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Multi-colored shades of betalains: recent advances in betacyanin chemistry

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Covering: 2001 to 2021

Betacyanins cover a class of remarkable natural red-violet plant pigments with prospective chemical and biological properties for wide-ranging applications in food, pharmaceuticals, and the cosmetic industry. Betacyanins, forming the betalain pigment group together with yellow betaxanthins, have gained much attention due to the increasing social awareness of the positive impact of natural products on human health. Betalains are commercially recognized as natural food colorants with preliminarily ascertained, but to be further investigated, health-promoting properties. In addition, they exhibit a remarkable structural diversity based on glycosylated and acylated varieties. The main research directions for natural plant pigments are focused on their structure elucidation, methods of their separation and analysis, biological activities, bioavailability, factors affecting their stability, industrial applications as a plant-based food, natural colorants, drugs, and cosmetics as well as methods for high-yield production and stabilization. This review covers period of the last two decades of betacyanin research. In the first part of the review, we present an updated classification of all known betacyanins and their derivatives identified by chemical means as well as by mass spectrometric and NMR techniques. In the second part, we review the current research reports focused on the chemical properties of the pigments (decarboxylation, oxidation, conjugation, and chlorination reactions as well as the acyl group migration phenomenon) and describe the semi-synthesis of natural and artificial fluorescent betalamic acid conjugates, showing various prospective research directions.

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1 Introduction

Betacyanins are unique, water-soluble, red-violet indoline- and dihydropyridine-derived nitrogen-containing natural plant pigments that occur in most families of the *Caryophyllales* order. Betacyanins, together with yellow-orange betaxanthins,

form a group of betalain pigments.[1] Simultaneously with anthocyanins, carotenoids, and chlorophylls, betalains are one of the most common plant pigments found in nature.[2,3] These compounds share betalamic acid as the main chromogenic structural unit condensed with *cyclo*-DOPA, forming betanidin or glycosylated *cyclo*-DOPA in other betacyanins as well as different amino acids or amines in betaxanthins.[4,5]

So far, betacyanins (Table 1) have been assigned to four structural groups comprising betanin-type, gomphrenin-type, amaranthin-type, and *Bougainvillea*-r-I-type,[6,7] the latter was recently renamed as melocactin-type.[8] However, for a convenient view on a wide spectrum of pigments, an expanded division with three additional betacyanin groups is proposed based on the sugar linkage: oleracin-type[9] (a very early result, not indepedently confirmed), apiocactin-type,[10–12] and *Bougainvillea*-v-type (here, we propose to name it as glabranin-type). In particular, the latter group of the pigments with the structures based on gomphrenin is important in the division system not only because of its less common 6-*O*-glycosylation pattern but also because of its unique and extremely complex profile of *ca.* 146 betacyanins tentatively detected in only one plant species, *Bougainvillea glabra* Choisy,[11] including nine definitively identified betacyanins by LC-ESI-MS and NMR.[13]

The schematic division of betacyanins into seven main structural groups based on their chemical structures is shown in Fig. 1. Betacyanins assigned to particular types differ in the attachment of the glucosyl moiety to the oxygen atom in the *ortho* position of *cyclo*-DOPA as well as the position of the substitution with additional glycosyl or glucuronosyl moieties.[6] The esterification of sugar moieties with organic acids such as ferulic, *p*-coumaric, caffeic, sinapic, and malonic acids is very common and leads to the formation of various acylated derivatives. The 15*R* diastereomers (the isoforms, epimers) of betacyanins are found in plants at much lower concentrations than the 15*S* forms and are regarded rather as artifacts due to epimerization, which takes place during the preparation of extracted plant samples.[14] The first studies on isomerization (as well as decarboxylation) mechanism in betacyanins were performed by Dunkelblum *et al.*[15] In more recent reports, the chromatographic differences in the 15*S*/15*R* diastereomers of decarboxylated betacyanins[16,17] as well as in the acyl migration products[18] were investigated.

Betanin (betanidin 5-*O*-β-D-glucopyranoside) is the main representative and most common betacyanin pigment found in the plant kingdom (Fig. 1 and 2A). In addition, betanin isolated from *Beta vulgaris* L. (beetroot, red beet) is the most studied pigment in the context of the antioxidant properties of betacyanins. Structurally, betanin and most of its derivatives are composed of aglycone (betanidin) linked by the β-glucosidic bond with the glucose unit at the C-5 carbon atom.[19,20] The chemical structures of additional compounds belonging to betanin-type betacyanins are shown in Fig. 2. Such compounds share

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a common betanidin backbone in their structures with an additional glucose moiety (betanin) along with (3-methyl-3-hydroxymethyl)glutaryl-, malonyl-, *E*-4-coumaroyl-, and *E*-feruloyl-moieties as well as the half sulphate ester in hylocerenin, phyllocactin, lampranthin I, lampranthin II, and prebetanin, respectively, but can be also partially oxidized (neobetanin) (Fig. 2).

Gomphrenin (betanidin 6-*O*-β-D-glucopyranoside), which is the isomeric pigment to betanin, is found at high concentrations in *Basella alba* L. fruits as well as in the leaves of its variety *Basella alba* var. 'Rubra' L.[21,22] According to our recent recommendation, we propose to rename gomphrenin I as "gomphrenin" in order to simplify the naming of its derivatives, especially generated by gomphrenin decarboxylation and oxidation.[16] In contrast to betanins, gomphrenins are characterized by the presence of a glucosyl moiety attached at the carbon C-6 (Fig. 1 and 3A).[21,22] Furthermore, the acylation of gomphrenin enables the formation of its derivatives such as *E*-4-coumaroyl-gomphrenin (former gomphrenin II, globosin), *E*-feruloyl-gomphrenin (former gomphrenin III, basellin), and *E*-sinapoyl-gomphrenin (gomphrenin IV, gandolin) (Table 1, Fig. 3B–D).[12] Gomphrenin pigments are of interest because their glucosylation position at the carbon C-6 should promote an interaction of the acylated residues with the carboxyl groups by increasing the intramolecular stabilization (by intramolecular stacking) of globosin and basellin[23] or other types of multiple acylated sophorosyl residues observed in glabranins. Betacyanins were also detected in red and purple *Gomphrena globosa* L. inflorescences. The most dominant betacyanins present in red *G. globosa* cultivars are amaranthin and celosianin but in violet species, gomphrenin and especially the acylated derivatives, globosin and basellin, are present.[24,25] In addition, other betacyanins were initially identified, namely, *Z*-4-coumaroyl-gomphrenin and *Z*-feruloyl-gomphrenin isomers as well as gandolin.[25,26]

Plant pigments belonging to the amaranthin-type group have a characteristic glucuronosylglucosyl moiety attached to the carbon C-5 in their structure. The most common example of these pigments is amaranthin [betanidin 5-*O*-β-(2′-*O*-β-glucuronosyl)glucoside] (Fig. 4A) isolated from plants of the *Amaranthaceae* family.[27,28] The other frequently detected pigments in the plants are betanin and 6′-*O*-malonyl-amaranthin.[29] Furthermore, sinapoyl-amaranthin has been tentatively identified in *G. globosa* petals.[30] Iresinin I [6′-*O*-(3″-hydroxy-3″-methyl) glutaryl-2′-*O*-glucuronosyl-betanin] and amaranthin are the most abundant pigments detected in *Iresine herbstii* Hook. ex Lindl leaves (Table 1).[27] Acylated amaranthin-based pigments, argentianin and celosianin, were also found in *Celosia* species[31] and the purple leaves of *I. herbstii* (Fig. 4).[25]

