Plant Physiology®

Plant Physiology, 2024, **196**, 446–460
https://doi.org/10.1093/plphys/kiae312
Advance access publication 3 June 2024
Research Article

Dopamine-derived pigments in nature: identification of decarboxybetalains in Amaranthaceae species

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Abstract

A unique family of decarboxylated betalains derived from dopamine has recently been discovered. Due to the lack of chemical standards, the existence and distribution of decarboxylated betalains in nature remain unknown. Traditional betalains contain L-dihydroxyphenylalanine as the starting point of the biosynthetic pathway and betalamic acid as a structural and functional unit, while the recently discovered betalains rely on dopamine. Here, 30 dopamine-derived betalains were biotechnologically produced, purified, and characterized, creating an unprecedented library to explore their properties and presence in nature. The maximum absorbance wavelengths for the pigments ranged between 461 and 485 nm. HPLC analysis showed retention times between 0.6 and 2.2 min higher than traditional betalains due to their higher hydrophobicity. The presence of decarboxybetalains in nature was screened using HPLC-ESI-Q-TOF mass spectrometry in various species of the Amaranthaceae family: beetroot (*Beta vulgaris* subsp. *vulgaris*), Swiss chard (*B. vulgaris* var. *cicla*), celosia (*Celosia argentea* var. *plumosa*), and quinoa (*Chenopodium quinoa*). The latter species had the highest content of decarboxybetalains (28 compounds in its POEQ-143 variety). Twenty-nine pigments were found distributed among the different analyzed plant sources. The abundance of decarboxybetalains demonstrated in this work highlights these pigments as an important family of phytochemicals in the order Caryophyllales.

Introduction

In nature, there exist only a few families of compounds derived from dopamine. This molecule is a catecholamine involved in the biosynthetic pathway of phytochemicals such as tetrahydroisoquinoline alkaloids (McMurtrev et al. 1980). However, plant pigments derived from dopamine are scarce, and only 2 molecules have been described, both belonging to the betalain group (Henarejos-Escudero et al. 2021). Betalains are nitrogenous, water-soluble compounds that constitute the main pigment of most plants belonging to the order Caryophyllales (Piatelli 1981; Moreno et al. 2008). These secondary metabolites have betalamic acid as a common structural and functional unit. Depending on the molecule with which it is condensed, there exist 2 types of betalains: betaxanthins, which present amino acids and amines linked to betalamic acid, and betacyanins, which are formed by the union of indoline-type molecules such as *cyclo*-dihydroxyphenylalanine (*cyclo*-DOPA) (Gandía-Herrero et al. 2010). The biosynthesis of these pigments involves the enzyme 4,5-DOPA-extradiol-dioxygenase (4,5-DODA), which is considered key to their formation (Gandía-Herrero and García-Carmona 2013). Betaxanthins show orange–yellow coloration, with maximum absorbance values around a wavelength of 480 nm, whereas betacyanins present violet coloration, with maximum absorbance values around 536 nm. The difference in coloration is due to the system of conjugated double bonds present in betalamic acid which, in the case of betacyanins, is extended along the aromatic ring of indoline. In addition, betaxanthins' fluorescence is much more intense than that of betacyanins (Gandía-Herrero et al. 2010).

Betalains have important functions in plant physiology, including the role in attracting animals for pollination (Stintzing and Carle 2004). The production of these compounds is regulated by numerous endogenous and exogenous factors, and once synthesized, they are accumulated in the vacuoles of epidermal and subepidermal plant cells (Xiao-Hong et al. 2009). Among the exogenous factors, there is evidence that salinity stress, drought, excess light and/or UV radiation, and low temperatures can substantially affect betalain production (Vogt et al. 1999; Ibdah et al. 2002; Hayakawa and Agarie 2010; Marchesini et al. 2014; Jain and Gould 2015). In fact, several studies have shown that reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) induce their production, since these pigments are able to neutralize them and thus reduce the damage caused by wounds and bacterial infiltrations (Sepúlveda-Jiménez et al. 2004; Wang et al. 2007). In addition, in some *Amaranthus* species subjected to excess light or UV radiation, betalains have been found to play an

Received April 2, 2024. Accepted May 10, 2024.

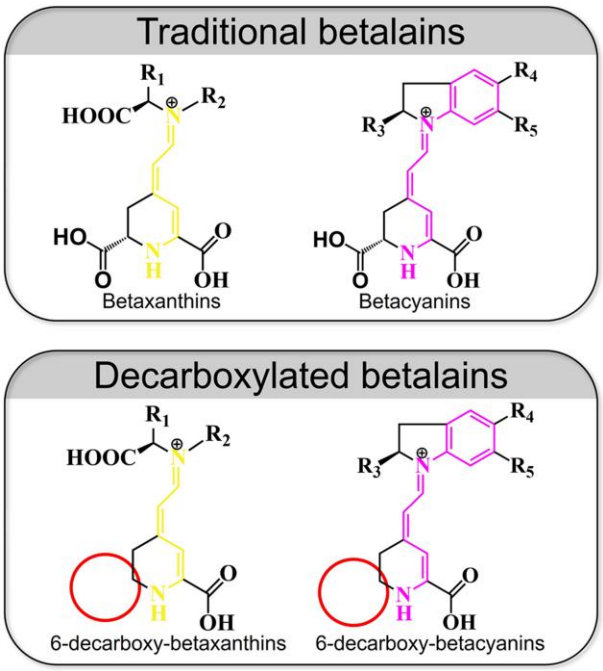

Figure 1. Chemical structures of traditional and decarboxylated betalains. In the latter, the absence of the carboxyl group at C_6 is indicated by a circle. In betaxanthins, R_1 and R_2 indicate *H* or the side chain of the amine group condensed with betalamic or 6-decarboxy-betalamic acid. In betacyanins, R_3, R_4, and R_5 indicate -COOH, -OH, or sugar groups.

important photoprotective role (Edreva 2005; Shao et al. 2013). Betalains also act as osmotically active solutes, and they can counteract osmotic disturbances and help plants adapt to extreme conditions (Jain and Gould 2015).

Recently, 2 members of a singular family of betalains, called decarboxybetalains, have been discovered, characterized by the presence of a decarboxylated betalamic acid (6-decarboxy-betalamic acid) derived from dopamine (Henarejos-Escudero, Hernández-García, et al. 2022). This structural unit is capable of originating betaxanthins and betacyanins without one of the carboxylic groups (-COOH) of the ring due to the elimination of a stereogenic carbon (Fig. 1).

The biosynthesis of traditional betalains begins with the hydroxylation of L-tyrosine by cytochrome P450-type enzymes (Sunnadeniya et al. 2016), to produce L-3,4-dihydroxyphenylalanine (L-DOPA). L-DOPA is the traditional substrate of the enzyme 4,5-DOPA-extradiol-dioxygenase (4,5-DODA), which catalyzes the opening of the aromatic ring and the formation of an aldehyde group to form the intermediate 4,5-seco-DOPA. This compound undergoes a spontaneous intramolecular condensation between its amino group and the enzymatically produced aldehyde group (-CHO), producing betalamic acid. This molecule may undergo spontaneous condensations with the amino groups of amines and amino acids to form betaxanthins or with indoline-type molecules such as *cyclo*-DOPA to form betacyanins (Gandía-Herrero and García-Carmona 2013; Gandía-Herrero and García-Carmona 2020) (Fig. 2).

The recent discovery of 2 decarboxylated betalains in the betalamic acid moiety allowed the determination of the dual character of the enzyme 4,5-DODA (Henarejos-Escudero, Martínez-Rodríguez, et al. 2022). Thus, it has been described that quinoa (*Chenopodium quinoa*) is capable of producing this variety of 6-decarboxylated betalains from dopamine, establishing a highly branched pathway that leads to the formation of traditional or decarboxylated betalains depending on the ratio of the catecholamines L-DOPA and dopamine. As a result, the incorporation of dopamine originates the formation of decarboxylated betalains such as DOPA-6-decarboxy-betaxanthin and dopamine-6-decarboxy-betaxanthin, being the 6-decarboxylated variants of dopaxanthin and miraxanthin-V, respectively, formed from L-DOPA (Fig. 2).

Traditional betalains can be found in roots, fruits, and leaves of plants of the order Caryophyllales (Gandía-Herrero and García-Carmona 2013). The most widely exploited betalain-producing plant crop is red beet (*Beta vulgaris* subsp. *vulgaris*), followed by the grains of *Amaranthus* sp. and the fruits of prickly pear (*Opuntia ficus-indica*) and *Hylocereus* spp. cactus (Azeredo 2009). They are also the pigments that give color to the flowers of *Celosia* sp., globe amaranth (*Gomphrena globosa*), rose moss (*Portulaca grandiflora*), *Bougainvillea* sp., pokeweed (*Phytolacca americana*), and purple ice plant (*Lampranthus productus*) (Gandía-Herrero et al. 2005a; Esquivel 2016). In addition, betalains are present in minor quantities in lesser-known sources such as ulluco (*Ullucus tuberosus*), pigeon berry (*Rivina humilis*), Malabar spinach (*Basella alba*), djulis (*Chenopodium formosanum*), Surinam purslane (*Talinum triangulare*), and *Eulychnia* (Martínez-Rodríguez et al. 2022). Other studies have shown that they are part of culturally important crops and foods, as is the case of quinoa (*C. quinoa*) (Escribano et al. 2017). Specifically, betalains are especially diverse in yellow quinoa varieties.

In this work, a search for decarboxylated betalains is performed in plant species of the family Amaranthaceae, belonging to the order Caryophyllales. Specifically, the presence of possible members of the singular family of pigments is screened in red and yellow varieties of beetroot (*B. vulgaris* subsp. *vulgaris*), Swiss chard (*B. vulgaris* var. *cicla*), and celosia (*Celosia argentea* var. *plumosa*) and in BGQ-24, BGQ-174, POEQ-143, and POQ-36 varieties of quinoa (*C. quinoa*) (Fig. 3).

Here, a metabolomic approach is applied to screen the presence of 6-decarboxy-betaxanthins in plants from the Amaranthaceae species. Reference standards of a wide range of decarboxylated betaxanthins were prepared "in-house" taking into account the structures of all the traditional betalains previously identified in plants (Gandía-Herrero and García-Carmona 2013). This metabolomic approach to the detection of these pigments substantially expands the number of phytochemicals present in relevant plant species and crops.

Results

Production and characterization of decarboxybetalains reference standards

Commercial reference standards are not available for members of the decarboxybetalain family. Therefore, prior to detection in natural sources, the 6-decarboxy-betaxanthins were obtained, purified, and characterized. 6-Decarboxy-betaxanthins were prepared in bioreactors containing dopamine as the enzyme substrate to produce 6-decarboxy-betalamic acid and amines or amino acids for its further condensation. The appearance of a yellow–orange coloration was indicative of the formation of 6-decarboxy-betaxanthins.

