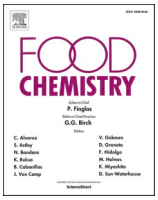
Food Chemistry 385 (2022) 132632

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Novel approach for purification of major betalains using flash chromatography and comparison of radical scavenging and antioxidant activities

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ARTICLE INFO

Keywords:
Betalains
Purification
Flash chromatography
HPLC
Antioxidant and radical scavenging activity
Chemical compounds studied in this article:
Betanin (PubChem CID: 12300103)
Neobetanin (PubChem CID: 102401026)
Vulgaxanthin I (PubChem CID: 135438599)
Indicaxanthin (PubChem CID: 6096870)

ABSTRACT

The present study focused on the development of a new purification protocol suitable for betanin and other major betalains, vulgaxanthin I, indicaxanthin and neobetanin, using flash chromatography which is a convenient and fast method to isolate unstable materials. Following preliminary tests, a gradient procedure using 0–60% acetonitrile, with 0.1% (v/v) formic acid as mobile phase, was selected for the purification. Different fractions were collected based on UV detection at 254 and 280 nm and purities were confirmed by reverse-phase HPLC analysis to be 97%, 95%, 79% and 52% for betanin, indicaxanthin, vulgaxanthin I, and neobetanin, respectively, with pigment yields ranging from 120 to 487 mg per 100 g of powdered raw material. Comparative assessment of antioxidant and radial scavenging properties of individual betalains indicated highest potential for betanin followed by neobetanin, vulgaxanthin I and indicaxanthin.

1. Introduction

Betalains are a group of natural pigments responsible for red, pink, and yellow-orange colours of plants belonging to the families of *Amaranthaceae* and *Cactaceae*. According to their chemical structure, two types of betalains can be distinguished: betacyanins such as betanin and isobetanin, and betaxanthins such as vulgaxanthin I and indicaxanthin. Betalains are present in abundance particularly in red beetroot, prickly pear and dragon fruit; however, total betalain content and betalain composition of any given plant species is distinctive and depends on different factors such as cultivar, plant part, ripening stage, salinity and farming practices (Celli & Brooks, 2017). Betalains have gained increasing attention in recent years not only due to their high tinctorial strength when used as food colourants but also due to their promising bioactive properties. It has been demonstrated that betalain extracts from different plant sources have stronger antioxidant and radical scavenging potential comparable to the typical antioxidants such as ascorbic acid, rutin, catechin and tocopherol (Slimen, Najar, & Abderrabba, 2017).

Recent studies on antioxidant activity suggested that betalains should be considered as a new category of dietary antioxidants, although there is inconsistency on whether betacyanins or betaxanthins are more potent. Gomphrenin-type betacyanins from *Amaranthacea* have been shown to reduce DPPH radical formation by 74%, whereas betaxanthins showed around 50% inhibition (Cai, Sun, & Corke, 2003). In contrast, the lipoperoxyl radical scavenging potential of betaxanthin-rich fractions were higher compared to betacyanin-rich fractions (Zakharova & Petrova, 1998). Most of the bioactivity studies have been conducted with crude plant extracts with little to no pigment purification (Čanadanović-Brunet et al., 2011; Georgiev et al., 2010; Vulić et al., 2014). Crude plant extracts contain not only betalains but also other bioactive compounds such as polyphenols, organic acids, and therefore, it is difficult to clearly allocate bioactivities to individual compounds. In addition to major betalain pigments, Mikołajczyk-Bator and Czapski (2017) identified neobetanin, a degradation product of betanin, as a strong antioxidant and established that increasing concentrations of

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https://doi.org/10.1016/j.foodchem.2022.132632
Received 19 July 2021; Received in revised form 2 March 2022; Accepted 3 March 2022
Available online 5 March 2022

neobetanin significantly increased antioxidant activity of beetroot juice. Despite the emerging evidence on betalain bioactivities, studies comparing the radical scavenging activities of individual betalains are scarce (Butera et al., 2002; Cai et al., 2003). Studying purified betalains is highly important in order to understand their individual potential and the detailed mechanisms by which they act on biological processes, and hence allow development of betalain-based novel applications.

Purification of individual betalains has been reported using different techniques, such as column chromatography and preparative HPLC (Kusznierewicz, Mróz, Koss-Mikołajczyk, & Namieśnik, 2021). The common challenges identified in the purification of phytochemicals including betalains, are the co-extraction of compounds bearing the same polarity, low purification yield, time consuming procedures and impacts on the stability of the pure compounds (Aznar & Rai, 2020). Gonçalves et al. (2012) compared seven different techniques previously used for betalain purification by other researchers (Butera et al., 2002; Stintzing, Schieber, & Carle, 2002) and concluded, based on yield and purity, that reversed-phase HPLC and ion exchange chromatography are the best methods to purify betanin from red beetroot. The purification yields using above methods were 14.7 ± 2.7 and 16.2 ± 2.9 mg per 100 g of raw material (red beetroot powder), respectively, with around 99% purity achieved with both methods. Butera et al. (2002) purified betanin and indicaxanthin from prickly pear using a gel filtration based method and showed purification yields of 5.12 ± 0.51 and 8.42 ± 0.51 mg per 100 g edible pulp, respectively. Similar to the above studies on betalain extraction, low yields of polyphenols were obtained using the column chromatography and preparative HPLC method. Minh et al. (2019) was able to purify 4.58 mg/g of gallic acid and methyl gallate mixture as well as 0.6 mg/g of fraxetin using dried methanol extract from stem bark of *Jatropha podagrica* by normal phase column chromatography method. Liu et al. (2020) purified 12 different polyphenols from 200 g of dried roots of *Heshouwu* (*Polygonum multiflorum* Thunb) using a combination of high-speed countercurrent chromatography and preparative HPLC which resulted in purification yields ranging from 4.1 to 20.2 mg. Although these methods demonstrated high compound purity, they are time-consuming, result in low purification yields, and require large quantities of raw materials and solvents.

Flash chromatography is a simple and robust column chromatography based technique that is heavily used in the pharmaceutical industry for drug discovery, peptide and antibiotic purification (Sandesh, Mudavath, Swapana, Balaiah, & Sharma, 2021). Compared to other chromatographic methods, flash chromatography uses relatively high flow rates with low pressure either by normal or reverse phase separation and high sample loading which contributes to good separation in a short time (Weber, Hamburger, Schafroth, & Potterat, 2011). Further, flash chromatography is easily applicable to scale up to meet industrial applications (Bickler, 2018). Whilst this method has been more frequently applied to purification of synthetic compounds, there is an increasing interest in utilizing flash chromatography as part of natural compound purification approaches. For example, flash chromatography was used as part of the fractionation workflow to purify four main bioactive compounds (two proanthocyanidins, *p*-hydroxybenzoic, and hyperoside) from extracts of *A. columbrina* leaves (Rodrigo Cavalcante de Araújo et al., 2019). Further, recent literature demonstrated the purification of quercetin and other individual compounds from *Chenopodium album* (Arora & Itankar, 2018), as well as rosmarinic acid in *Origanum majorana* (Hossain, Camphuis, Aguiló-Aguayo, Gangopadhyay, & Rai, 2014). Therefore, flash chromatography is considered a comparatively effective technique to achieve high yields using low solvent and raw material input. Detection and characterization of isolated compounds poses a challenge, however, high resolution mass spectrometry (HRMS) has been highlighted as a powerful technique that can be used to identify and confirm identity of natural compounds without the need for authentic standards. Moreover, HRMS is widely used due to its key features such as fast scanning speed, wide mass range, high sensitivity, high-quality resolution and accurate molecular weight (Alvarez-Rivera, Ballesteros-Vivas, Parada-Alfonso, Ibañez, & Cifuentes, 2019).