Most of the pigments from the two *Bougainvillea* groups have sophorosyl moieties linked to carbons C-5 or C-6 of the basic skeleton of betanidin, expanded further by glucosyl, rhamnosyl, apiosyl, or xylosyl moieties.[13,26,32–35] A complex mixture of betacyanins that differs in acyl-oligoglycoside units and exists mostly in the 6-*O*-glycosylated forms of betanidin was initially identified in the purple bracts of *B. glabra*.[13] However, due to the complex mixture of a huge number of betacyanins present in *B. glabra*,[34] its profile has not been completely characterized. The examples of bougainvillein-type betacyanins are bougainvillein-r-I (melocactin-type) and bougainvillein-v (glabranin-type) (Table 1, Fig. 5A and B). Similar betacyanins were recently found within *Melocactus* species.[8] The most abundant pigment, melocactin, previously named 'bougainvillein-r-I', was identified as betanidin 5-*O*-β-sophoroside (Fig. 5A). The presence of feruloylated and sinapoylated melocactins as well as melocactin's malonylated derivative, mammillarinin, was also noted (Fig. 5C).[8] In some *Mammillaria* species, mammillarinin [betanidin 5-*O*-(6′-*O*-malonyl)-β-sophoroside] was reported as the dominant pigment.[36]

Two red-violet acylated betacyanins, oleracins I and II, have been found in *Portulaca oleracea* L. Upon hydrolysis in alkaline conditions, oleracins gave ferulic acid and two newly detected pigments that were identified as 5-*O*-β-cellobiosides of

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Table 1 List of betacyanins (with proposed new trivial names[a]) identified by chemical methods; LC-MS and NMR in different plant sources

No.	Name	Trivial name/proposed new name[a]	*m/z* $[M + H]^+$	Chem.	LC-MS	NMR	Plant sources(Chem.)/(LC-MS)/(NMR)	Ref. (Chem.)/(LC-MS)/(NMR)
1	Betanidin 5-*O*-β-glucoside	Betanin	551	+	+	+	*B. vulgaris*/*G. globosa*/*B. vulgaris*	47 and 48/24/24
2	Betanidin	Betanidin	389	+	+	+	*B. vulgaris*/*B. glabra*/*B. vulgaris*	49/50/50
3	2-Decarboxy-betanin		507	–	+	+	*B. vulgaris*	51
4	14,15-Dehydro-betanin	Neobetanin	549	+	+	+	*B. vulgaris*	52 and 53
5	6′-*O*-Sulfate-betanin	Prebetanin	631	+	+	+	*3x P. americana*; *B. vulgaris*/*B. vulgaris*/	54/10/54
6	2-Decarboxy-phyllocactin		593	–	+	+	*B. vulgaris*	51
7	2-Decarboxy-betanidin		345	+	+	–	*C. acinaciformis*	55/51
8	2′-*O*-β-Apiosyl-betanin	Apiocactin[a]	683	–	+	+	*H. ocamponis*	11
9	5″-*O*-*E*-Sinapoyl-apiocactin		889	+	+	–	*2x H. ocamponis*/*Melocactus* spp.	11/8, 11
10	4′-*O*-Malonyl-betanin		637	+	+	–	*H. ocamponis*	11
11	5″-*O*-*E*-Feruloyl-apiocactin		859	+	+	+	*P. americana*	10
12	6′-*O*-Malonyl-betanin	Phyllocactin	637	–	+	+	*S. buckleyi*/*P. hybridus*	38/48
13	2′-*O*-β-Apiosyl-phyllocactin		683	–	+	+	*S. buckleyi*	38
14	2′-*O*-β-(5″-*O*-*E*-Feruloyl)-apiosyl-phyllocactin		945	–	+	–	*S. buckleyi*	38
15	6′-*O*-(3″-Hydroxy-3″-methylglutaryl)-betanin	Hylocerenin	695	–	+	+	*H. polyrhizus*	56
16	Betanidin 5-*O*-(6′-*O*-*E*-4-coumaroyl-β-glucoside)	Lampranthin I	(697)	+	–	–	*Lampranthus* sp.	57
17	Betanidin 5-*O*-β-(6′-*O*-*E*-feruloyl)-glucoside	Lampranthin II	727	+	+	+	*Lampranthus* sp./*G. globosa*/*L. sociorum*	57/24/58
18	3′-*O*-Sulfate-betanin	Rivinianin	(631)	+	–	–	*R. humilis*	59
19	Betanidin 5-*O*-β-sophoroside	Bougainvillein-r-I; melocactin[a]	713	+	+	+	*3x Boug.* Mrs. Butt'/*Melocactus* spp./	32/8, 11/11
20	(Caffeoyl and/or coumaroyl)-bougainvillein-r I	Bougainvillein-r-II, III, IV, V	–	+	–	–	*Boug.* Mrs. Butt	32
21	Betanidin 5-*O*-(2′-*O*-β-glucuronosyl)-glucoside	Amaranthin	727	+	+	+	*A. tricolor*/*Amaranthus* sp./*C. cristata*	60/27/61
22	2″-*O*-*E*-4-Coumaroyl-amaranthin	Celosianin I; argentianin[a]	873	–	+	–	*C. cristata*	25 and 27
23	2″-*O*-*E*-Feruloyl-amaranthin	Celosianin II; celosianin[a]	903	–	+	+	*C. cristata*	27 and 58/58
24	6′-*O*-Malonyl-amaranthin	Celoscristatin	813	+	+	+	*C. cristata*	29
25	4′-*O*-Malonyl-amaranthin		813	–	+	–	*C. cristata*	29
26	6′-*O*-(3″-Hydroxy-3″-methylglutaryl)-amaranthin	Iresinin I	871	+	+	+	*I. herbstii*	61/27/61
27	4′-*O*-Malonyl-bougainvillein-r I		799	–	+	–	*Mammillaria* spp.	36
28	6′-*O*-Malonyl-bougainvillein-r I	Mammillarinin	799	–	+	+	*Mammillaria* spp.	36
29	2-Decarboxy-mammillarinin		755	+	+	–	*Mammillaria* spp.	36
30	17-Decarboxy-mammillarinin		755	–	+	–	*Mammillaria* spp.	36
31	17-Decarboxy-bougainvillein-r I		669	–	+	–	*Mammillaria* spp.	36
32	Feruloyl-bougainvillein-r I		889	–	+	–	*M. crystallinum* L.; *B. vulgaris* (Swiss chard)	62 and 63
33	(Dehydrated phyllocactin)		619	–	+	–	*U. tuberosus*	64
35	Betanidin 6-*O*-β-D-sophoroside	Bougainvillein-v; glabranin[a]	713	–	+	+	*B. glabra* v. *sander.*	13/33
36	6″-*O*-Rhamnosyl-glabranin		859	+	–	–	*B. glabra* v. *sander.*	65
37	6′-*O*-*E*-Caffeoyl-glabranin	Cafglabranin[a]	875	–	+	+	*B. glabra*	13
38	6′-*O*-*E*-4-Coumaroyl-glabranin	Coumglabranin[a]	859	–	+	+	*B. glabra*	13
39	6″-*O*-*E*-4-Coumaroyl-glabranin		859	–	+	+	*B. glabra*	13
40	6′,6″-Di-*O*-*E*-4-coumaroyl-glabranin	Bicoumglabranin[a]	1005	–	+	+	*B. glabra*	13

Table 1 (*Contd.*)

