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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,

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

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

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

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, trypsine, 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 × *g* at 4°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 L mol^{-1} cm^{-1} at $\lambda = 538$ nm for betanin and 48,000 L mol^{-1} cm^{-1} at $\lambda = 480$ 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).

2.2.3.1. ABTS assay. To generate a radical stock solution, ABTS powder was dissolved in aqueous $Na_2S_2O_8$ (4.45 mmol L^{-1}) to obtain a concentration of 7 mmol/L and left in the dark at ambient temperature for 24 h. Before measurements, the ABTS stock solution was diluted in water until the absorbance reached to 0.8 ± 0.05 at λ=734. This ABTS solution (1 mL) was mixed with different volumes of beetroot extracts selected in such a way that the measured absorbance values were within a linear range of the assay (1 – 10 µL for RB and 20 – 160 µL for ChB and YB). All reactions were carried out in 48-well plates. The absorbance of the mixtures was measured at 734 nm after 10 min. The amount of ABTS radicals scavenged by the tested beetroot sample in reaction media was calculated using the Beer-Lambert-Bouguer Law (Beer's Law) according to the equation: [µmol]=[((A$_0$-A$_{10}$) ×V×1000))/(ε×l)], where A$_0$ is the initial absorbance of the radical solution; A$_{10}$ is the absorbance of the radical solution after reaction time; V is the final volume or reaction mixture [mL]; l is the optical path length [cm], and ε is the ABTS molar extinction coefficient (16,000 L mol^{-1} cm^{-1} at λ = 734). The data were used to generate a linear relationship between the different amounts of tested beetroot samples calculated as grams of dry lyophilizates in the reaction mixtures and the number of ABTS scavenged µmoles. The slopes of the straight lines were then used to express the total antioxidant activity and mean of how many ABTS µmoles were scavenged by 1 g of tested sample.

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

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

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

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

2.2.4. Betanin and vulgaxanthin I isolation

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

2.2.5. Profiling of betalains by HPTLC

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

2.2.6. Profiling betalains and antioxidants by HPLC

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

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

2.2.7. LC-Q-Orbitrap HRMS analysis

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

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

The external mass calibration and the quadrupole calibration were carried out daily. For the calibration, a mixture containing *n*-butylamine, caffeine, Met-Arg-Phe-Ala (MRFA), and Ultramark 1621 was used. All beetroot extracts (30% aqueous ethanol) were run in triplicate followed by injecting the blank H_2O/EtOH (7:3, v/v) sample. The injection volume was 10 µL.

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

3. Results and discussion

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

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

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

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

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

beetroot cultivar was a rich source of red-violet betacyanins as well as yellow betaxanthins, which agreed with previous studies (Sawicki et al., 2016; Slatnar, Stampar, Veberic, & Jakopic, 2015). The results indicated a strong negative correlation between the lightness parameter (L^*) and total betalain content in the extracts (Pearson coefficient r = -0.95), which agreed with other studies (Prietio-Santiago et al., 2020).

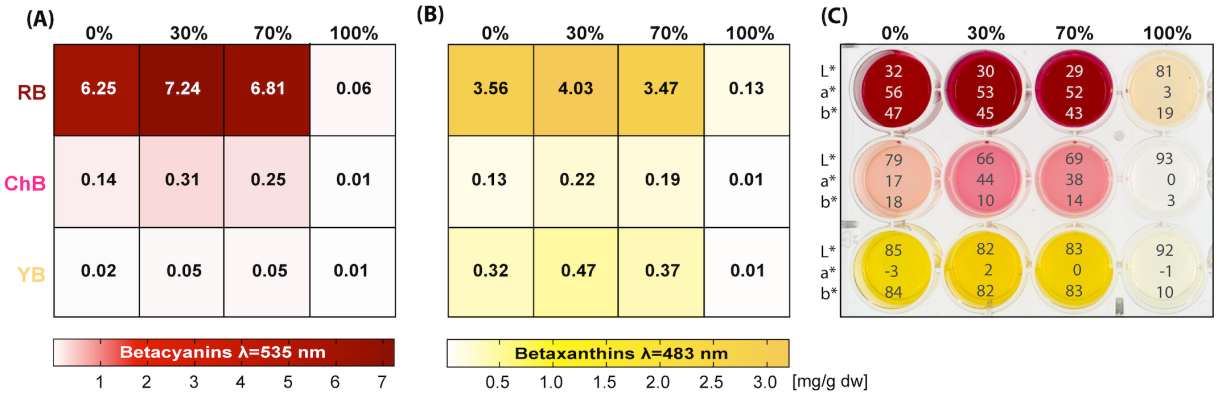

Fig. 1. The average content of (A) betacyanins and (B) betaxanthins in red beetroot (RB), Chioggia beetroot (ChB) and yellow beetroot (YB) extracts set with (C) photographs of these extracts. The solvents for beetroot extraction contained 1% formic acid and different amounts of ethanol (0, 30, 70 and 100%) in water. Betalain levels [mg/g dw] were determined using spectrophotometry in three parallel extracts. The standard deviation of the calculated values did not exceed 7%. The colour parameters (L^*, a^*, b^*) were determined from photographs of extracts with the use of a digital colorimeter.