The present study aimed to develop a flash column chromatography-based protocol for the purification of betanin from red beetroot which is not currently available, and to apply the method to isolate other major pigments, vulgaxanthin I and indicaxanthin, from yellow beetroot and prickly pear sources, respectively. Furthermore, we aimed for the isolation of neobetanin, a major betanin degradation product that frequently evolves during storage, which has not been attempted earlier. Ultimately, the comparative assessment of the four purified betalains with regards to their antioxidant and radical scavenging activities is targeted to provide more detailed information on the potential of individual betalains.

2. Materials and methods

2.1. Chemicals and materials

Red and yellow beetroot (*Beta vulgaris*, subsp. *vulgaris*, Conditiva group), originating from UK and US sources, respectively, were kindly provided by Biopowder Ltd., Milton Keynes, UK in dried and powdered form (moisture content < 2% (w/w)). Yellow prickly pear (*Opuntia ficus-indica*) samples (freeze dried) were sourced from Spain (Miguel Hernandez University of Elche, Alicante, Spain). Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl hydrate, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium peroxodisulfate, sodium acetate-trihydrate, iron (III)-chloride-hexahydrate, 2,4,6-tri(2-pyridyl)-s-triazine, 1,10-phenanthroline, ferrous sulfate heptahydrate, 30% hydrogen peroxide, sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate dehydrate were purchased from Sigma-Aldrich (Dorset, UK). All other chemicals and solvents were purchased from Fisher Scientific (Loughborough, UK). All aqueous solutions were prepared using deionized water purified by Milli-Q water purification system (Millipore, MA, USA).

2.2. Sample preparation

Extraction of betalains was carried out using ultrasound-assisted extraction as recently described (Fernando et al., 2021). This method uses 30% (v/v) ethanol instead of pure water to extract betalains since pure water can cause severe difficulties during the solute separation by filtration stage, due to the co-extraction of mucilaginous compounds such as pectin (Fernando et al., 2021). Moreover, the removal of water is more challenging than alcohol when concentrating the samples and pure water could lead to degradation of pigments in the extract.

Briefly, beetroot and prickly pear powder samples (10 g) were mixed with 250 mL of 30% (v/v) ethanol. The mixture was vortexed for 2 min, then placed in an ultrasonic bath (XUBA3, Grant Instruments, UK) and sonicated at 44 kHz for 30 min at 30 °C. The mixture was subsequently centrifuged for 10 min at 3500 × g and the supernatant decanted. The residue was re-extracted with the same amount of solvent for maximum recovery of pigments. The extracts were filtered using 0.22 μm pore membrane filter and narrowed down to 20% of initial volume using Genevac centrifugal evaporator (SP industries Inc., Warminster, Pennsylvania). Extracts were stored at −20 °C until further use. Initial betalain content of the crude extracts was determined using spectrophotometry (Specord 210 plus, Analytik Jena, Germany) and calculated using extinction coefficient values for 60,000 $cm^{-1}M^{-1}$ at λ_{max} 540 nm and 48,000 $cm^{-1}M^{-1}$ at λ_{max} 480 nm for betacyanin and betaxanthin, respectively. The total amount of betalain (in mg per g sample) was calculated by combining the values for betacyanin and betaxanthin.

2.3. Purification of betalains

2.3.1. Preparative high performance liquid chromatography (Reversed-phase)

Initially, the purification of betanin was performed using the Agilent 1260 preparative HPLC system (Santa Clara, United States) with a reverse-phase Kinetex® 5 μm C18 100 Å, AXIA packed LC Column 100 × 21.2 mm (Part number: OOD-4601-PO-AX) following a method described by Gonçalves et al. (2012) with modifications. The program consisted of 40 min gradient elution with 0.1% (v/v) acetic acid in water (solvent A) and 0.1% acetic acid in 60% acetonitrile (solvent B) with a flow rate of 5 mL/min. A sample volume of 5 mL (concentrated red beetroot extract), which is the maximum volume with this equipment, was used per run. The chromatographic conditions were defined as follows: gradient elution mode with 2–20% B for 30 min, 20–100% B for 5 min, and 100–5% B for the last 5 min. Elution monitoring was performed at 280 nm. Eluted fractions were stored separately at −20 °C until used for identification and lyophilization. The fractions were identified as betanin, betaxanthin and a mixture of degraded products of betanin (neobetanin, 15-decarboxy-betanin, 2-decarboxyisobetanin, and 2-decarboxyneobetanin), based on the HPLC/MS analysis. Further, UV–Vis monitoring of three main fractions showed absorbance maxima (λ_{max}) at 530 nm, 479 nm, and 478 nm (data not shown) which is characteristic to betacyanins, betaxanthins and degraded products of betanin, respectively.

2.3.2. Flash chromatography

Thin layer chromatography was used to pre-determine the separation of betalains in two different solvent systems. Briefly, red beetroot extract was run in acetonitrile (0 – 100 % v/v) with 0.1% formic acid or with acetic acid on TLC Silica gel 60 F_{254} aluminium plates (Merck, Darmstadt, Germany). The retention factor of the compounds was calculated by dividing travel distance by the solvent front. The solvent system containing 60% acetonitrile with 0.1% formic acid showed the best separation (Fig. S1), and was therefore used with flash chromatography to purify the betalains.

Concentrated extracts of red beetroot, yellow beetroot, and yellow prickly pear were submitted to a reversed-phase flash chromatography procedure using a KP-C18-HS Biotage SNAP cartridge that was mounted on a fully automated Biotage Isorela system (Isorela One, Biotage, Sweden). This reverse-phased cartridge was suitable for separation of polar compounds and the cartridge was packed with silica, amorphous (irregular). Average particle size was 50 μm with 100 Å pore diameter and 500 m^2/g surface area with good resolution. For the purification, 15 mL of concentrated extract was loaded onto the cartridge. The elution process was performed at a flow rate of 50 mL/min. The chromatographic procedure was run using a linear-gradient solvent system with mobile phase A with water and 0.1% formic acid, and mobile phase B with acetonitrile and 0.1% formic acid. The elution started with 1 column volume (CV) of 0% (v/v) of solvent B for equilibrating the cartridge and then eluted with 10 CV of the solvent B starting from 0% to 60% (v/v). UV absorption was monitored at 254 nm and 280 nm wavelength. Eluted fractions (20 mL) were stored separately at −20 °C until further use. Flash purification process was repeated a minimum of three times per compound.