Once all the bioreactors were processed, HPLC analytical studies were carried out to verify the presence of 6-decarboxy-betalamic acid and to search for the 6-decarboxy-betaxanthins of interest. The formation of the compounds in the bioreactors

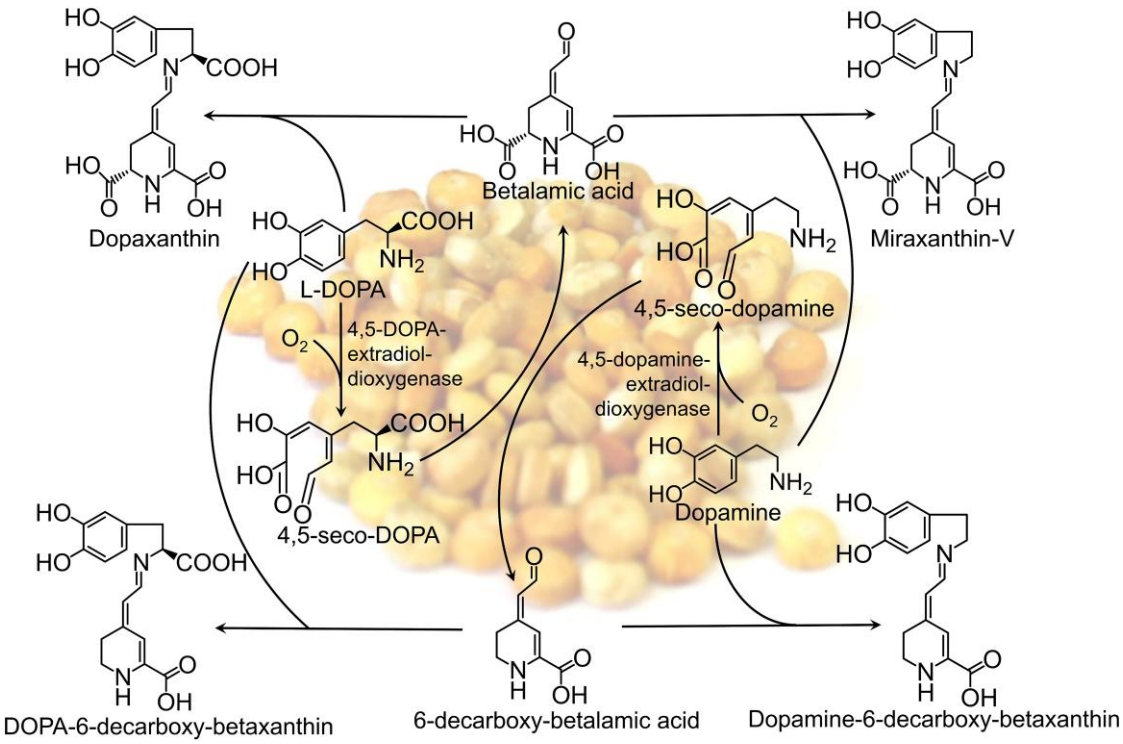

Figure 2. Dual biosynthetic pathway, initially described in *C. quinoa*, able to produce traditional or decarboxylated betalains with the participation of a single ring-opening enzyme. DOPA, dihydroxyphenylalanine.

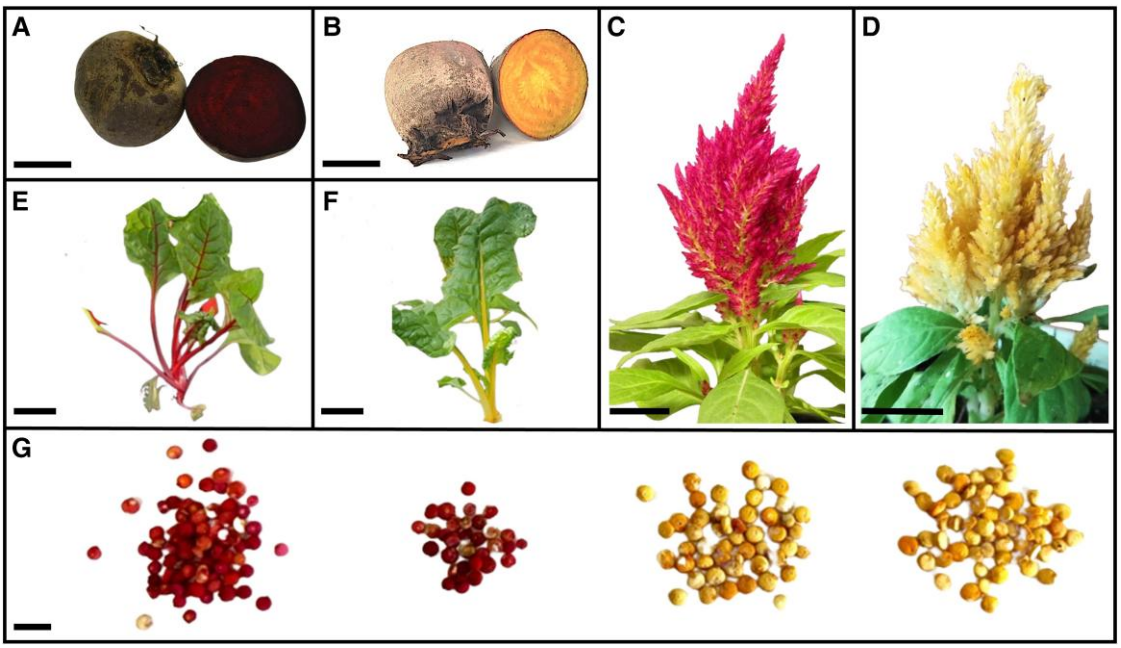

Figure 3. Plant sources used in this work. Red **A)** and yellow **B)** beetroots, red **C)** and yellow **D)** celosia plants, red **E)** and yellow **F)** Swiss chards, and colored quinoa **G)** of varieties BGQ-24, BGQ-174, POEQ-143, and POQ-36 (from left to right). Images were digitally extracted for comparison. Scale bars: 4 cm **A, B)**; 2 cm **C, D)**; 4 cm **E, F)**; 0.5 cm **G)**.

was monitored at different wavelengths according to the absorbance maxima of each molecule: 405 nm for 6-decarboxy-betalamic acid and 480 nm for 6-decarboxy-betaxanthins. After individual analysis of each bioreactor, the formation of the structural unit of the decarboxylated betalains, 6-decarboxy-betalamic acid, was observed in all of them. This molecule presented a retention time (RT) of 17.66 min, similar in all bioreactors, detected at a wavelength of 405 nm (Supplementary Fig. S1). The dopamine-6-decarboxy-betaxanthin molecule (RT = 16.70 min at 480 nm), formed by condensation between 6-decarboxy-betalamic

Table 1. Experimental data obtained for the pure decarboxylated betalains used in this work

Betalain	HPLC RT (min)	λ_{max} (nm)	TOF exact mass		
			Theoretical (*m/z*)	Experimental (*m/z*)	Δppm
(1) Ethanolamine-6-decarboxy-betaxanthin	10.42	466	211.1077	211.1082	2.36845932
(2) Glycine-6-decarboxy-betaxanthin	10.06	467	225.0870	225.0869	−0.44427266
(3) Putrescine-6-decarboxy-betaxanthin	10.05	466	238.1550	238.1550	0.00000000
(4) Alanine-6-decarboxy-betaxanthin	12.20	468	239.1026	239.1036	4.18230500
(5) γ-Aminobutyric acid-6-decarboxy-betaxanthin	13.16	477	253.1183	253.1192	3.55564967
(6) Serine-6-decarboxy-betaxanthin	9.14	461	255.0975	255.0977	0.78401396
(7) Histamine-6-decarboxy-betaxanthin	9.91	468	261.1346	261.1346	0.00000000
(8) Proline-6-decarboxy-betaxanthin	13.76	479	265.1183	265.1185	0.75438021
(9) Valine-6-decarboxy-betaxanthin	16.80	469	267.1339	267.1343	1.49737641
(10) Norvaline-6-decarboxy-betaxanthin	17.59	469	267.1339	267.1343	1.49737641
(11) Threonine-6-decarboxy-betaxanthin	10.63	469	269.1132	269.1142	3.71590840
(12) Phenylethylamine-6-decarboxy-betaxanthin	23.83	469	271.1441	271.1452	4.05688341
(13) Hydroxyproline-6-decarboxy-betaxanthin	9.55	479	281.1132	281.1136	1.42291433
(14) Leucine-6-decarboxy-betaxanthin	20.31	469	281.1496	281.1501	1.77841263
(15) Isoleucine-6-decarboxy-betaxanthin	19.85	470	281.1496	281.1501	1.77841263
(16) Asparagine-6-decarboxy-betaxanthin	8.33	468	282.1084	282.1095	3.89921037
(17) Tyramine-6-decarboxy-betaxanthin	18.67	469	287.1390	287.1392	0.69652677
(18) Glutamine-6-decarboxy-betaxanthin	9.40	469	296.1241	296.1257	5.40314010
(19) Lysine-6-decarboxy-betaxanthin	9.06	469	296.1605	296.1610	1.68827376
(20) Glutamic acid-6-decarboxy-betaxanthin	11.10	468	297.1081	297.1098	5.72182313
(21) Methionine-6-decarboxy-betaxanthin	16.87	469	299.1060	299.1064	1.33731854
(22) Dopamine-6-decarboxy-betaxanthin	16.70	469	303.1339	303.1346	2.30921055
(23) Histidine-6-decarboxy-betaxanthin	8.16	469	305.1244	305.1258	4.58829251
(24) Methionine sulfoxide-6-decarboxy-betaxanthin	9.55	469	315.1009	315.1010	0.31735866
(25) Phenylalanine-6-decarboxy-betaxanthin	21.05	471	315.1339	315.1343	1.26930172
(26) Methoxytyramine-6-decarboxy-betaxanthin	19.06	470	317.1496	317.1502	1.89185167
(27) Arginine-6-decarboxy-betaxanthin	9.86	469	324.1666	324.1669	0.92545006
(28) Tyrosine-6-decarboxy-betaxanthin	16.28	473	331.1288	331.1288	0.00000000
(29) DOPA-6-decarboxy-betaxanthin	14.49	485	347.1238	347.1249	3.16889824
(30) Tryptophan-6-decarboxy-betaxanthin	21.83	473	354.1448	354.1469	5.92977788

The RT obtained by HPLC, the maximum absorbance wavelength (λ_{max}), and the exact mass determined by HPLC-ESI-Q-TOF-MS for each molecule are indicated. Reversed-phase chromatography was performed with a C-18 column by using a linear gradient with water and acetonitrile, both supplemented with 0.05% TFA (*v/v*), as the mobile phase

acid and dopamine, was also detected. The difference between the different bioreactors was the compound added for the condensation with 6-decarboxy-betalamic acid. Thus, the presence of additional compounds, detected at 480 nm among the different bioreactors, indicated the possible formation of the expected molecules.

