Orbitrap-HRMS techniques. Additionally, we compared the total antioxidant activity in both the cell-free and cellular systems. Betalain contribution to antioxidant activity was also determined using post-column derivatization and it was found that in the case of red beetroot it is about 50%. Photometric measurements are recommended for a simple and inexpensive analysis of the total betacyanin and betaxanthin content. Liquid chromatography techniques produced more precise information on phytochemical composition in the tested samples. The combination of liquid chromatography with high-resolution mass spectrometry produced the largest amount of quantitative and qualitative data; in beetroot samples sixty-four phytochemicals have been identified therefore, this approach is recommended for more detailed metabolomics studies.

## Keywords:

Betalains, Antioxidant activity, Beetroot, HPTLC, HPLC-DAD, Q-Orbitrap-HRMS

## 1. Introduction

Betalains are an unusual class of pigments that are found in certain families within the *Pentapetalae* order Caryophyllales, where they replace the more common anthocyanins (Brockington, Walker, Glover, Soltis, & Soltis, 2011). These water-soluble nitrogen-containing pigments can be divided into two major structural groups, red-violet betacyanins and yellow-orange betaxanthins (Slimen, Najar, & Abderrabba, 2017). Betacyanins can be further classified by their chemical structures into four kinds: betanin-type, amaranthin-type, gomphrenin-type, and bougainvillein-type (Polturak & Aharoni, 2018). Betalains are of great interest as food colorants in industrial applications. Since these compounds are relatively stable over a wide pH range (3–7), they may be used to impart colour and improve the appearance of numerous food products (Azeredo, 2009). In addition to colour, betalains also have great potential as functional food ingredients employed in the food and medical industries due to their diverse health-promoting effects. Betalains and betalain-rich diets are not only nontoxic but may also prove to be a promising alternative to supplement therapies for oxidative stress-, inflammation-, and dyslipidaemia-related diseases such as stenosis of the arteries, atherosclerosis, hypertension, and cancer (Rahimi, Abedimanesh, Mesbah-Namina,

51 & Ostadrahimi, 2019). Due to their toxicological safety, availability, low cost,  
52 biodegradability, and potentially beneficial health effects, including betalains in food and  
53 allied industries could pave the way in overcoming concerns about health risks from artificial  
54 colours. The growing interest in this group of pigments justifies the search for various  
55 methods to characterize them and research their bioavailability and stability. Various types of  
56 methods for studying this group of pigments include mainly spectrophotometric and  
57 chromatographic techniques. The most popular and straightforward approach to quantify  
58 betalains is photometric measurement (Stintzing & Carle, 2007; Chauhan, Sheth, Rathod,  
59 Suhagia, & Maradia, 2013; Sandate-Flores et al., 2016). However, this method has some  
60 limitations (Schwartz, Hildenbrand, & von Elbe, 1981), which is why it is often used to  
61 supplement chromatographic methods (Stintzing, Schieber, & Carle, 2003; Kugler, Graneis,  
62 Stintzing, & Carle, 2007). For the separation of betalains, the HPLC technique is most often  
63 and successfully used. Additionally, the use of mass spectrometry (MS) coupled with HPLC  
64 complements the use of diode-array detectors (DAD) and enables identification of separated  
65 pigments (Stintzing & Carle, 2007; Chauhan et al., 2013). In many botanical laboratories,  
66 analysis of phytochemicals is most often performed using HPTLC, but for betalains, such an  
67 optimized method is still not available.

68 There are many articles in the literature on the analysis of samples containing  
69 betalains. However, these are most often either original articles describing the results of  
70 analyses of a given set of samples performed with one method or review articles comparing  
71 the use of multiple methods for different samples derived from different experiments. In our  
72 article, we present a different approach aimed at comparative evaluation of different methods  
73 performed for the same set of samples to provide a guide to help scientists as well as  
74 supplement and nutraceutical producers to choose the appropriate analytical solutions  
75 depending on the assumed goal and the available equipment.

76 The purpose of this study was to present known procedures and propose new solutions  
77 to assess the health-promoting potential of plants, food products, nutraceuticals, and dietary  
78 supplements containing betalains. This paper describes methods of varying degrees of  
79 sophistication used for the determination and profiling of phytochemicals, including betalains.  
80 Additionally, we propose four procedures for determination of total antioxidant activity as an  
81 important biomarker of quality of sample containing betalains. We also suggest a new way of  
82 converting and expressing the total antioxidant activity value. The new method for  
83 determining the contribution of betalains to the antioxidant potential of tested samples on the  
84 basis of HPLC antioxidant profiles is also shown. The lack of commercially available betalain  
85 standards often makes quantitative analysis difficult, therefore in the article we propose a  
86 new, simple method for isolating betanin and vulgaxanthin I from red beetroot juice using the  
87 SPE technique. Thin-layer chromatography is a technique widely applied to the fingerprinting  
88 of complex mixtures (e.g., those of botanical origin) in many laboratories. However, in the  
89 case of samples containing betalains, this method was used very rarely due to the very polar  
90 nature of these pigments. In our research, we present a solution to this problem by proposing  
91 the use of an appropriate TLC stationary phase, which guarantees obtaining a high-quality  
92 betalain profiles. In this study, we also indicate a potential of using LC-Q-Orbitrap HRMS  
93 and Compound Discoverer software to identify various phytochemical classes, including  
94 betalains and their degradation products. The type and quality of data generated in these  
95 methods are presented on the basis of analysing three beetroot cultivars with different  
96 pigmentations. Beetroots were selected for the research because, next to prickly pear, they are  
97 the main edible source of betalains (Rahimi et al., 2019). Betalains found in red beetroot are  
98 used to dye a number of food products, such as ice cream, wine, jams, marmalade, and  
99 yoghurt. Red beetroot is also one of the most widely planted root vegetables in Poland and is  
100 quite popular in Europe (Sawicki, Bączek, & Wiczkowski, 2016).

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## 2. Materials and methods

### 2.1. Chemicals and reagents

Reagents of analytical, HPLC or MS grade, including acetonitrile, ethanol, methanol, water, formic acid and trifluoroacetic acid, and reagents for antioxidant activity determination, including 2,2'-azinobis(3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1 picrylhydrazyl (DPPH), Folin-Ciocalteu's phenol reagent (FC), gallic acid, quercetin, McCoy's 5A cell culture medium, foetal bovine serum, trypsin, and streptomycin-penicillin solution, were purchased from Sigma Aldrich (St. Louis, MO, USA).

### 2.2. Methods

#### 2.2.1. Plant material and extracts preparation

Three beetroot (*Beta vulgaris* L. subsp. *vulgaris*) cultivars with different pigmentations, red-coloured "Red Ball" (RB), yellow-coloured "Boldor" (YB), and white and pink striped "Chioggia" (ChB), were received in October 2019 courtesy of a local vegetable and fruit wholesaler Lilu Fruits® (Gdansk, Poland). Each beetroot sample was chopped into small pieces, placed into a plastic bag, immediately shock-frozen in liquid nitrogen, and lyophilized. The ground, freeze-dried beetroot samples were extracted with four different solvents of varying polarities: distilled water, 30% aqueous ethanol, 70% aqueous ethanol, and ethanol. Each solvent was acidified with formic acid (1%). Briefly, 0.1 g of beetroot powder was extracted with 1.5 mL of the above-mentioned solvents. The mixture was then sonicated for 1 min and centrifuged (Centrifuge 5415R, Eppendorf, Wesseling, Germany) for 5 min ( $13,200 \times g$  at  $4^\circ\text{C}$ ). The supernatant was collected, and the extraction step was repeated with a new portion of solvent (1.5 mL). The combined supernatants (~3 mL) were used for further analyses.

#### 2.2.2. Photometric quantification of betalains and colour measurement

Quantification of betacyanins and betaxanthins was performed according to the assay by Stintzing, Schieber, and Carle (2003). The pigment extracts were diluted with McIlvaine buffer to obtain absorption values of  $0.8 \leq A \leq 1.0$  at their respective analytical wavelengths. Betacyanin and betaxanthin content was calculated as betanin and vulgaxanthin I equivalents, respectively using extinction coefficient  $60,000 \text{ L mol}^{-1} \text{ cm}^{-1}$  at  $\lambda = 538 \text{ nm}$  for betanin and  $48,000 \text{ L mol}^{-1} \text{ cm}^{-1}$  at  $\lambda = 480 \text{ nm}$  for vulgaxanthin I. All absorbance measurements were performed with a Nanodrop 2000c (Thermo Scientific, USA).

The colour parameters ( $L^*$ ,  $a^*$ ,  $b^*$ ) were assessed using photographs of beetroot extracts placed in 24-well plates and with the use of a digital colorimeter available as standard computer software (macOS Mojave 10.14.6). The photo of the plate was taken with a CAMAG TLC visualizer 2.

#### 2.2.3. Total antioxidant activity determination

The total beetroot extract antioxidant activity (30% aqueous ethanol) was determined by standard assays employing ABTS and DPPH radicals and the cellular antioxidant activity test (CAA) as described previously (Baranowska et al., 2018) with minor modifications, as well as with Folin-Ciocalteu (FC) reagent according to the standard ISO 14502-1:2005 method. All absorbance measurements were performed with a TECAN Infinite M200 spectrophotometer (Tecan Group Ltd., Switzerland).