No.	Name	Trivial name/proposed new name[a]	*m/z* [M + H]$^+$	Chem.	LC-MS	NMR	Plant sources(Chem.)/(LC-MS)/(NMR)	Ref. (Chem.)/ (LC-MS)/(NMR)
41	2″-*O*-[(6″-*O*-*E*-4-Coumaroyl)]-glucosyl-cafglabranin		1183	–	+	+	*B. glabra*	13
42	2″-*O*-Glucosyl-bicoumglabranin		1167	–	+	+	*B. glabra*	13
43	2″-*O*-[(6″-*O*-*E*-4-Coumaroyl)]-sophorosyl-cafglabranin		1345	–	+	+	*B. glabra*	13
44	Feruloyl-dihexosyl-betanidin		889	–	+	–	*M. amoenus*	8
45	Sinapoyl-dihexosyl-betanidin		919	–	+	–	*M. amoenus*	8
47	Cityl-(caffeoyl or 4-coumaroyl)-amaranthin	Suaedin	–	+	–	–	*S. fruticosa*	66
48	2″-*O*-*E*-Sinapoyl-amaranthin	Lindenin[a]	933	–	+	–	*G. globosa*; *I. lindenii*	25, 30 and 67
49	Betanidin 6-*O*-β-glucoside	Gomphrenin I; gomphrenin[a]	551	+	+	+	*G. globosa*; *B. alba*	68/24/24, 69
50	6′-*O*-*E*-4-Coumaroyl-gomphrenin I	Gomphrenin II; globosin[a]	697	+	+	+	*G. globosa*	68/24, 25/24
51	6′-*O*-*E*-Feruloyl-gomphrenin I	Gomphrenin III; basellin[a]	727	+	+	+	*G. globosa*	68/24, 25/24
52	6′-*O*-*E*-Sinapoyl-gomphrenin I	Gomphrenin IV; gandolin[a]	757	–	+	–	*G. globosa*	25 and 26
53	*E*-Isomer of gomphrenin II		697	–	+	–	*G. globosa*	30
54	*E*-Isomer of gomphrenin III		727	–	+	–	*G. globosa*	30
55	Betanidin 5-*O*-β-(*E*-feruloyl)-cellobioside	Oleracin I	(889)	+	–	–	*P. oleracea*	9 and 60
	Number of betacyanins identified by a given method			22	50	31		

betanidin and isobetanidin (Fig. 1E).[9] Five other yellow oleracins were also detected in *P. oleracea*; however, in contrast to oleracins I and II, they did not possess betalamic acid in their structures.[37]

Betacyanins belonging to the apiocactin-type group contain the rare branched pentose, apiose, bound to the carbon C-2′ of betanin. Pigments such as 2′-*O*-β-apiosyl-betanin (apiocactin) and/or its acylated derivatives (Table 1, Fig. 6A–C) have been found in fruits of pokeberry and its corresponding suspension and callus cultures (*Phytolacca americana* L.), Christmas cactus flowers (*Schlumbergera x buckleyi*), and *Hylocereus* species.[10,11,38] The chemical structure of the basic apiocactin-type betacyanin present in *Hylocereus* species was confirmed as 2′-*O*-β-apiosyl-betanin by Wybraniec *et al.*[11] In addition, in the same study, acylated compounds with sinapoyl and feruloyl residues attached to C-5″ of the apiose moiety were identified as 5″-*O*-*E*-sinapoyl-apiocactin and 5″-*O*-*E*-feruloyl-apiocactin.[11] Similarly, 5″-*O*-*E*-feruloyl-apiocactin was identified earlier in *P. americana* cultures by Schliemann *et al.*[10] Another betacyanin, 2′-*O*-β-(5″-*O*-*E*-feruloyl)-apiosyl-phyllocactin, which is the first betacyanin example containing both an aliphatic and an aromatic acyl residue, was tentatively detected in Christmas cactus flower extracts together with other apiocactin derivatives[38] and later in *Hylocereus* species.[11]

2 Characterization of betacyanins

2.1 Sources of betacyanin pigments

There are several known edible sources of betacyanins (Table 1). These include red and yellow beetroots (*B. vulgaris* ssp. vulgaris),[1] *B. alba* fruits,[39] *G. globosa* flowers,[13,30] grainy amaranth (*Amaranthus* sp.),[27] grains of *Chenopodium quinoa*,[40] leaves of *A. hortensis* var. *rubra*,[41] and fruits/flowers of cacti genera (*Opuntia*, *Hylocereus*, *Mammillaria*, *Melocactus*, and *Myrtillocactus spec.*).[8,36,42–45] Pokeberry (*Phytolacca americana* L.) is another source of betacyanins but it has been forbidden as a food colorant due to the presence of toxic saponins and lectins.[46]

Compounds tentatively identified as betacyanin-like are also found in some higher fungi such as *Amanita muscaria* (fly agaric).[70,71] The less common edible sources are Ulluco tubers (*Ullucus tuberosus*)[64,72] as well as fruits and berries of pigeonberry (*Rivina humilis*).[73]

Table 1 presents a detailed summary of the identified betacyanins in different plant sources along with the methods of their identification. Most of the chemical methods were applied in the early decades of betalain research based on the typical reactions of hydrolysis and derivatization, such as permethylation and partial degradation, resulting in the generation of diagnostic products, which can be identified and can confirm certain parts of the chemical structure of a starting compound. The chemical structures of 31 naturally occurring betacyanins were definitively identified by NMR methods, whereas a huge group of over 187 betacyanins (including pigments from their richest source, *B. glabra*) were detected by LC-MS techniques and still await the final confirmation. In addition, structures of 18 betacyanin derivatives were also

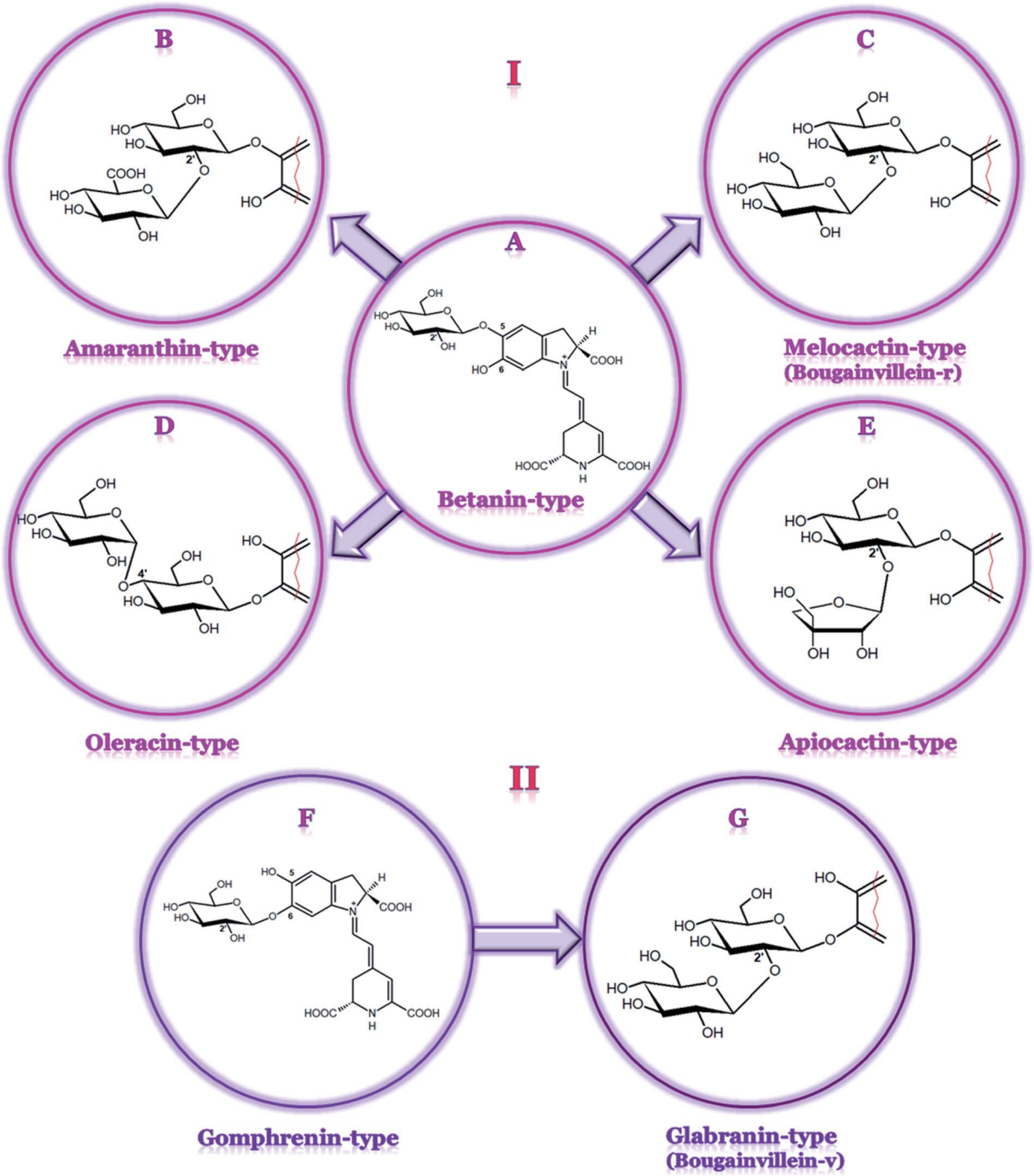


Fig. 1 A new comprehensive classification of betacyanin pigments belonging to the betanin-type (A); amaranthin-type (B); melocactin-type (C); oleracin-type (D); apiocactin-type (E); gomphrenin-type (F); and glabranin-type (G). The structures of betacyanins belonging to the (B–E) types are based on betanin (I), while the structures of glabranins are based on the gomphrenin backbone (II).