3.2. Total antioxidant activity determination

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

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

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

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

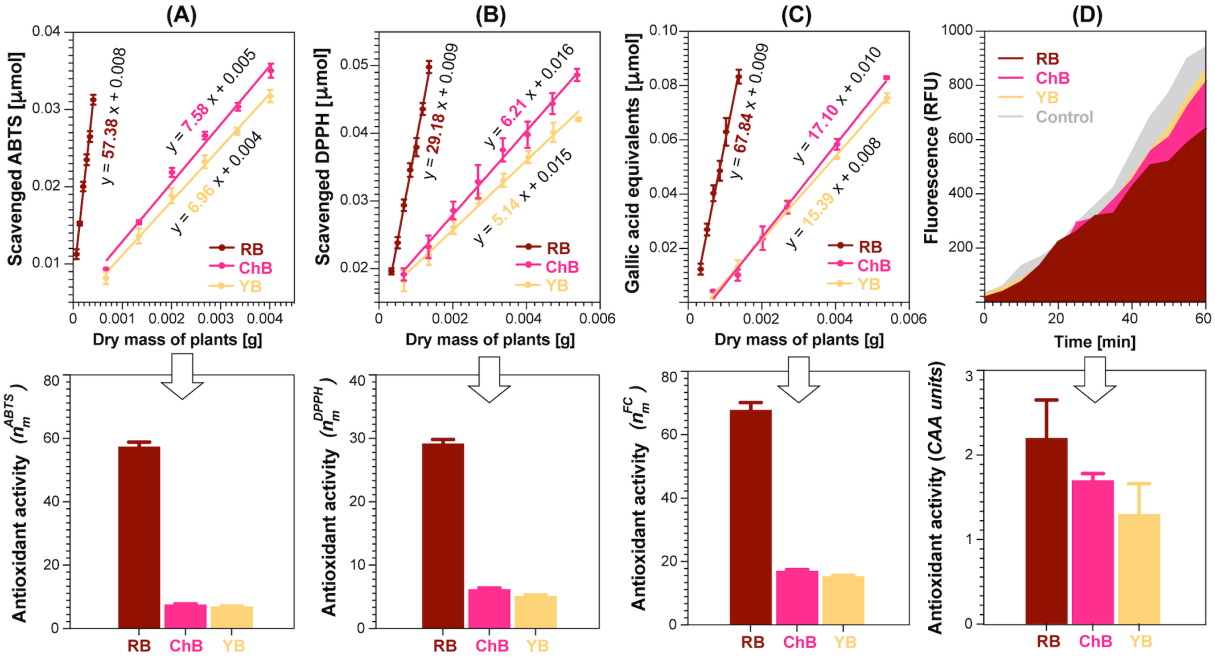

Fig. 2 Graphs showing the beetroot extract antioxidant activity determination method using tests with (A) ABTS radicals, (B) DPPH radicals, (C) Folin-Ciocalteu reagent, and (D) cellular antioxidant activity assay (upper panel). The bar graphs show total antioxidant activity expressed as coefficients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

3.3. Profiling betalains by HPTLC

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.

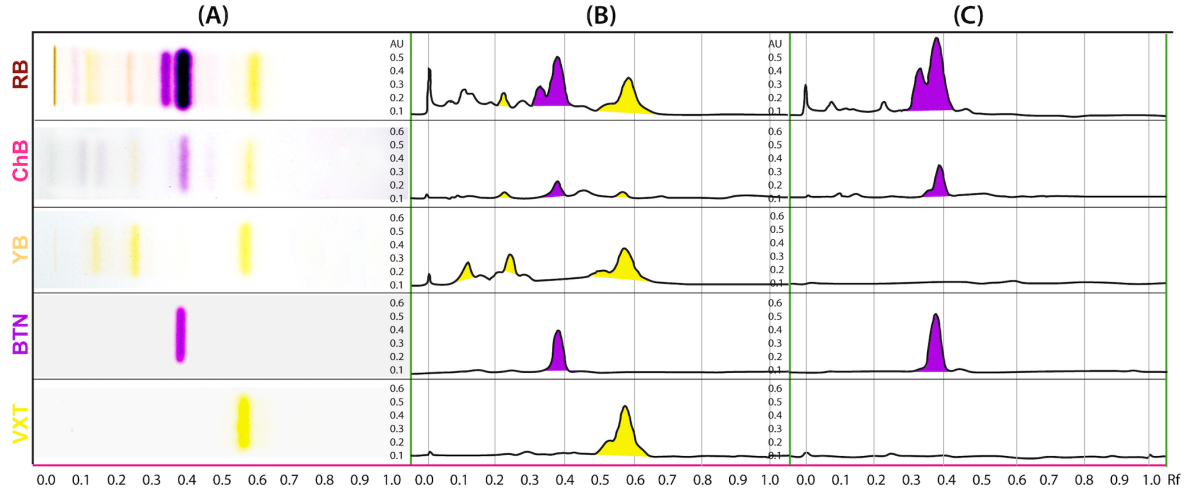

Fig. 3 HPTLC profiles registered under (A) white light and (B) densitograms at 480 nm and (C) 535 nm of extracts from red beetroot (RB), Chioggia beetroot (ChB) and yellow beetroot (YB) and two isolated standards: betanin (BTN) and vulgaxanthin I (VXT). For RB, the extract was diluted twice before dosing.