151 2.2.3.1. *ABTS* assay. To generate a radical stock solution, ABTS powder was dissolved in  
152 aqueous  $\text{Na}_2\text{S}_2\text{O}_8$  ( $4.45 \text{ mmol L}^{-1}$ ) to obtain a concentration of  $7 \text{ mmol/L}$  and left in the dark  
153 at ambient temperature for 24 h. Before measurements, the ABTS stock solution was diluted  
154 in water until the absorbance reached to  $0.8 \pm 0.05$  at  $\lambda=734$ . This ABTS solution ( $1 \text{ mL}$ ) was  
155 mixed with different volumes of beetroot extracts selected in such a way that the measured  
156 absorbance values were within a linear range of the assay ( $1 - 10 \mu\text{L}$  for RB and  $20 - 160 \mu\text{L}$   
157 for ChB and YB). All reactions were carried out in 48-well plates. The absorbance of the  
158 mixtures was measured at  $734 \text{ nm}$  after 10 min. The amount of ABTS radicals scavenged by  
159 the tested beetroot sample in reaction media was calculated using the Beer-Lambert-Bouguer  
160 Law (Beer's Law) according to the equation:  $[\mu\text{mol}] = [((A_0 - A_{10}) \times V \times 1000) / (\epsilon \times l)]$ , where  $A_0$   
161 is the initial absorbance of the radical solution;  $A_{10}$  is the absorbance of the radical solution  
162 after reaction time;  $V$  is the final volume or reaction mixture [ $\text{mL}$ ];  $l$  is the optical path length  
163 [ $\text{cm}$ ], and  $\epsilon$  is the ABTS molar extinction coefficient ( $16,000 \text{ L mol}^{-1} \text{ cm}^{-1}$  at  $\lambda = 734$ ). The  
164 data were used to generate a linear relationship between the different amounts of tested  
165 beetroot samples calculated as grams of dry lyophilizates in the reaction mixtures and the  
166 number of ABTS scavenged  $\mu\text{moles}$ . The slopes of the straight lines were then used to  
167 express the total antioxidant activity and mean of how many ABTS  $\mu\text{moles}$  were scavenged  
168 by  $1 \text{ g}$  of tested sample.

170 2.2.3.2. *DPPH* assay. The freshly prepared DPPH methanolic stock solutions ( $5 \text{ mmol L}^{-1}$ )  
171 were diluted in methanol before measurements until absorbance reached  $1.0 \pm 0.05$  at  $515 \text{ nm}$ .  
172 This DPPH solution ( $1 \text{ mL}$ ) was mixed with different volumes of beetroot extracts selected in  
173 such a way that the measured absorbance values were within the range of  $0.2 - 0.9$  at  $515 \text{ nm}$   
174 ( $10 - 60 \mu\text{L}$  for RB and  $40 - 300 \mu\text{L}$  for ChB and YB). Each reaction mixture was placed in a  
175 separate well of a 48-well plate, and after 10 min the absorbance was measured at  $515 \text{ nm}$ .  
176 The amount of DPPH radicals scavenged by the tested beetroot sample present in reaction  
177 media was calculated using the same equation as for ABTS, but in this case,  $\epsilon$  was the DPPH  
178 molar extinction coefficient ( $11,240 \text{ L mol}^{-1} \text{ cm}^{-1}$  at  $\lambda = 515$ ). The total antioxidant activity of  
179 the tested samples was expressed as the slope of linear relationship between the dry  
180 lyophilizate weight [ $\text{g}$ ] present in the reaction mixtures and the number of scavenged DPPH  
181  $\mu\text{moles}$ .

183 2.2.3.3. *FC* assay. The commercial FC reagent was diluted with water at a ratio of  $1:9$  (v/v).  
184 The different volumes of beetroot extracts ( $20 - 120 \mu\text{L}$  for RB and  $100 - 400 \mu\text{L}$  for ChB  
185 and YB) were mixed in separate wells of 48-well plates with a FC reagent solution ( $500 \mu\text{L}$ ).  
186 After 5 min, a water solution of sodium carbonate ( $7.5\% \text{ w/v}$ ) was added to reach a final  
187 reaction mixture volume of  $1 \text{ mL}$ . The reactants were mixed, and the absorbance was  
188 measured at  $765 \text{ nm}$  after 1 h. The results were calculated using of a gallic acid calibration  
189 curve. The antioxidant activity of the tested extracts was expressed as the slope of the line  
190 from the relationship between the dry weight of lyophilizates [ $\text{g}$ ] and the amount of gallic acid  
191 equivalents [ $\mu\text{mol}$ ] present in the reaction mixtures after 60 min.

193 2.2.3.4. *CAA test*. A commercially available OxiSelect™ Cellular Antioxidant Activity Assay  
194 Kit (Cell Biolabs Inc., San Diego, CA, USA) was used to determine cellular antioxidant  
195 activity. HT29 cells (human colon adenocarcinoma, ATCC, USA) were cultured and treated  
196 as described previously (Koss-Mikołajczyk, Kusznierevicz, & Bartoszek, 2019). Briefly,  
197 HT29 cells were seeded in 96-well tissue culture clear bottom black plates ( $10\,000$  cells/well  
198 in  $0.15 \text{ mL}$  of McCoy's medium). After the cells reached 90% confluence, they were treated  
199 for 1 h with  $0.05 \text{ mL}$  of fluorescent probe (DCFH-DA) and  $0.05 \text{ mL}$  of quercetin standard  
200 solution ( $31.3 - 2000 \mu\text{M}$  – standard curve) or  $0.05 \text{ mL}$  of tested plant extracts (diluted with



201 PBS to a final concentration of 1% v/v). The procedure strictly followed the manufacturer's  
202 recommendations ([http://www.cellbiolabs.com/sites/default/files/STA-349-cellular-](http://www.cellbiolabs.com/sites/default/files/STA-349-cellular-antioxidant-activity-assay-kit.pdf)  
203 [antioxidant-activity-assay-kit.pdf](http://www.cellbiolabs.com/sites/default/files/STA-349-cellular-antioxidant-activity-assay-kit.pdf)), and the results are presented as quercetin equivalents (QE  
204 [ $\mu\text{mol/g d.w.}$ ])  
205

#### 206 2.2.4. *Betain and vulgaxanthin I isolation*

207 Betain (BTN) and vulgaxanthin I (VXT) were isolated from freshly squeezed red  
208 beetroot juice using solid phase extraction (SPE). A Bakerbond SPE Octadecyl C18 Polar  
209 Plus column (1000 mg, 6 mL, J.T. Baker) was pre-conditioned with methanol (6 mL) and  
210 water (6 mL). Next, 1 mL of red beetroot juice was applied to the column. In first step of  
211 elution, 3 mL of water was used. As soon as the eluate leaving the column turned yellow, the  
212 VXT fraction was collected (Fig. S1). When the yellow fraction left the bed, a 15% aqueous  
213 methanol solution (3 mL) was dosed into the column. As the dark purple fraction left the  
214 column, the BTN fraction was collected. The VXT and BT concentrations in the collected  
215 fractions were determined photometrically by measuring the absorbance at 480 and 538 nm,  
216 respectively, and using Beer's law and appropriate extinction coefficients. To generate  
217 calibration curves, pigment fractions were diluted with the appropriate mobile phase in the  
218 range of 10 – 90% and analysed by high performance thin layer chromatography (HPTLC) or  
219 HPLC.  
220

#### 221 2.2.5. *Profiling of betalains by HPTLC*

222 HPTLC was used to profile betalain. Beetroot extracts (30% aqueous ethanol) and  
223 different dilutions of isolated fractions with BTN and VXT were applied in the form of bands  
224 (10 mm, 6  $\mu\text{L}$ ) using a spraying technique with a 25  $\mu\text{L}$  syringe on TLC silica gel 60 RP-18  
225 F<sub>254S</sub> aluminium plates (10 x 20 cm) (Merck, Darmstadt, Germany) using an automatic  
226 sampler TLC 4 (CAMAG, Muttenz, Switzerland). For red beetroot, the extract was diluted  
227 twice before dosing. After applying the samples and standards, the chromatograms were  
228 developed in a CAMAG ADC2 automatic developing chamber using a mobile phase  
229 containing water, acetonitrile, and trifluoroacetic acid (80:20:2 v/v/v). The developing  
230 distance was 80 mm from the lower edge of the plate. The chromatograms were documented  
231 using a CAMAG TLC visualizer 2 in white light, and the plates were scanned using a  
232 CAMAG TLC scanner at 480 and 535 nm. Finally, R<sub>f</sub> values, fingerprint profiles, and  
233 densitograms were recorded by visionCATS CAMAG HPTLC software. The content of  
234 yellow and red pigments in beetroot extracts was calculated from the peak areas at 484 and  
235 535 nm and with VXT and BTN calibration curves, respectively.  
236

#### 237 2.2.6. *Profiling betalains and antioxidants by HPLC*

238 Profiles of betalains and antioxidants were obtained for beetroot extracts (30%  
239 aqueous ethanol) and different dilutions of isolated fractions with BTN and VXT with a  
240 HPLC-DAD system (Agilent Technologies, Wilmington, DE, USA) connected to a Pinnacle  
241 PCX Derivatization Instrument (Pickering Laboratories Inc., Mountain View, California,  
242 USA) and UV-VIS detector (Agilent Technologies, Wilmington, DE, USA). The  
243 chromatographic separation conditions were as follows: SynergiTM Hydro-RP A column  
244 (150 x 4,5 mm, 4  $\mu\text{m}$ , Phenomenex); mobile phase: A – water with formic acid (1%), B –  
245 acetonitrile with formic acid (1%); elution programme: 0 min – 100% A; 20 min – 30% B; 25  
246 min – 100% B; flow rate – 0.8 mL/min; and injection volume – 10  $\mu\text{L}$ . For red beetroot, the  
247 extract was diluted four times before injection. The chromatograms were registered at 270,  
248 470 and 535 nm in a DAD detector. The eluate stream from the DAD detector was directed to  
249 the post-column derivatization instrument. Post-column derivatization with ABTS was  
250 performed according to Kusznierevich, Piasek, Bartoszek, and Namieśnik (2011a) and

251 Kusznerewicz, Piasek, Bartoszek, and Namieśnik (2011b) with slight modifications. The  
252 methanolic ABTS solution stream (1 mM) was introduced to the eluate stream at a rate of 0.1  
253 mL/min and then directed to the reaction loop (1 mL, 130°C). The antioxidant profiles were  
254 recorded on a UV-Vis detector at 734 nm. Major betalains were identified using UV-Vis  
255 spectra and elution order. VXT and BTN calibration curves were used to quantify  
256 betaxanthins and betacyanins at 470 and 535 nm, respectively. The antioxidant activity of the  
257 separated compounds was quantified as a sum of areas under the negative peaks at 734 nm  
258 during analyses by HPLC with post-column derivatization with ABTS.

