

Analysis of Red Amaranth (*Amaranthus cruentus* L.) Betalains by LC-MS

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Abstract. Betalains are natural pigments responsible for the colour of flowers, bracts, stems, and leaves of genus *Amaranthus*, and they are bioactive compounds. Due to their great chemical diversity, the determination and confident identification of betalains are still challenges and of great interest as they are natural colourants for health benefit. By high-performance liquid chromatography (LC) coupled to tandem mass spectrometry (MS), the catalog of betalains in red amaranth (*Amaranthus cruentus* L.) was defined. In total, 30 betacyanins and 13 betaxanthins were found. Mass measurements for the parent ion and for its fragments with errors of less than 5 parts per million (ppm) achieved the recommended criteria for identification of compounds. This work defines the main betalains in red amaranth (*A. cruentus*) and valorizes the leaves and inflorescence of red amaranth as betalain pigments sources.

Keywords: Amaranth; betacyanins; betaxanthins; high-performance liquid chromatography; tandem mass spectrometry.

Resumen. Las betalaínas son pigmentos naturales responsables del color en las flores, brácteas, tallos y hojas del género *Amaranthus* y también son compuestos bioactivos. Debido a su amplia diversidad química, la identificación y determinación confiable de betalaínas siguen siendo desafíos y son de gran interés puesto que son colorantes naturales beneficiosos para la salud. Por medio de cromatografía de líquidos de alta eficiencia acoplada a espectrometría de masas en tándem se definió el catálogo de betalaínas en “amaranto rojo” (*Amaranthus cruentus* L.). En total se encontraron 30 betacianinas y 13 betaxantinas. Las mediciones de las masas del ion molecular y sus fragmentos con errores menores a 5 partes por millón (ppm) garantizaron los criterios recomendados para la identificación de compuestos. Este trabajo define las principales betalaínas del amaranto rojo (*A. cruentus*) y valora las hojas e inflorescencias del amaranto rojo como fuente de pigmentos betalaínicos.

Palabras clave: Amaranto; betacianinas; betaxantinas; cromatografía de líquidos de alta eficiencia; espectrometría de masas en tándem.

Introduction

The betalains, naturally occurring pigments in most of the families of the plant order Caryophyllales [1], fungi of the genus *Amanita* [2], and proteobacterium *Gluconacetobacter diazotrophicus* [3], are responsible for the red-violet and yellow-orange colour found in flowers, bracts, fruits, stems, leaves, and roots [4]. The genus *Amaranthus* belongs to the order Caryophyllales and to the largest eudicot clade with C4-based photosynthetic pathway species and consists of nearly 75 species found worldwide [5-7]. Amaranth was sacred to and eaten by Aztecs [6]. Examination of an archaeological grain of amaranth found in Coxcatlan cave in Puebla, Mexico, has revealed that *Amaranthus hypochondriacus* L. and *Amaranthus cruentus* L. were harvested for thousands of years [8]. Despite the genetic diversity among the large number of species in the genus *Amaranthus*, the *A. hypochondriacus* is the major crop cultivated in Mexico.

The discoveries on the high content in high-quality protein, essential minerals including iron, calcium, magnesium, phosphorus, and potassium, and unsaturated fatty acids in amaranth (*Amaranthus* spp) overcoming other cereals such as wheat, maize, or rice made important to introduce amaranth and its products into the market [9,10]. Besides Mexico's traditional amaranth foods such as "Alegria" that is popped amaranth seeds confection, there is an array of consumer food products including breakfast cereals, baked items, sprouts, and betalains pigments [11]. Thus, molecular characterizing amaranth species may facilitate the amaranth breeding to feed the ever-growing consumer demand for amaranth.

Betanin, which is a betalain, was first isolated from "red beetroot" (*Beta vulgaris* L.) in 1918 by Schudel [12] and approved as natural food colourant E162 in defined amounts specified by the Food and Drug Administration (FDA). Betalains from amaranth were approved as a natural food colourant in China as well (National Food Safety Standard Use for Food Additive, GB2760-2011). Betalains are divided into two classes the betacyanins and betaxanthins. Red-violet betacyanins are an array of compounds that appear as betalamic acid condensed with either *cyclo*-DOPA or glycosylated *cyclo*-DOPA that can be further decorated with added glucosyl or glucuronosyl groups or subjected to acylation of sugar moieties, decarboxylation by removing carboxyl groups, and dehydrogenation, whereas yellow-orange betaxanthins are compounds with frameworks displaying the betalamic acid condensed with an imino or amino group of amino acids [4, 13,14].

Betacyanins are organized in at least seven distinctive groups that differ in attaching glucosyl groups to the oxygen atoms in the *ortho* position on the *cyclo*-DOPA moiety as well as in the position of the glycosyl- or glucuronosyl-linkage moieties [4, 15]: betanin-type, gomphrenin-type, amaranthin-type, melocactin-type (*Bougainvillea*-r-I-type), oleracin-type, apiocactin-type, and glabranin-type (*Bougainvillea*-v-type). Amaranthin is the main betacyanin in the genus *Amaranthus*. Besides the betacyanin behaviour as pigment, biological activities have been reported for betalains such as anti-inflammatory-, antioxidant-, and immunomodulatory-activities and cancer chemoprotective properties [16, 17].