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 ($Rf = 0.58$) 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 ± 0.13 and 4.467 ± 0.047 mg/g dw, respectively. Lower levels of these pigments were found in ChB (0.2627 ± 0.0037 and 0.2097 ± 0.0039 mg/g dw,

464 respectively). For YB, only yellow betaxanthins were detected, with a total content of 0.378 ±
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.
471
472 *3.4. Profiling betalains and antioxidants by HPLC*
473
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 λ_{max} 536 nm,
484 were detected. Vulgaxanthin I with λ_{max} 471 nm was the dominant betaxantin present in all
485 beetroot extracts studied. A prominent peak of yellow-orange neobetanin (λ_{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 ± 0.13, 0.416 ± 0.014, and 0.212 ± 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 ± 0.11 and 0.275 ± 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%).

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

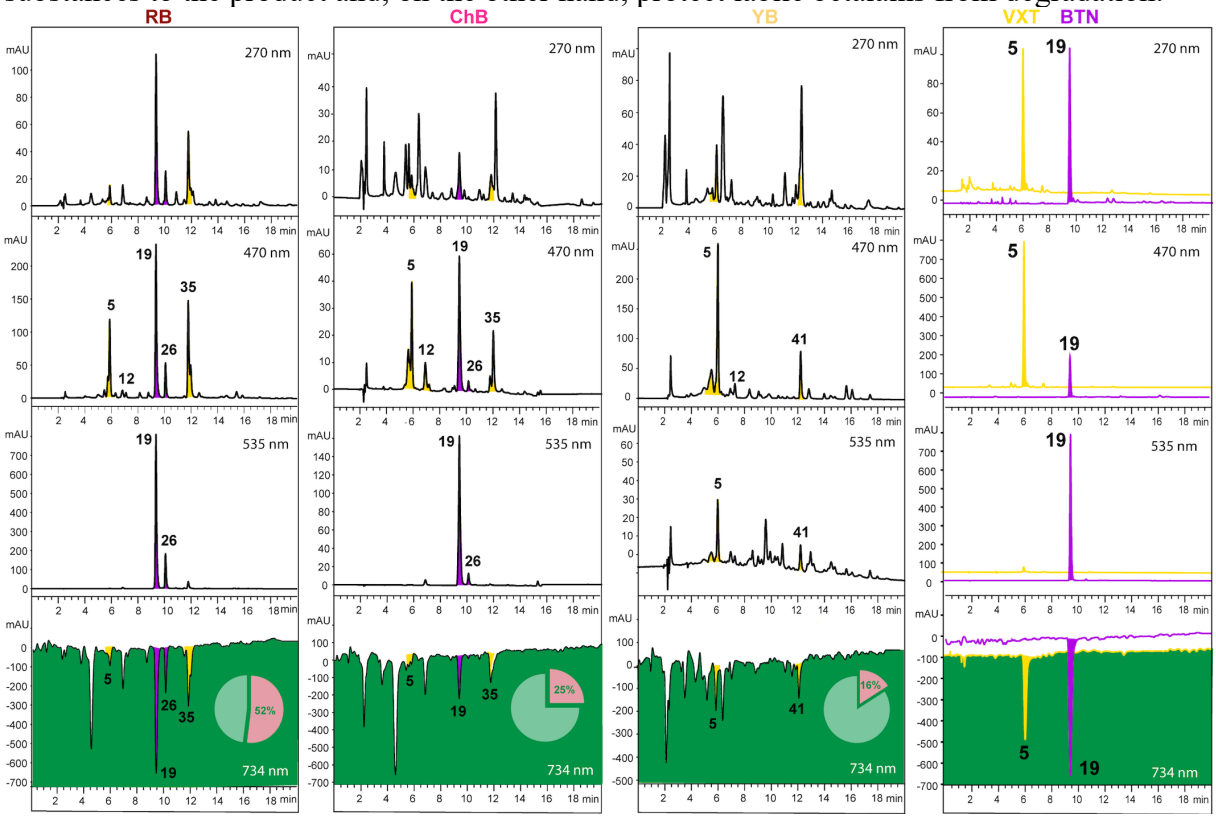

Fig. 4 HPLC profiles of extracts from red beetroot (RB), Chioggia beetroot (ChB) and yellow beetroot (YB) and two isolated standards, betanin (BTN) and vulgaxanthin I (VXT), registered before (270 nm, 470 nm, 535 nm) and after (734 nm) post-column derivatization with ABTS. In the antioxidant profiles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

3.5. Tentative phytochemical identification by LC-HRMS

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

Table 1

Phytochemicals tentatively identified by LC-Q-Orbitrap-HRMS in red beet (RB), Chioggia beet (ChB) and yellow beet (YB) extracts.