259

### 260 2.2.7. LC-Q-Orbitrap HRMS analysis

261 Separation was performed on a Dionex Ultimate 3000 UHPLC system (Thermo  
262 Scientific™, Dionex, San Jose, CA, USA) equipped with a 4.6 × 100 mm, 3.5 μm Agilent  
263 Eclipse Plus C18 column held at a temperature of 30°C. The mobile phase was composed of  
264 A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. A gradient elution was  
265 performed at a flow rate of 600 μL/min, according to the following gradient profile: the initial  
266 mobile phase composition was 5% eluent B, was linearly increased up to 35% in 17 min, and  
267 then the composition of eluent B was increased to 80% in 3 min. The column equilibration  
268 time was 5 min.

269 The chromatographic system was coupled to a Q Exactive™ Focus quadrupole-Orbitrap mass  
270 spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a heated electrospray  
271 ionisation source (HESI II). Detection was performed using a Q-Exactive mass spectrometer.  
272 The HESI parameters in positive polarity were as follows: sheath gas flow rate, 35; auxiliary  
273 gas flow rate, 15; sweep gas flow rate, 2; spray voltage, 3.5 kV; capillary temperature, 275°C;  
274 S-lens RF level, 50; and heater temperature, 450°C. The full-scan analysis parameters were as  
275 follows: resolution, 70,000; AGC target, 1e6; max IT, auto; and scan range, 120-1200. The  
276 data-dependent MS<sup>2</sup> parameters were as follows: resolution, 35,000; isolation window, 2.0  
277 m/z; normalized collision energy, 20-50-80; AGC target, 1e6; max IT, auto.

278 The external mass calibration and the quadrupole calibration were carried out daily. For the  
279 calibration, a mixture containing *n*-butylamine, caffeine, Met-Arg-Phe-Ala (MRFA), and  
280 Ultramark 1621 was used. All beetroot extracts (30% aqueous ethanol) were run in triplicate  
281 followed by injecting the blank H<sub>2</sub>O/EtOH (7:3, v/v) sample. The injection volume was 10  
282 μL.

283 To perform untargeted analysis on betalains and phenolic compounds, a customized  
284 database containing exact masses of several classes of Caryophyllales phytochemicals was  
285 created using Compound Discoverer software (v. 2.1, Thermo, Waltham, USA). The database  
286 included betacyanins, betaxanthins and their derivatives, and products of their degradation as  
287 well as flavonoids and phenolic acids (100 compounds). The exact masses and chemical  
288 formulas were combined using Excel 2019 (Microsoft). For each sample, raw data from three  
289 consecutive injections and from the blank sample were processed by Compound Discoverer.  
290 Raw data from three experimental replicates and a blank sample were processed using the  
291 workflow presented in Fig. S2. The customized database was implemented using mass list  
292 features to automatically match extracted compound m/z ratios in the database.

293

## 294 3. Results and discussion

295

### 296 3.1. Determination of pigment extraction efficiency by photometric betalain quantification 297 and colour measurements

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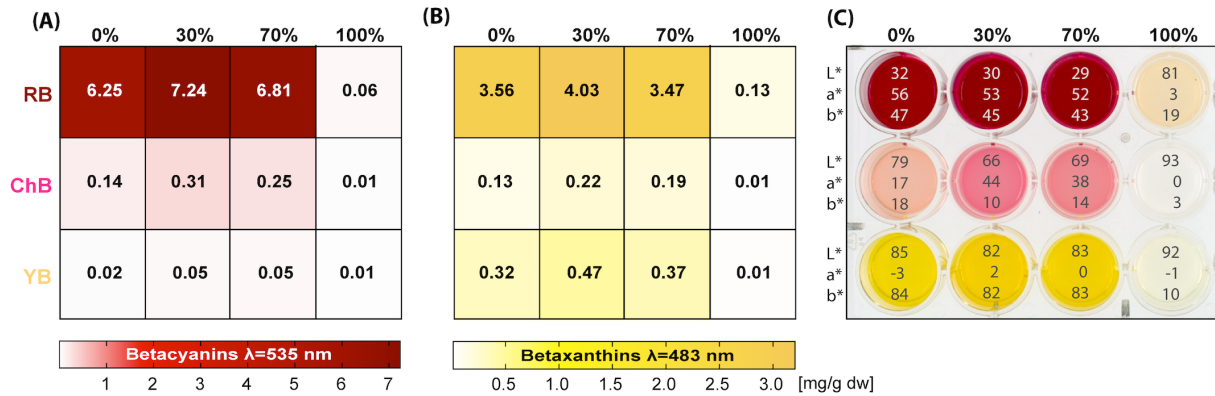
299 The extraction efficiency of phytochemicals from plant materials depends on the  
300 extraction method and affects the phytochemical profile of the final sample. Additionally, the

301 different polarities of the extracted compounds and the influence of the complex sample  
302 matrix make it difficult to find universal extraction conditions that could be used for each type  
303 of plant material. Therefore, due to the diversity of raw materials used in the research, for  
304 each type of beetroot, four parallel extractions were performed with extractants having  
305 different polarities, regulated by changes in the proportions of water and ethanol. According  
306 to Fu et al. (2020), adding ethanol or methanol to water is generally necessary to thoroughly  
307 extract betalain pigments and improve the extraction yield. We chose ethanol in this study  
308 because it enabled further analysis of extracts in biological systems such as cell culture.  
309 Because betalains are stable at pH 3 – 7 (Castro-Enriquez et al., 2020), the extraction medium  
310 was additionally acidified with formic acid.

311 Betalain extraction efficiency from freeze-dried beetroots was determined from the  
312 extract colour intensity and using the most popular and simple spectrophotometric method.  
313 Visual extract colour assessment enabled a quick sample comparison without the use of  
314 measuring devices. Using only these observations, extracts with the highest and the lowest  
315 pigment concentrations could be identified. Such a visual colour assessment could be  
316 supported by spectrophotometers with the possibility of CIELAB space parameter ( $L^*$ ,  $a^*$ ,  $b^*$ ,  
317  $C^*$ ) measurements or by using photographs of extracts and measuring the colour with digital  
318 colorimeters available as standard computer software. For the tested beetroot extracts, all  
319 colour differences were visually appreciable by human eyes. The parameters  $L^*$ ,  $a^*$ , and  $b^*$   
320 were additionally estimated for photographs of these extracts (**Fig. 1C**) using a digital  
321 colorimeter. The  $L^*$  parameter, which indicated lightness read from 0 (completely opaque or  
322 black) to 100 (completely transparent or white). The darkest samples were approximately 30,  
323 while the brightest samples were approximately 90. The positive value of the  $a^*$  parameter,  
324 which indicated redness, was the highest for the most red aqueous or aqueous ethanol extracts  
325 from red beetroots (52 – 56). The Chioggia beetroot extracts were pink and had  $a^*$  parameter  
326 in the range of 17 – 44. The same extracts from yellow cultivars of beetroots had the lowest  
327  $a^*$  parameter (-3 – 2), but the highest  $b^*$  parameter (82 – 85) for which a positive value  
328 indicated yellowness. The results showed that this approach may be helpful in assessing the  
329 effectiveness of betalain extraction as well as studying pigment stability (Prieto-Santiago,  
330 Cavia, Alonso-Torre, & Carrillo, 2020). The disadvantage of this approach, however, is the  
331 lack of information on the betalain content in the sample. However, such data on the total  
332 betacyanin and betaxanthin content could be obtained by simple spectrophotometric  
333 measurements. This method uses the Beer-Lambert-Bouguer Law and the values of the  
334 appropriate extinction coefficients. The choice of the appropriate molar absorption coefficient  
335 depends on the type of betalain groups that are present in the test sample at the highest  
336 concentration level. If the sample contains amaranthin-type, betanin-type or gomphrenin-type  
337 betacyanins, the molar absorptivity values ( $\epsilon$ ) of amaranthin ( $5.66 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$  at 536  
338 nm), betanin, ( $6.00 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$  at 538 nm) and gomphrenin I ( $5.06 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$   
339 at 540 nm) would be used for the calculation (Cai, Sun, & Corke, 2005). When determining  
340 the total betaxanthin content, the yellow vulgaxanthin I absorption coefficient ( $4.80 \times 10^4 \text{ L}$   
341  $\text{mol}^{-1} \text{ cm}^{-1}$  at 480 nm) is most often used.