Purification of each of the previously produced compounds was performed by preparative HPLC. The purity of all of them (tentatively identified as decarboxylated betalains) was verified by an additional analysis in analytical HPLC (Supplementary Fig. S2). The RTs obtained were those identified in the initial analysis described above, having succeeded in purifying the compounds of interest. All RT values are shown in Table 1. The degree of purity of the pigments was verified by 3-dimensional chromatograms as shown in Supplementary Fig. S3. The presence of a single compound and the absence of impurities were observed in the UV–visible (vis) spectrum using a wide range of wavelengths (350 to 800 nm).

The absorbance spectra of the 30 decarboxybetalains were analyzed using the analytical HPLC diode array detector (DAD) (Supplementary Fig. S4). The results obtained showed a λ_{max} range of 461 to 485 nm for the 6-decarboxy-betaxanthins, responsible for their yellow color. The specific values for λ_{max} obtained for each compound are also shown in Table 1.

After purification, each compound was analyzed by HPLC-ESI-Q-TOF-MS. Thus, the exact molecular mass and the formula and identity of each pigment derived from 6-decarboxy-betalamic acid were determined. The experimental mass (*m/z*) and Δppm results obtained are shown in Table 1, and the formula of the single compounds is summarized in Table 2. The MS–MS data obtained showed the loss of carboxyl groups in all pigments (Fig. 4A and Supplementary Fig. S5A). In addition, the isotopic distribution profile obtained in each case showed a strong association between the proposed formula and the mass spectra of each molecule (Fig. 4B and Supplementary Fig. S5B). The data confirmed the existence of decarboxylated betalains, and each mass supported the identity of the compounds derived from decarboxylated betalamic acid and the added amine, in agreement with the Δppm ranges previously established as valid (Sivaperumal et al. 2015). Therefore, a library of 30 6-decarboxy-betaxanthin reference standards was obtained to detect these compounds in natural sources.

6-Decarboxylated betaxanthin profiles in Amaranthaceae species

Aqueous extracts of plant species of the Amaranthaceae family were prepared and analyzed by HPLC-ESI-Q-TOF-MS using the same HPLC detection method used for the individual 6-decarboxy-betaxanthin standards. The resulting extracted ion chromatograms (EICs) were analyzed considering the reference standards prepared (by exact masses and RTs).

The extracts of the 2 varieties of Swiss chard (*B. vulgaris* var. *cicla*), red and yellow, showed different 6-decarboxy-betaxanthin profiles. Specifically, in the red Swiss chard variety, DOPA-6-decarboxy-betaxanthin (**29**) was identified. The presence of this molecule was detected both by its exact mass and in the DAD chromatogram at 480 nm (Supplementary Table S1). Meanwhile,

Table 2. Chemical formula for the compounds

Betalain	Chemical formula
(**1**) Ethanolamine-6-decarboxy-betaxanthin	$C_{10}H_{14}N_2O_3$
(**2**) Glycine-6-decarboxy-betaxanthin	$C_{10}H_{12}N_2O_4$
(**3**) Putrescine-6-decarboxy-betaxanthin	$C_{12}H_{19}N_3O_2$
(**4**) Alanine-6-decarboxy-betaxanthin	$C_{11}H_{14}N_2O_4$
(**5**) γ-Aminobutyric acid-6-decarboxy-betaxanthin	$C_{12}H_{16}N_2O_4$
(**6**) Serine-6-decarboxy-betaxanthin	$C_{11}H_{14}N_2O_5$
(**7**) Histamine-6-decarboxy-betaxanthin	$C_{13}H_{16}N_4O_2$
(**8**) Proline-6-decarboxy-betaxanthin	$C_{13}H_{17}N_2O_4$
(**9**) Valine-6-decarboxy-betaxanthin	$C_{13}H_{18}N_2O_4$
(**10**) Norvaline-6-decarboxy-betaxanthin	$C_{13}H_{18}N_2O_4$
(**11**) Threonine-6-decarboxy-betaxanthin	$C_{12}H_{16}N_2O_5$
(**12**) Phenylethylamine-6-decarboxy-betaxanthin	$C_{16}H_{18}N_2O_2$
(**13**) Hydroxyproline-6-decarboxy-betaxanthin	$C_{13}H_{17}N_2O_5$
(**14**) Leucine-6-decarboxy-betaxanthin	$C_{14}H_{20}N_2O_4$
(**15**) Isoleucine-6-decarboxy-betaxanthin	$C_{14}H_{20}N_2O_4$
(**16**) Asparagine-6-decarboxy-betaxanthin	$C_{12}H_{15}N_3O_5$
(**17**) Tyramine-6-decarboxy-betaxanthin	$C_{16}H_{18}N_2O_3$
(**18**) Glutamine-6-decarboxy-betaxanthin	$C_{13}H_{17}N_3O_5$
(**19**) Lysine-6-decarboxy-betaxanthin	$C_{14}H_{21}N_3O_4$
(**20**) Glutamic acid-6-decarboxy-betaxanthin	$C_{13}H_{16}N_2O_6$
(**21**) Methionine-6-decarboxy-betaxanthin	$C_{13}H_{18}N_2O_4S$
(**22**) Dopamine-6-decarboxy-betaxanthin	$C_{16}H_{18}N_2O_4$
(**23**) Histidine-6-decarboxy-betaxanthin	$C_{14}H_{16}N_4O_4$
(**24**) Methionine sulfoxide-6-decarboxy-betaxanthin	$C_{13}H_{18}N_2O_5S$
(**25**) Phenylalanine-6-decarboxy-betaxanthin	$C_{17}H_{18}N_2O_4$
(**26**) Methoxytyramine-6-decarboxy-betaxanthin	$C_{17}H_{20}N_2O_4$
(**27**) Arginine-6-decarboxy-betaxanthin	$C_{14}H_{21}N_5O_4$
(**28**) Tyrosine-6-decarboxy-betaxanthin	$C_{17}H_{18}N_2O_5$
(**29**) DOPA-6-decarboxy-betaxanthin	$C_{17}H_{18}N_2O_6$
(**30**) Tryptophan-6-decarboxy-betaxanthin	$C_{19}H_{19}N_3O_4$

in the yellow Swiss chard variety, the presence of 5 decarboxylated betalains was detected based on their exact masses: ethanolamine-6-decarboxy-betaxanthin (**1**), glycine-6-decarboxy-betaxanthin (**2**), glutamic acid-6-decarboxy-betaxanthin (**20**), dopamine-6-decarboxy-betaxanthin (**22**), and DOPA-6-decarboxy-betaxanthin (**29**). However, only molecule **29** was identified in the DAD chromatogram at 480 nm (Supplementary Table S2), indicating a higher abundance of this compound in the extract.

As for the beetroot (*B. vulgaris* subsp. *vulgaris*) extracts, different results were also obtained for each of the 2 varieties analyzed, red and yellow. In red beetroot, the presence of DOPA-6-decarboxy-betaxanthin (**29**) was detected by exact mass analysis. Moreover, the molecule abundance allowed its detection by the HPLC-DAD (Supplementary Table S3). On the other hand, yellow beetroot mass spectrometry analysis revealed the presence of glycine-6-decarboxy-betaxanthin (**2**) and glutamine-6-decarboxy-betaxanthin (**18**). However, these molecules were not detected in the chromatograms at 480 nm (Supplementary Table S4), thus suggesting that their presence in the extract was relegated to trace amounts.

With respect to the extracts of red celosia (*C. argentea*), 6 decarboxylated betalains were identified by their exact masses: ethanolamine-6-decarboxy-betaxanthin (**1**), glycine-6-decarboxy-betaxanthin (**2**), putrescine-6-decarboxy-betaxanthin (**3**), serine-6-decarboxy-betaxanthin (**6**), histamine-6-decarboxy-betaxanthin (**7**), and glutamine-6-decarboxy-betaxanthin (**18**). Only the abundance of molecule **6** was enough the make it detectable in DAD chromatograms at 480 nm (Supplementary Table S5) in this plant. In comparison, the yellow variety of this species presented a higher number of decarboxylated betalains detected through exact masses: ethanolamine-6-decarboxy-betaxanthin (**1**), glycine-6-decarboxy-betaxanthin (**2**), proline-6-decarboxy-betaxanthin (**8**), hydroxyproline-6-decarboxy-betaxanthin (**13**), isoleucine-6-decarboxy-betaxanthin (**15**), glutamine-6-decarboxy-betaxanthin (**18**), lysine-6-decarboxy-betaxanthin (**19**), phenylalanine-6-decarboxy-betaxanthin (**25**), methoxytyramine-6-decarboxy-betaxanthin (**26**), arginine-6-decarboxy-betaxanthin (**27**), DOPA-6-decarboxy-betaxanthin (**29**), and tryptophan-6-decarboxy-betaxanthin (**30**). Among the 12 molecules, only the betaxanthin **29** could be detected by absorbance at 480 nm (Supplementary Table S6).

Finally, in relation to quinoa (*C. quinoa*) varieties, different results were obtained for each of the extracts analyzed. In the quinoa variety BGQ-24 (red grains), up to 26 decarboxylated betalains were identified through their exact masses: ethanolamine-6-decarboxy-betaxanthin (**1**), glycine-6-decarboxy-betaxanthin (**2**), putrescine-6-decarboxy-betaxanthin (**3**), alanine-6-decarboxy-betaxanthin (**4**), γ-aminobutyric acid-6-decarboxy-betaxanthin (**5**), serine-6-decarboxy-betaxanthin (**6**), histamine-6-decarboxy-betaxanthin (**7**), proline-6-decarboxy-betaxanthin (**8**), valine-6-decarboxy-betaxanthin (**9**), norvaline-6-decarboxy-betaxanthin (**10**), threonine-6-decarboxy-betaxanthin (**11**), phenylethylamine-6-decarboxy-betaxanthin (**12**), hydroxyproline-6-decarboxy-betaxanthin (**13**), leucine-6-decarboxy-betaxanthin (**14**), isoleucine-6-decarboxy-betaxanthin (**15**), tyramine-6-decarboxy-betaxanthin (**17**), glutamine-6-decarboxy-betaxanthin (**18**), lysine-6-decarboxy-betaxanthin (**19**), glutamic acid-6-decarboxy-betaxanthin (**20**), dopamine-6-decarboxy-betaxanthin (**22**), histidine-6-decarboxy-betaxanthin (**23**), methionine sulfoxide-6-decarboxy-betaxanthin (**24**), phenylalanine-6-decarboxy-betaxanthin (**25**), tyrosine-6-decarboxy-betaxanthin (**28**), DOPA-6-decarboxy-betaxanthin (**29**), and tryptophan-6-decarboxy-betaxanthin (**30**). Among them, up to 10 were found in quantities enough to be detected in the UV–vis chromatograms at 480 nm: **5**, **8**, **9**, **13**, **17**, **22**, **23**, **24**, **28**, and **29** (Supplementary Table S7).