confirmed by NMR and 61 tentatively were identified by LC-MS methods (Fig. 7).

2.2 Biological properties of betacyanins

Numerous health-promoting activities are attributed to betacyanin pigments, including antioxidative,[6,74] chemopreventive,[75] and anti-inflammatory[76,77] properties. Furthermore, it was noted that betacyanins protect low-density lipoproteins (LDL) against oxidative damage by reacting with the LDL polar groups.[78] In addition, betanin present in red beets prevents DNA from damage in lymphocytes and hepatocytes.[79] Betanin also shows neuroprotective effects, improves cognitive functions and reduces oxidative stress caused by D-galactose in the brain of mice by increasing the level of antioxidant enzymes and reducing lipid peroxidation.[80]

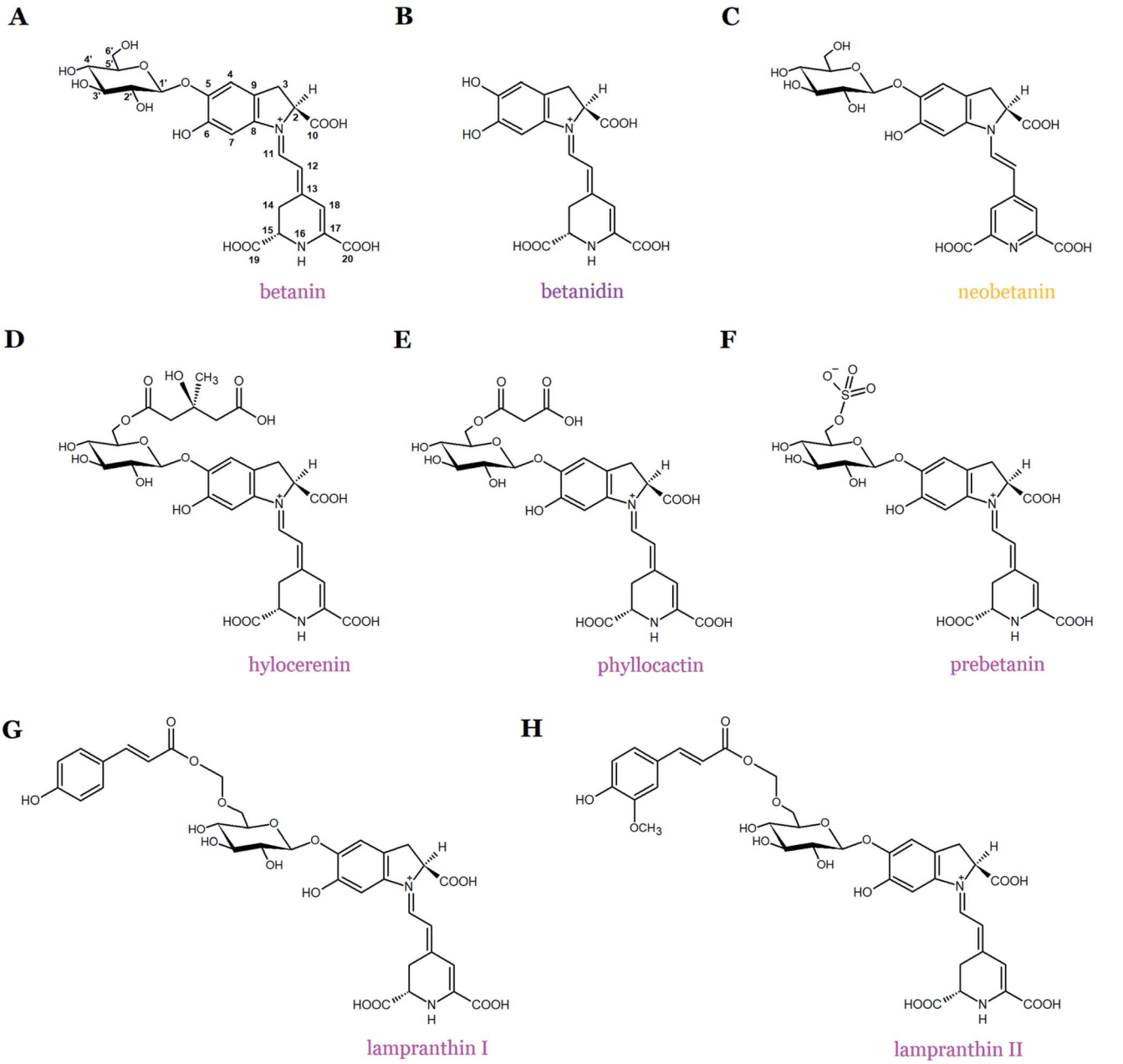


Fig. 2 Chemical structures of betanin-type betacyanins.

Some betacyanin pigments have been shown to have higher antioxidant activity compared to typical natural antioxidants such as ascorbic acid,[81] rutin,[82] catechin,[83] β-carotene,[84] and α-tocopherol.[6,85] In a test of the antioxidant activity expressed in TROLOX equivalents, betacyanins extracted from red beet exhibited 1.5–2.0 times higher free radical scavenging activity than some anthocyanins such as cyanidine-3-*O*-glucoside and cyanidine above pH 4.[86,87]

The effect of the hydroxyl group position on the antioxidant activity of the betacyanins bearing the conjugated phenol moiety was studied. Three non-natural regioisomeric phenolic betacyanins (common name: *o*-, *m*-, *p*-OH-pBeets) were semi-synthesized by coupling betalamic acid with appropriate aminophenols in water according to a procedure described by Schliemann and co-authors[88] and their antioxidant activity was studied.[89,90] The results showed that the *meta* isomer exhibits greater antiradical activity than most of the betalains, flavonoids, and anthocyanins. This result may be explained by the fact that the phenolic moiety is not conjugated with the diazapolymethine system but both groups are prone to further oxidation and can stabilize the radicals by resonance. In addition, the N–H imine bond present in the *meta* regioisomeric structure is the preferred site for oxidation, whereas $1e^-$ oxidation of the phenolic groups in *p*- and *o*-OH-pBeet results in the formation of semi-quinone and leads to lower values of the potential energy (Ep) compared to *m*-OH-pBeet.[89,90]