342 For the beetroot extracts in this study, the total betacyanin and betaxanthin content determined  
343 by this photometric method are presented as the contents of betanin and vulgaxanthin I  
344 equivalents, respectively (**Fig. 1 A, B**). In all cases, 30% aqueous ethanol extracted the  
345 greatest pigment amount, therefore this kind of extracts were chosen for further research. The  
346 highest betacyanin content was found in red beetroot ( $7.24 \pm 0.17 \text{ mg/g dw}$ ), followed by  
347 Chioggia beetroot ( $0.314 \pm 0.022 \text{ mg/g dw}$ ) and yellow beetroot ( $0.0456 \pm 0.0011 \text{ mg/g dw}$ ).  
348 The red beetroot also contained the greatest yellow betaxanthin content ( $4.03 \pm 0.18 \text{ mg/g}$   
349  $\text{dw}$ ), while the yellow cultivar had approximately eight times less ( $0.472 \pm 0.011 \text{ mg/g dw}$ ).  
350 Chioggia beetroot extracts had the lowest pigment amount ( $0.224 \pm 0.015 \text{ mg/g dw}$ ). The red

351 beetroot cultivar was a rich source of red-violet betacyanins as well as yellow betaxanthins,  
 352 which agreed with previous studies (Sawicki et al., 2016; Slatnar, Stampar, Veberic, &  
 353 Jakopic, 2015). The results indicated a strong negative correlation between the lightness  
 354 parameter ( $L^*$ ) and total betalain content in the extracts (Pearson coefficient  $r = -0.95$ ), which  
 355 agreed with other studies (Prieto-Santiago et al., 2020).  
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359 **Fig. 1.** The average content of (A) betacyanins and (B) betaxanthins in red beetroot (RB),  
 360 Chioggia beetroot (ChB) and yellow beetroot (YB) extracts set with (C) photographs of these  
 361 extracts. The solvents for beetroot extraction contained 1% formic acid and different amounts  
 362 of ethanol (0, 30, 70 and 100%) in water. Betalain levels [mg/g dw] were determined using  
 363 spectrophotometry in three parallel extracts. The standard deviation of the calculated values  
 364 did not exceed 7%. The colour parameters ( $L^*$ ,  $a^*$ ,  $b^*$ ) were determined from photographs of  
 365 extracts with the use of a digital colorimeter.

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### 367 3.2. Total antioxidant activity determination

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369 The antioxidant content in foods and the related total antioxidant capacity have  
 370 emerged as prominent aspects of food quality and functionality and are used frequently to  
 371 promote the consumption of food types with high antioxidant potential. Since betalains are  
 372 known antioxidants, the total antioxidant activity of the products that contain them is often  
 373 regarded as an important biomarker of health-related quality (Sawicki et al., 2016; Slimen et  
 374 al., 2017; Wang, Jayaprakasha, & Patil, 2020).

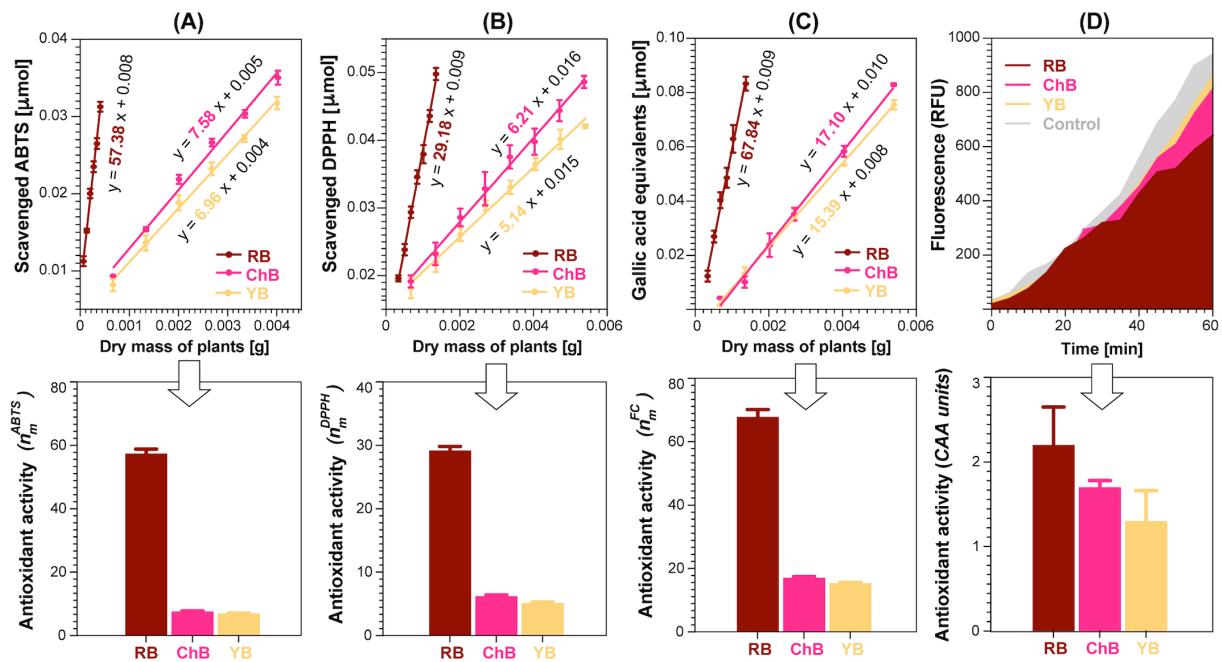
375 A study compared three of most common cell-free procedures and one cellular test used for  
 376 the analysing betalain-containing products. ABTS and DPPH assays have been widely used in  
 377 determining the free radical scavenging activity of extracts as well as pure compounds. In  
 378 these tests, antioxidant capacity is usually characterized by the EC50 value (the concentration  
 379 necessary to reduce 50% of radicals) or by the Trolox equivalent antioxidant capacity (TEAC)  
 380 index. We propose a new different method for determining the total antioxidant activity,  
 381 which is independent of the initial radical concentration and does not require a standard. This  
 382 approach determined the stoichiometric value for steady-state oxidation-reduction reactions in  
 383 pure compounds (Baranowska et al., 2018). For beetroot extracts, the calculated antioxidant  
 384 activity described the number of oxidant molecules reduced by antioxidants derived from 1 g  
 385 of lyophilizates after 10 min of reaction in ABTS and DPPH tests. These values were  
 386 calculated within a linear range of the assay and were expressed as the slope of a line  
 387 describing the relationship between the number of reduced oxidants and different amounts of  
 388 tested samples calculated as grams of dry weight in the reaction mixtures. The concentration  
 389 responses obtained after a 10 min reaction time, calculated from their antioxidant activities  
 390 are presented in **Fig. 2 A, B**. The method using Folin-Ciocalteu reagent was also considered  
 391 in this study. It is based on the transfer of electrons in an alkaline medium from compounds



392 with active hydroxyl groups to phosphomolybdenic phosphotungstic acid complexes to form  
 393 blue-coloured complexes. The reducing ability in this case was expressed as the number of  
 394 gallic acid molecules that formed blue complex equivalents and were derived from 1 g of dry  
 395 matter of the plant under study (Fig. 2 C). The results indicated the same trend for the three  
 396 tests. The antioxidant activity of the tested RB extracts was approximately four to eight times  
 397 greater than that of the ChB and YB extracts, which showed similar levels of antioxidant  
 398 activity. The same trend was observed for the total betalain content, which could indicate that  
 399 this group of compounds mainly affected the total beetroot antioxidant activity (Pearson  
 400 coefficient  $r=0.99$ ). A positive correlation between betalain content and total antioxidant  
 401 activity was also observed for beetroots in other studies (Koss-Mikołajczyk, Kusznerewicz,  
 402 Wiczkowski, Sawicki, & Bartoszek, 2019; Sawicki et al., 2016).

403 It is difficult to predict antioxidant activity *in vivo* based only on chemical assays  
 404 conducted under non-physiological conditions. The cellular antioxidant activity test was  
 405 designed to mimic biological conditions (pH, temperature), and it considers the  
 406 bioavailability, biodistribution, and cellular metabolism of the tested antioxidants. The CAA  
 407 test results (Fig. 2 D) agreed with chemical test results; RB showed the strongest antioxidant  
 408 potential, followed by ChB, and the least active was YB. The differences between samples  
 409 were not as pronounced as in the spectrophotometric methods, probably due to the different  
 410 bioavailabilities of red and yellow betalain pigments and other antioxidant components  
 411 present in the tested extracts.

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415 **Fig. 2** Graphs showing the beetroot extract antioxidant activity determination method using  
 416 tests with (A) ABTS radicals, (B) DPPH radicals, (C) Folin-Ciocalteu reagent, and (D)  
 417 cellular antioxidant activity assay (upper panel). The bar graphs show total antioxidant  
 418 activity expressed as coefficients (lower panel of graphs). The results are the means  $\pm$  SD of  
 419 three independent determinations. ( $n_m^{\text{ABTS}}$  -  $\mu\text{moles}$  of ABTS reduced by compounds derived  
 420 from 1 g of lyophilizates,  $n_m^{\text{DPPH}}$  -  $\mu\text{moles}$  of DPPH reduced by compounds derived from 1 g  
 421 of lyophilizates,  $n_m^{\text{FC}}$  -  $\mu\text{moles}$  of gallic acid equivalents derived from 1 g of lyophilizates  
 422 which reduce FC reagent, CAA units -  $\mu\text{moles}$  of quercetin equivalents derived from 1 g of  
 423 lyophilizates which inhibit DCFH probe oxidation).