The flash chromatograms of each purified compound using crude extracts as input material are shown in Fig. S2. For example, a total of 19 fractions were collected using red beetroot extract while 22 fractions were collected using yellow beetroot extract (Tables S1–S4). All the fractions were analysed by HPLC/MS, described under section 2.4, and the fractions bearing the same mass and spectral characteristics were combined together. The combined fractions were then evaporated using a Genevac centrifugal evaporator to remove the solvent and freeze-dried.

2.4. Identification of purified betalains

Identity and purity of the target compounds in collected fractions were determined with HPLC/MS and accurate mass methods.

HPLC/MS method described in Fernando et al. (2021) was used to identify the purified fractions. The HPLC (LC-2010 HT) coupled with a 2020 quadrupole mass spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a 4.6 mm × 250 mm, 5 μm Phenomenex Gemini C18 column was used for the analysis. Run conditions were as follows: gradient elution mode started with 5–25% B for 15 min, 25–70% B for 4 min and 70–5% for the last 7.10 min. The injection volume was 10 μL and the flow rate of 0.95 mL/min was used. Betacyanins and betaxanthins were monitored at 536 nm and 486 nm, respectively. HPLC elution profiles of the purified fractions were identified using *m/z* values and retention times from the literature (Hayet et al., 2014).

The Maxis Impact HD Mass Spectrometer (Bruker MaXis impact, Bremen, Germany) was used to confirm the results of analytical HPLC/MS characterization in purified fractions by determining the accurate molecular formula of the compounds. Samples were submitted to electrospray ionization (ESI) mass spectroscopy and a positive ion ESI mass spectrum was recorded.

Based on HPLC/MS identification, fractions of major betalains from different flash chromatography runs were pooled (betanin, neobetanin, vulgaxanthin I and indicaxanthin). Acetonitrile was evaporated from the samples using Genevac centrifugal evaporator followed by lyophilization (Labconco FreeZone 2.5, Labconco cooperation, Kansas City, United States). Freeze dried samples were stored at −20 °C until further analysis.

2.5. Assessment of purity of the purified betalains

2.5.1. Sugar analysis of purified fractions

Presence of sugars in purified fractions was determined using $UFLC_{XR}$ system (Shimadzu, Kyoto, Japan) with evaporative light scattering detector (ELSD). The samples were separated on a Grace Davison Prevail Carbohydrate Es column (5 μm, 250 mm × 4.6 mm) using isocratic conditions. The mobile phase was 75% acetonitrile (v/v) and the flow rate was 0.5 mL/min with a sample volume of 10 μL. The operating parameters for ELSD were signal, 0.00, gain 4 and pressure 350 kPa with the 40 °C drift tube temperature. Peak identification and quantification were carried out based on the comparison of retention times with external standards of sucrose, glucose and fructose ranging between 100 and 3000 μg/mL.

2.5.2. Polyphenol analysis of purified fractions

The presence of polyphenols in purified fractions was tested using HPLC/MS method described by Ifie, Marshall, Ho and Williamson (2016). The same HPLC/MS instrument described in 2.4 section was used for the analysis. The mobile phase consisted of 0.5% (v/v) formic acid in water (solvent A) and a mixture of acetonitrile, water, and formic acid (50:49.5:0.5, v/v) (solvent B), with a flow rate of 0.5 mL/min. The gradient elution started with 8% B and was increased to 18% B at 5.32 min, 32% B at 27.36 min, 60% B at 42.56 min reaching 100% B at 49.04 min, held at 100% B for 6.08 min and returning to initial conditions for 4.52 min, with total run time of 60.04 min. Presence of polyphenols was checked using DAD data and *m/z* values taken from the literature (Hayet et al., 2014).

2.6. Antioxidant activity assays

Purified betalains were subjected to antioxidant and radical scavenging assays using Trolox equivalent antioxidant capacity (TEAC), Ferric reducing antioxidant power (FRAP), 2,2,-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and hydroxyl radical scavenging activity assays. Stock solutions (5 mg/mL) of each purified compound were prepared in 30% ethanol and further diluted according to the assay

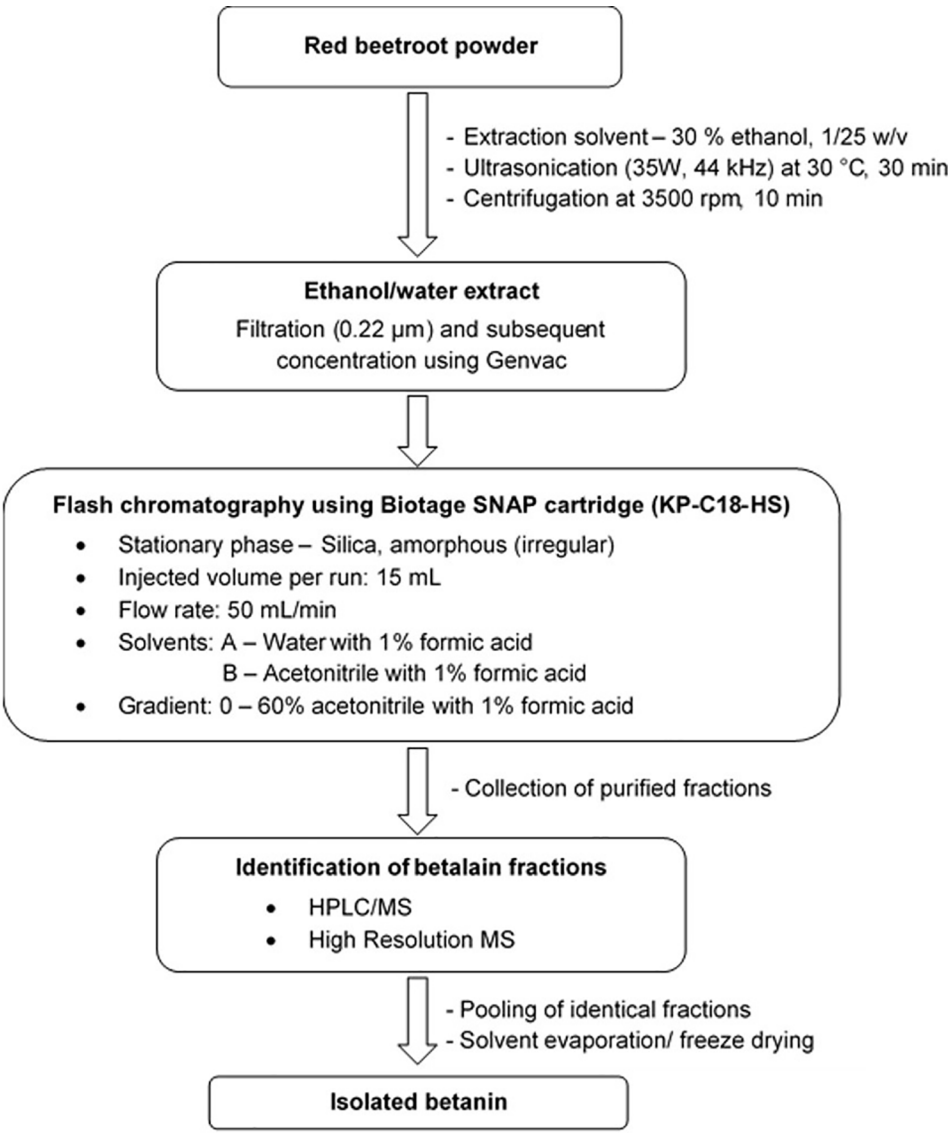

Fig. 1. Workflow of extraction, purification, and identification of betanin using flash chromatography method.

requirement (between 0 and 100 µg/mL concentrations).