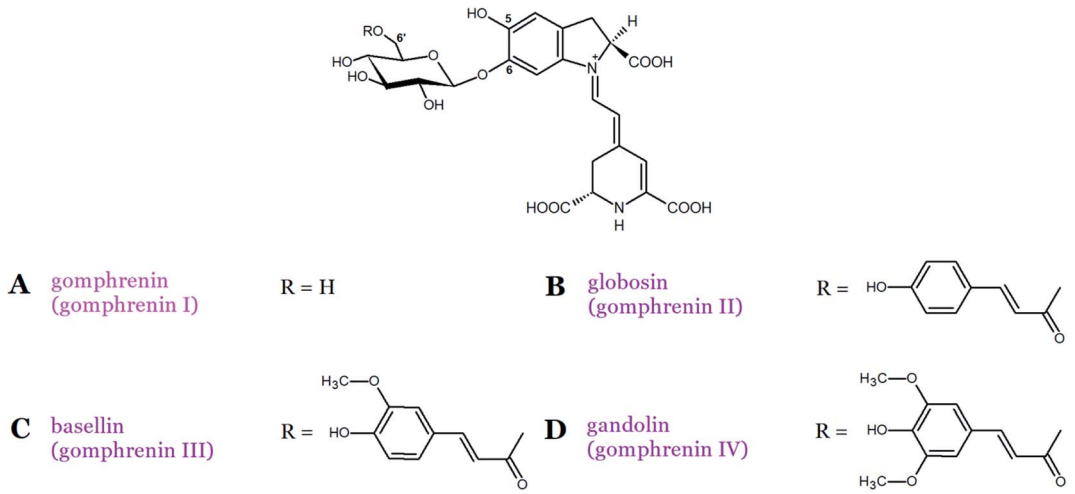


Fig. 3 Chemical structures of basic betacyanins (A–D) belonging to gomphrenin-type.

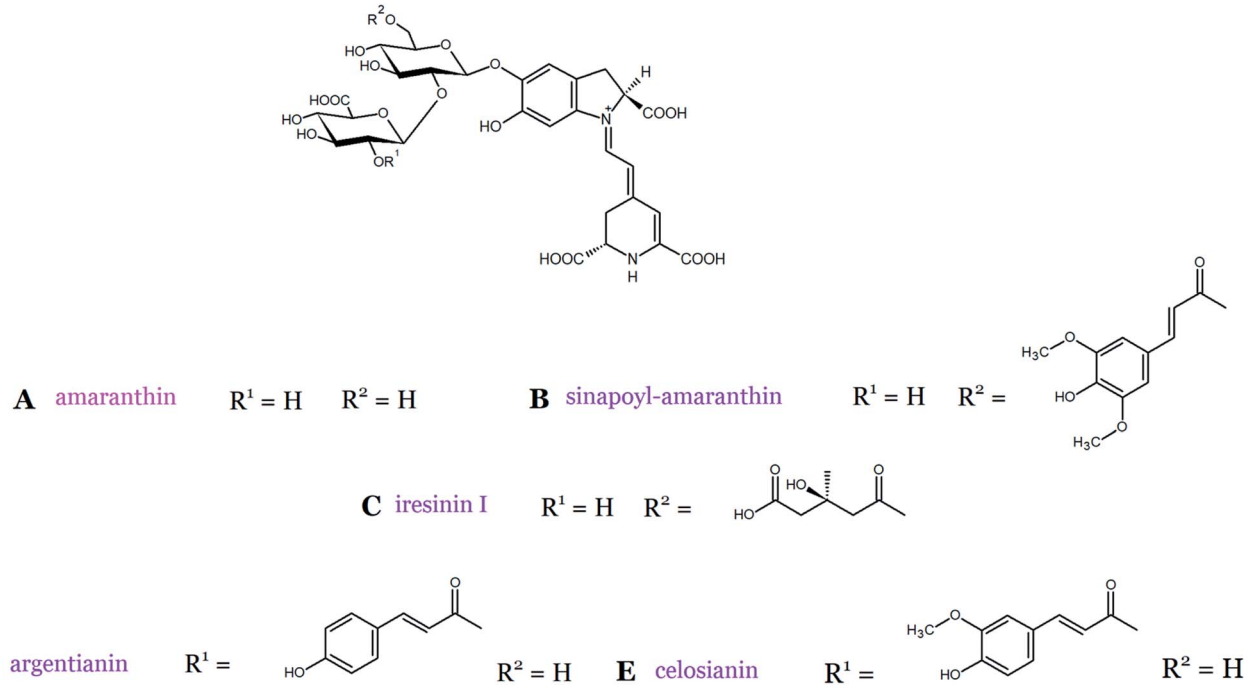


Fig. 4 Chemical structures of betacyanins (A–E) belonging to amaranthin-type.

Betacyanins have been shown to actively participate in free radical scavenging and consequently, their consumption may lead to chemoprevention and delay of the development of cancer tissues.[75] Purified betanin shows strong inhibition of melanoma cancer cell proliferation[91] and excellent growth inhibition of MCF-7 (breast), HCT-116 (colon), AGS (stomach), SF-268 (CNS), and NCI-H460 (lungs) cancer cell lines with IC_{50} values of 162, 142, 158, 164, and 147 μg mL^{-1}, respectively.[92] It has also been found that betanin induces dose- and time-dependent apoptosis of cells in the human chronic myelogenous leukemia (K562) cell line.[93]

In vitro experiments revealed that betalains extracted from *B. vulgaris* juice show pro-apoptotic effects on activated neutrophils and inhibit the neutrophil oxidative metabolism.[94] In addition, pilot clinical trials have shown that short-term treatment with red beet extract improves joint function in people with knee joint discomfort.[76,95]

2.3 Applications of betacyanin pigments

In Fig. 8, betacyanin applications are schematically presented. Natural plant extracts containing betacyanin pigments are increasingly used in food technology as a safe alternative to synthetic food colorants. According to Title 21 of the Code of

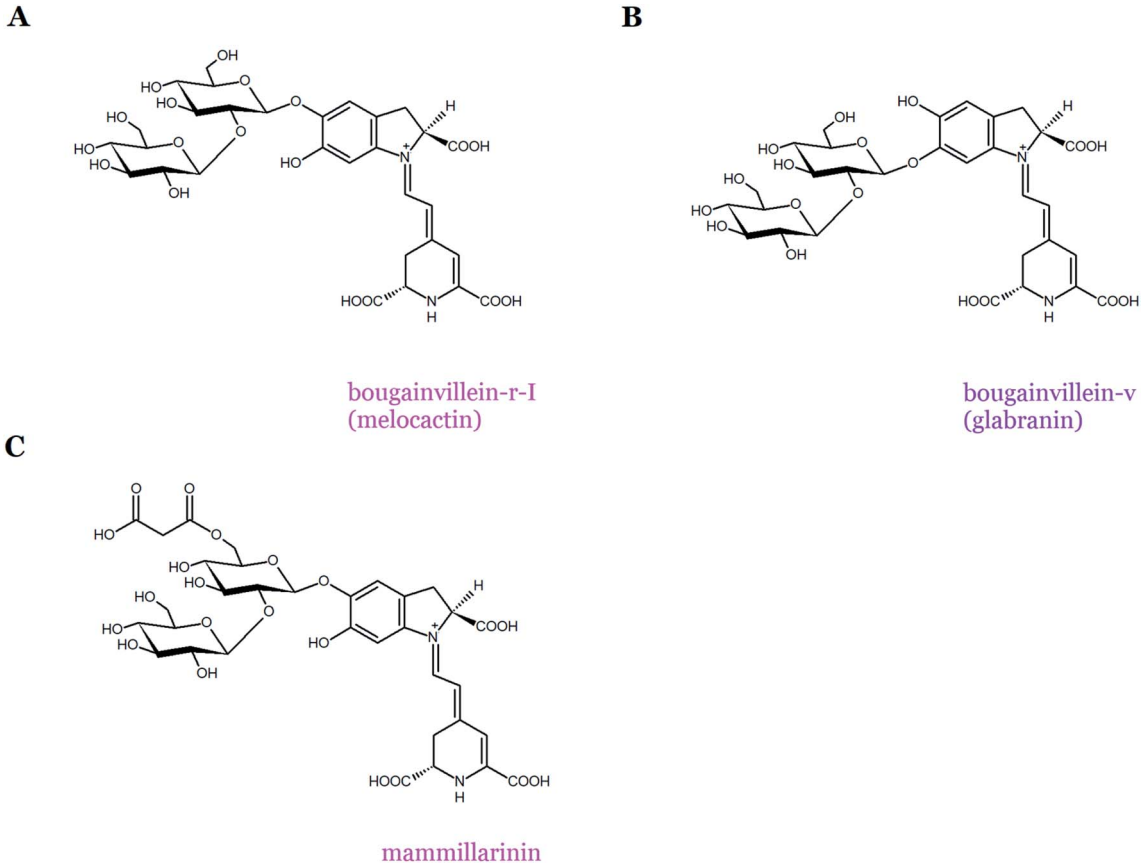
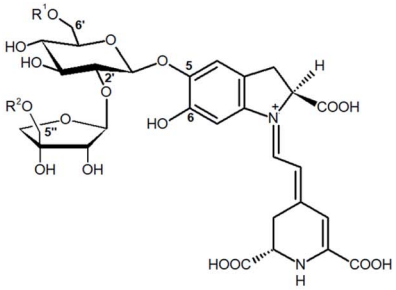