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)	$C_{15}H_{16}N_4O_6$	349.11425	349.11343	2.35	+	+	+	Kugler et al., 2007
2	2.17	Asparagine-betaxanthin (vulgaxanthin III)	$C_{13}H_{15}N_3O_7$	326.09828	326.09738	2.77	+	+	+	Kugler et al., 2007
3	2.21	Serine-betaxanthin	$C_{12}H_{14}N_2O_7$	299.08738	299.08658	2.67	+	+	+	Kugler et al., 2007
4	2.23	Arginine-betaxanthin	$C_{15}H_{21}N_5O_6$	368.15646	368.15536	2.97	+	+	+	Amaya-Cruz et al., 2019
5	2.23	Glutamine betaxanthin (vulgaxanthin I)	$C_{14}H_{17}N_3O_7$	340.11393	340.11307	2.53	+	+	+	Kugler et al., 2007
6	2.24	Etanolamine-betaxanthin	$C_{11}H_{14}N_2O_5$	255.09754	255.09689	2.56	+	-	+	Kugler et al., 2007
7	2.24	Isoglycine-betaxanthin (isoportulacaxanthin III)	$C_{11}H_{12}N_2O_6$	269.07682	269.07608	2.74	+	-	+	Nemzer et al., 2011
8	2.68	Hydroxybenzoic acid	$C_7H_6O_3$	139.03897	139.03874	1.65	-	+	+	Waldron et al.,1997
9	2.89	Aspartic acid-betaxanthine (miraxanthin II)	$C_{13}H_{14}N_2O_8$	327.08229	327.08151	2.38	+	-	+	Kugler et al., 2007
10	3.03	Glycine-betaxanthin (portulacaxanthin III)	$C_{11}H_{12}N_2O_6$	269.07682	269.07605	2.85	+	-	+	Kugler et al., 2007
11	3.51	Threonine-betaxanthin	$C_{13}H_{16}N_2O_7$	313.10302	313.10226	2.44	+	-	+	Kugler et al., 2007
12	3.66	Glutamic acid-betaxanthin (vulgaxanthin II)	$C_{14}H_{16}N_2O_8$	341.09795	341.09717	2.28	+	+	+	Kugler et al., 2007
13	4.79	Alanine-betaxanthin	$C_{12}H_{14}N_2O_6$	283.09247	283.09186	2.15	+	-	+	Kugler et al., 2007
14	4.83	17-Decarboxy-betanidyn	$C_{17}H_{17}N_2O_6^+$	345.10812	345.10733	2.28	+	+	+	Nemzer et al., 2011
15	5.09	17-Decarboxy-isobetanidyn	$C_{17}H_{17}N_2O_6^+$	345.10812	345.10733	2.28	+	+	+	Nemzer et al., 2011
16	5.29	γ-Aminobutric acid-betaxanthin	$C_{13}H_{16}N_2O_6$	297.10811	297.10757	1.81	+	+	+	Kugler et al., 2007
17	5.35	Vanillic acid	$C_8H_8O_4$	169.04954	169.04932	1.30	-	-	+	Waldron et al., 1997
18	5.42	15-Decarboxy-betanidin	$C_{17}H_{17}N_2O_6^+$	345.10812	345.10733	2.28	+	+	+	Nemzer et al., 2011
19	5.54	Betanin	$C_{24}H_{26}N_2O_{13}$	551.15077	551.14948	2.35	+	+	-	Nemzer et al., 2011
20	5.54	Betanidin	$C_{18}H_{16}N_2O_8$	389.09795	389.09729	1.69	+	-	+	Nemzer et al., 2011
21	5.54	17-Decarboxy neobetanin	$C_{23}H_{24}N_2O_{11}$	505.14529	505.1442	2.16	+	-	-	Nemzer et al., 2011
22	5.55	17-Decarboxy-betanin	$C_{23}H_{26}N_2O_{11}$	507.16094	507.16077	0.34	+	-	-	Nemzer et al., 2011
23	5.60	Prebetanin	$C_{24}H_{26}N_2O_{16}S$	631.10758	631.10583	2.77	+	+	-	Nemzer et al., 2011
24	5.95	Proline-betaxanthin (indicaxanthin)	$C_{14}H_{16}N_2O_6$	309.10811	309.10757	1.74	+	-	+	Kugler et al., 2007
25	6.08	Caffeic acid	$C_9H_8O_4$	181.04954	181.04921	1.82	-	+	-	Waldron et al., 1997
26	6.10	Isobetanin	$C_{24}H_{26}N_2O_{13}$	551.15077	551.14948	2.35	+	+	-	Nemzer et al., 2011
27	6.14	Isoprebetanin	$C_{24}H_{26}N_2O_{16}S$	631.10758	631.10638	1.90	+	-	-	Nemzer et al., 2011
28	6.36	Dopa-betaxanthin (dopaxanthin)	$C_{18}H_{18}N_2O_8$	391.11360	391.11267	2.37	-	-	+	Kugler et al., 2007
29	6.17	Syringic acid	$C_9H_{10}O_5$	199.06009	199.05978	1.58	+	-	+	Wang et al., 2020
30	6.61	2-Decarboxy-betanidin	$C_{17}H_{17}N_2O_6^+$	345.10812	345.10733	2.28	+	+	+	Nemzer et al., 2011
31	6.61	5-Caffeoyl quinic acid	$C_{16}H_{18}O_9$	355.10237	355.1015	2.44	+	-	-	Wang et al., 2019
32	6.68	17-Decarboxy-isobetanin	$C_{22}H_{26}N_2O_{11}$	507.16094	507.1601	1.66	+	-	-	Nemzer et al., 2011
33	6.89	2,17-Bidecarboxy-neobetanin	$C_{22}H_{24}N_2O_9$	461.15545	461.1546	1.85	+	-	-	Nemzer et al., 2011