The quinoa variety BGQ-174 (red grains) showed the presence of 18 decarboxylated betalains, as identified according to their exact masses: ethanolamine-6-decarboxy-betaxanthin (**1**), glycine-6-decarboxy-betaxanthin (**2**), alanine-6-decarboxy-betaxanthin (**4**), γ-aminobutyric acid-6-decarboxy-betaxanthin (**5**), serine-6-decarboxy-betaxanthin (**6**), histamine-6-decarboxy-betaxanthin (**7**), proline-6-decarboxy-betaxanthin (**8**), valine-6-decarboxy-betaxanthin (**9**), norvaline-6-decarboxy-betaxanthin (**10**), threonine-6-decarboxy-betaxanthin (**11**), leucine-6-decarboxy-betaxanthin (**14**), isoleucine-6-decarboxy-betaxanthin (**15**), tyramine-6-decarboxy-betaxanthin (**17**), glutamine-6-decarboxy-betaxanthin (**18**), dopamine-6-decarboxy-betaxanthin (**22**), phenylalanine-6-decarboxy-betaxanthin (**25**), tyrosine-6-decarboxy-betaxanthin (**28**), and DOPA-6-decarboxy-betaxanthin (**29**). Among them, up to 8 decarboxylated betalains were detected in the chromatograms obtained at 480 nm: **1**, **5**, **6**, **8**, **9**, **17**, **28**, and **29** (Supplementary Table S8).

This extraordinary number of phytochemicals detected by mass spectrometry and UV–vis analysis of the extracts was also found when analyzing yellow varieties of quinoa. In the quinoa variety POEQ-143 (yellow grains), 28 decarboxylated betalains were identified following their exact masses: ethanolamine-6-decarboxy-betaxanthin (**1**), glycine-6-decarboxy-betaxanthin (**2**), putrescine-6-decarboxy-betaxanthin (**3**), alanine-6-decarboxy-betaxanthin (**4**), γ-aminobutyric acid-6-decarboxy-betaxanthin (**5**), serine-6-decarboxy-betaxanthin (**6**), histamine-6-decarboxy-betaxanthin (**7**), proline-6-decarboxy-betaxanthin (**8**), valine-6-decarboxy-betaxanthin (**9**), norvaline-6-decarboxy-betaxanthin (**10**), threonine-6-decarboxy-betaxanthin (**11**), phenylethylamine-6-decarboxy-betaxanthin (**12**), hydroxyproline-6-decarboxy-betaxanthin (**13**), leucine-6-decarboxy-betaxanthin (**14**), isoleucine-6-decarboxy-betaxanthin (**15**), asparagine-6-dec

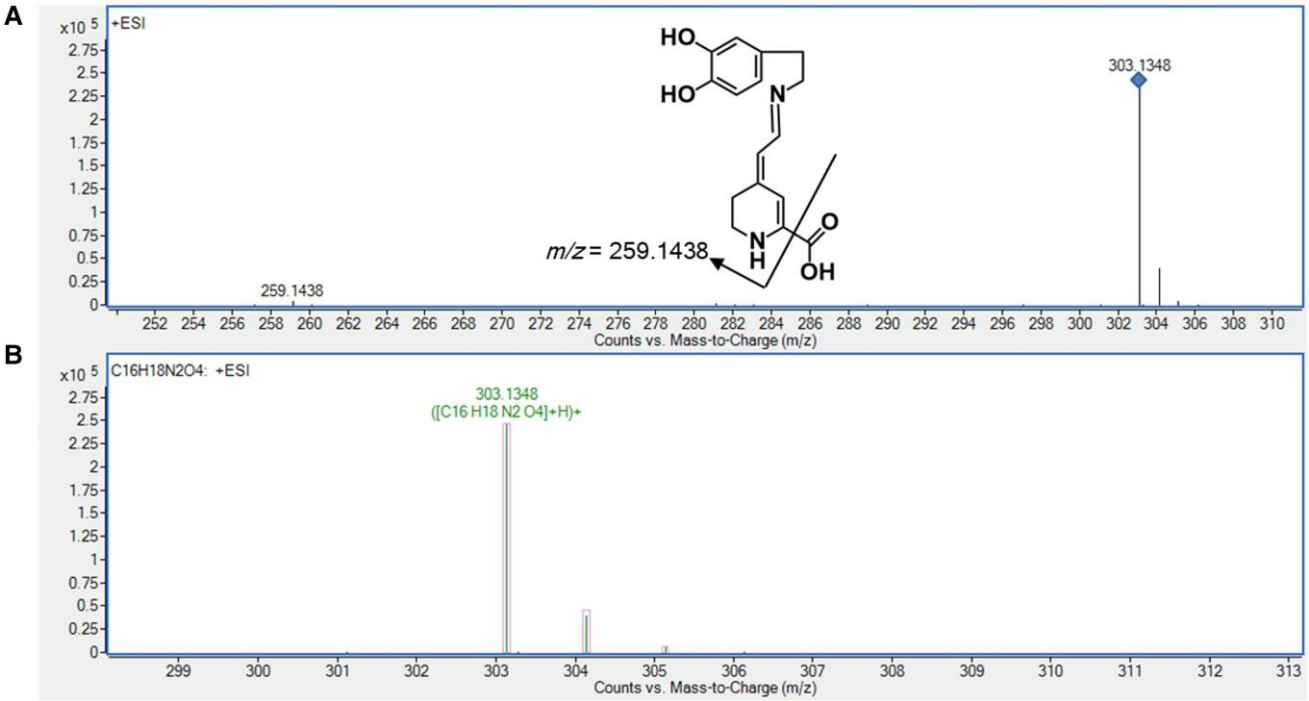

Figure 4. ESI-Q-TOF-MS-MS analysis of dopamine-6-decarboxy-betaxanthin. Fragmentation mass spectra **A)** and adjustment to theoretical molecular formula considering accurate mass and isotopic distribution profile **B)** are shown. Fragmentation spectra and analysis for all the pigments can be found in Supplementary Fig. S5. ESI, Electrospray ionization.

arboxy-betaxanthin (**16**), tyramine-6-decarboxy-betaxanthin (**17**), glutamine-6-decarboxy-betaxanthin (**18**), lysine-6-decar boxy-betaxanthin (**19**), glutamic acid-6-decarboxy-betaxanthin (**20**), dopamine-6-decarboxy-betaxanthin (**22**), histidine-6-de carboxy-betaxanthin (**23**), methionine sulfoxide-6-decarboxy-betaxanthin (**24**), phenylalanine-6-decarboxy-betaxanthin (**25**), arginine-6-decarboxy-betaxanthin (**27**), tyrosine-6-decarboxy-betaxanthin (**28**), DOPA-6-decarboxy-betaxanthin (**29**), and tryptophan-6-decarboxy-betaxanthin (**30**). This extraordinary number of compounds was also supported by the visible chromatograms where up to 15 were in quantities enough to be detected in the chromatograms obtained at 480 nm: **1**, **2**, **5**, **6**, **8**, **10**, **11**, **13**, **15**, **17**, **19**, **20**, **25**, **28**, and **29** (Supplementary Table S9).

In the same manner, the quinoa variety POQ-36 (yellow grains) showed the presence of 23 decarboxylated betalains as identified by their exact masses: ethanolamine-6-decarboxy-betaxanthin (**1**), glycine-6-decarboxy-betaxanthin (**2**), putrescine-6-decarb oxy-betaxanthin (**3**), alanine-6-decarboxy-betaxanthin (**4**), γ-aminobutyric acid-6-decarboxy-betaxanthin (**5**), serine-6-decar boxy-betaxanthin (**6**), histamine-6-decarboxy-betaxanthin (**7**), proline-6-decarboxy-betaxanthin (**8**), valine-6-decarboxy-betax anthin (**9**), norvaline-6-decarboxy-betaxanthin (**10**), threonine-6-decarboxy-betaxanthin (**11**), phenylethylamine-6-decarboxy-betaxanthin (**12**), hydroxyproline-6-decarboxy-betaxanthin (**13**), leucine-6-decarboxy-betaxanthin (**14**), isoleucine-6-decarboxy-betaxanthin (**15**), tyramine-6-decarboxy-betaxanthin (**17**), glutamine-6-decarboxy-betaxanthin (**18**), glutamic acid-6-decarboxy-betaxanthin (**20**), dopamine-6-decarboxy-betaxanthin (**22**), phenylalanine-6-decarboxy-betaxanthin (**25**), tyrosine-6-decarboxy-betaxanthin (**28**), DOPA-6-decarboxy-betaxanthin (**29**), and tryptophan-6-decarboxy-betaxanthin (**30**). Among them, 7 decarboxylated betalains were detected in the chromatograms at 480 nm: **5**, **6**, **8**, **17**, **20**, **22**, and **29** (Supplementary Table S10). The results obtained from all the natural sources used are summarized in Table 3. As can be seen, these results imply the discovery of a whole family of betalains in nature, expanding the number of plausible molecules derived from dopamine.

Pigment data analysis by machine learning

Once the decarboxylated pigment profile of each species was identified, the data were screened by machine learning. After performing the distance analysis, the hierarchical clustering obtained showed the existence of 5 different clusters (Fig. 5A): C1, composed only of the quinoa variety BGQ-174; C2, composed of all the other quinoa varieties (POEQ-143, BGQ-24, and POQ-36); C3, composed of the yellow celosia; C4, composed of the red celosia; and C5, composed of the red and yellow varieties of Swiss chard and beetroot, thus clustering all the extracts derived from *B. vulgaris*. The distance map indicated higher similarity between the different quinoa varieties with respect to the rest of the species, being represented as a separate group from the rest of the plants analyzed (Fig. 5B). The MDS (multidimensional scaling) representation showed that the plants more similar to each other belonged to the same species (*B. vulgaris*, *C. argentea*, or *C. quinoa*) (Fig. 5C). The RadViz diagram showed that all quinoa varieties and the yellow varieties of celosia and Swiss chard were found in the center of the circumference, while the 2 beetroot varieties, and the red varieties of Swiss chard and celosia, were located on the perimeter (Fig. 5D). *C. quinoa* was the species that presented a higher amount of decarboxylated betalains, compared with the rest of the plants analyzed (Fig. 5E).