Most efforts have been focused on quantifying betacyanins and betaxanthins using multicomponent spectrometric analysis in the presence of interfering substances [18]. Despite multiple betacyanins and betaxanthins are present in the plant tissue samples, the concentration of betacyanins based on betanin and betaxanthins based on vulgaxanthin-I give in the total betalains content [19]. High-Performance Liquid Chromatography coupled with a diode array detector (DAD) has simplified to survey different betacyanins and betaxanthins, and LC coupled to tandem MS has been led by recording the *m/z* value of the parent ion and its tandem fragmentation to distinguish different betacyanins and betaxanthins, and its derivatives. Here, we used LC-MS to define the betalain composition in red amaranth (*A. cruentus*) to valorize the leaves and inflorescences as betalain pigments source.

Experimental

Plant material

The *A. cruentus* seeds were originally collected in Valladolid, Yucatan in 2021, and identified by Ph.D. Ivonne Sánchez del Pino (Taxonomist) at Scientific Research Center of Yucatán (CICY). *A. cruentus* plants were grown at the outdoor experimental garden of CICY. The leaves and inflorescences were collected from

60-days-old *A. cruentus* plants, immediately frozen in liquid nitrogen, lyophilized at -51 °C for 72 h, ground to powder with a blade mill, and stored at -20 °C until use.

Chemicals and reagents

Methanol (MeOH) and water (H₂O) HPLC-grade were from Tedia (Fairfield, USA). Methanol and water LC-MS-grade were obtained from Tedia (Fairfield, USA). Acetic acid LC-MS-grade was obtained from Fisher Scientific (Waltham, USA). Red beet extract was obtained from Sigma-Aldrich (Cat. # 901266, St. Louis, MO, USA) and used as reference material (RM). Sorbent BondElut-C18 was purchased from Agilent Technologies (40 mm particle size, Santa Clara, CA, USA).

Betalain extraction from plants tissues

Total betalains were extracted from 100 mg of tissue powder. After 400 mg of sorbent BondElut-C18 was ground with tissue powder with mortar and pestle at room temperature, the mix was packed in an empty SPE cartridge. Next, elution was performed with 9 mL of H₂O made 0.1 % acetic acid, and then with 9 mL of MeOH:H₂O (1:1, v/v) made 0.1 % acetic acid using a vacuum manifold system (Visiprep SPE Vacuum Manifold, Sigma-Aldrich, United States), both eluents were collected separately and evaporated. The residues were resuspended in 1 mL of H₂O made 0.1 % acetic acid and transferred to an amber vial (MS-grade) for LC-MS analyses.

High-Performance Liquid Chromatography (LC) coupled with LTQ-Orbitrap Mass Spectrometry (MS)

The analysis by LC-MS Orbitrap was performed using an Ultimate 3000 UHPLC (Thermo Scientific, Waltham, MA, USA) coupled to an UV/Vis detector (Ultimate 3000 UV/VIS detector, Dionex), and to a LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a Heated Electrospray Ionization interface (HESI-II, Thermo Fisher Scientific, Waltham, MA, USA). A 20 mL aliquot of the sample was injected, transferred to a stainless steel loop and then to a Hypersil GOLD C18 column (100 × 2.1 mm, 1.9 mm particle size) at a flow rate of 300 mL/min. Sample was eluted from the column at a flow rate of 300 mL/min using a binary gradient composed of H₂O made 0.1% acetic acid (solvent A) and MeOH made 0.1% acetic acid (solvent B). The gradient settings were as follows: 0-30 min from 5% to 100%, isocratic for 10 min, then returning to 5% and isocratic for 10 min for column reconditioning. UV/Vis spectra data were collected at 480 and 540 nm. MS spectra data were acquired in positive mode: MS1 resolution 60,000, scan range 100-1500 *m/z*; MS2 resolution 60,000, scan range 100-600 *m/z*. Higher-energy collisional dissociation (HCD) was used as a fragmentation method by applying 50 eV. Peak areas of each compound based in MS1 mode using the extracted ion chromatograms were determined with Xcalibur 4.1 software (Thermo Scientific, USA). Graphs were obtained using GraphPad Prism version 9.0 (GraphPad, San Diego, CA, USA).