34	7.06	2,17-Bidecarboxy-betanin/isobetanin	$C_{22}H_{27}N_2O_9^+$	463.17110	463.17007	2.23	+	-	-	Nemzer et al., 2011
35	7.32	Neobetanin	$C_{24}H_{24}N_2O_{13}$	549.13512	549.13416	1.75	+	+	-	Wang et al., 2020
36	7.42	2-Decarboxy neobetanin	$C_{23}H_{24}N_2O_{11}$	505.14529	505.1442	2.16	+	-	-	Nemzer et al., 2011
37	7.49	Betalamic acid	$C_9H_9NO_5$	212.05535	212.05516	0.91	+	+	-	Kujala et al., 2001
38	7.71	2-Decarboxy-isobetanidin	$C_{17}H_{17}N_2O_6^+$	345.10812	345.10757	1.59	-	-	+	Nemzer et al., 2011
39	7.73	trans-*p*-Coumaric acid	$C_9H_8O_3$	165.05463	165.05432	1.85	-	+	-	Waldron et al., 1997
40	7.77	Tyrosine-betaxanthin (portulacaxanthin II)	$C_{18}H_{18}N_2O_7$	375.11868	375.11789	2.11	+	+	+	Nemzer et al., 2011
41	7.79	Dopamine-betaxanthin (miraxanthin V)	$C_{17}H_{18}N_2O_6$	347.12377	347.12308	1.98	+	+	+	Nemzer et al., 2011
42	7.92	Ferulic/isoferulic acid isomer	$C_{10}H_{10}O_4$	195.06519	195.0649	1.49	+	+	+	Waldron et al., 1997
43	8.15	Tetrahydroxy-biindolyl	$C_{16}H_{12}N_2O_4$	297.08699	297.08643	1.88	-	+	-	Kujala et al., 2002
44	8.34	Methionine-betaxanthin	$C_{14}H_{18}N_2O_6S$	343.09584	343.09497	2.54	+	-	+	Kugler et al., 2007
45	8.38	3-Caffeoyl quinic acid	$C_{16}H_{18}O_9$	355.10237	355.1015	2.44	+	+	+	Wang et al., 2019
46	8.43	Valine-betaxanthin	$C_{14}H_{18}N_2O_6$	311.12377	311.12302	2.40	+	+	+	Nemzer et al., 2011
47	9.00	cis-*p*-Coumaric acid	$C_9H_8O_3$	165.05463	165.05432	1.85	+	+	-	Waldron et al., 1997
48	9.21	Tyramine-betaxanthin (miraxanthin III)	$C_{17}H_{18}N_2O_5$	331.12885	331.12796	2.70	+	-	+	Kugler et al., 2007
49	9.61	3-Metoxy-tyramine-betaxanthin	$C_{18}H_{20}N_2O_6$	361.13942	361.13840	2.82	+	-	+	Kugler et al., 2007
50	9.75	Ferulic/isoferulic acid isomer	$C_{10}H_{10}O_4$	195.06519	195.06485	1.74	+	+	+	Waldron et al., 1997
51	9.78	Feruloylglucose	$C_{16}H_{20}O_9$	357.11801	357.11700	2.81	+	+	+	Kujala et al., 2002
52	10.14	Leucin-isobetaxanthin (isovulgaxanthin IV)	$C_{15}H_{20}N_2O_6$	325.13942	325.13855	2.67	+	-	+	Nemzer et al., 2011
53	10.22	6'-*O*-Feruloyl-2'-*O*-glucosyl-betanin/isobetanin	$C_{40}H_{45}N_2O_{21}^+$	889.25093	889.24841	2.84	+	-	+	Nemzer et al., 2011
54	10.35	2-Decarboxy-2,3-dehydro-neobetanin	$C_{23}H_{22}N_2O_{11}$	503.12964	503.12839	2.49	+	-	-	Nemzer et al., 2011
55	10.70	Leucine-betaxanthin (vulgaxanthin IV)	$C_{15}H_{20}N_2O_6$	325.13942	325.13855	2.67	+	+	+	Kugler et al., 2007
56	11.05	Isoleucin-betaxanthin	$C_{15}H_{20}N_2O_6$	325.13942	325.13855	2.67	+	+	+	Nemzer et al., 2011
57	11.45	2-Decarboxy-betanin/isobetanin	$C_{23}H_{26}N_2O_{11}$	507.16094	507.15988	2.10	+	-	-	Nemzer et al., 2011
58	11.53	Phenylalanine-betaxanthin	$C_{18}H_{18}N_2O_6$	359.12376	359.12280	2.67	+	-	+	Kugler et al., 2007
59	11.46	Tetrahydroxy-biindolyl	$C_{16}H_{12}N_2O_4$	297.08698	297.08627	2.38	+	-	-	Kujala et al., 2002
60	11.80	6'-*O*-Feruloyl-betanin/isobetanin	$C_{34}H_{35}N_2O_{16}^+$	727.19811	727.19598	2.93	+	+	-	Nemzer et al., 2011
61	12.03	Tryptophan-betaxanthin	$C_{20}H_{19}N_3O_6$	398.13467	398.1337	2.43	+	+	+	Kugler et al., 2007
62	13.12	Ferulic/isoferulic acid isomer	$C_{10}H_{10}O_4$	195.06519	195.06485	1.74	+	+	+	Waldron et al., 1997
63	17.44	N-trans-Feruloyltyramine (Moupinamide)	$C_{18}H_{19}NO_4$	314.13869	314.13794	2.38	-	+	+	Kujala et al., 2002
64	17.91	N-trans-Feruloyl-3-metoxytyramine (N-trans-Feruloylhomovanillyalamine)	$C_{19}H_{21}NO_5$	344.14925	344.14841	2.45	+	+	+	Kujala et al., 2002