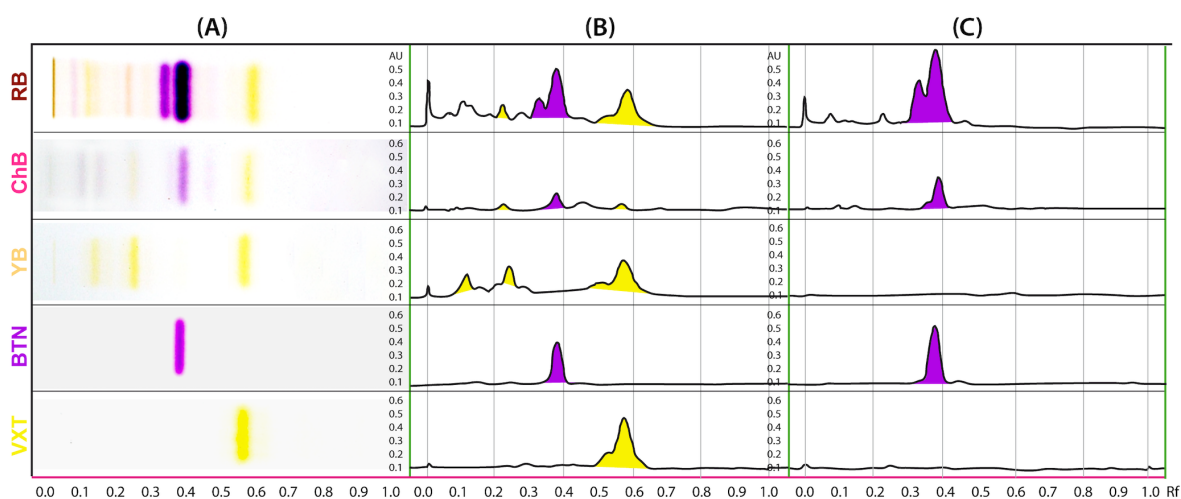
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426 3.3. Profiling betalains by HPTLC

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Thin layer chromatography (TLC) is an important planar chromatographic technique widely used as a cost-effective method for rapidly analysing different phytochemical groups. Currently, the modern high-performance version of this technique (HPTLC), combined with automated sample application and densitometric scanning, is sensitive, reliable, and suitable for use in qualitative and quantitative analysis. Unfortunately, due to the very polar nature of betalains, this technique is rarely used to profile these compounds. In the literature, there have been limited reports of the use of this technique for separating betalains (Bilyk, 1981; Rodriguez, Vidallon, Mendoza, & Reyes, 2016; Sunnadeniya et al., 2016; Viloria-Matos, Moreno-Alvarez, & Hidalgo-Báez, 2001). In these studies, betalains were separated on cellulose-coated plates, and the resulting chromatograms were of rather poor quality. In our research, we propose reversed-phase (RP) betalain pigment separation using a plate coated with octadecyl-modified silica (C18). To the best of our knowledge, this is the first report presenting such an HPTLC procedure for betalain profiling. **Fig. 3** presents HPTLC profiles and densitograms for beetroot extracts and standard solutions.



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**Fig. 3** HPTLC profiles registered under (A) white light and (B) densitograms at 480 nm and (C) 535 nm of extracts from red beetroot (RB), Chioggia beetroot (ChB) and yellow beetroot (YB) and two isolated standards: betanin (BTN) and vulgaxanthin I (VXT). For RB, the extract was diluted twice before dosing.

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The major betacyanins and betaxanthins in the samples were well separated and visible in white light as purple and yellow bands, respectively. Betanin ( $R_f = 0.38$ ) and isobetanin ( $R_f = 0.35$ ) were the major betacyanins in the RB and ChB extracts. In yellow beetroot, vulgaxanthin I ( $R_f = 0.58$ ) was the main betalain. This betaxanthin was also detected in the remaining beetroot extracts. Betanin and vulgaxanthin I identity was confirmed by comparison with standards isolated from red beetroot juice by the SPE. The HPTLC profile also indicated the presence of other betalains, but their identity should be confirmed by appropriate standards or with the use of a TLC-MS interface and mass spectrometer. The isolated betanin and vulgaxanthin I were also used for quantifying major betacyanins and betaxanthins in beetroot extracts with appropriate calibration curves and densitograms. The total betacyanin and betaxanthin contents were similar to those determined by spectrophotometric methods. The average betacyanin and betaxanthin contents in RB determined by the HPTLC were  $6.30 \pm 0.13$  and  $4.467 \pm 0.047$  mg/g dw, respectively. Lower levels of these pigments were found in ChB ( $0.2627 \pm 0.0037$  and  $0.2097 \pm 0.0039$  mg/g dw,

464 respectively). For YB, only yellow betaxanthins were detected, with a total content of  $0.378 \pm$   
465  $0.073$  mg/g dw.

466 Using the densitograms recorded at 480 and 535 nm, it was also possible to calculate the  
467 contribution of specific pigment concentrations to the total betaxanthin or betacyanin content  
468 (the sum of the individual compounds). Such a quantitative approach did not require a  
469 standard, but it required the determination of the total betaxanthin and betacyanin content in  
470 the tested extract by the aforementioned spectrophotometric method.

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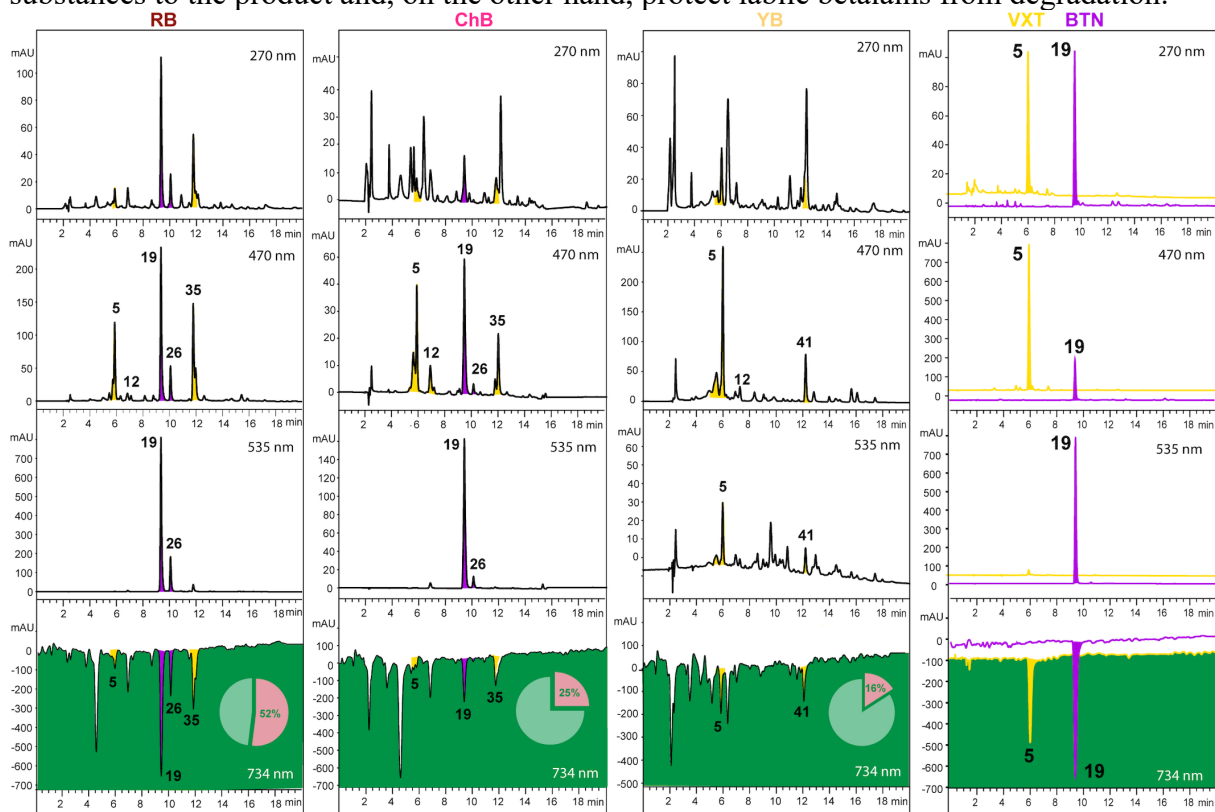
#### 472 3.4. Profiling betalains and antioxidants by HPLC

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474 HPLC is widely used method for analysing betalains. The most popular stationary  
475 phase is octadecyl-modified silica (C18), ensuring adequate betalain efficiency and retention,  
476 as well as sufficient resolution. Since betalains exist in aqueous solution in various forms of  
477 ionisation at altered pH values, conventional acidic eluents with or without buffers can help  
478 regulate their separation. Betaxanthin and betacyanin detection is most often performed with  
479 photodiode array detectors (DAD) at wavelengths of 470 and 535 nm, respectively, as well as  
480 MS detectors. Due to the lack of commercially available standards, predominant betalains are  
481 often identified from their UV-Vis or MS spectra, the order of elution, and literature data. **Fig.**  
482 **4** shows example HPLC profiles from beetroot extracts recorded at 270, 470 and 535 nm. For  
483 RB and ChB extracts, two major violet betacyanins, betanin and isobetanin, with  $\lambda_{\max}$  536 nm,  
484 were detected. Vulgaxanthin I with  $\lambda_{\max}$  471 nm was the dominant betaxanthin present in all  
485 beetroot extracts studied. A prominent peak of yellow-orange neobetanin ( $\lambda_{\max} = 462$  nm),  
486 which has been frequently detected in *B. vulgaris* L. roots (Nemezer et al., 2011; Sawicki et  
487 al., 2016; Slatnar et al., 2015), was also observed in the RB and ChB chromatograms. Major  
488 betacyanin and betaxanthin contents were calculated as betanin and vulgaxanthin I  
489 equivalents, respectively, using calibration curves for the isolated compounds. The total  
490 betaxanthin contents were  $3.79 \pm 0.13$ ,  $0.416 \pm 0.014$ , and  $0.212 \pm 0.010$  mg/g dw for RB, YB  
491 and ChB, respectively. Betacyanins were detected only in RB and ChB, where their total  
492 contents amounted to  $6.49 \pm 0.11$  and  $0.275 \pm 0.011$ , respectively.