Results and discussion

Betalains determination in commercial reference material (RM)

Given that reference betalains are unavailable, RM got from Sigma-Aldrich was used as a reference to identify betanin chromatographic peaks accurately and to further characterize the chromatographic behavior of other unavailable betalains. The HPLC chromatogram of the RM obtained at 540 nm wavelength showed two high absorbing peaks with retention time (Rt) of 3.93 and 6.76 min, respectively (Fig. 1(A)). Both peaks were baseline separated and displayed an intense [M+H]⁺ ion at *m/z* 551.1482, as expected for betanin and isobetanin, and MS2 spectra got with HCD 50 eV were almost same. The two peaks yielded the MS2 ion at *m/z* 389.09, suggesting the presence of hexoside (Fig. 1(B) and 1(C)). In betanin-type betalains, the glucoside is attached through the C5-oxygen [20]. Isobetanin is the epimer of betanin at the chiral center of C15, and greater Rt value has been reported for isobetanin than betanin that is explained to some extent by slightly less polar isobetanin than betanin based on dipole moment estimation [21, 22]. The baseline separation of the two peaks, MS1, and MS2 spectra data lead to confident identification of betanin and isobetanin.

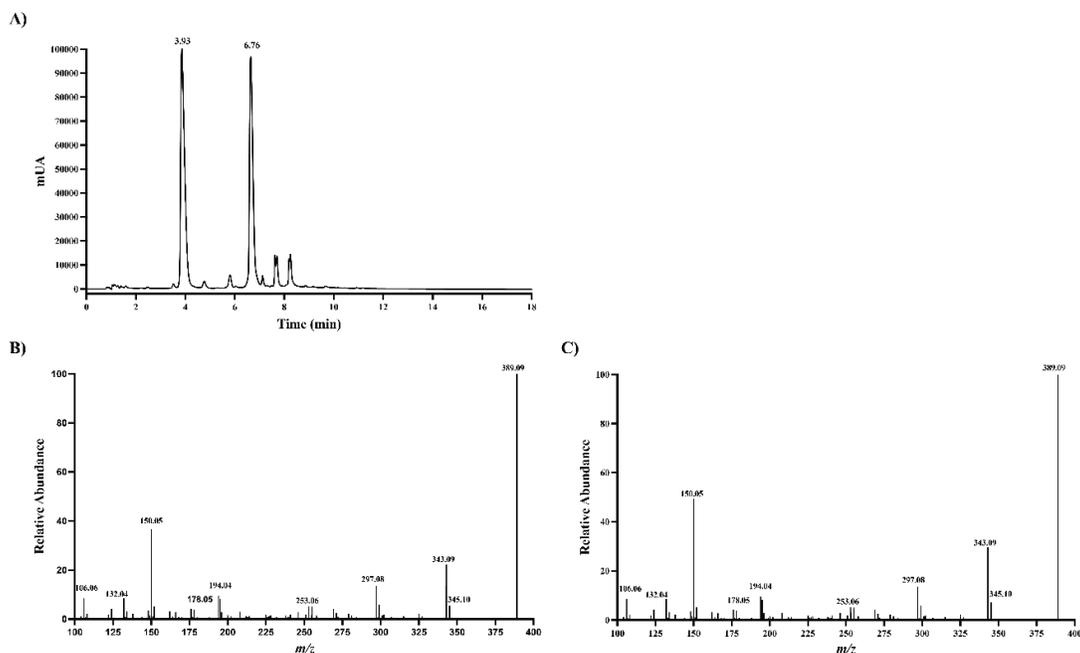


Fig. 1. LC-MS analysis of RM. Liquid chromatography-ultraviolet chromatogram at 540 nm (A). MS2 spectra were obtained with HCD 50 eV for peak at 3.93 min (betanin) (B) and 6.76 min (isobetanin) (C).

Identification and relative quantification of 43 betalains by LC-MS

Next, we used betanin as a reference, recording the absorbance at 540 nm in-line with LC-MS analysis, to find out betalains in leaves and inflorescences samples from the red amaranth (*A. cruentus*). Red amaranth plant parts were almost, if not all, red colours. Stem, petioles, and leaves, as well inflorescences display red colours (Fig. 2). A catalog of betalains consisting of 68 betalains chromatographically separated and confident identified by Xie and Chen (2021) [23] from *Chenopodium formosanum* cultivars was also used as a reference since C18 column was used in this study as well. Overall, the high of the absorbing peaks in the leaves were lower than in the pair peaks in inflorescences, and the highest absorbing peaks matched to the peaks 1 and 2, which are the major compounds in both chromatograms, corresponding to amaranthin and isoamaranthin (Fig. 3(A) and 3(B)). The $[M+H]^+$ ion at m/z 727.1803 and the MS2 ions after fragmented with 50 eV in HCD showed in the Fig. 3(C) and 3(D) were similar to the data from the literature for amaranthin isolated from stems and inflorescences of Amaranthaceae plants including the genus *Amaranthus* [20, 24, 25], petal of *Gomphrena globosa* [26], and inflorescences of *Celosia argentea* var. *cristata* and *Celosia argentea* var. *plumosa* [27]. The lower retention got for both amaranthin and isoamaranthin than betanin agrees with the previously reported data [23, 24]. The fact that R_t value for amaranthin is smaller than that of betanin can be explained by the glucuronic acid attached to the glucose moiety. In amaranthin-type betalains, the glucuronic acid is attached through the C2-oxygen of the glucose moiety.



Fig. 2. Red amaranth (*Amaranthus cruentus* L.) plant.