Fig. 5 Chemical structures of betacyanins belonging to both the bougainvillein groups: melocactin-type (bougainvillein-r-I (A) and mammillarinin (C)) and glabranin-type (bougainvillein-v) (B).

Federal Regulations part 73.40 of the Food and Drug Administration (FDA), USA, and under the E-162 code in the European Union, beetroot powder rich in betanin is permitted as a natural red food colorant.[3] Food preparations obtained in different industrial conditions can significantly vary in their stability and coloration due to the presence and the composition of betacyanin degradation products. Betalains are stable at pH ranging from 3 to 7 and they are suitable for dyeing low acidic and neutral foods. In addition, they may be stabilized by ascorbic acid. In contrast, due to instability at pH over 3, the application of anthocyanins in the coloration of such foods is not possible. Furthermore, the use of ascorbic acid also facilitates the degradation of anthocyanins. For this reason, the utilization of betalain pigments instead of anthocyanins for coloring food with a high amount of vitamin C or vitamin C-supplemented products seems to be more favorable.[96,97] Beetroot extracts are utilized to emphasize the redness of dairy products such as tomato soups, sauces, pastes, desserts, jams, sweets, and jelly beans. They are also used to protect meat from discoloration and to extend its shelf-life.[98] However, due to the unpleasant flavor of beetroot extracts due to their geosmin and pyrazine derivatives content, a membrane process during juice concentration is needed to be applied for commercial red beet application.[14]

Due to health-related benefits, betalains are also regarded as natural dietary supplements.[99] The effect of food supplements containing betalain-rich extracts (a betalain-rich supplement of red beetroot and a betacyanin-rich supplement of *Opuntia stricta*) on different atherosclerotic risk factors were tested among coronary artery disease patients. The results showed that the levels of glucose, total cholesterol, homocysteine, triglyceride, and low-density lipoprotein (LDL) of 48 male patients were decreased. Furthermore, betalain-rich supplements taken at a safe dose of 50 mg betalain/betacyanins for 2 week interventions reduced the systolic and diastolic blood pressures.[100] In the past 20 years, a number of food and nutraceutical preparations have been developed from quinoa (*Chenopodium quinoa* Willd.) as well. Several clinical studies have demonstrated that quinoa supplementation exerts prominent effects on the cardiovascular, gastrointestinal, and metabolic health of humans.[101]

Natural plant pigments were also tested in the context of solar cell applications as a cheaper, faster, low energy, and environment-friendly alternative to dyes based on ruthenium complexes.[102,103] Betanin, 2,17-bidecarboxy-betanin, vulgaxanthin I, extracted from red beetroot extract and betanidin-6-*O*-(6′,6″-di-*O*-*E*-4-coumaroyl)-β-sophoroside from *Bougainvillea* were also tested toward the application as dye-sensitized solar cells (DSSC). The construction of betalain-based DSSC is



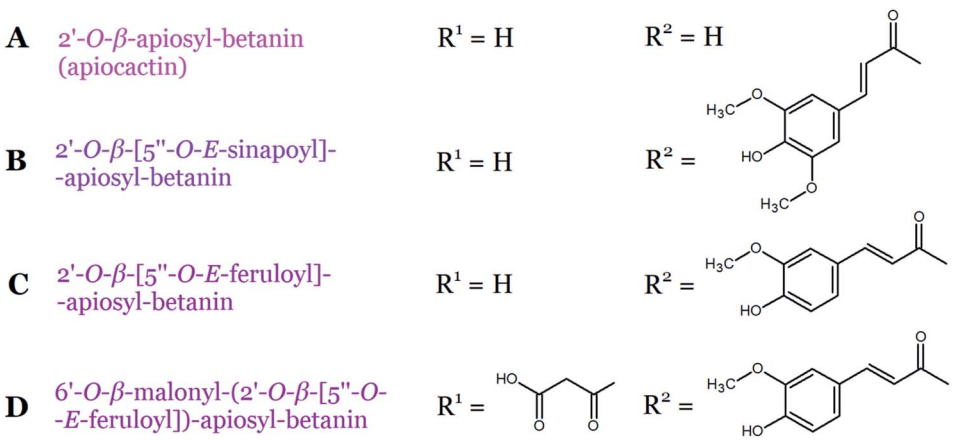


Fig. 6 Chemical structures of betacyanins (A–D) belonging to apiocactin-type.

Fig. 7 The number of naturally-occurring betacyanins and semi-synthesized betacyanins derivatives identified chemically, by NMR techniques and detected by LC-MS methods.

possible due to the carboxylic groups in betalains, which serve as anchors after the immersion of TiO_2 electrodes in betalain aqueous solutions at pH ≈ 3. The quantum yield of electron injection in betacyanin-based and betaxanthin-based DSSC is about 50% and 70%, respectively. In the case of betaxanthin-based DSSC, the total sunlight conversion efficiency was however smaller because of the blue-shifted absorption band.[104] The electron injection from betanin to TiO_2 is a two-electron, one-proton process.[105,106]

Enhanced light-harvesting and the photoconversion efficiency of nanocrystalline TiO_2 sensitized with betanin was observed and revealed the self-assembly of betanin on the surface. Due to the fact that an aggregated betanin sensitizer improved the performance in DSSC, the mechanism of improved electron injection and collection in DSSCs with more aggregation as compared to monomeric betanin need to be regarded.[107]

Betanin is capable of injecting up to two electrons per photon absorbed into the ZnO conduction band in less than 15 ps.[108] Betanin was also used for the light-harvesting process in ultra-stable ZnO nanocrystals modified with a carboxylate oligoethylene glycol shell system studied for utilization in H_2 production under visible light irradiation.[109]

The photophysical properties of betanin in aqueous and alcoholic solutions were determined and formation of betanin electronically excited species was studied. Betanin has a short S_1 state lifetime (π, π^*) (6.4 ps in water), mainly determined by the efficient $S_1 \rightarrow S_0$ radiationless relaxation, which probably requires a strong geometry change. Other processes, such as photoproduct formation or $S_1 \rightarrow T_1$ intersystem crossing have

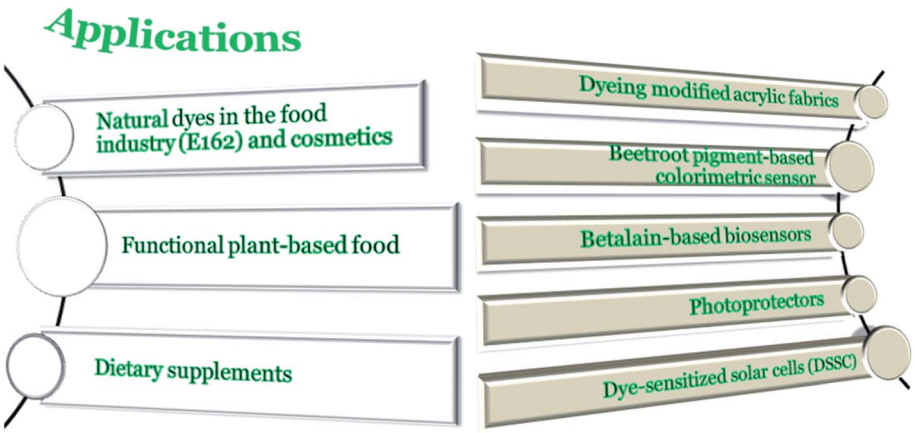


Fig. 8 Overview of various betacyanin pigment applications.