493 During beetroot extract HPLC-DAD analysis, post-column derivatization of analytes  
494 with ABTS reagent was performed. As in the ABTS colorimetric tests, the reduction reaction  
495 led to a significant shift in the UV-visible spectrum, resulting in ABTS reagent absorption  
496 change (discolouration). This dependence could also be used as a quantitative measurement of  
497 the antioxidant potential of individual analytes separated by HPLC (Kusznierewicz et al.,  
498 2011a; Kusznierewicz et al., 2011b). This approach was used here for detecting antioxidant  
499 phytochemicals in the beetroot extract chromatographic profiles (**Fig. 4**, bottom  
500 chromatograms). The presence of antioxidants in the eluate caused negative peaks in the  
501 chromatogram recorded after derivatization at 734 nm. By adding the area of the antioxidant  
502 negative peaks, it was possible to estimate the total antioxidant activity of the tested sample.  
503 Unlike spectrophotometric bulk tests, this approach did not take into account possible  
504 synergistic or antagonistic effects between phytochemicals. Comparing the summed negative  
505 peak areas per gram of sample, a similar relationship between the total antioxidant activity of  
506 the tested beetroots was observed as in the ABTS spectrophotometric test. The total negative  
507 peak area per gram of tested RB extracts was approximately eight times greater than that of  
508 ChB and YB extracts, which showed a similar level. Chromatographic profiling coupled with  
509 chemical post-detection not only revealed the individual reducing analytes but also enabled  
510 quantification of their input into the antioxidant potential of the sample. In **Fig. 4** (bottom  
511 panel), the percentage contribution of betalains to the total beetroot sample antioxidant  
512 activities presented as a pie graph. The greatest betalain contribution to the beetroot sample  
513 antioxidant potential was observed for RB (52%), followed by ChB (25%) and YB (16%).

514 Profiles obtained after derivatization indicated the presence of antioxidants in beetroot  
 515 extracts other than betalains. Their content, as well as the content of betalain, was the highest  
 516 in the RB extract. They were probably compounds belonging to flavonoids or phenolic acids,  
 517 the presence of which has also been observed frequently in beetroot (Kujala, Loponen, &  
 518 Pihlaja, 2001; Kujala, Vienola, Klika, Loponen, & Pihlaja, 2002; Waldron, Ng, Parker, &  
 519 Parr, 1997; Wang, Jayaprakasha, & Patil, 2019). Such a high content of antioxidants other  
 520 than betalains in the extracts justified use of crude plant extracts rather than the isolated  
 521 pigment fraction in dietary supplements or functional food. The additional presence of other  
 522 naturally occurring antioxidants could, on the one hand, provide additional bioactive  
 523 substances to the product and, on the other hand, protect labile betalains from degradation.



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 526 **Fig. 4** HPLC profiles of extracts from red beetroot (RB), Chioggia beetroot (ChB) and yellow  
 527 beetroot (YB) and two isolated standards, betanin (BTN) and vulgaxanthin I (VXT),  
 528 registered before (270 nm, 470 nm, 535 nm) and after (734 nm) post-column derivatization  
 529 with ABTS. In the antioxidant profiles (at 734 nm), pie graphs of the betalain input to the  
 530 total antioxidant activity calculated on the basis of the negative peak area are included. For  
 531 RB, the extract was diluted four times before injection.

### 532 533 534 3.5. Tentative phytochemical identification by LC-HRMS

536 Betalain identification is challenging due to the low accessibility of high-quality  
 537 standards that may be used as references. Knowing that mass spectrometry measurement with  
 538 a precision of four decimal places allows us to predict the molecular formula, the  
 539 identification process can be carried out by comparing the experimental MS data to the results  
 540 from previous betalain studies in the literature. Since searching the literature is often a time-  
 541 consuming process, this study shows the advantages of creating local databases that can be  
 542 processed by specialized software.