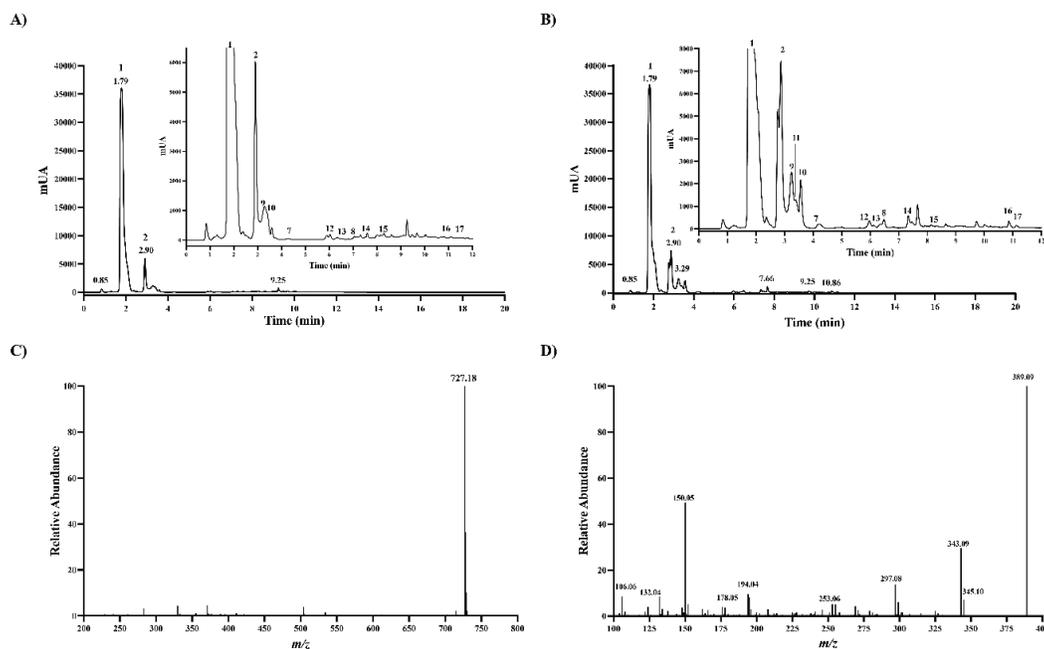


Fig. 3. LC-MS analysis of red amaranth samples. Liquid chromatography-ultraviolet chromatogram got at 540 nm in leaves samples (A) and inflorescence samples (B). The inset shows the expanded region of the chromatogram with retention time of 1-12 min. (C) MS1 spectra got for peak 1. (D) MS2 spectra got with HCD 50 eV for peak 1.

Based on the mass measurement accuracy on Orbitrap Elite, 43 betalains were identified with mass measurement errors of less than 5 parts per million (ppm) (Table 1). MS2 spectra data obtained for betalains were used to confirm the accurate betanin identification in amaranth samples and to compare with MS2 spectra

data previously reported for samples of *Chenopodium formosanum* cultivars [23], *Melocactus* species [28], *Beta vulgaris* cultivars [29, 30], and *Amaranthus* species [24]. Among the betalains in the samples of red amaranth, six amarantin-type, two gomphrenin-type, 22 betanin-type, and 13 betaxanthins were detected. Amarantin-type includes amarantin, isoamarantin that is the epimer of amarantin, and amarantin-derivatives (Fig. 4). Gomphrenin-type includes gomphrenin-I and its epimer. Betanin-type include prebetanin, betanin, betanidin, isobetanin, phyllocactin, isophyllocactin, lampranthin II, isolampranthin II, and betanin-derivatives. It has been reported that decarboxylation and oxidation of betacyanins produce betacyanin-derivatives, usually strengthened by high temperatures [2, 31, 32]. When comparing the betacyanin-derivatives, we found a smaller number of amarantin-derivatives than betanin-derivatives. Betaxanthins that were identified in red amaranth include amine-derived betaxanthins, for instance, histamine-, tyramine-, and dopamine-betaxanthin. Non-polar amino acid-derived betaxanthins, for instance, tryptophan-, phenylalanine-, leucine-, alanine-, and proline-betaxanthin, and polar amino acid-derived betaxanthins such as serine-, threonine-, and glutamine-betaxanthin were also identified.