The methods described in Han, Hernández Álvarez, Maycock, Murray and Boesch (2021) were used to perform TEAC and FRAP assays. Briefly, $ABTS^{\cdot+}$ was prepared by reacting ABST stock solution with potassium peroxodisulfate. ABTS radical scavenging activity was measured adding 300 µL of diluted ABTS radical solution into 10 µL of sample. Absorbance values were recorded after 6 min incubation at 734 nm. For the FRAP assay, $FeCl_3$ solution in sodium acetate buffer was prepared adding 2, 4, 6-tris (2-pyridyl)-s-triazine (TPTZ) solution and acetate buffer (FRAP reagent). Reducing capability was measured mixing 10 µL of sample with 300 µL FRAP reagent. Absorbance was monitored at 594 nm after incubation at 37 °C for 15 min. Trolox standard curve was used in both assays and results were expressed as mg Trolox equivalents per g purified pigments.

The DPPH assay was conducted based on the method described by Yu et al. (2019) with some modifications. Briefly, 0.1 mM DPPH solution in methanol (200 µL) was added to 20 µL of sample and incubated for 30 min in the dark at room temperature. Absorption was measured at 517 nm. The hydroxyl radical scavenging activity assay (HORSA) was carried out using the method described by Arise et al. (2016). Samples and 1,10 phenanthroline (3 mM) were dissolved in 0.1 M phosphate buffer, pH 7.4, while $FeSO_4$ and 0.01% hydrogen peroxide were each prepared in distilled water. Scavenging activity was measured adding 50 µL of sample/standard or buffer into the 96-well plate followed by 50 µL of 1, 10-phenanthroline and 50 µL of $FeSO_4$. Then, 50 µL of H_2O_2 was added into each well to start the reaction and measured the absorbance at 536 nm every 10 min for a period of 1 h at 37 °C. The absorbance of blank (without H_2O_2) and control (without sample) was also determined. Antioxidant activities were given as percentage DPPH and HORSA scavenging and calculated as: [(blank absorbance – sample absorbance)/ (blank absorbance)] × 100. All plate based absorbance measurements were conducted using a Tecan Spark™ 10 M multimode microplate reader (TECAN, Männedorf, Switzerland).

2.7. Folin-Ciocalteu method

The Folin-Ciocalteu method described by Fernando et al. (2021) was used with different dilutions of purified compounds (0 – 100 µg/mL). Gallic acid was used as the reference standard. Briefly, 10 µL of sample or gallic acid standard was mixed with 40 µL of 10 % (v/v) Folin reagent and 150 µL of 4% (w/v) sodium carbonate followed by a 30 min incubation at room temperature in the dark. Absorbance was measured at 765 nm using Tecan microplate reader.

Table 1
Yield, purity (%), calculated *m/z* values, observed HRMS molecular formula and *m/z* values of purified betalains obtained through flash chromatography.

Compound	Molecular weight (g/mol)	Yield (mg) using 10 g raw material	Formula from HRMS	Calculated [M + H] (*m/z*)	Observed [M + H] (*m/z*)	% purity based on HPLC
Betanin[a]	550.5	48.7 ± 2.12	M + H	551.1508	551.1507	96.85
Neobetanin[a]	548.5	18.7 ± 1.69	M - CH_2OH + H + Na^+	541.1070	541.1524	51.86
Vulgaxanthin I[a]	339.3	30.2 ± 2.89	M + H_2O + H + Na^+	381.0910	381.0788	79.17
Vulgaxanthin I[b]		12.0 ± 1.06				61.55
Indicaxanthin[c]	308.3	40.9 ± 2.26	M + H	309.1082	309.1069	95.32

Source: a – red beetroot powder, b- yellow beetroot powder, c- yellow prickly pear powder.

2.8. Data analysis

The data are reported as mean ± standard deviation of three or more independent experiments and graphs were drawn using GraphPad Prism version 9.0 for Windows. One-way and two-way ANOVA with Tukey's post-hoc multiple comparison test were applied to determine the statistical significance among the different purified compounds and within the different treatments of purified compounds at $p < 0.05$. ChemDraw Ultra version 12.0.2. for Windows were used to draw the chemical structures and calculate the exact mass values.

3. Results and discussion

3.1. Purification of betalains

Betanin purification was carried out by preparative HPLC method in the first instance to assess the yield and purity of the compound. The chromatogram of preparative HPLC showed three main peaks with retention times of 2.52, 5.92, and 33.74 min (Fig. S3), corresponding to betanin, betaxanthins and degraded products of betanin respectively.

The total betalain content (betacyanin and betaxanthin) of initial red beetroot extract was 8.67 ± 0.30 mg/g and the yield of the purified betanin using preparative HPLC was 0.37 ± 0.01 mg/g which corresponds to 4.2% of the initial betalain content based on the UV–Vis spectrophotometric measurements. The betanin yield achieved here was similar to da Silva et al. (2019) who reported a betanin yield of 0.384 mg/g. However, this yield is fairly low and preparative HPLC is a lab-intensive and time-consuming process to obtain purified betalains. The aim of the present study was to develop a quantitative purification method for betalains, which can deliver a cost- and resource effective solution for betalains in amounts that are adequate for further experiments i.e. identification and bioactivity confirmation. Therefore, automated flash chromatography was selected as a fast, effective and economical method compared with other purification techniques, suitable for separating large quantities. Flash chromatography has indeed been successfully applied to purification of groups of compounds as well as individual compounds. For example, using this technique, Arora and Itankar (2018) obtained yields up to 7.34 mg/g purified flavonoid from *Chenopodium album* and Hossain et al. (2014) demonstrated the successful purification of rosmarinic acid and arbutin from *Origanum majorana L.* To our knowledge, this method has not been applied for betalain purification.