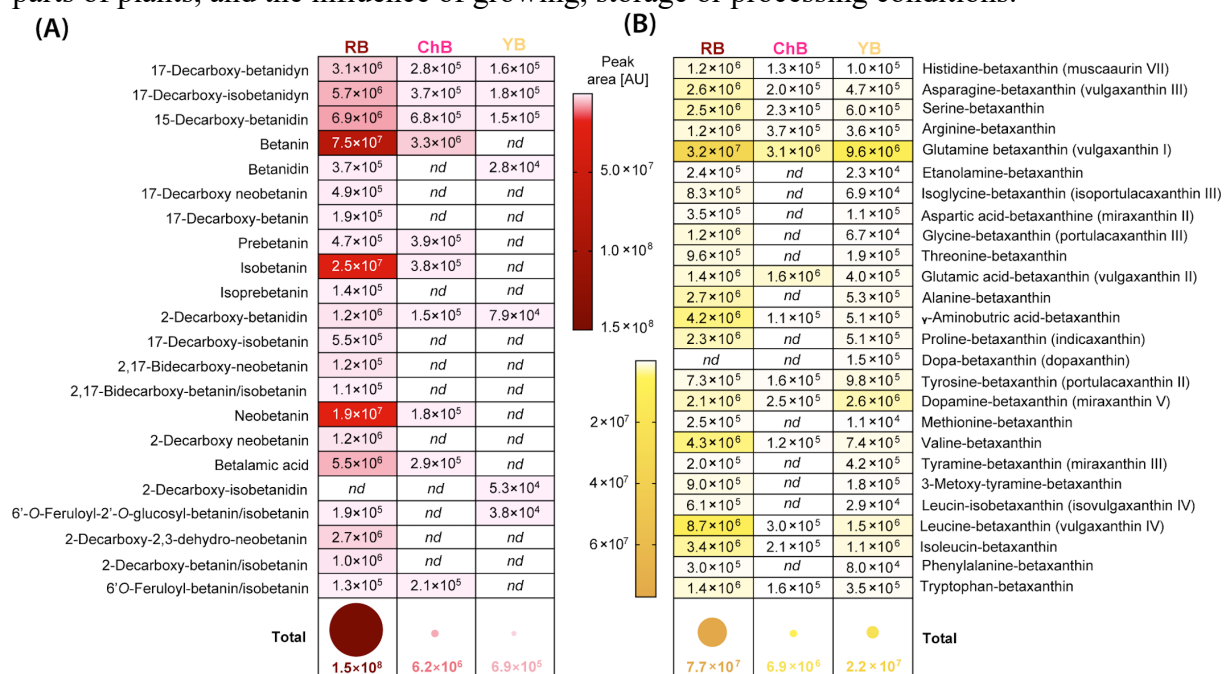
543 **Table 1**  
 544 Phytochemicals tentatively identified by LC-Q-Orbitrap-HRMS in red beet (RB), Chioggia  
 545 beet (ChB) and yellow beet (YB) extracts.  
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No.	RT (min)	Component name	Molecular formula	Expected mass (Da)	Observed mass (Da)	Mass error (ppm)	Beet Varieties			Ref.
							RB	ChB	YB	
1	1.93	Histidine-betaxanthin (muscaaurin VII)	C <sub>15</sub> H <sub>16</sub> N <sub>4</sub> O <sub>6</sub>	349.11425	349.11343	2.35	+	+	+	Kugler et al., 2007
2	2.17	Asparagine-betaxanthin (vulgaxanthin III)	C <sub>15</sub> H <sub>15</sub> N <sub>5</sub> O <sub>7</sub>	326.09828	326.09738	2.77	+	+	+	Kugler et al., 2007
3	2.21	Serine-betaxanthin	C <sub>12</sub> H <sub>14</sub> N <sub>2</sub> O <sub>7</sub>	299.08738	299.08658	2.67	+	+	+	Kugler et al., 2007
4	2.23	Arginine-betaxanthin	C <sub>15</sub> H <sub>21</sub> N <sub>5</sub> O <sub>6</sub>	368.15646	368.15536	2.97	+	+	+	Amaya-Cruz et al., 2019
5	2.23	Glutamine betaxanthin (vulgaxanthin I)	C <sub>14</sub> H <sub>17</sub> N <sub>3</sub> O <sub>7</sub>	340.11393	340.11307	2.53	+	+	+	Kugler et al., 2007
6	2.24	Etanolamine-betaxanthin	C <sub>11</sub> H <sub>14</sub> N <sub>2</sub> O <sub>5</sub>	255.09754	255.09689	2.56	+	-	+	Kugler et al., 2007
7	2.24	Iso glycine-betaxanthin (isoportulacaxanthin III)	C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>6</sub>	269.07682	269.07608	2.74	+	-	+	Nemzer et al., 2011
8	2.68	Hydroxybenzoic acid	C <sub>7</sub> H <sub>6</sub> O <sub>3</sub>	139.03897	139.03874	1.65	-	+	+	Waldron et al., 1997
9	2.89	Aspartic acid-betaxanthine (miraxanthin II)	C <sub>13</sub> H <sub>14</sub> N <sub>2</sub> O <sub>8</sub>	327.08229	327.08151	2.38	+	-	+	Kugler et al., 2007
10	3.03	Glycine-betaxanthin (portulacaxanthin III)	C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>6</sub>	269.07682	269.07605	2.85	+	-	+	Kugler et al., 2007
11	3.51	Threonine-betaxanthin	C <sub>13</sub> H <sub>16</sub> N <sub>2</sub> O <sub>7</sub>	313.10302	313.10226	2.44	+	-	+	Kugler et al., 2007
12	3.66	Glutamic acid-betaxanthin (vulgaxanthin II)	C <sub>14</sub> H <sub>16</sub> N <sub>2</sub> O <sub>8</sub>	341.09795	341.09717	2.28	+	+	+	Kugler et al., 2007
13	4.79	Alanine-betaxanthin	C <sub>12</sub> H <sub>14</sub> N <sub>2</sub> O <sub>6</sub>	283.09247	283.09186	2.15	+	-	+	Kugler et al., 2007
14	4.83	17-Decarboxy-betanidin	C <sub>17</sub> H <sub>17</sub> N <sub>2</sub> O <sub>6</sub> <sup>+</sup>	345.10812	345.10733	2.28	+	+	+	Nemzer et al., 2011
15	5.09	17-Decarboxy-isobetaniidyn	C <sub>17</sub> H <sub>17</sub> N <sub>2</sub> O <sub>6</sub> <sup>+</sup>	345.10812	345.10733	2.28	+	+	+	Nemzer et al., 2011
16	5.29	γ-Aminobutric acid-betaxanthin	C <sub>13</sub> H <sub>16</sub> N <sub>2</sub> O <sub>6</sub>	297.10811	297.10757	1.81	+	+	+	Kugler et al., 2007
17	5.35	Vanillic acid	C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>	169.04954	169.04932	1.30	-	-	+	Waldron et al., 1997
18	5.42	15-Decarboxy-betanidin	C <sub>17</sub> H <sub>17</sub> N <sub>2</sub> O <sub>6</sub> <sup>+</sup>	345.10812	345.10733	2.28	+	+	+	Nemzer et al., 2011
19	5.54	Betanin	C <sub>24</sub> H <sub>26</sub> N <sub>2</sub> O <sub>13</sub>	551.15077	551.14948	2.35	+	+	-	Nemzer et al., 2011
20	5.54	Betanidin	C <sub>18</sub> H <sub>16</sub> N <sub>2</sub> O <sub>8</sub>	389.09795	389.09729	1.69	+	-	+	Nemzer et al., 2011
21	5.54	17-Decarboxy neobetaniin	C <sub>23</sub> H <sub>24</sub> N <sub>2</sub> O <sub>11</sub>	505.14529	505.1442	2.16	+	-	-	Nemzer et al., 2011
22	5.55	17-Decarboxy-betanin	C <sub>23</sub> H <sub>26</sub> N <sub>2</sub> O <sub>11</sub>	507.16094	507.16077	0.34	+	-	-	Nemzer et al., 2011
23	5.60	Prebetanin	C <sub>24</sub> H <sub>26</sub> N <sub>2</sub> O <sub>16</sub> S	631.10758	631.10583	2.77	+	+	-	Nemzer et al., 2011
24	5.95	Proline-betaxanthin (indicaxanthin)	C <sub>14</sub> H <sub>16</sub> N <sub>2</sub> O <sub>6</sub>	309.10811	309.10757	1.74	+	-	+	Kugler et al., 2007
25	6.08	Caffeic acid	C <sub>8</sub> H <sub>6</sub> O <sub>4</sub>	181.04954	181.04921	1.82	-	+	+	Waldron et al., 1997
26	6.10	Isobetaniin	C <sub>24</sub> H <sub>26</sub> N <sub>2</sub> O <sub>13</sub>	551.15077	551.14948	2.35	+	+	-	Nemzer et al., 2011
27	6.14	Isoprebetanin	C <sub>24</sub> H <sub>26</sub> N <sub>2</sub> O <sub>16</sub> S	631.10758	631.10638	1.90	+	-	-	Nemzer et al., 2011
28	6.36	Dopa-betaxanthin (dopaxanthin)	C <sub>18</sub> H <sub>18</sub> N <sub>2</sub> O <sub>8</sub>	391.11360	391.11267	2.37	-	-	+	Kugler et al., 2007
29	6.17	Syringic acid	C <sub>9</sub> H <sub>10</sub> O <sub>5</sub>	199.06009	199.05978	1.58	+	-	+	Wang et al., 2020
30	6.61	2-Decarboxy-betanidin	C <sub>17</sub> H <sub>17</sub> N <sub>2</sub> O <sub>6</sub> <sup>+</sup>	345.10812	345.10733	2.28	+	+	+	Nemzer et al., 2011
31	6.61	5-Caffeoyl quinic acid	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	355.10237	355.1015	2.44	+	-	-	Wang et al., 2019
32	6.68	17-Decarboxy-isobetaniin	C <sub>22</sub> H <sub>26</sub> N <sub>2</sub> O <sub>11</sub>	507.16094	507.1601	1.66	+	-	-	Nemzer et al., 2011
33	6.89	2,17-Bidecarboxy-neobetaniin	C <sub>22</sub> H <sub>24</sub> N <sub>2</sub> O <sub>9</sub>	461.15545	461.1546	1.85	+	-	-	Nemzer et al., 2011
34	7.06	2,17-Bidecarboxy-betanin/isobetaniin	C <sub>22</sub> H <sub>27</sub> N <sub>2</sub> O <sub>9</sub> <sup>+</sup>	463.17110	463.17007	2.23	+	-	-	Nemzer et al., 2011
35	7.32	Neobetaniin	C <sub>24</sub> H <sub>24</sub> N <sub>2</sub> O <sub>13</sub>	549.13512	549.13416	1.75	+	+	-	Wang et al., 2020
36	7.42	2-Decarboxy neobetaniin	C <sub>23</sub> H <sub>24</sub> N <sub>2</sub> O <sub>11</sub>	505.14529	505.1442	2.16	+	-	-	Nemzer et al., 2011
37	7.49	Betalamic acid	C <sub>9</sub> H <sub>9</sub> NO <sub>5</sub>	212.05535	212.05516	0.91	+	+	-	Kujala et al., 2001
38	7.71	2-Decarboxy-isobetaniin	C <sub>17</sub> H <sub>17</sub> N <sub>2</sub> O <sub>6</sub> <sup>+</sup>	345.10812	345.10757	1.59	-	-	+	Nemzer et al., 2011
39	7.73	trans- <i>p</i> -Coumaric acid	C <sub>8</sub> H <sub>6</sub> O <sub>3</sub>	165.05463	165.05432	1.85	-	+	+	Waldron et al., 1997
40	7.77	Tyrosine-betaxanthin (portulacaxanthin II)	C <sub>18</sub> H <sub>18</sub> N <sub>2</sub> O <sub>7</sub>	375.11868	375.11789	2.11	+	+	+	Nemzer et al., 2011
41	7.79	Dopamine-betaxanthin (miraxanthin V)	C <sub>17</sub> H <sub>18</sub> N <sub>2</sub> O <sub>6</sub>	347.12377	347.12308	1.98	+	+	+	Nemzer et al., 2011
42	7.92	Ferulic/isoferulic acid isomer	C <sub>10</sub> H <sub>10</sub> O <sub>4</sub>	195.06519	195.0649	1.49	+	+	+	Waldron et al., 1997
43	8.15	Tetrahydroxy-biindolyl	C <sub>16</sub> H <sub>12</sub> N <sub>2</sub> O <sub>4</sub>	297.08699	297.08643	1.88	-	+	-	Kujala et al., 2002
44	8.34	Methionine-betaxanthin	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O <sub>6</sub> S	343.09584	343.09497	2.54	+	-	+	Kugler et al., 2007
45	8.38	3-Caffeoyl quinic acid	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	355.10237	355.1015	2.44	+	+	+	Wang et al., 2019
46	8.43	Valine-betaxanthin	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O <sub>6</sub>	311.12377	311.12302	2.40	+	+	+	Nemzer et al., 2011
47	9.00	cis- <i>p</i> -Coumaric acid	C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>	165.05463	165.05432	1.85	+	+	-	Waldron et al., 1997
48	9.21	Tyramine-betaxanthin (miraxanthin III)	C <sub>17</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub>	331.12885	331.12796	2.70	+	-	+	Kugler et al., 2007
49	9.61	3-Methoxy-tyramine-betaxanthin	C <sub>18</sub> H <sub>20</sub> N <sub>2</sub> O <sub>6</sub>	361.13942	361.13840	2.82	+	-	+	Kugler et al., 2007
50	9.75	Ferulic/isoferulic acid isomer	C <sub>10</sub> H <sub>10</sub> O <sub>4</sub>	195.06519	195.06485	1.74	+	+	+	Waldron et al., 1997
51	9.78	Feruloylglucose	C <sub>16</sub> H <sub>20</sub> O <sub>9</sub>	357.11801	357.11700	2.81	+	+	+	Kujala et al., 2002
52	10.14	Leucin-isobetaxanthin (isovulgaxanthin IV)	C <sub>15</sub> H <sub>20</sub> N <sub>2</sub> O <sub>6</sub>	325.13942	325.13855	2.67	+	-	+	Nemzer et al., 2011
53	10.22	6'- <i>O</i> -Feruloyl-2'- <i>O</i> -glucosyl-betanin/isobetaniin	C <sub>40</sub> H <sub>45</sub> N <sub>2</sub> O <sub>21</sub> <sup>+</sup>	889.25093	889.24841	2.84	+	-	+	Nemzer et al., 2011
54	10.35	2-Decarboxy-2,3-dehydro-neobetaniin	C <sub>23</sub> H <sub>22</sub> N <sub>2</sub> O <sub>11</sub>	503.12964	503.12839	2.49	+	-	-	Nemzer et al., 2011
55	10.70	Leucine-betaxanthin (vulgaxanthin IV)	C <sub>15</sub> H <sub>20</sub> N <sub>2</sub> O <sub>6</sub>	325.13942	325.13855	2.67	+	+	+	Kugler et al., 2007
56	11.05	Isoleucin-betaxanthin	C <sub>15</sub> H <sub>20</sub> N <sub>2</sub> O <sub>6</sub>	325.13942	325.13855	2.67	+	+	+	Nemzer et al., 2011
57	11.45	2-Decarboxy-betanin/isobetaniin	C <sub>23</sub> H <sub>26</sub> N <sub>2</sub> O <sub>11</sub>	507.16094	507.15988	2.10	+	-	-	Nemzer et al., 2011
58	11.53	Phenylalanine-betaxanthin	C <sub>18</sub> H <sub>18</sub> N <sub>2</sub> O <sub>6</sub>	359.12376	359.12280	2.67	+	-	+	Kugler et al., 2007
59	11.46	Tetrahydroxy-biindolyl	C <sub>16</sub> H <sub>12</sub> N <sub>2</sub> O <sub>4</sub>	297.08698	297.08627	2.38	+	-	-	Kujala et al., 2002
60	11.80	6'- <i>O</i> -Feruloyl-betanin/isobetaniin	C <sub>34</sub> H <sub>35</sub> N <sub>2</sub> O <sub>16</sub> <sup>+</sup>	727.19811	727.19598	2.93	+	+	-	Nemzer et al., 2011
61	12.03	Tryptophan-betaxanthin	C <sub>20</sub> H <sub>19</sub> N <sub>3</sub> O <sub>6</sub>	398.13467	398.1337	2.43	+	+	+	Kugler et al., 2007
62	13.12	Ferulic/isoferulic acid isomer	C <sub>10</sub> H <sub>10</sub> O <sub>4</sub>	195.06519	195.06485	1.74	+	+	+	Waldron et al., 1997
63	17.44	N-trans-Feruloyltyramine (Moupinamide)	C <sub>18</sub> H <sub>19</sub> NO <sub>4</sub>	314.13869	314.13794	2.38	-	+	+	Kujala et al., 2002
64	17.91	N-trans-Feruloyl-3-methoxytyramine (N-trans-Feruloylhomovanillylamine)	C <sub>19</sub> H <sub>21</sub> NO <sub>5</sub>	344.14925	344.14841	2.45	+	+	+	Kujala et al., 2002