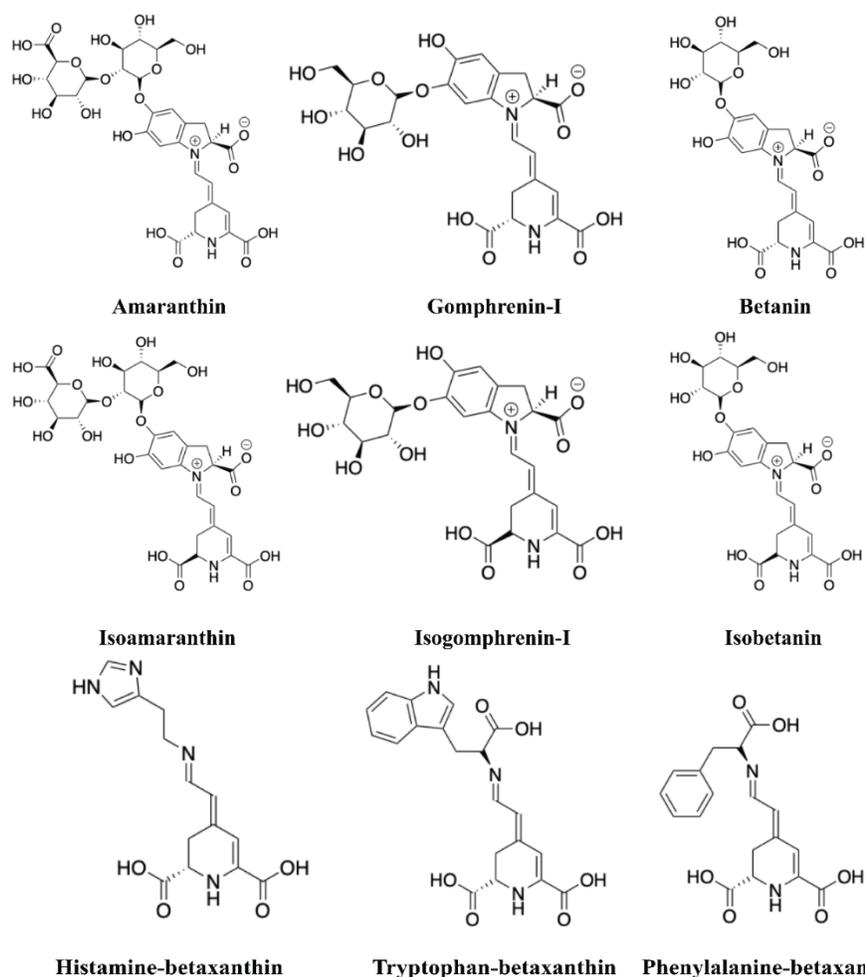


Fig. 4. Chemical structures of the main components of betalains in the red amaranth (*A. cruentus* L.)

To reveal the most abundant betalains in red amaranth, relative quantification was performed since no commercially available analytical standards for betalains were available. Peak areas of each betalain in MS1 mode using the extracted ion chromatograms based on the precursor ion $[M+H]^+$ were measured and \log_{10} -transformed (Fig. 5). The heat map highlights the most abundant betacyanins and betaxanthins (the darker the colour, the higher the concentration). The amaranthin with the highest abundance in the red amaranth samples agrees with previous studies [20, 25, 33]. Amaranthin was four times more abundant in the inflorescences than in leaves and was nearly 70 % of the betacyanin composition. Isoamaranthin, which is the epimer of amaranthin, was also detected but is less abundant than amaranthin in both inflorescences and leaves of red amaranth. Amaranthin and its epimer isoamaranthin were reported as abundant betacyanins and widely distributed in thirty-seven species of eight genera of *Amaranthus* [24]. Among the forty accessions studied by Cai et al. (2001) [24], amaranthin and isoamaranthin were displayed up to 91.5 and 2.8 % in average of the betacyanin composition. The amaranthin-derivatives abundance was higher than that of betanin-derivatives; we found three and ten times more amaranthin-derivatives than betanin-derivatives in inflorescences than in leaves, respectively. This finding agrees with the fact that betacyanin-derivatives were generated spontaneously from betacyanins, thus more abundance of amaranthin than betanin can result in higher abundance of either 17-decarboxy-amaranthin, decarboxy-(iso)amaranthin, and neo-amaranthin than betanin-derivatives. The main betaxanthins in leaves and inflorescences of red amaranth were histamine-betaxanthin, non-polar amino acid-derived betaxanthins (tryptophan-, phenylalanine-, leucine-, alanine-, proline-betaxanthin) and polar amino acid-derived betaxanthins (serine-, threonine-, glutamine-betaxanthin), which form about 90 % of the betaxanthin's composition. A comparison of individual betaxanthins abundance revealed that there was a higher abundance in leaves than in inflorescences. These findings suggest that both betacyanins and betaxanthins can be produced in red amaranth.