Discussion

Obtention of decarboxybetalain standards

Decarboxylated betalains are a singular family of dopamine-derived plant pigments that have hitherto been unknown

Table 3. Identification of decarboxylated betalains in plants of the family Amaranthaceae

Decarboxylated betalain	Natural sources
(**1**) Ethanolamine-6-decarboxy-betaxanthin	Yellow Swiss chard, red and yellow celosia, BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**2**) Glycine-6-decarboxy-betaxanthin	Yellow Swiss chard, yellow beetroot, red and yellow celosia, BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**3**) Putrescine-6-decarboxy-betaxanthin	Red celosia, BGQ-24, POEQ-143, and POQ-36
(**4**) Alanine-6-decarboxy-betaxanthin	BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**5**) γ-Aminobutyric acid-6-decarboxy-betaxanthin	BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**6**) Serine-6-decarboxy-betaxanthin	Red celosia, BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**7**) Histamine-6-decarboxy-betaxanthin	Red celosia, BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**8**) Proline-6-decarboxy-betaxanthin	Yellow celosia, BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**9**) Valine-6-decarboxy-betaxanthin	BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**10**) Norvaline-6-decarboxy-betaxanthin	BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**11**) Threonine-6-decarboxy-betaxanthin	BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**12**) Phenylethylamine-6-decarboxy-betaxanthin	BGQ-24, POEQ-143, and POQ-36
(**13**) Hydroxyproline-6-decarboxy-betaxanthin	Yellow celosia, BGQ-24, POEQ-143, and POQ-36
(**14**) Leucine-6-decarboxy-betaxanthin	BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**15**) Isoleucine-6-decarboxy-betaxanthin	Yellow celosia, BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**16**) Asparagine-6-decarboxy-betaxanthin	POEQ-143
(**17**) Tyramine-6-decarboxy-betaxanthin	BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**18**) Glutamine-6-decarboxy-betaxanthin	Yellow beetroot, red and yellow celosia, BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**19**) Lysine-6-decarboxy-betaxanthin	Yellow celosia, BGQ-24, and POEQ-143
(**20**) Glutamic acid-6-decarboxy-betaxanthin	Yellow Swiss chard, BGQ-24, POEQ-143, and POQ-36
(**21**) Methionine-6-decarboxy-betaxanthin	-
(**22**) Dopamine-6-decarboxy-betaxanthin	Yellow Swiss chard, BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**23**) Histidine-6-decarboxy-betaxanthin	BGQ-24 and POEQ-143
(**24**) Methionine sulfoxide-6-decarboxy-betaxanthin	BGQ-24 and POEQ-143
(**25**) Phenylalanine-6-decarboxy-betaxanthin	Yellow celosia, BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**26**) Methoxytyramine-6-decarboxy-betaxanthin	Yellow celosia
(**27**) Arginine-6-decarboxy-betaxanthin	Yellow celosia and POEQ-143
(**28**) Tyrosine-6-decarboxy-betaxanthin	BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**29**) DOPA-6-decarboxy-betaxanthin	Red and yellow Swiss chard, red beetroot, yellow celosia, BGQ-24, BGQ-174, POEQ-143, and POQ-36
(**30**) Tryptophan-6-decarboxy-betaxanthin	Yellow celosia, BGQ-24, POEQ-143, and POQ-36

(Henarejos-Escudero et al. 2021; Henarejos-Escudero, Martínez-Rodríguez, et al. 2022). To explore the possible presence of multiple members of this family in nature, the molecules need to be obtained and purified. In this way, the pigments obtained may serve as reference standards to determine their presence in natural sources of interest within the order Caryophyllales. For this purpose, decarboxybetalains were prepared in microbial bioreactors (Guerrero-Rubio et al. 2019) and purified by preparative HPLC. Then, the identity of the compounds was verified by HPLC-ESI-Q-TOF-MS analysis. The biotechnological production of betalains allowed its obtention individually in an efficient and scalable manner for its subsequent characterization (Guerrero-Rubio et al. 2019). Recent studies have shown that it is possible to produce betalains through bacterial cultures using the enzyme 4,5-DODA from the bacterium *Gluconacetobacter diazotrophicus* (GdDODA) (Contreras-Llano et al. 2019). This proteobacterium, not directly related to the order Caryophyllales, is present in roots and stems of sugar cane (*Saccharum officinarum*) and can generate the enzyme dioxygenase, which catalyzes the same reaction as those of plant origin (Contreras-Llano et al. 2019). This enzyme shows better stability, higher activity, and higher affinity than other DODAs cloned from plants such as *B. vulgaris* (Guerrero-Rubio et al. 2019).

Thus, the production of betalains in large quantities can be carried out by establishing biofactories of *Escherichia coli* bacteria transformed with the gene expressing GdDODA. The biofactories produce betalamic acid from L-DOPA or 6-decarboxy-betalamic acid from dopamine. Once obtained, and after the chemical condensation with selected amines or amino acids, the formation of betalains, both traditional and decarboxylated, is possible. By using this methodology, the 12 decarboxylated betalains described to date have been produced and characterized (Henarejos-Escudero, Hernández-García, et al. 2022), being only 2 of them (DOPA-6-decarboxy-betaxanthin and dopamine-6-decarboxy-betaxanthin) detected in nature (Henarejos-Escudero et al. 2021).

All the reactors prepared showed a yellow–orange coloration indicative of the presence of the expected betaxanthins: dopamine-6-decarboxy-betaxanthin and decarboxylated betaxanthin of interest. These were obtained by the addition of nontransformable amino acids and amines. The presence of dopamine-6-decarboxy-betaxanthin is due to the dopamine added to the medium for the formation of 6-decarboxy-betalamic acid, since the 2 molecules can condense to yield the pigment. Thus, dopamine competes with the other amine or amino acid added. In this way, a library of dopamine-derived plant pigments was tentatively obtained, including previously described pigments (Henarejos-Escudero, Hernández-García, et al. 2022) and novel ones. Sodium ascorbate was added to the medium as an antioxidant to prevent the degradation or oxidation of dopamine and the pigments (Guerrero-Rubio et al. 2019). Initial screening of the bioreactors by HPLC confirmed the possibility of forming a wide range of decarboxylated betalains (Henarejos-Escudero et al. 2021).

Compared with the traditional betalains previously described (Guerrero-Rubio et al. 2020), the decarboxylated betalains containing the same amino acid or amine moiety eluted from the HPLC column between 0.6 and 2.2 min later (Table 4). This fact indicates that the produced molecules presented a higher affinity for the C-18 column matrix employed due to their higher

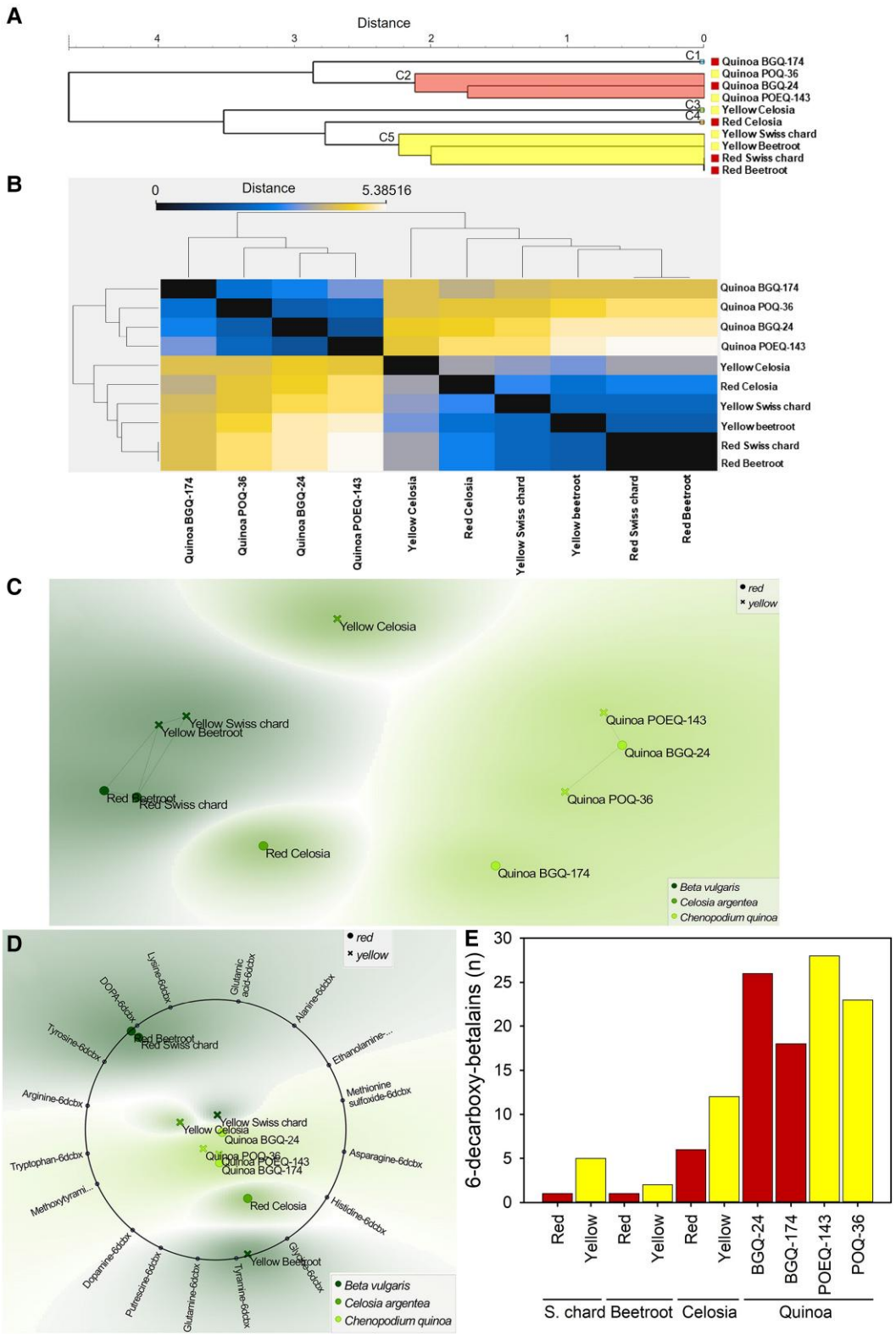

Figure 5. Analysis of pigment profiles found in the natural sources by machine learning. **A)** Hierarchical clustering, **B)** distance map, **C)** multidimensional scaling representation (MDS), and **D)** RadViz diagram. The number of pigments found in each plant source is shown **E)**.

hydrophobic nature produced by the absence of a carboxyl group in their structure (Henarejos-Escudero, Hernández-García, et al. 2022).