547  
 548 For the purposes of this study, MS data gathered from multiple studies concerning  
 549 phytochemicals from betalain-synthesizing plants were implemented as a mass list in a local

550 database using Compound Discoverer software (CD). CD software revealed 475 compounds,  
 551 of which 64 were identified and are shown in **Table 1** in order of retention time (RT).  
 552 **Table 1** also includes the molecular formula, theoretical and experimental mass, and presence  
 553 of each compound in the three beetroot varieties. Monoisotopic positive ion  $[M+H]^+$  mass  
 554 was used for identification and compared to spectroscopic data for compounds previously  
 555 described in the literature. The mass errors between the expected and observed exact masses  
 556 of the identified compounds did not exceed 3 ppm. The identified compounds were mainly  
 557 classified as betacyanins and their derivatives (22) and betaxanthins (26). Other compounds  
 558 found in beetroot extracts were phenolic acids (12), bisindoles (2), and two phenolic amides.  
 559 To the best of our knowledge, this is the first report about beetroots with the greatest number  
 560 of identified secondary metabolites. The betacyanin diversity in red beetroot was similar to  
 561 that reported by Nemzer et al. (2011), who identified 28 betacyanins and 17 betaxanthins.  
 562 Few betalain derivatives found by Nemzer et al. (2011) did not occur in red beetroot. A  
 563 similar number of betaxanthins was also identified by Kugler, Graneis, Stintzing, and Carle  
 564 (2007), who detected the presence of 24 betaxanthins in red and yellow beetroots.

565 CD software normalized peak areas for each of the detected signals. This  
 566 information was used for comparative compound semi-quantification in different samples.  
 567 This approach could be useful, especially since betalain standards are not available. Thus, it  
 568 was not possible to adequately correct the analyte peak area in the mass chromatogram for a  
 569 possible difference in MS responses. For beetroot sample comparison, semi-quantification of  
 570 betaxanthins, betacyanins, and their derivatives was performed based on their peak areas, as  
 571 shown in **Figure 5**. The results indicated a relationship similar to that observed in other  
 572 methods between the total content of red and yellow pigments in the three beetroot varieties.  
 573 However, in this case, we also obtained information on the quantitative differences between  
 574 all detected compounds, which may be of great importance when comparing the profile of  
 575 betalains and their derivatives when testing different plant varieties, different morphological  
 576 parts of plants, and the influence of growing, storage or processing conditions.



577 **Fig. 5** Mean values of peak areas of (A) betacyanins and their derivatives and (B)  
 578 betaxanthins, which were obtained from the extracted ion chromatograms registered during  
 579 LC-Q-Orbitrap-HRMS analyses of red beetroot (RB), Chioggia beetroot (ChB) and yellow  
 580 beetroot (YB) extracts.  
 581  
 582

#### 583 4. Conclusion

584 This study presented a set of methods to assess the health-promoting potential of  
585 plants, food products, nutraceuticals, and dietary supplements containing betalains. The type  
586 and quality of data generated in these methods were evaluated from analysing samples of  
587 three beetroot cultivars with different pigmentations.

588 The most popular photometric measurements are recommended for a quick,  
589 simple, and inexpensive analysis of the total betacyanin and betaxanthin content. This method  
590 can be useful for the fast control of betalain levels in different samples, for example, when  
591 selecting the best extraction parameters or studying the effects of storage or processing. Such  
592 spectrophotometric measurements do not require additional reagents and expensive  
593 equipment, but the results give only an estimated value of the total betalain content.

594 Spectrophotometric methods can also be used to determine the total sample  
595 antioxidant activity. However, additional use of appropriate indicators is required, *e.g.*, ABTS  
596 and DPPH radicals or the Folin-Ciocalteu reagent. We stress that these batch methods employ  
597 oxidants with no physiological relevance, which is their major limitation. Nonetheless, they  
598 have a number of advantages, such as being inexpensive, quick and easy to perform, and in  
599 many situations, such as in food production, they are sufficient to compare the antioxidant  
600 potentials of different samples. Additionally, a number of reducing phytochemicals are indeed  
601 valuable bioactive compounds, so their monitoring along the food or dietary supplement  
602 production chain is increasingly recognized as an important issue, especially in the so-called  
603 functional food industry (Bartoszek, Kusznierevich, & Namieśnik, 2014). Furthermore,  
604 comparing the levels of antiradical activity of different samples may be easier using the new  
605 method of calculation and expression of this activity proposed in this article, which is  
606 independent of the initial radical concentration and does not require reference substance.  
607 Despite wide use of these chemical antioxidant activity assays, their ability to predict *in vivo*  
608 activity is limited for a number of reasons. In this study, the cellular antioxidant activity assay  
609 was proposed as an alternative procedure for total antioxidant potential determination in  
610 betalain-containing samples. The cell culture model better represents the complexity of  
611 biological systems than popular chemistry antioxidant activity assays and is an important tool  
612 for screening foods, phytochemicals, and dietary supplements for potential biological activity  
613 (Wolfe & Liu, 2007).

614 Liquid chromatography provides more precise information about phytochemical  
615 composition of the tested samples. Two liquid chromatography techniques, HPLC and  
616 HPTLC, were used for profiling betalains. In both methods separation of phytochemicals was  
617 performed using the reversed phase system. In the case of the TLC technique, this approach to  
618 betalain pigments was presented for the first time. Both methods require the availability of the  
619 apparatus, although in the case of TLC, it is possible to use a simplified procedure in the  
620 manual version. These techniques provide betalain fingerprints, that allow for more precise  
621 comparative analysis of the tested samples. Additionally, DAD detectors and appropriate  
622 standards enable quantitative and qualitative analysis of both pigments and other colourless  
623 analytes with chromophore groups. The problem of the lack of commercially available  
624 betalain standards can be solved by isolating the main compounds representing betacyanin  
625 and betaxanthin groups using the simple SPE procedure proposed in the article.

626 HPLC-DAD can also be coupled with a post-column derivatization system fed  
627 with an appropriate indicator, *e.g.*, ABTS. This method enables profiling of individual  
628 reducing compounds in complex mixtures following their chromatographic separation from  
629 the matrix. This is a great advantage in that it provides both chromatographic profiles and  
630 corresponding fingerprints of antioxidants (including unknowns) along with quantitative  
631 determination of antioxidant activity from individual compounds. This approach also makes it  
632 possible to estimate the contribution of the betalains fraction to the total antioxidant activity

633 of sample, which was presented here for the first time. Although this method requires  
634 additional equipment (it may be a commercial post-column derivatization system or an  
635 additional HPLC pump), it enables monitoring of antioxidant changes (degradation of native  
636 and possible formation of new ones) during processing or storage. This is especially important  
637 for plant-based products with health-related properties.

638 The combination of liquid chromatography with high-resolution mass spectrometry  
639 (HRMS) is a powerful tool enabling the comprehensive, qualitative, and quantitative analysis  
640 of various metabolites. This study discussed beetroot extract analysis using LC-Q-Orbitrap  
641 HRMS and Compound Discoverer software. The results indicated a high potential for  
642 applying such an approach to identifying various phytochemical classes, including betalains  
643 and their degradation products. Using CD software ensures flexible workflows and statistical  
644 analyses. Another valuable feature is the ability to search self-generated local databases of  
645 compounds based on literature data, which is particularly useful for researchers working with  
646 specific groups of phytochemicals. Processing the raw MS data with CD software enabled  
647 rapid detection, identification, and quantification of betalains in several beetroot samples  
648 simultaneously. This approach can be useful for verifying the presence of the expected health-  
649 promoting compounds and monitoring changes in their composition in food at different stages  
650 of production. It can also be a great tool for more advanced metabolomics research. However,  
651 the high cost of hardware and software must be considered.

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