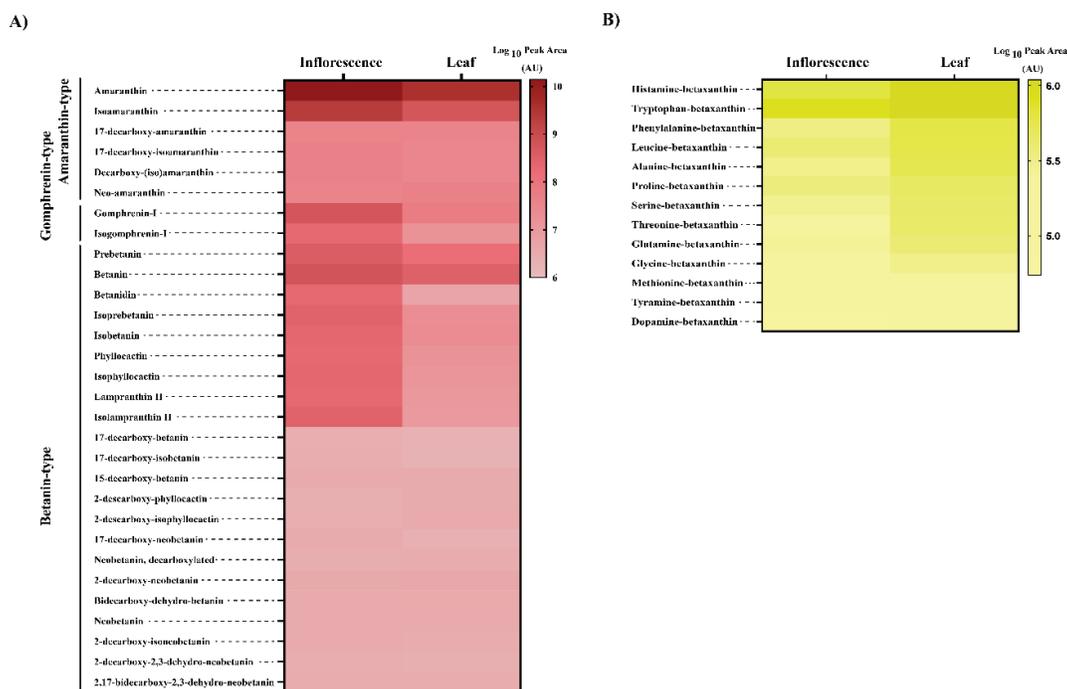


Fig. 5. Relative abundances of main betalains in red amaranth. **(A)** Heat map of relative abundances calculated in \log_{10} for main betacyanins. **(B)** Heat map of relative abundances calculated in \log_{10} for main betaxanthins. The colour bar indicates the \log_{10} -transformed peak area values.

Given that red amaranth used in this study was red colour in almost, if not all, tissues including stems, leaves, and inflorescences, the betacyanins and betaxanthins detected imply that red amaranth is supported by a betalains biosynthetic pathway. There have been studies focused on improving the production of betalains in plants that have revealed the L-tyrosine use and key genes responsible for the overaccumulation of betacyanins and betaxanthins [34, 35]. Betalains are synthesized from L-tyrosine that is hydroxylated to L-DOPA by a group of redundant tyrosine hydroxylases (CYP76AD1/5/6/15). L-DOPA is further converted to 4,5-seco-DOPA by L-DOPA 4,5-dioxygenase (DODA), and then to betalamic acid after spontaneous intramolecular condensation occurs in the unstable 4,5-seco-DOPA [35, 36]. L-DOPA can be alternatively oxidized to *cyclo*-DOPA by CYP76AD1/3. When the betalamic acid is conjugated with *cyclo*-DOPA, the red-violet betacyanins are generated, whereas the conjugation with imino or amino group of amino acids generates yellow betaxanthins. Wang and co-workers (2017) [34], using red beet varieties that produce high yield of betacyanins and yellow beet varieties that produce high yields of betaxanthins, have found that accumulation of betacyanins needs high concentration of L-tyrosine whereas betaxanthins requires lowering to convert L-DOPA to *cyclo*-DOPA. As most research on amaranth has been focused on nutrient values, our LC-MS analysis of the leaves and inflorescences allowed the characterization of the 43 betalains in red amaranth. This data expands the information for betalain content in *A. cruentus* got with spectrophotometric techniques [18]. New products and technologies based on the use of *A. cruentus* have been generated recently [37, 38].

Conclusions

In the present study, we tried to valorize the red amaranth (*A. cruentus* L.) as betalain pigments source and develop the LC-MS platform to monitor the composition of betalains. Consequently, betalains from the leaves and inflorescences were identified and analyzed by LC-MS to determine the composition of betalains. Our findings define the main betalains in the leaves and inflorescences of red amaranth (*A. cruentus* L.), consisting of six amaranthin-type, two gomphrenin-type, 22 betanin-type, and 13 betaxanthins. LC coupled with high-resolution mass spectrometry allowed to identify the betalains with mass measurement errors of less than 5 ppm. Betacyanins-derivatives were included in the identified betalains. The wide variety of betalains content underlines the importance of using leaves and inflorescences as sources of colourants. The composition of betalains in red amaranth can allow the future characterization of these colourants. This study served as a platform to monitoring the composition of betalains useful for revealing their impact in key biological and pharmacological activities.

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Table 1. Chromatographic and MS data for betalains in red amaranth samples.