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

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

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

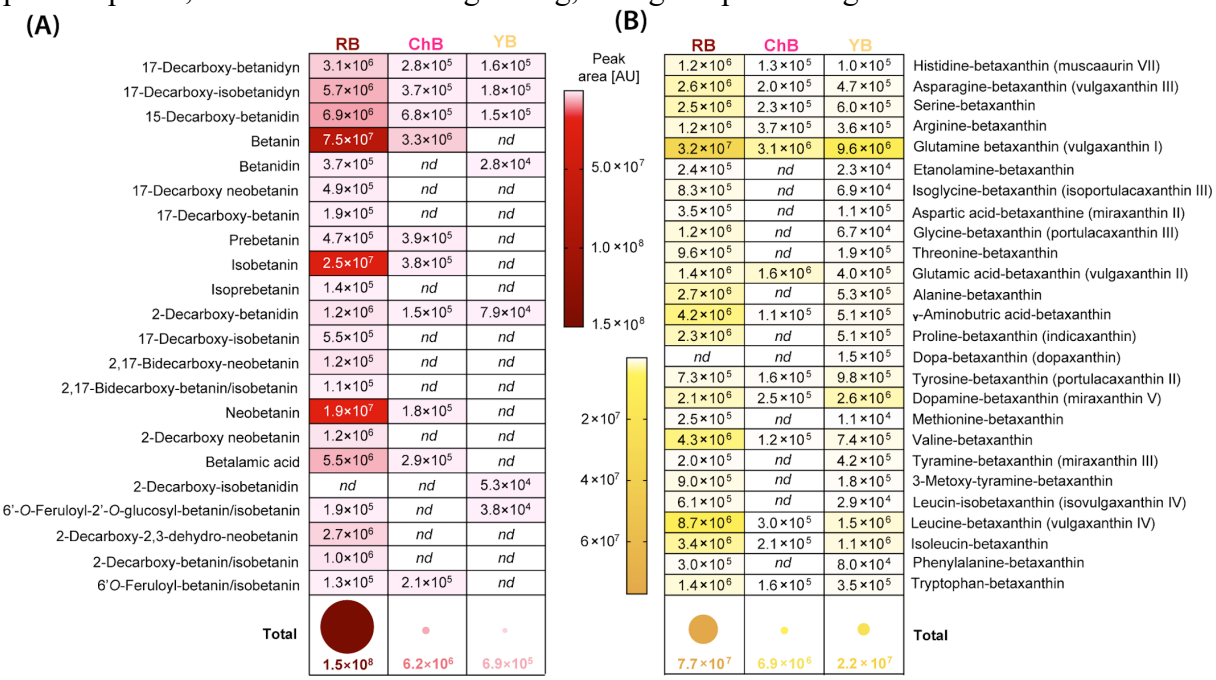

Fig. 5 Mean values of peak areas of (A) betacyanins and their derivatives and (B) betaxanthins, which were obtained from the extracted ion chromatograms registered during LC-Q-Orbitrap-HRMS analyses of red beetroot (RB), Chioggia beetroot (ChB) and yellow beetroot (YB) extracts.

4. Conclusion

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

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

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

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

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

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

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

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References

Amaya-Cruz, D. M., Pérez-Ramírez, I. F., Delgado-García, J., Mondragón-Jacobo, C., Dector-Espinoza, A., & Reynoso-Camacho, R. (2019). An integral profile of bioactive compounds and functional properties of prickly pear (*Opuntia ficus indica* L.) peel with different tonalities. *Food Chemistry, 278*, 568–578.

Azeredo, H. M. C. (2009). Betalains: Properties, sources, applications and stability – A review. *International Journal of Science & Technology, 44*, 2365–2376.

Baranowska, M., Suliborska, K., Chrzanowski, W., Kusznierewicz, B., Namieśnik, J., & Bartoszek, A. (2018). The relationship between standard reduction potentials of catechins and biological activities involved in redox control. *Redox Biology, 17*, 355–366. https://doi.org/10.1016/j.redox.2018.05.005.

Bartoszek, A., Kusznierewicz, B., & Namieśnik, J. (2014). HPLC-coupled post-column derivatization aims at characterization and monitoring of plant phytocomplexes, not at assessing their biological properties, *Journal of Food Composition and Analysis, 33*, 220–223. https://doi.org/10.1016/j.jfca.2013.08.001.