been reported as virtually absent. In the absence of triplet excited state formation, the fast light-to-heat conversion was observed, supporting the consideration that betanin is a photoprotector *in vivo*.[110,111]

Due to the presence of the phenolic moiety within the betanin structure, which may react with $^1O_2$, the colorimetric detection of reactive oxygen species is possible. Efficient singlet oxygen quenching by betanin in deuterated water was reported. The capacity of betanin to quench $^1O_2$ supports the photoprotective role of betanin *in vivo*.[112]

In another study, a red beetroot pigment-based colorimetric sensor for detecting copper ions in drinking water was developed. In addition, an application for quantitative and visual colorimetric analysis was designed for the Android system smartphone. When Cu^{2+} concentration in drinking water increased, the red beet pigment solution gradually changed from bright purple to orange-red as it formed a complex by chelating Cu^{2+}. The linear range of this detection system ranged from 4 to 20 μM and the limit of detection was 0.84 μM.[113]

Betanin can also compete with synthetic dyes in terms of the depth and color stability. The possibility of dying of modified acrylic fabrics with betanin was investigated. It was found that the optimal conditions during 45 min dyeing of acrylic fabrics with betanin were 50 °C and pH 5. The modified materials were resistant to color loss under the influence of water and the addition of cobalt(II) sulphate(VI) ($CoSO_4$) ensured light resistance.[114]

Attempts are being made to develop cheap, green, and easy-to-use betalain-based biosensors. A good example is a need for the early detection of *Bacillus* species. The highly stable and virulent *B. anthracis* has been considered as a dangerous bioterrorism agent after the anthrax attack took place in 2001.[115] Another threat is pathogenic *B. cereus* that grows on food.[116] In order to detect the *Bacillus* species early, research on the application of betanin as a ligand in the new europium(III) complex, sensitive to calcium dipicolinate, CaDPA (the major component of the bacterial spore core) was conducted. In the presence of Eu(III) ions, betanin was converted into a water-soluble, non-luminescent orange complex. The addition of CaDPA changes the orange color of the [Eu(betanin)$^+$] aqueous solution to magenta. The limit of detection of CaDPA is about 2.06×10^{-6} mol L^{-1}. In addition, the complex is sensitive to CaDPA but not to other structurally similar aromatic, pyridinic, and acidic ligands.[117,118]

Beetroot extracts are under consideration as a mild alternative to organometallic reductants, such as $NaBH_4$ and N_2H_4, and as stabilizing (growth-limiting) agents. Beetroot extract was exploited for the bottom-up synthesis of metal nanoparticles, leading to broad size and shape distributions.[119]

3 Chemistry of betacyanins and betalamic acid derivatives

3.1 Overview of betacyanin chemical reactions

Taking into account the pro-health nature of betacyanin pigments, their diversity, structural characteristics, and light-absorption properties, there is a growing need to supplement and broaden the knowledge on their chemistry. Understanding the reactions that betacyanins undergo may contribute to the determination of their fate and distribution in the human body, increase their stability, the development of betalain-based biosensors, screening assays and tailor-made probes, the preparation of betalain-based potential medicinal substances or cosmetics. In the following part of this contribution, we sum up the current knowledge obtained during the last decade on the chemical properties of betacyanins as well as new perspectives of the semi-synthesis of betalamic acid derivatives with extended chromophoric systems. The list of betacyanin derivatives semi-synthesized from plant sources and betacyanin degradation or chemical conversion products, with their structural identification by LC-MS and NMR methods, is presented in Table 2.

3.1.1 Thermal decarboxylation and dehydrogenation of betacyanins. Considering the significant impact of organoleptic characteristics on food selection and consumer acceptance, color is one of the most important attributes of foods as it is considered as a quality indicator.[120,121] Nowadays, the complex processing of most foodstuffs may cause some unwanted changes in their visual appearance, contributing to the fading

Table 2 List of betacyanin derivatives semi-synthesized from plant sources and betacyanin degradation or chemical conversion products, with their structural identification by LC-MS and NMR methods