#	Compound	Retention time (Rt)	Relative Rt to betanin	Chemical Formula	Theoretical m/z [M+H]	Observed m/z [M+H]	Mass error (ppm)	Fragments
<i>Amaranthin-type</i>								
1	Amaranthin	1.85	0.53	C ₃₀ H ₃₄ N ₂ O ₁₉	727.1826	727.1803	-2.3	389.09, 343.09, 297.08, 194.04, 150.05
2	Isoamaranthin	2.70	0.78	C ₃₀ H ₃₄ N ₂ O ₁₉	727.1826	727.1806	-2.0	389.09, 343.09, 297.08, 194.04, 150.05
3	17-decarboxy-amaranthin	3.07	0.89	C ₂₉ H ₃₄ N ₂ O ₁₇	683.1930	683.1918	1.2	389.09, 343.09, 297.08, 194.04, 150.05
4	17-decarboxy-isoamaranthin	6.05	1.76	C ₂₉ H ₃₄ N ₂ O ₁₇	683.1930	683.1915	1.5	389.09, 343.09, 297.08, 194.04, 150.05
5	Decarboxy-(iso)amaranthin	7.68	2.23	C ₂₉ H ₃₄ N ₂ O ₁₇	683.1930	683.1922	0.8	389.09, 343.09, 297.08, 194.04, 150.05
6	Neo-amaranthin	7.25	2.11	C ₃₀ H ₃₂ N ₂ O ₁₉	725.1672	725.1628	4.3	389.09, 343.09, 297.08, 194.04, 150.05
<i>Gomphrenin-type</i>								
7	Gomphrenin-I	4.06	1.18	C ₂₄ H ₂₆ N ₂ O ₁₃	551.1508	551.1475	-3.26	389.09, 343.09, 297.08, 194.04, 150.05
8	Isogomphrenin-I	6.87	2.00	C ₂₄ H ₂₆ N ₂ O ₁₃	551.1508	551.1473	-3.53	389.09, 343.09, 297.08, 194.04, 150.05
<i>Betanin-type</i>								
9	Prebetanin	3.19	0.93	C ₂₄ H ₂₆ N ₂ O ₁₆ S	630.1003	630.0961	-4.24	389.09, 343.09, 297.08, 194.04, 150.05
10	Betanin	3.93	1.00	C ₂₄ H ₂₆ N ₂ O ₁₃	551.1508	551.1483	-2.51	389.09, 343.09, 297.08, 194.04, 150.05
11	Betanidin	3.44	1.00	C ₁₈ H ₁₆ N ₂ O ₈	389.0979	389.0941	-3.77	389.09, 343.09, 297.08, 194.04, 150.05
12	Isoprebetanin	6.01	1.75	C ₂₄ H ₂₆ N ₂ O ₁₆ S	630.1003	630.0979	-2.39	389.09, 343.09, 297.08, 194.04, 150.05
13	Isobetanin	6.76	1.92	C ₂₄ H ₂₆ N ₂ O ₁₃	551.1508	551.1481	-2.69	389.09, 343.09, 297.08, 194.04, 150.05
14	Phyllocactin	7.71	2.25	C ₂₇ H ₂₉ N ₂ O ₁₆	638.1596	638.1574	-2.17	389.09, 343.09, 297.08, 194.04, 150.05
15	Isophyllocactin	8.46	2.47	C ₂₇ H ₂₉ N ₂ O ₁₆	638.1596	638.1574	-2.23	389.09, 343.09, 297.08, 194.04, 150.05
16	Lampranthin II	11.00	3.21	C ₃₄ H ₃₄ N ₂ O ₁₆	727.1989	727.1946	-4.31	389.09, 343.09, 297.08, 194.04, 150.05
17	Isolampranthin II	11.34	3.31	C ₃₄ H ₃₄ N ₂ O ₁₆	727.1989	727.1946	-4.29	389.09, 343.09, 297.08, 194.04, 150.05
18	17-decarboxy-betanin	5.83	1.70	C ₂₃ H ₂₆ N ₂ O ₁₁	507.1609	507.1574	-3.52	345.10, 299.10, 194.04, 150.05, 106.06

Table 1. Chromatographic and MS data for betalains in red amaranth samples. (Continued)