The spectrophotometric characterization of the produced molecules is in agreement with the published data of decarboxylated betalains (Henarejos-Escudero, Hernández-García, et al. 2022). Previous studies established that the λ_{max} of traditional betalains is in the range of 464 to 480 nm (Gandía-Herrero et al. 2005b; Gandía-Herrero et al. 2010). The results obtained in this work showed slightly wider λ_{max} ranges for the decarboxylated

Table 4. Comparison of RTs of traditional and decarboxylated betalains. Reversed-phase chromatography was performed with a 250 × 4.6 mm C-18 column packed with 5 μm particles. A linear gradient using water with 0.05% (v/v) TFA as solvent A and acetonitrile with 0.05% (v/v) TFA as solvent B was performed

Trivial name	Traditional betalain	RT (min)	Decarboxylated betalain	RT (min)	References
Indicaxanthin	Proline-betaxanthin	12.65	Proline-6-decarboxy-betaxanthin	13.76	(Guerrero-Rubio et al. 2020)
…	Valine-betaxanthin	15.45	Valine-6-decarboxy-betaxanthin	16.8	(Guerrero-Rubio et al. 2020)
…	Phenylethylamine-betaxanthin	22.10	Phenylethylamine-6-decarboxy-betaxanthin	23.83	(Guerrero-Rubio et al. 2020)
Vulgaxanthin IV	Leucine-betaxanthin	18.82	Leucine-6-decarboxy-betaxanthin	20.31	(Guerrero-Rubio et al. 2020)
Vulgaxanthin III	Asparagine-betaxanthin	6.17	Asparagine-6-decarboxy-betaxanthin	8.33	(Guerrero-Rubio et al. 2020)
Vulgaxanthin I	Glutamine-betaxanthin	8.21	Glutamine-6-decarboxy-betaxanthin	9.40	(Guerrero-Rubio et al. 2020)
Vulgaxanthin II	Glutamic acid-betaxanthin	9.82	Glutamic acid-6-decarboxy-betaxanthin	11.10	(Guerrero-Rubio et al. 2020)
Miraxanthin-V	Dopamine-betaxanthin	15.3	Dopamine-6-decarboxy-betaxanthin	16.70	(Henarejos-Escudero, Martínez-Rodríguez, et al. 2022)
Miraxanthin I	Methionine sulfoxide-betaxanthin	8.67	Methionine sulfoxide-6-decarboxy-betaxanthin	9.55	(Guerrero-Rubio et al. 2020)
…	Phenylalanine-betaxanthin	20.08	Phenylalanine-6-decarboxy-betaxanthin	21.05	(Guerrero-Rubio et al. 2020)
Dopaxanthin	DOPA-betaxanthin	13.91	DOPA-6-decarboxy-betaxanthin	14.49	(Guerrero-Rubio et al. 2020)
…	Tryptophan-betaxanthin	20.28	Tryptophan-6-decarboxy-betaxanthin	21.83	(Guerrero-Rubio et al. 2020)

pigments compared with traditional betalains (Supplementary Fig. S4 and Table 1). The fragmentation data obtained revealed the loss of at least 1 carboxyl group in all molecules (Fig. 4 and Supplementary Fig. S5), which is expected based on previous MS–MS fragmentation studies of betalains (Contreras-Llano et al. 2019). The determination of the exact mass and the expected isotopic distribution profile for each molecule allowed the unequivocal identification of the proposed formula for the decarboxylated betalains produced and purified, extending to 33 the number of decarboxybetalains characterized to date, with respect to the 12 described in early works (Henarejos-Escudero, Hernández-García, et al. 2022).

Discovery of decarboxybetalains in nature

Decarboxylated betalains have only been detected in quinoa grains (Henarejos-Escudero et al. 2021). Specifically, DOPA-6-decarboxy-betaxanthin and dopamine-6-decarboxy-betaxanthin are the only decarboxylated betalains detected in natural sources to date. Due to the promiscuity of the 4,5-DODA enzyme and its broad presence within the order Caryophyllales, there is a likelihood that 6-decarboxylated betalains may be present in more species. However, due to the focus on traditional betalains, coupled with the lack of reference standards, the detection of decarboxylated betalains has not been possible. Despite metabolomic studies (Qian et al. 2023), decarboxylated betalains have not been discovered in nature excluding the 2 pigments mentioned above.

After the purification and identification of decarboxybetalains, the pigments were used as reference standards to carry out screenings in natural sources. Betalains are found as secondary metabolites in plants within some families of the order Caryophyllales, such as the family Amaranthaceae. Many other compounds are found in plant extracts, which hinder the identification of these molecules which may be present in low concentrations or even in traces (Azeredo 2009). Therefore, the 10 selected plant extracts of the Amaranthaceae family were analyzed by TOF mass spectrometry in an ambitious attempt to search for decarboxybetalains by using the produced standards.

Regarding the results obtained, it should be noted that TOF detection based on exact mass identification is more sensitive than the detection by UV–vis absorbance. Thus, in some cases, the pigments were not detected in the DAD chromatograms at 480 nm, but its detection through exact mass allowed to confirm its presence accurately. In the same manner, if in addition to finding the molecules in the TOF mass spectrometry analysis, they were also in the chromatograms at 480 nm, it was considered that this pigment was found in a higher amount. The concentration of such molecules in the extracts was considered higher with respect to those compounds that were only detected by exact mass.

After analyzing each extract individually, it was determined that quinoa, in its yellow grain variety POEQ-143, was the natural source with the highest number of decarboxylated betalains (28), followed by the red grain variety BGQ-24 (26) (Fig. 5E). Likewise, the yellow grain variety POQ-36 and the red grain variety BGQ-174 also presented a wide range of the studied molecules (23 and 18 pigments, respectively). The presence of decarboxylated betaxanthins is the main reason for the yellow grain coloring, and their mixture with traditional betacyanins generates the red tones shown by the BGQ-174 and BGQ-24 varieties (Escribano et al. 2017). In this regard, the decarboxylated pigments DOPA-6-decarboxy-betaxanthin and dopamine-6-decarboxy-betaxanthin were detected in all the quinoa varieties analyzed, where it is reported that their respective traditional analogs, dopaxanthin and dopamine-betaxanthin, are also present. Likewise, proline-6-decarboxy-betaxanthin was detected in grains POEQ-143 and POQ-24, one of the few varieties of this species where its carboxylated analog proline-betaxanthin has also been detected (Escribano et al. 2017). Thus, the results obtained in the present work show the presence of the 3 decarboxylated analog pigments of the only 3 traditional betaxanthins described until now in *C. quinoa* (dopaxanthin, dopamine-betaxanthin, and proline-betaxanthin) (Escribano et al. 2017). Regarding the structures proposed in this work and taking into account the scarce data available in the literature, 28 decarboxylated betaxanthins and 3 traditional pigments (1:9 ratio) have been detected until now in quinoa.

With respect to the Swiss chard extracts, the pigment DOPA-6-decarboxy-betaxanthin was identified in both yellow and red varieties. The red Swiss chard extract only had DOPA-6-decarboxy-betaxanthin, being the extract with the fewest amount of 6-decarboxy-betaxanthins detected, together with

red beetroot. Interestingly, glutamine-6-decarboxy-betaxanthin, an analogous decarboxylated betalain to vulgaxanthin I, which is a characteristic pigment of beetroots (Stintzing et al. 2002), was detected in the yellow variety of this plant. Five decarboxylated betalains identified in *B. vulgaris* appear together with their analogous traditional pigments previously detected in this plant (ethanolamine-betaxanthin, glycine-betaxanthin, glutamine-betaxanthin, glutamic acid-betaxanthin, and dopamine-betaxanthin) (Kugler et al. 2004; Gandía-Herrero and García-Carmona 2013). Considering the data from previous studies together with those presented in this work, and focusing on the proposed structures, 21 traditional betaxanthins and 6 decarboxylated analogs (4:1 ratio) have been detected until now in the species *B. vulgaris*. Finally, the molecules methoxytyramine-6-decarboxy-betaxanthin and tryptophan-6-decarboxy-betaxanthin were identified in yellow celosia. In this case, the analogous traditional betalains, methoxytyramine-betaxanthin and tryptophan-betaxanthin, have also been detected in this species (Schliemann et al. 2001). As in the case of quinoa, due to the scarce information in the literature on the search for betaxanthins in *C. argentea*, 15 decarboxylated betaxanthins and only 3 traditional analogs (1:5 ratio) have been detected until now in this species.

The yellow varieties of the plants considered in this work presented a higher number of 6-decarboxy-betaxanthins. The detected molecules may contribute to the color exhibited by these plants, while the number and quantity of the compounds are lower in the violet-toned varieties of the same species. In red varieties, the yellow color provided by the detected 6-decarboxy-betaxanthins is concealed by the betacyanins present in higher quantities. Thus, the color of red varieties of Swiss chard and beetroot is due to violet pigments such as betanin (Kujala et al. 2002). Likewise, the red color of celosia inflorescences and quinoa grains BGQ-24 and BGQ-174 is due to the betalain amaranthin, the reported main pigment of these plants (Escribano et al. 2017; Polturak and Aharoni 2018).

The 6-decarboxy-betaxanthin profile of the studied plants was compared by machine learning. The analysis is focused on the identification of patterns or anomalies and allowed a deeper understanding of the obtained data. The hierarchical clustering first divided the extracts into 2 groups, one for quinoas and another for the rest of the extracts, suggesting that quinoas 6-decarboxy-betaxanthin profile is unique (Fig. 5, A, B, and C).

The 3 species used in this work were distributed in 5 clusters, where *C. quinoa* and *C. argentea* varieties were allocated in 4 clusters (Fig. 5A). Meanwhile, Swiss chard (*B. vulgaris* var. *cicla*) and beetroot (*B. vulgaris* subsp. *vulgaris*) were grouped together in 1 common cluster. This indicates that pigment analysis itself was able to identify the simplicity and similarity of these samples, all belonging to the same species (*B. vulgaris*).

In addition, within the same species, it was possible to observe differences corresponding to the different varieties, with those of the same color grouping together (Fig. 5C). Thus, within the species *B. vulgaris*, the red varieties are close to each other, as are the yellow varieties among them. Similarly, the yellow variety of *C. argentea* is dissimilar to its own red variety in the analysis. In the case of *C. quinoa*, the 6-decarboxy-betaxanthin profile of the red grains BGQ-174 appears to be more distant with respect to the rest of the quinoa varieties. This analysis objectively indicates that the pigment profile varies according to species but also coloration depends on other factors. Furthermore, when several decarboxylated betalains are included as variables in a RadViz diagram, quinoas are allocated in the center of the diagram, an indication of their numerous pigments in comparison with the other species such as beetroot, whose peripheral distribution indicates the detection of fewer and specific pigments (Fig. 5D).