In the present study, flash chromatography was initially used to purify betanin from red beetroot extracts. The method optimized for betanin was subsequently applied to separate neobetanin, vulgaxanthin I and indicaxanthin from red beetroot, yellow beetroot and yellow prickly pear extracts, respectively. Preliminary experiments using TLC were conducted to test solvent effects on separation effectiveness of the target compounds since it is a simple and rapid method to generate separation fingerprints of crude extracts. The mobile phase that resulted in R_f (retention factor) values between 0.2 and 0.5 was selected for flash separation (Bedikou, Logesh, Niamké, & Dhanabal, 2020). R_f values indicate the solubility of the particular compound in the solvent by measuring the compound movement on the TLC plate. The solvent system composed of 60% acetonitrile with 0.1% formic acid (v/v) showed better separation compared to other tested solvent systems Fig. S2. Acetonitrile with 0.1% formic acid showed better separation compared to the equivalent with 0.1% acetic acid addition. It has been well documented that formic acid is ideal for improving the peak shape and separation and is available in higher purity compared to acetic acid which can be easily degraded and contaminated (Do, Nguyen, Nguyen, & Le, 2020).

The purification workflow is shown in Fig. 1. In the present study reversed-phase 50 μM C18 SNAP cartridge was used for the purification and this high surface silica cartridge provides improved loading capacity and effective separation of betalains from crude extracts. Due to

unavailability of detectors for the visible range in this instrument, the UV region was used to monitor the separation of compounds. Betalains are typical organic compounds, which have characteristic bands in visible as well as UV regions. Therefore, both regions can be used for efficient detection and separation of the pigments. The average purification yields using flash chromatography were 4.87, 1.87, 3.02 and 4.09 mg/g for betanin, neobetanin, vulgaxanthin I and indicaxanthin, respectively (Table 1). Compared to the preparative HPLC, purification via flash chromatography has resulted in a 13.2 times higher betanin yield. In this case flash chromatography is providing a faster, cheaper and equivalent separation performance compared to preparative HPLC due to several features such as shorter and wider cartridge dimensions with larger particle sizes (25 or 50 μm), which can withstand a higher sample load with reduced operating pressure at the elevated flow rates as well as reusability of cartridges with comparatively low replacement and operating costs. Furthermore, it is possible to scale up flash chromatography separation to industrial-scale compared to the other pigment separation techniques such as preparative HPLC. The effective scale up of flash purification can be achieved when controlling factors such as sample concentration, solvent, form of sample (dry or liquid), stationary phase and solvent flow linear velocity.

The betanin as well as indicaxanthin yields from the present study were higher compared to Butera et al. (2002) who used gel filtration on a Sephadex G-25 column and obtained 5.12 mg betanin and 8.42 mg indicaxanthin from 100 g edible pulp of red and yellow prickly pear respectively. Further, current betanin yield was higher than Gonçalves et al. (2012) who purified betanin using seven different purification methods including normal and reverse phase chromatography, as well as aqueous two-phase extraction methods, resulting in purification yields ranging between 4.9 ± 1.4 – 20.5 ± 2.4 mg/100 g of red beetroot powder. Vulgaxanthin I yield in the present study was 3.7 times higher compared to Tesoriere, Fazzari, Angileri, Gentile and Livrea (2008), who used liquid chromatography on a Sephadex G-25 column (80 × 2.2 cm) and obtained 81.6 mg/100 g from red beetroot, which was quantified by spectrophotometry without indication of purity. Overall, results of the present study indicate superior purification yield using flash chromatography when compared to other betalain purification methods.

3.2. Identification of purified betalain fractions

In the present study several analytical techniques such as HPLC-DAD (Diode-Array Detector), quadrupole MS and HRMS were used for the determination and profiling of betalains while confirming the purity of the isolated compounds that were obtained from flash chromatography. These are standard analytical techniques utilized in characterizing of chemical compounds. Initial identification of the purified fractions was performed using HPLC/MS. Further, HPLC-DAD chromatograms were monitored at 536 nm and 486 nm for betacyanin and betaxanthin, respectively (Fig. 2) in order to calculate the purity of the isolated compounds. Additionally, chromatographic profiles of betalains in crude extracts (red beetroot, yellow beetroot and yellow prickly pear) were monitored at 536 nm and 486 nm (Fig. S4) to compare the purification effectiveness. Purity of the isolated compounds was calculated using peak area of DAD chromatograms observed at 536 nm for betanin and 486 nm for neobetanin, vulgaxanthin I and indicaxanthin (Table 1). A high purity (>95%) was noted for betanin and indicaxanthin while vulgaxanthin I showed only 79% purity under these conditions. The purity of neobetanin was found to be 52% with approximately 10% of betanin and isobetanin identified in the sample.

Furthermore, identification of purified betalain fractions was performed using high resolution mass spectroscopy (HRMS) which is shown for betanin in Fig. S5. The pseudo-molecular ion having an *m/z* value of