#	Compound	Retention time (Rt)	Relative Rt to betanin	Chemical Formula	Theoretical m/z [M+H]	Observed m/z [M+H]	Mass error (ppm)	Fragments
19	17-decarboxy-isobetainin	7.69	2.24	C ₂₃ H ₂₆ N ₂ O ₁₁	507.1609	507.1587	-2.24	345.10, 299.10, 194.04, 150.05, 106.06
20	15-decarboxy-betanin	8.29	2.42	C ₂₃ H ₂₆ N ₂ O ₁₁	507.1609	507.1574	-3.57	345.10, 299.10, 194.04, 150.05, 106.06
21	2-descarboxy-phyllactin	9.21	2.69	C ₂₆ H ₂₉ N ₂ O ₁₄	593.1613	593.1578	-3.51	345.10, 299.10, 194.04, 150.05, 106.06
22	2-descarboxy-isophylloactin	9.41	2.74	C ₂₆ H ₂₉ N ₂ O ₁₄	593.1613	593.1589	-2.43	345.10, 299.10, 194.04, 150.05, 106.06
23	17-decarboxy-neobetainin	3.62	1.06	C ₂₃ H ₂₄ N ₂ O ₁₁	505.1453	505.142	-3.31	343.09, 297.08, 194.04, 150.05, 106.06
24	Neobetainin, decarboxylated	6.6	1.92	C ₂₃ H ₂₄ N ₂ O ₁₁	505.1453	505.1415	-3.83	343.09, 297.08, 194.04, 150.05, 106.06
25	2-decarboxy-neobetainin	6.71	1.96	C ₂₃ H ₂₄ N ₂ O ₁₁	505.1453	505.1421	-3.24	343.09, 297.08, 194.04, 150.05, 106.06
26	Bidecarboxy-dehydro-betanin	7.61	2.22	C ₂₂ H ₂₄ N ₂ O ₉	461.1555	461.1508	-4.71	299.10, 297.08, 194.04, 150.05, 106.06
27	Neobetainin	8.31	2.42	C ₂₄ H ₂₄ N ₂ O ₁₃	549.1351	549.1306	-4.50	387.07, 297.08, 194.04, 150.05, 106.06
28	2-decarboxy-isoneobetainin	8.39	2.45	C ₂₃ H ₂₄ N ₂ O ₁₁	505.1453	505.1404	-4.90	343.09, 297.08, 194.04, 150.05, 106.06
29	2-decarboxy-2,3-dehydro-neobetainin	10.06	2.93	C ₂₃ H ₂₃ N ₂ O ₁₁	503.1296	503.1271	-2.50	341.07, 297.08, 194.04, 150.05, 106.06
30	2,17-bidecarboxy-2,3-dehydro-neobetainin	11.14	3.25	C ₂₂ H ₂₂ N ₂ O ₉	459.1398	459.1354	-4.41	297.08, 269.09, 251.08, 223.08, 195.09

Table 1. Chromatographic and MS data for betalains in red amaranth samples. (Continued)

#	Compound	Retention time (Rt)	Relative Rt to betanin	Chemical Formula	Theoretical m/z [M+H]	Observed m/z [M+H]	Mass error (ppm)	Fragments
<i>Betaxanthins</i>								
31	Histamine-betaxanthin	9.04	2.64	C ₁₄ H ₁₆ N ₄ O ₄	305.1244	305.1206	-3.76	287.12, 261.13, 211.07, 194.04, 150.05, 106.06
32	Tryptophan-betaxanthin	10.12	2.95	C ₂₀ H ₁₉ N ₃ O ₆	398.1340	398.1318	-2.19	269.07, 223.07, 211.07, 194.04, 150.05, 106.06
33	Phenylalanine-betaxanthin	10.15	2.96	C ₁₈ H ₁₈ N ₂ O ₆	359.1230	359.1199	-3.15	315.13, 313.11, 211.07, 194.04, 150.05, 106.06
34	Leucine-betaxanthin (Vulgaxanthin IV)	10.01	2.92	C ₁₅ H ₂₀ N ₂ O ₆	325.1387	325.1361	-2.56	281.14, 251.13, 211.07, 194.04, 150.05, 106.06
35	Alanine-betaxanthin	2.02	0.59	C ₁₂ H ₁₄ N ₂ O ₆	283.0920	283.0887	-3.30	237.08, 211.07, 194.04, 150.05, 106.06
36	Proline-betaxanthin (Indicaxanthin)	3.04	0.89	C ₁₄ H ₁₆ N ₂ O ₆	309.1075	309.1047	-2.82	265.11, 211.07, 194.04, 150.05, 106.06
37	Serine-betaxanthin	4.77	1.39	C ₁₂ H ₁₄ N ₂ O ₇	299.0869	299.0831	-3.84	255.09, 253.09, 211.07, 194.04, 150.05, 106.06
38	Threonine-betaxanthin	1.52	0.44	C ₁₃ H ₁₆ N ₂ O ₇	313.1022	313.1004	-1.83	269.09, 267.09, 211.07, 194.04, 150.05, 106.06
39	Glutamine-betaxanthin (Vulgaxanthin I)	1.20	0.35	C ₁₄ H ₁₇ N ₃ O ₇	340.1134	340.1102	-3.20	323.08, 277.08, 211.07, 194.04, 150.05, 106.06
40	Glycine-betaxanthin (Portulacaxanthin II)	6.49	1.89	C ₁₁ H ₁₂ N ₂ O ₆	269.0756	269.0738	-1.78	331.12, 239.11, 211.07, 194.04, 150.05, 106.06
41	Methionine-betaxanthin	7.30	2.13	C ₁₄ H ₁₈ N ₂ O ₆ S	343.095	343.0916	-3.36	315.09, 269.09, 211.07, 194.04, 150.05, 106.06
42	Tyramine-betaxanthin (Miraxanthin III)	8.64	2.52	C ₁₇ H ₁₈ N ₂ O ₅	331.1281	331.1239	-4.21	287.13, 239.11, 211.07, 194.04, 150.05, 106.06
43	Dopamine-betaxanthin (Miraxanthin V)	7.67	2.24	C ₁₇ H ₁₈ N ₂ O ₆	347.123	347.1213	-1.75	255.11, 211.07, 194.04, 150.05, 106.06