These results support the hypothesis that the DODA enzymes of plant species different from quinoa may have dual activity toward L-DOPA and dopamine, thus being able to accumulate traditional and 6-decarboxylated pigments (Henarejos-Escudero, Martínez-Rodríguez, et al. 2022). This fact opens horizons in the determination of pigment profiles in plants of the order Caryophyllales, where the existence of 6-decarboxylated betalains has been so far unknown.

C. quinoa, a species rich in decarboxylated betalains

Quinoa was the plant source with the highest presence of decarboxylated betalains of the studied species (Fig. 5E). This fact is in agreement with the results of our previous work since it was in varieties of quinoa grains where the dual activity of the 4,5-DODA was first demonstrated (Henarejos-Escudero, Martínez-Rodríguez, et al. 2022). DOPA-6-decarboxy-betaxanthin and dopamine-6-decarboxy-betaxanthin were detected in all quinoa varieties tested, supporting previous research by Henarejos-Escudero et al. (2021).

Betalains show multiple physiological functions in the plants that produce them and participate in the responses to biotic and abiotic stresses. In this sense, quinoa was originally cultivated in the Andean region, from where this sample was obtained, and is considered a species with great resistance to a multitude of adverse conditions. *C. quinoa* stands out for its ability to survive and grow in nutrient-poor, high-salinity, or very dry environments. In addition, quinoa is resistant to extreme temperatures and can withstand high concentrations of heavy metals in the soil and high levels of UV-B light irradiation (Hinojosa et al. 2018). The results obtained in this work substantially increase the number of secondary metabolites detected and identified in quinoa with bioactive potential. Its presence may help quinoa plants in the tolerance of this species to the numerous abiotic stresses mentioned.

In addition, betalains present potent bioactive properties and health benefits. This has increased the interest in their identification in nature, production, and characterization, and they are the subject of numerous in vitro and in vivo studies (Martínez-Rodríguez et al. 2022). Within the wide range of health-promoting properties of betalains, it is known for their antioxidant, anti-inflammatory, antitumor, antidiabetic, antimalarial, and cardio- and neuroprotective effects (Martínez-Rodríguez et al. 2022). Previous studies highlighted DOPA-6-decarboxy-betaxanthin (present in all quinoa varieties tested), tryptophan-6-decarboxy-betaxanthin, and glutamic acid-6-decarboxy-betaxanthin (both present in 3 of the 4 quinoa varieties tested) as excellent in vitro antioxidants, with Trolox equivalent antioxidant capacity (TEAC) values of 18.9, 18.3, and 14.6, respectively (Henarejos-Escudero, Hernández-García, et al. 2022). In fact, the TEAC values of the 6-decarboxy-betaxanthins were higher than those previously described for traditional betalains (Guerrero-Rubio et al. 2020). In addition, the molecules phenylalanine-6-decarboxy-betaxanthin (found in BGQ-24, BGQ-174, POEQ-143, and POQ-36), glutamic acid-6-decarboxy-betaxanthin, tryptophan-6-decarboxy-betaxanthin, phenylethylamine-6-decarboxy-betaxanthin (BGQ-24, POEQ-143, and POQ-36), and methionine sulfoxide-6-decarboxy-betaxanthin (BGQ-24 and POEQ-143) presented high antioxidant capacity in vivo when tested in the animal model *Caenorhabditis elegans*

(Henarejos-Escudero, Hernández-García, et al. 2022). The mentioned decarboxylated betalains, supplemented at 25 μM, were able to significantly reduce the oxidative stress of the animals between 69% and 99%. Likewise, the molecules phenylalanine-6-decarboxy-betaxanthin and DOPA-6-decarboxy-betaxanthin, both present in all the quinoa varieties analyzed, were able to increase the lifespan of the nematodes by 6.2% and 7.0%, respectively (Henarejos-Escudero, Hernández-García, et al. 2022). The aforementioned bioactive molecules, detected in this work in multiple natural sources, some of them edible, could contribute to increase the health-promoting properties of the plants that produce them.

Conclusions

Twenty-nine decarboxylated betalains were identified in nature, of which 27 of them had not been identified previously. The results showed that the presence of these pigments is more general than expected, with representatives in all the plant species analyzed. In quinoa, a total of 26, 18, 28, and 23 pigments were detected in the varieties BGQ-24, BGQ-174, POEQ-143, and POQ-36, respectively, being the richest natural source of these described metabolites. A total of 30 decarboxylated betalains were obtained, purified, and characterized, of which 21 of them had not been characterized previously. All molecules showed a yellow coloration, with absorbance spectra in the range of the betalains already described in the literature. The exact mass of each of the decarboxybetalains has been confirmed, allowing their use as reference standards to be used for the identification of these compounds in plant extracts. This allowed a screen of 6-decarboxy-betaxanthins in plants belonging to the Amaranthaceae family: Swiss chard, beetroot, celosia, and quinoa, both in red and yellow varieties. The results obtained demonstrate the existence of the decarboxylated betalains in nature with a high variety of structures.

The findings of this work represent evidence of the presence of this recently discovered family of betalains in nature. The results obtained increase the number of secondary metabolites identified in plants, some of them with high resistance tolerance to stresses (e.g. quinoa). In addition to the taxonomic interest presented of the detection of betalains (in the plant kingdom, they only present have been found in the order Caryophyllales), the findings described here show present evolutionary interest. The question of coevolution of the dopamine based biosynthetic pathway alongside with the production of DOPA-derived carboxylated betalains has been opened, and the existence of both families of compounds may be based on the duplication and neofunctionalization of preexisting plant dioxygenases of unknown function (Brockington et al. 2015) and in chemical reactions supported by the existence of free amines available in the plant tissues to be colored (Gandía-Herrero and García-Carmona 2020). The physiological relevance of the decarboxybetalains may rely on their color and antioxidant properties, common characteristics of other water-soluble pigments as anthocyanins and carboxylated betalains. The present manuscript describes the characterization and identification in nature of molecules of physiological relevance produced from a biosynthetic pathway with dopamine as a starting point. The metabolites, hitherto unknown or ignored, have been identified, thanks to the development of standards. Therefore, it opens the possibility of expanding the search for these and other related molecules to all the plant families within the order Caryophyllales.

Materials and methods

Chemical reagents

Sodium ascorbate, isopropyl β-D-1-thioagalactopyranoside (IPTG), kanamycin (Km), chloramphenicol (Cn), trifluoroacetic acid (TFA), sodium hydroxide (NaOH), Luria–Bertani (LB) culture medium, and sodium acetate ($NaC_2H_3O_2$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The solvents used for HPLC (water and acetonitrile) were purchased from Thermo Fisher Scientific (Dublin, Ireland). For the production of the different decarboxylated betalains, the following compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA): 3,4-dihydroxy-L-phenylalanine (L-DOPA), pyrrolidin-2-carboxylic acid (L-proline), 2-amino-4-carbamoylbutanoic acid (L-glutamine), (2S)-2-amino-4-methylsulfinylbutanoic acid (L-methionine sulfoxide), 2-aminopentanedioic acid (L-glutamic acid), 4-(2-aminoethyl)benzene-1,2-diol (dopamine), (2S)-2-amino-3-(1H-indole-3-yl)propanoic acid (L-tryptophan), 2-phenylethylamine (L-phenylethylamine), (2S)-2-amino-3-phenylpropanoic acid (L-phenylalanine), 4-(2-aminoethyl)phenol (tyramine), (2S,4R)-4-hydroxypyrrolidine-2-carboxylic acid (L-hydroxy proline), 2-amino-3-(4-hydroxyphenyl)propanoic acid (L-tyrosine), 2-aminoacetic acid (L-glycine), 2,4-diamino-4-oxobutanoic acid (L-asparagine), (2S)-2-amino-4-methylpentanoic acid (L-leucine), 4-aminobutanoic acid (γ-aminobutyric acid), (2S)-2-amino-3-methylbutanoic acid (L-valine), (2S,3S)-2-amino-3-methylpentanoic acid (L-isoleucine), (2S)-2-aminopentanedioic acid (L-alanine), 2-amino-4-methylsulfanilutanoic acid (L-methionine), 2-aminopentanoic acid (L-norvaline), 2-amino-3-(1H-imidazol-5-yl)propanoic acid (L-histidine), 2-amino-3-hydroxypropanoic acid (L-serine), (2S)-2,6-diaminohexanoic acid (L-lysine), 2-aminoethanol (ethanolamine), (2S,3R)-2-amino-3-hydroxybutanoic acid (L-threonine), 2-amino-5-[(diaminomethylidene)amino]pentanoic acid (L-arginine), butane-1,4-diamine (putrescine), 2-(1H-imidazol-5-yl)ethanamine (histamine), 4-(2-aminoethyl)-2-methoxyphenol (3-methoxytyramine).

Bacterial strains and plasmids

For the biotechnological production of betalains in bioreactors, *E. coli* Rosetta 2 (DE3) bacteria, previously transformed by the insertion of the expression vector pET28 containing the 4,5-DODA enzyme gene from the proteobacterium *G. diazotrophicus* (GdDODA, WP_012222467.1) (primers and restriction sites: GdDODA-F [5′TATATATACATATGACACCGGTGCCGGAA] and GdDODA-R [5′ATATATATCTCGAGTTAAATCGGGGTTGC]), was used (Contreras-Llano et al. 2019).

Plant material

All plants used in this work belong to the Amaranthaceae family. The red and yellow varieties of beetroot (*B. vulgaris* subsp. *vulgaris*), celosia (*C. argentea* var. *plumosa*), and Swiss chard (*B. vulgaris* var. *cicla*) were grown and obtained from local producers in Murcia (Southwestern Spain). The different varieties of quinoa (*C. quinoa*) grains (POQ-36, POEQ-143, BGQ-24, and BGQ-174) used were obtained from the germplasm bank of the National Agricultural University—La Molina (Lima, Peru).