Bilyk, A. (1981). Thin-layer chromatographic separation of beet pigments. *Journal of Food Sciences, 46*, 298–299. https://doi.org/10.1111/j.1365-2621.1981.tb14588.x.

Brockington, S. F., Walker, R. H., Glover, B. J., Soltis, P. S., & Soltis, D.E. (2011). Complex pigment evolution in the Caryophyllales. *New Phytologist, 190*, 854–864. https://doi.org/10.1111/j.1469-8137.2011.03687.x.

Cai, Y-Z., Sun, M., & Corke, H. (2005). Characterization and application of betalain pigments from plants of the Amaranthaceae, *Trends in Food Science & Technology*, *16*, 370–376. https://doi.org/10.1016/j.tifs.2005.03.020.

682 Castro-Enríquez, D. D., Montaño-Leyva, B., Del Toro-Sánchez, C. L., Juaréz-Onofre, J. E.,
683 Carvajal-Millan, E., Burruel-Ibarra, S. E., Tapia-Hernández, J. A., Barreras-Urbina, C. G., &
684 Rodríguez-Félix, F. (2020). Stabilization of betalains by encapsulation—a review. *Journal of*
685 *Food Science and Technology, 57*, 1587–1600. https://doi.org/10.1007/s13197-019-04120-x.
686 Chauhan, S. P., Sheth, N. R., Rathod, I. S., Suhagia, B. N., & Maradia, R. B. (2013). Analysis
687 of betalains from fruits of Opuntia species. *Phytochemistry Review*, 12, 35–45.
688 https://doi.org/10.1007/s11101-012-9248-2.
689 Fu, Y., Shi, J., Xie, S.-Y., Zhang, T.-Y., Soladoye, O. P., & Aluko, R. E. (2020). Red beetroot
690 betalains: perspectives on extraction, processing, and potential health benefits. *Journal of*
691 *Agricultural and Food Chemistry, 68*, 11595−11611.
692 https://doi.org/10.1021/acs.jafc.0c04241.
693 Koss-Mikołajczyk, I., Kusznierewicz, B., & Bartoszek, A. (2019). The relationship between
694 phytochemical composition and biological activities of differently pigmented varieties of
695 berry fruits; comparison between embedded in food matrix and isolated anthocyanins. *Foods,*
696 *25*, 1−14. https://doi.org/10.3390/foods8120646.
697 Koss-Mikołajczyk, I., Kusznierewicz, B., Wiczkowski, W., Sawicki, T., & Bartoszek, A.
698 (2019). The comparison of betalain composition and chosen biological activities for
699 differently pigmented prickly pear (*Opuntia ficus-indica*) and beetroot (*Beta vulgaris*)
700 varieties. *International Journal of Food Sciences and Nutrition, 70*, 442−452.
701 https://doi.org/10.1080/09637486.2018.1529148.
702 Kugler, F., Graneis, S., Stintzing, F. C., & Carle, R. (2007). Studies on betaxanthin profiles of
703 vegetables and fruits from the Chenopodiaceae and Cactaceae, *Zeitschrift fur Naturforschung*
704 *C, 62c*, 311−318. https://doi.org./10.1515/znc-2007-5-601.
705 Kujala, T., Loponen, J., & Pihlaja, K. (2001). Betalains and phenolics in red beetroot (*Beta*
706 *vulgaris*) peel extracts: extraction and characterization. *Verlag der Zeitschrift für*
707 *Naturforschung, 56 c*, 343–348. https://doi.org/10.1515/znc-2001-5-604.
708 Kujala, T. S., Vienola, M. S., Klika, K. D., Loponen, J. M., & Pihlaja, K. (2002) Betalain and
709 phenolic compositions of four beetroot (*Beta vulgaris*) cultivars. *European Food Research*
710 *and Technology, 214*, 505–510. https://doi.org/10.1007/s00217-001-0478-6.
711 Kusznierewicz, B., Piasek, A., Bartoszek, A., & Namiesnik, J. (2011a). Application of a
712 commercially available derivatization instrument and commonly used reagents to HPLC on-
713 line determination of antioxidants. *Journal of Food Composition and Analysis, 24*, 1073–
714 1080. https://doi.org/10.1016/j.jfca.2011.01.010.
715 Kusznierewicz, B., Piasek, A, Bartoszek, A., & Namiesnik, J. (2011b). The optimisation of
716 analytical parameters for routine profiling of antioxidants in complex mixtures by HPLC
717 coupled post-column derivatisation. *Phytochemical Analysis, 22*, 392–402.
718 https://doi.org/10.1002/pca.1294.
719 Nemzer, B., Pietrzkowski, Z., Spórna, A., Stalica, P., Thresher, W., Michałowski, T., &
720 Wybraniec, S. (2011). Betalainic and nutritional profiles of pigment-enriched red beet root
721 (*Beta vulgaris* L.) dried extracts. *Food Chemistry, 127*, 42–53.
722 https://doi.org/10.1016/j.foodchem.2010.12.081.
723 Polturak, G., & Aharoni, A. (2018). "La Vie en Rose": Biosynthesis, sources, and
724 applications of betalain pigments. *Molecular Plant, 11*, 7−22.
725 https://doi.org/10.1016/j.molp.2017.10.008.
726 Prieto-Santiago, V., Cavia, M. M., Alonso-Torre, S. R., & Carrillo, C. (2020). Relationship
727 between color and betalain content in different thermally treated beetroot products. *Journal of*
728 *Food Science and Technology, 57*, 3305–3313. https://doi.org/10.1007/s13197-020-04363-z.
729 Rahimi, P., Abedimanesh, S., Mesbah-Namina, S. A., & Ostadrahimi, A. (2019). Betalains,
730 the nature-inspired pigments, in health and diseases. *Critical Reviews in Food Science and*
731 *Nutrition, 59*, 2949–2978. https://doi.org/10.1080/10408398.2018.1479830.