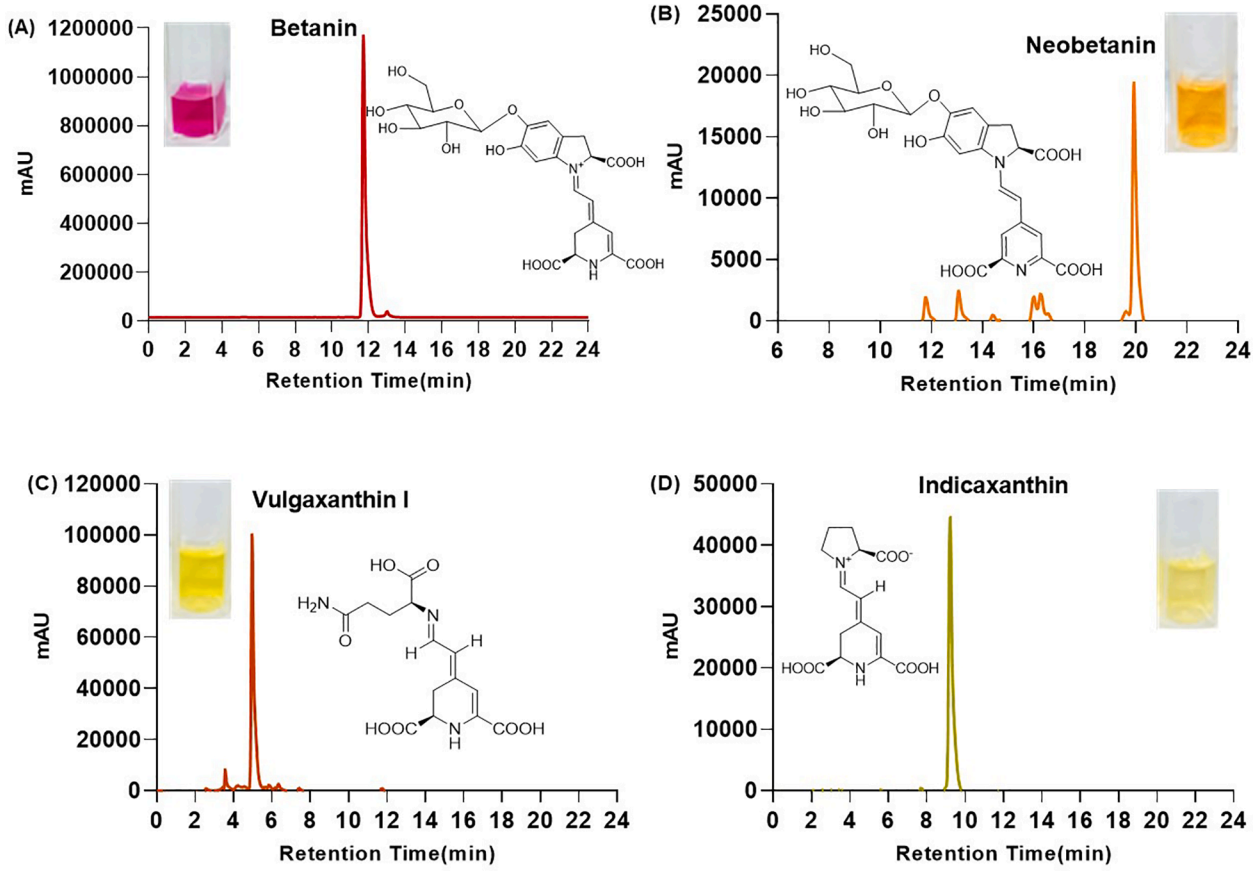

Fig. 2. HPLC-DAD chromatograms of purified betalains (A) betanin at 536 nm, (B) neobetanin, (C) vulgaxanthin I, and (D) indicaxanthin at 486 nm. Chromatographic conditions: mobile phase A - water/formic acid (98:2 v/v), B – methanol; flow rate: 0.95 mL/min; column oven: 40 °C.

551.1507 which corresponds to betanin and having a fragmentation ion with an *m/z* of 389.0973 corresponding to betanidin, can be observed in the MS spectrum and provided confirmation of betanin presence. The same fragmentation pattern of betanin was observed in studies conducted by Gonçalves et al. (2012) and da Silva et al. (2019). Table 1 shows the calculated *m/z* values using ChemDraw (Calculated *m/z*) and observed *m/z* values in HRMS of all purified betalains confirming the identity and purity of the collected fractions.

In the present study, reverse-phase cartridge which contains a hydrophobic stationary phase (C18) and hydrophilic mobile phases (water/ acetonitrile) was used to perform flash chromatography. Under these conditions, hydrophilic sugars can pass through the column and elute first which is evident by shorter retention times of sugars in comparison to betalain pigments. In order to ensure absence of sugars

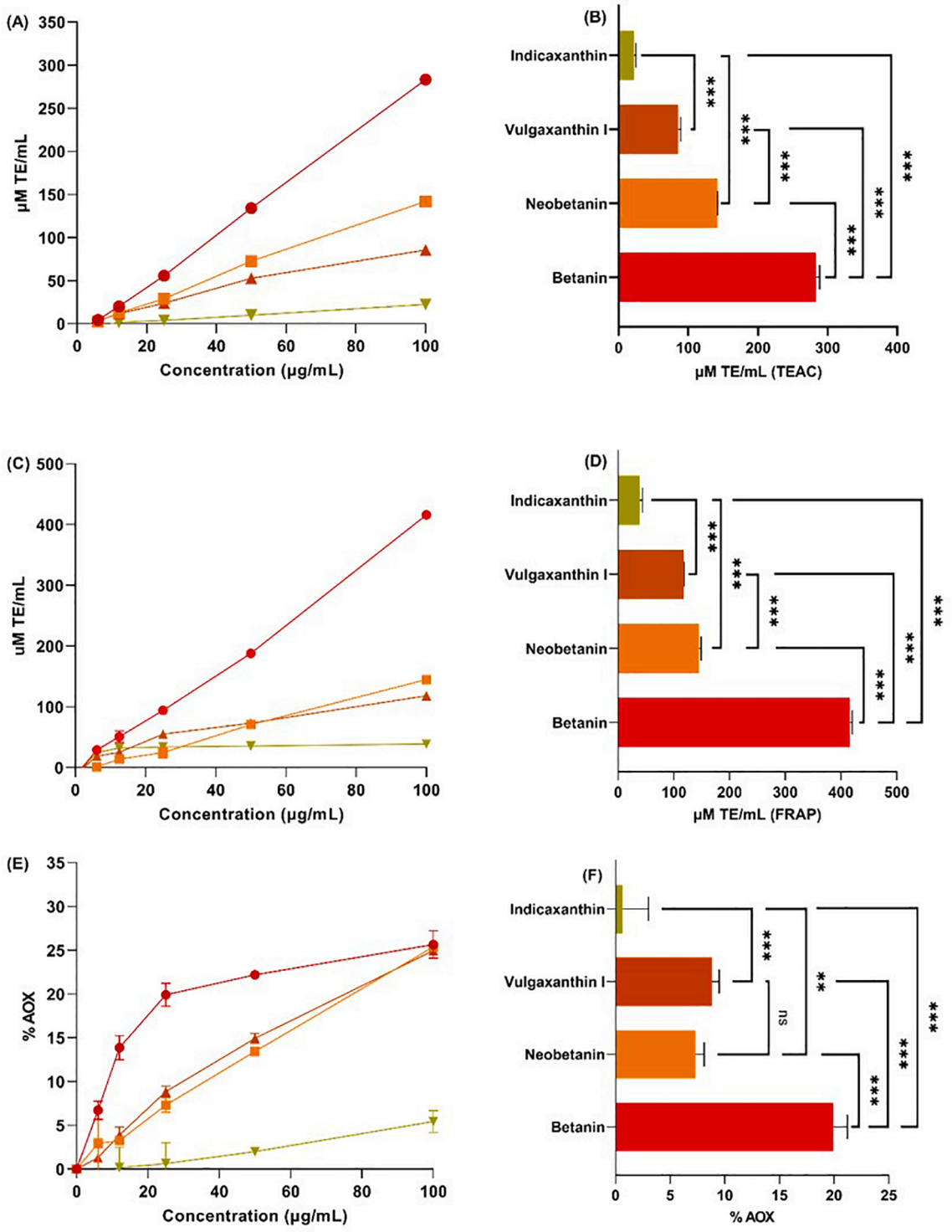

Fig. 3. Dose-dependent antioxidant activity of purified betalains demonstrating (A) ABTS + radical scavenging activity, (C) Ferric reducing antioxidant power (FRAP), and (E) DPPH radical scavenging activity. Comparison of ABTS + radical scavenging activity (B), FRAP (D) at 100 µg/mL and DPPH (F) at 25 µg/mL of individual betalains. Data are presented as mean (A, C, E) and mean with SD (B, D, F) of at least three independent experiments performed in duplicate. * indicates significant difference, Tukey's test: *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

that might interfere with subsequent analyses, purified betalain samples were analysed via HPLC-ELSD. The results demonstrate that all samples were free from sugars except vulgaxanthin I which was isolated from both red and yellow beetroot extracts, which showed a sucrose peak in the purified sample (318.03 ± 22.5 mg/g). Attempts to remove sugars from the yellow beetroot extract prior to flash chromatography e.g. using SPE cartridge (Oasis MAX 3 cc Vac cartridge, 60 mg sorbent, Waters Corporation, U.K), or adjustment of elution gradient during chromatography run were unsuccessful which is likely due to the similar polarities of vulgaxanthin I and sucrose resulting in co-elution, which is particularly evident with low pigment concentrations in the initial extract. Indeed, the yield and purity of vulgaxanthin I from yellow beetroot were significantly lower ($p < 0.05$) compared to red beetroot extract (yield: 12 vs 30.2 mg/g; purity: 61 vs 79%) with starting concentrations of total betaxanthins in yellow beetroot being 6.27 times lower compared to red beetroot.