Production of decarboxylated betalains by microbial bioreactors

Decarboxylated betalains were produced using microbial bioreactors of transformed *E. coli* (Guerrero-Rubio et al. 2019). *E. coli* cultures were grown in LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride) at a concentration of 20 g L^{-1}

and supplemented with Km (100 μg mL^{-1}) and Cn (34 μg mL^{-1}). First, a 5 mL starter culture was inoculated with *E. coli* Rosetta 2 transformed with the plasmid containing the 4,5-DODA gene from *G. diazotrophicus* (Contreras-Llano et al. 2019; Guerrero-Rubio et al. 2019). The starter culture was incubated overnight under orbital shaking at 150 rpm and 37 °C. Then, the starter culture was added to 500 mL of fresh LB and incubated under the same conditions until the optical density at 600 nm ($OD_{600\ nm}$) was 0.8 to 1. Next, 4,5-DODA enzyme expression was induced by the addition of IPTG (1 mM), incubated overnight under orbital shaking at 150 rpm and 20 °C. The culture was then centrifuged at 4 °C and 5,000 rpm for 10 min, and the supernatant was discarded. Subsequently, the cell pellet was resuspended in sterile water, transferred to Falcon tubes, and centrifuged again at 4 °C and 7,500 rpm for 10 min. The supernatant was removed, and each cell pellet was resuspended with 20 mL of sterile water supplemented with sodium ascorbate (10 mM) and dopamine (1 mM). The production of the different decarboxylated betalains was started by adding the corresponding amino acids or amine at a concentration of 10 mM, except for tyrosine, which was added at a concentration of 1.5 mM, due to its limited solubility (Henarejos-Escudero, Hernández-García, et al. 2022). The pH of the bioreactors was measured and adjusted, if necessary, to a pH value around 6.0 and then incubated under orbital shaking at 150 rpm at 20 °C for 72 h. Finally, the bioreactors were centrifuged at 4 °C and 7,500 rpm for 10 min, and the supernatant was stored at −20 °C until purification.

Purification of decarboxylated betalains by preparative HPLC

The decarboxylated betalains produced were purified for use as reference standards. For this purpose, the bioreactor supernatants, previously filtered through a filter with a pore size of 0.2 μm and a diameter of 25 mm, were purified using a Shimadzu Nexera Prep HPLC apparatus (Kyoto, Japan), equipped with an LC-20AP pump and an SPD-M40 PDA detector. Reversed-phase chromatography was carried out using a 250 × 21.2 mm Kinetex 5 μL/C-18 column (Phenomenex, Torrance, CA, USA). The purification procedure was performed as published by Henarejos-Escudero, Hernández-García, et al. (2022). The mobile phase was water and acetonitrile (both supplemented with 0.05% [v/v] TFA), and the gradient started with 0% acetonitrile and reached 35% after 25 min. The pump flow rate was 25 mL min^{-1} and the injection volume was 5 mL. Fractions containing the pigments were collected, and the TFA present was neutralized to pH 5.5 to 6.0 with a solution of NaOH prior to the elimination of acetonitrile solvent by rotary evaporation. The concentrated fractions containing the 6-decarboxy-betaxanthins of interest were stored at −20 °C until their characterization or use as reference molecules.

HPLC analysis

Analysis of decarboxylated betalains was carried out using a Shimadzu LC-10A analytical-type HPLC apparatus (Kyoto, Japan) equipped with an SPD-M10A photodiode array detector. Reversed-phase chromatography was performed with a 250 × 4.6 mm Kinetex C-18 column packed with 5 μm particles (Phenomenex, Torrance, CA, USA). All samples were analyzed at least 3 times. Following the procedure described in the literature (Henarejos-Escudero, Hernández-García, et al. 2022), a linear gradient using water with 0.05% (v/v) TFA as solvent A and acetonitrile with 0.05% (v/v) TFA as solvent B was performed. The method was applied for 25 min, from 0% of solvent B to reach 35% of solvent B, with a flow rate of 1 mL min^{-1}. The column temperature was maintained at 30 °C and the injection volume used was 50 μL. Then, the obtained chromatograms of the pure betalains and their absorbance spectra were plotted using SigmaPlot software (version 14.0) from Systat Software Inc. (Palo Alto, CA, USA).

Preparation of plant extracts

Aqueous extracts from natural sources were carried out in 2 different ways depending on the type of plant species used. In both cases, 100 mM sodium acetate buffer pH 5 supplemented with 10 mM sodium ascorbate was used (Escribano et al. 2017). For beetroot, Swiss chard, and celosia extracts, the root, stalk, and flowers, respectively, were collected. Subsequently, the plant samples were soaked in ice-cold buffer and crushed with a pestle in a mortar. The volume of buffer added to each sample was 250 μL per 0.08 g of plant sample (wet weight). The extracts were then centrifuged twice at 10,000 rpm for 5 min to remove plant debris.

Meanwhile, for quinoa grain extracts (POQ-36, POEQ-143, BGQ-24, and BGQ-174 varieties), a ratio of 400 μL of buffer per 0.2 g of plant sample was used. The pigments were extracted by soaking the grains in buffer and vortexing without grinding the samples. Finally, the solids were removed by centrifugation at 10,000 rpm for 2 min. All extracts were stored at −20 °C until further analysis.

Mass spectrometry analysis (HPLC-ESI-Q-TOF-MS)

In order to identify the decarboxylated betalains, they were first modeled with ChemBioDraw software (version 12.0) from PerkinElmer Inc. (Waltham, MA, USA). The expected structures were obtained, and the molecular weights and chemical formulas were calculated. This guided the search for ions in mass spectrometry analyses, carried out by using an Agilent 6550 Q-TOF-MS spectrometer equipped with a dual electrospray ionization interface (Agilent JetStream Dual ESI). Nitrogen was used as the drying gas at 200 °C, with a flow rate of 15 L min^{-1} and the nebulizer pressure set at 50 psi. The sheath gas temperature and flow rate were 300 °C and 12 L min^{-1}, respectively. The mass spectrometer was operated in the positive mode. The capillary spray, nozzle, fragmentor, and octopole 1 RF Vpp voltages were 4000, 500, 350 and 750 V, respectively. Centroid data in the 50 to 1000 *m/z* range were acquired for MS scans in 2 GHz Extended Dynamic Range High Resolution mode with 4 spectra/s, 250 ms/spectrum, and 2026 transients/spectrum. Spectra were recorded in both MS Scan and Target MS–MS mode using the *m/z* values for the compounds of interest. Reference masses at 121.0509 and 922.0098 were used for mass correction during the analysis. Data analysis was performed with MassHunter Qualitative Analysis Navigator software (Agilent Technologies, Rev. B.08.00). The chromatographic method used was the same as described for the HPLC analyses (HPLC analysis section). The samples of pure decarboxylated betalains (standards) were analyzed together with the natural extracts.

Data analysis

The expected nature of the 6-decarboxy-betaxanthins obtained in the bioreactors and purified as standards was verified in all cases by HPLC-ESI-Q-TOF-MS. After that, they were used to explore the presence of decarboxylated betalains in the different extracts from natural sources. For this purpose, peaks with the same

molecular mass (*m*/*z*) and RT with an error of ±0.2 min with respect to the standards were searched for. Each detected peak had an experimental mass (*m*/*z*), which was used for the calculation of Δppm with respect to the theoretical mass of the tentative molecule by using equation 1. The determination of Δppm values served as an elemental composition confirmation method, accepting values between −5 and 5 ppm (Ferrer et al. 2005; Sivaperumal et al. 2015) for the unambiguous identification of the different compounds.

$$\Delta\text{ppm} = \left(\frac{\text{Experimental mass} - \text{Theoretical mass}}{\text{Theoretical mass}}\right) \times 1{,}000{,}000.$$

Equation 1. Calculation of Δppm for the determination of the presence of decarboxylated betalains.

Data analysis by machine learning

The data were analyzed by unsupervised machine learning using the software Orange data mining (version 3.35.0) available at orangedatamining.com (Bioinformatics Lab, University of Ljubljana, Slovenia) (Demšar et al. 2013). The distance analysis was performed using the normalized Euclidean method, and then hierarchical clustering and MDS representation of the data were performed. The distances calculated from the data were also visualized with a heat map. Finally, a RadViz diagram was made to project the multidimensional data set in a 2-dimensional space.

Accession numbers

Sequence data from this article can be found in the GenPept (NCBI) data libraries under accession number WP_012222467.1 (GdDODA).

Acknowledgments

The authors are grateful to Dr. Alejandro Torrecillas (SAI, University of Murcia, Spain) for skillful technical assistance in mass spectrometry experiments.

Author contributions

F.G.-H. conceived the original screening and research plans. F.G.-H. supervised the experiments and supervised and completed the writing. P.M.-R., P.H.-E., D.J.P.-L., S.H.-G., and M.A.G.-R. performed the experiments. F.G.-H. and P.M.-R. conceived the project, designed the experiments, analyzed the data, and wrote the manuscript with the contributions of all the authors. L.R.G.-P. provided the quinoa grains. F.G.-H. agrees to serve as the author responsible for contact and ensures communication.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. HPLC analysis of the bioreactor medium used to produce alanine-6-decarboxy-betaxanthin.

Supplementary Figure S2. Chemical structures and HPLC analysis of the library of 30 decarboxylated betalains used in this work.

Supplementary Figure S3. 3D chromatograms obtained using the HPLC-DAD of the pure decarboxylated betalains used in this work.

Supplementary Figure S4. Absorbance spectra of the 30 decarboxylated betalains used in this work.

Supplementary Figure S5. ESI-Q-TOF-MS-MS analysis of the 30 pure pigments used in the present work.

Supplementary Table S1. Detection of decarboxylated betalains in red Swiss chard.

Supplementary Table S2. Detection of decarboxylated betalains in yellow Swiss chard.

Supplementary Table S3. Detection of decarboxylated betalains in red beetroot.

Supplementary Table S4. Detection of decarboxylated betalains in yellow beetroot.

Supplementary Table S5. Detection of decarboxylated betalains in red celosia.

Supplementary Table S6. Detection of decarboxylated betalains in yellow celosia.

Supplementary Table S7. Detection of decarboxylated betalains in quinoa (variety BGQ-24).

Supplementary Table S8. Detection of decarboxylated betalains in quinoa (variety BGQ-174).

Supplementary Table S9. Detection of decarboxylated betalains in quinoa (variety POEQ-143).

Supplementary Table S10. Detection of decarboxylated betalains in quinoa (variety POQ-36).

Funding

This work was supported by the Spanish Ministry of Science and Innovation, project PID2021-122896NB-I00 (MCI/AEI/10.13039/501100011033/FEDER, UE). P.M.-R. holds a contract financed by Séneca Foundation - Agency of Science and Technology of the Region of Murcia (21587/FPI/21). P.H.-E. enjoyed a contract financed by the University of Murcia (Spain).

Conflict of interest statement. Some of the authors launched in 2022 the company Betaelegans Biotech, a spin-off from the public academic institution Universidad de Murcia (Spain), primarily devoted to the synthesis of betalain standards. This has not biased or influenced the conception, analysis, or writing of the present research. The authors declare no other competing financial interest.

Data availability

The structures for the 30 molecules described in this work have been deposited in the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) under the external ID "decarboxybetalainX," making X reference to the numbering assigned throughout the present text.

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