Rodriguez, E. B., Vidallon, M. L. P., Mendoza, D. J. R., & Reyes, T. R. (2016). Health-promoting bioactivities of betalains from red dragon fruit (Hylocereus polyrhizus (Weber) Britton and Rose) peels as affected by carbohydrate encapsulation. *Journal of the Science of Food and Agriculture, 96*, 4679–4689. https://doi.org/10.1002/jsfa.7681.

Sandate-Flores, L., Rodríguez-Rodríguez, J., Calvo-Segura, S., Mayorga-Martínez, A., Parra-Saldívar, R., & Chuck-Hernández, C. (2016). Evaluation of different methods for betanin quantification in pitaya (*Stenocereus* spp.), Agro FOOD Industry Hi Tech, 27, 20-24.

Sawicki, T., Bączek, N., & Wiczkowski, W. (2016). Betalain profile, content and antioxidant capacity of red beetroot dependent on the genotype and root part. *Journal of Functional Foods, 27*, 249–261. https://doi.org/10.1016/j.jff.2016.09.004.

Schwartz, S. J., Hildenbrand, B. E., & von Elbe J. H. (1981). Comparison of spectrophotometric and HPLC methods to quantify betacyanins. *Journal of Food Science*, *46,* 296-297. https://doi.org/10.1111/j.1365-2621.1981.tb14587.x.

Slatnar, A., Stampar, F. Veberic, R., & Jakopic, J. (2015). HPLC-MS^n identification of betalain profile of different beetroot (*Beta vulgaris* L. *ssp*. *vulgaris*) parts and cultivars. *Journal of Food Science, 80*, C1952–C1958. https://doi.org/10.1111/1750-3841.12977.

Slimen, I. B., Najar,T., & Abderrabba, M. (2017). Chemical and antioxidant properties of betalains. Journal of Agricultural and Food Chemistry. 65, 675−689. https://doi.org/10.1021/acs.jafc.6b04208.

Stintzing, F. C., Schieber, A., & Carle, R. (2003). Evaluation of colour properties and chemical quality parameters of cactus juices. *European Food Research and Technology, 216*, 303–311. https://doi.org/10.1007/s00217-002-0657-0.

Stintzing, F. C., & Carle, R. (2007) Betalains – emerging prospects for food scientists. *Trends in Food Science & Technology,*18, 514–525. https://doi.org/10.1016/j.tifs.2007.04.012.

Sunnadeniya, R., Bean, A., Brown, M., Akhavan, N., Hatlestad, G., Gonzalez, A., Symonds, V. V., Lloyd, A. (2016). Tyrosine hydroxylation in betalain pigment biosynthesis is performed by cytochrome P450 enzymes in beets (*Beta vulgaris*). *PLoS ONE, 11*(2): e0149417. https://doi.org/10.1371/journal.pone.0149417.

Viloria-Matos, A., Moreno-Alvarez, M. J. & Hidalgo-Báez, D. (2001). Isolation and identification of betacyanin from fruits of *Opuntia boldinghii* Br. et R. by HPTLC. *Ciencia y Tecnologia Alimentaria, 3*, 140–143. https://doi.org/10.1080/11358120109487720

Waldron, K. W., Ng, A., Parker, M. L., & Parr, A. J. (1997). Ferulic acid dehydrodimers in the cell walls of *Beta vulgaris* and their possible role in texture. *Journal of the Sciences of Food and Agriculture, 74*, 221–228. https://doi.org/10.1002/(SICI)1097-0010(199706)74:2<221::AID-JSFA792>3.0.CO;2-Q

Wang, J., Jayaprakasha, G. K., & Patil, B.S. (2019). Chemometric characterization of 30 commercial thermal and cold processed juices using UPLC-QTOF-HR-MS fingerprints. *LWT - Food Science and Technology, 114*, 108322. https://doi.org/10.1016/j.lwt.2019.108322.

Wang, J., Jayaprakasha, G. K., & Patil, B. S. (2020). UPLC-QTOF-MS fingerprinting combined with chemometrics to assess the solvent extraction efficiency, phytochemical variation, and antioxidant activities of *Beta vulgaris* L. *Journal of Food and Drug Analysis, 28*, 23e36. https://doi.org/10.38212/2224-6614.1056

Wolfe, K. L., & Liu, R. H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry, 55*, 8896–8907. https://doi.org/10.1021/jf0715166.