3.3. Antioxidant activity assays

The antioxidant and radial scavenging potential of betalains has been highlighted by a number of studies, however, very few have utilized purified betalains (Cai et al., 2003; Gandia-Herrero, Escribano, & Garcia-Carmona, 2016). The focus of previous research was on betanin and indicaxanthin, and to the best of our knowledge, none of the studies included purified vulgaxanthin I and neobetanin. The current study therefore focused on evaluating the antioxidant properties of purified betanin compared to neobetanin, vulgaxanthin I and indicaxanthin using several commonly used *in vitro* assays that assess radical scavenging (ABTS, DPPH, hydroxyl radical) and iron reduction capacities (FRAP). Given the different mechanisms and radical probes as well as limitations of each individual assay, it is of advantage to run several assays to determine the overall antioxidant capacity of a particular compound (Mu et al., 2018).

Scavenging effects of purified betalains with different radical systems are shown in Fig. 3. ABTS + and DPPH radical scavenging assays determine the reducing capabilities of antioxidants either by electron transfer or radical quenching via H atom transfer while FRAP assay measures the ability of an antioxidant to transfer one electron to reduce the Fe^{3+} into Fe^{2+} (Nimse & Pal, 2015). All purified betalain compounds appeared to exhibit a dose-dependent response in radical scavenging assays (ABTS+, DPPH and FRAP). When compared at 100 μg/mL, betanin showed significantly higher ABTS + and FRAP reducing antioxidant activity ($p < 0.001$) than neobetanin, vulgaxanthin I and indicaxanthin (Fig. 3B and 3D). Current results are in agreement with Butera et al. (2002) who demonstrated that betanin was more potent scavenger of ABTS + radicals compared to indicaxanthin.

Similar to the above results, betanin showed higher DPPH scavenging activity compared with other betalains, however, a plateau can be observed at 25 μg/mL and above (Fig. 3E). Olszowy and Dawidowicz (2018) examined the possible usage of ABTS + and DPPH assay to estimate the antioxidant activity of coloured compounds. They exhibited that reliable results from the above assays can be obtained if a test compound does not coincide with the wavelength used in the assay. The DPPH assay used a 517 nm wavelength to monitor the radical scavenging activity and the absorption maxima (λ_{max}) of betanin is ~ 536 nm. Therefore, absorbance scans with and without DPPH radicals were examined (Fig. S6) to assess the possible colour interference of purified betanin. The results showed that higher concentrations of betanin interfered with the assay results while concentrations <25 μg/mL showed less interference. The possible reason could be that any remaining betanin which does not react with DPPH radicals at the moment of the measurement also absorbs at 517 nm wavelength and therefore does not allow the exact monitoring of DPPH radical formation (Olszowy & Dawidowicz, 2018).

All other purified samples were tested with and without DPPH radicals and they did not show any interference (data not shown). Indeed, when compared at 25 μg/mL, the DPPH response pattern of betanin is similar to ABTS+ and FRAP assays indicating superior radical scavenging activity to all other betalains ($p < 0.001$; Fig. 3F). In addition, we tested the application of HORSA assay to assess hydroxyl radical scavenging properties of betalains, however, due to wavelength interference at 536 nm it was only possible to monitor vulgaxanthin I and

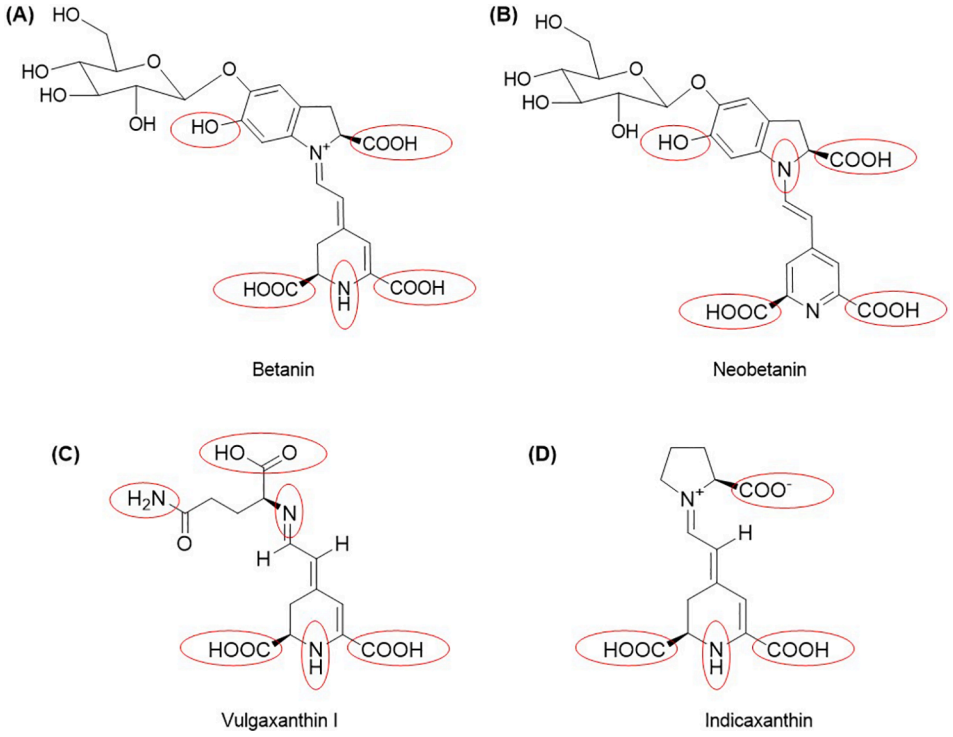

Fig. 4. Structures of purified betalains with potential active groups marked in red circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

indicaxanthin. In line with other radical scavenging assays, vulgaxanthin I showed 31.4% higher hydroxyl radical scavenging activity compared to indicaxanthin (data not shown). These results highlight limitations of absorbance based assays when testing pigmented samples, and alternative methods such as Electron Spin Resonance (ESR) would be advantageous. In fact, a recent study conducted by Esatbeyoglu et al. (2014) demonstrated a dose-dependent scavenging of betanin against DPPH-, galvinoxyl-, superoxide- and hydroxyl-radicals using ESR. In summary, antioxidant activity of purified betalains in the present study decreased in the following order betanin > neobetanin > vulgaxanthin I > indicaxanthin.

The chemical structure of the betalain molecules greatly influence their antioxidant activity. The structures of the betalains marked with potentially active groups are shown in Fig. 4. Generally, higher antioxidant activity in betalains is connected with the increasing number of hydroxyl groups and the presence of other hydrogen donation groups such as amino (–NH), and thiol (-SH) groups (Cai et al., 2003) as well as imino and acyl groups (Esatbeyoglu, Wagner, Schini-Kerth, & Rimbach, 2015). Further, the glycosylation pattern influences the radical scavenging abilities of betalains, e.g. 6-*O*-glycosylated betacyanins have shown higher scavenging capabilities compared to the 5-*O*-glycosylated betacyanins (Cai et al., 2003; Esatbeyoglu et al., 2015). Gandía-Herrero, Escribano and García-Carmona (2010) emphasized that the radical scavenging activity of betalains not only link with the number of hydroxyl groups present in the molecule but also with the electronic resonance system supported between two nitrogen atoms. In the case of betanin as the most studied pigment, its high antioxidant activity has been linked with the hydrogen and electron donation ability when changing from cationic state to different deprotonated states in basic solutions (Gandía-Herrero et al., 2010; Slimen et al., 2017).

To the best of our knowledge, radical scavenging capacities of vulgaxanthin I and neobetanin have not been demonstrated so far. Vulgaxanthin I is formed by conjugation of betalamic acid with glutamine (Fig. 4C). Neobetanin is a degradation product of betanin formed by removal of two hydrogen atoms from the betanin structure (Fig. 4B). In principle, both vulgaxanthin I and neobetanin are good radical scavengers while vulgaxanthin I has more active groups than neobetanin. However, in the current study, neobetanin showed higher radical scavenging activity compared to vulgaxanthin I. The reason could be that the presence of indoline-like substructures (Fig S7.B) may significantly enhance the radical scavenging properties of the neobetanin (Gandía-Herrero et al., 2010). Further, the presence of the phenolic hydroxyl group in neobetanin can significantly enhance the radical scavenging activity in comparison to vulgaxanthin I (Gandía-Herrero et al., 2010). The lower potency of neobetanin compared to betanin could be explained by the ability of betanin to create a stable carbocation through the electron resonance system shared between the imino and the tetrahydropyridine groups (Fig. S8. A) as well as stabilization of tetrahydropyridine through hydrogen bonds with adjacent carboxylic groups (Slimen et al., 2017).

To summarize, present data demonstrate that among betaxanthins, vulgaxanthin I was more potent compared to indicaxanthin. The reason could be that the charged imino group present in indicaxanthin can decrease the scavenging activity of the molecule. Overall, betacyanins (betanin, neobetanin) are more potent than betaxanthins (vulgaxanthin I, indicaxanthin) due to the presence of the indole substructure (Fig. S8. B) and hydroxyl group.

The Folin assay is the most commonly used method to evaluate the total polyphenol content in plant and food extracts (Sánchez-Rangel, Benavides, Heredia, Cisneros-Zevallos, & Jacobo-Velázquez, 2013). The lack of specificity of the Folin assay is well known, as the Folin reagent can be reduced by other compounds such as reducing sugars (glucose, fructose), dehydroascorbic acid and amino acids present in a sample (Sánchez-Rangel et al., 2013). The current study examined whether betalains could react with the Folin reagent and therefore affect the specificity of the Folin assay to determine the polyphenol content in a mixture. As shown in Fig. 5, betanin demonstrated a strong dose-dependent increase in signal in contrast to neobetanin, vulgaxanthin I and indicaxanthin. When compared at 50 μg/mL, the corresponding values for PP content were 70.1, 11.7, 6.99, 8.08 μg GAE/mL for betanin, neobetanin, vulgaxanthin I and indicaxanthin, respectively. It can be assumed that the results are a good representation of betalain response as polyphenol analysis with HPLC/MS confirmed the absence of polyphenols in the purified betanin and indicaxanthin samples (data not shown). In the case of purified vulgaxanthin I, trace amounts (<1%) of catechin and epichatechin were found while neobetanin contained trace amounts (<1%) of *n-trans*-feruloyltyramine; a lower accuracy can therefore not be excluded for these two compounds. Based on these findings, it needs to be taken into account that the application of the Folin assay to determine the TPC in betalain-containing samples may be significantly confounded, in particularly in samples with high betanin content.

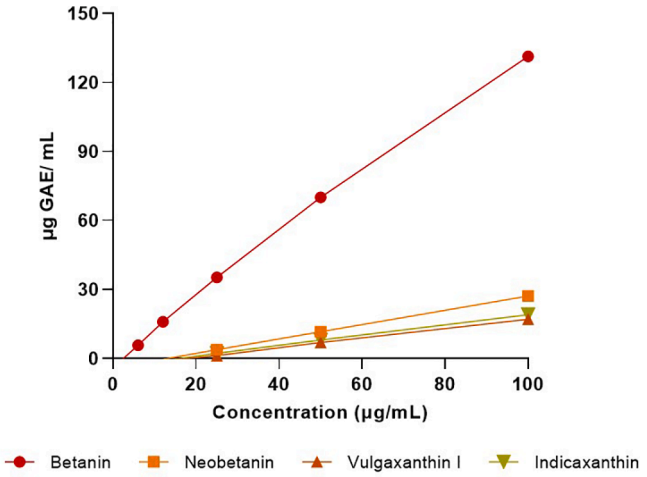

Fig. 5. Dose-dependent response of purified betalains in the Folin assay.

4. Conclusions

The flash chromatography system is capable of purifying betanin, neobetanin, vulgaxanthin I, and indicaxanthin from crude extracts of beetroot and prickly pear sources effectively, using a single chromatographic step. Given the current purification yields of 1.87 – 4.87 mg/g and percentage purity of betanin, neobetanin, vulgaxanthin I, and indicaxanthin of 97%, 52%, 79%, and 95%, flash chromatography demonstrates strong potential with significant advantages compared to other methods. Further work should be done to increase purity of vulgaxanthin I and neobetanin, and apply the method to purification of other betalains. Importantly, all purified betalains demonstrated antioxidant and free radical scavenging activities, with betanin showing much stronger capacity as compared to the other betalains. Overall, betacyanins were more potent as antioxidants in comparison to betaxanthins which indicates a strong structure-function relationship of different betalains, and further studies should investigate the relevance of these findings.

CRediT authorship contribution statement

Ganwarige Sumali N. Fernando: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Natalia N. Sergeeva:** Methodology, Validation, Writing – review & editing, Supervision. **Maria J. Frutos:** Methodology, Writing – review & editing. **Lisa J. Marshall:** Validation, Writing – review & editing, Supervision. **Christine Boesch:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was partially supported by N8 AgriFood consortium. GSNF is funded by a Commonwealth PhD Scholarship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132632.

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