No.	Name	Trivial name or abbreviation	m/z $[M + H]^+$	LC-MS	NMR	Plant sources (Chem.)/(LC-MS)/(NMR)	References (Chem.)/(LC-MS)/(NMR)
56	2-Decarboxy-betanin	2-dBt	507	+	+	*B. vulgaris*/*H. polyrhizus*	126/127
57	15-Decarboxy-betanin	15-dBt	507	+	–	*B. vulgaris*	122
58	17-Decarboxy-betanin	17-dBt	507	+	+	*B. vulgaris*/*H. polyrhizus*	122/127
59	2,17-Bidecarboxy-betanin	2,17-dBt	463	+	+	*B. vulgaris*/*H. polyrhizus*	126/127
60	2,15,17-Tridecarboxy-betanin	2,15,17-dBt	419	+	–	*B. vulgaris*	126
61	2-Decarboxy-neobetanin	2-dNBt	505	+	–	*B. vulgaris*	126
62	2,17-Bidecarboxy-neobetanin	2,17-dNBt	461	+	–	*B. vulgaris*	126
63	2,15,17-Tridecarboxy-neobetanin	2,15,17-dNBt	417	+	–	*B. vulgaris*	126
64	2,17-Bidecarboxy-xanbetanin	2,17-dXBt	461	+	–	*B. vulgaris*	52
65	2-Decarboxy-xanneobetanin	2-dXNBt	503	+	+	*B. vulgaris*	52
66	2,17-Bidecarboxy-xanneobetanin	2,17-dXNBt	459	+	+	*B. vulgaris*	52
67	2,15,17-Tridecarboxy-xanneobetanin	2,15,17-dXNBt	415	+	+	*B. vulgaris*	52
68	18-Chloro-betanin	Bt-Cl	585	+	+	*B. vulgaris*	156
69	18-Chloro-17-decarboxy-betanin	17-dBt-Cl	541	+	+	*B. vulgaris*	156
70	18-Chloro-2,17-bidecarboxy-betanin	2,17-dBt-Cl	497	+	+	*B. vulgaris*	156
71	18-Chloro-2-decarboxy-betanin	2-dBt-Cl	541	+	–	*B. vulgaris*	156
72	18-Chloro-15-decarboxy-betanin	15-dBt-Cl	541	+	–	*B. vulgaris*	156
73	Cysteinyl-2-decarboxy-xanbetanin	2-dXBt-Cys	624	+	–	*B. vulgaris*	149
74	2-Decarboxy-xanbetanin	2-dXBt	505	+	–	*B. vulgaris*	149
75	*N*-Methyl-phenyl-betalain	mepBeet	301	+	+	*B. vulgaris*	165
76	*N*-Aryl-phenyl-betalains	dipBeet	363	+	+	*B. vulgaris*	165
77	2,4-Dimethylpyrrole-betalain	BeetBlue	289	+	+	*B. vulgaris*	166
78	7-Amino-4-methylcoumarin-betalain	cBeet120	369	+	+	*B. vulgaris*	167
79	7-Amino-4-trifluoromethylcoumarin-betalain	cBeet151	423	+	+	*B. vulgaris*	167
80	7-Amino-4-methylcoumarin-betalain	BtC	369	+	–	*B. vulgaris*	168
81	17-Decarboxy-neobetanin	17-dNBt	505	+	–	*H. polyrhizus*	125
82	2-Decarboxy-phyllocactin	2-dPc	593	+	+	*H. polyrhizus*	125/127
83	17-Decarboxy-phyllocactin	17-dPc	593	+	+	*H. polyrhizus*	125/127
84	2,17-Bidecarboxy-phyllocactin	2,17-dPc	549	+	+	*H. polyrhizus*	125/127
85	2,15,17-Tridecarboxy-phyllocactin	2,15,17-dPc	505	+	–	*H. polyrhizus*	125
86	2-Decarboxy-hylocerenin	2-dHc	651	+	+	*H. polyrhizus*	125/127
87	17-Decarboxy-hylocerenin	17-dHc	651	+	–	*H. polyrhizus*	125
88	2,17-Bidecarboxy-hylocerenin	2,17-dHc	607	+	+	*H. polyrhizus*	125/127
89	2,15,17-Tridecarboxy-hylocerenin	2,15,17-dHc	563	+	–	*H. polyrhizus*	125
90	2-Decarboxy-neophyllocactin	2-dNPc	591	+	–	*H. polyrhizus*	125
91	17-Decarboxy-neophyllocactin	17-dNPc	591	+	–	*H. polyrhizus*	125
92	2,17-Bidecarboxy-neophyllocactin	2,17-dNPc	547	+	–	*H. polyrhizus*	125
93	2,15,17-Tridecarboxy-neophyllocactin	2,15,17-dNPc	503	+	–	*H. polyrhizus*	125
94	2-Decarboxy-neohylocerenin	2-dNHc	649	+	–	*H. polyrhizus*	125
95	17-Decarboxy-neohylocerenin	17-dNHc	649	+	–	*H. polyrhizus*	125
96	2,17-Bidecarboxy-neohylocerenin	2,17-dNHc	605	+	–	*H. polyrhizus*	125
97	2,15,17-Tridecarboxy-neohylocerenin	2,15,17-dNHc	561	+	–	*H. polyrhizus*	125
98	2-Decarboxy-xanbetanidin	2-dXBd	343	+	–	*B. alba*	140
99	2,17-Bidecarboxy-xanbetanidin	2,17-dXBd	299	+	–	*B. alba*	140
100	2-Decarboxy-xangomphrenin;	2-dXGp	505	+	–	*B. alba*	140
101	2,17-Bidecarboxy-xangomphrenin	2,17-dXGp	461	+	–	*B. alba*	140
102	2-Decarboxy-xanneobetanidin	2-dXNBd	341	+	–	*B. alba*	140
103	2,17-Bidecarboxy-xanneobetanidin	2,17-dXNBd	297	+	–	*B. alba*	140
104	2-Decarboxy-xanneogomphrenin	2-dXNGp	503	+	–	*B. alba*	140
105	2,17-Bidecarboxy-xanneogomphrenin	2,17-dXNGp	459	+	–	*B. alba*	140
106	2,15,17-Tridecarboxy-xanneogomphrenin	2,15,17-dXNGp	415	+	–	*B. alba*	140
107	Cysteinyl-betanidin	Cys-Bd	508	+	–	*B. alba*	149
108	Cysteaminyl-betanidin	CSH-Bd	464	+	–	*B. alba*	149
109	*N*-Acetyl-cysteinyl-betanidin	NAC-Bd	550	+	–	*B. alba*	149
110	Dithiothreitol-betanidin	DTT-Bd	541	+	–	*B. alba*	149
111	Cysteinyl-gomphrenin	Cys-Gp	670	+	–	*B. alba*	149
112	Cysteaminyl-gomphrenin	CSH-Gp	626	+	–	*B. alba*	149
113	Dithiothreitol-gomphrenin	DTT-Gp	703	+	–	*B. alba*	149
114	*N*-Acetyl-cysteinyl-gomphrenin	NAC-Gp	712	+	+	*B. alba*	149
115	Glutathionyl-betanidin	GSH-Bd	694	+	+	*B. alba*	140
116	Glutathionyl-gomphrenin	GSH-Gp	856	+	–	*B. alba*	140
	Number of betacyanin derivatives identified by a given method			18	6		

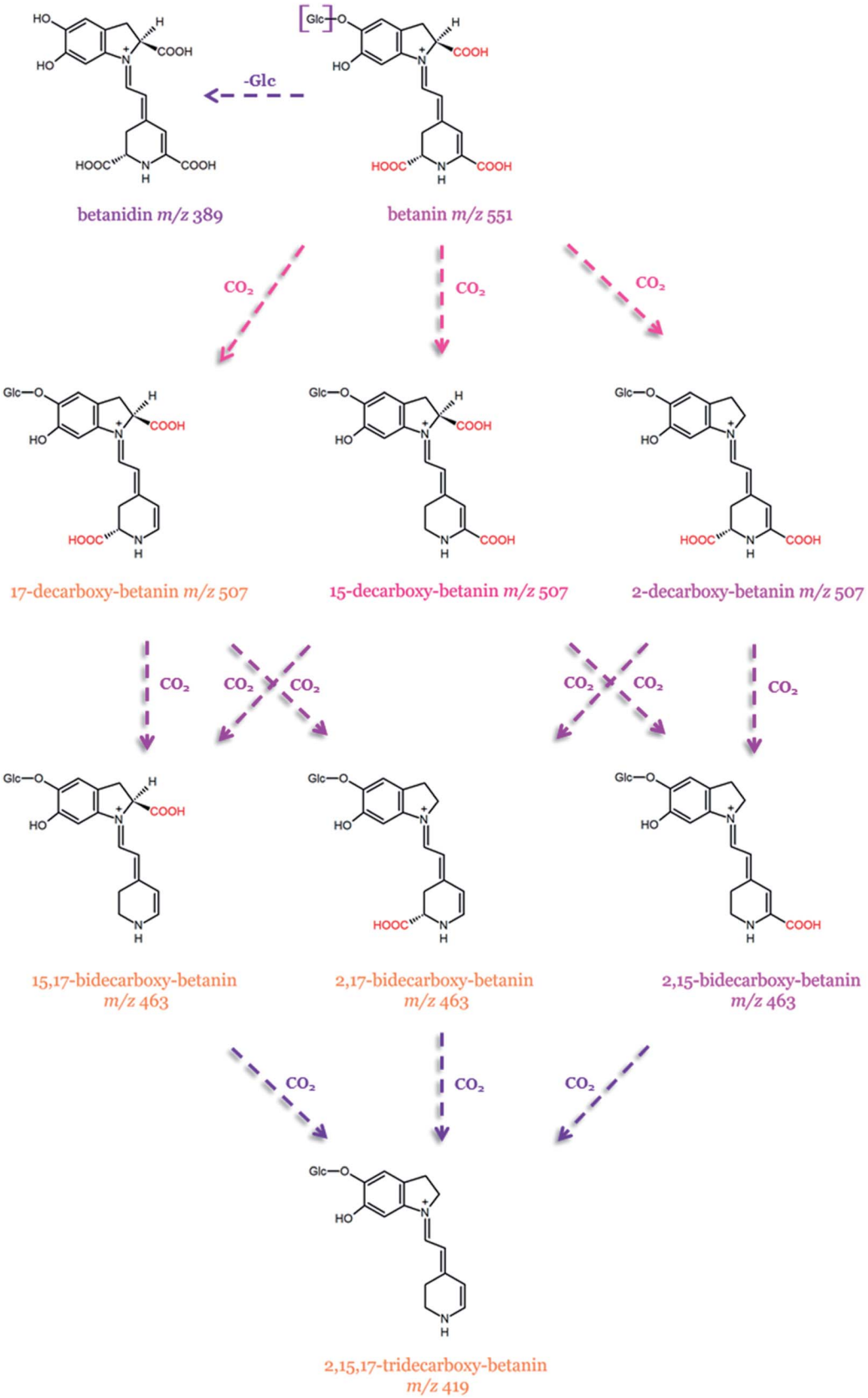


Fig. 9 Possible pathways of betanin transformation by decarboxylation, dehydrogenation, and deglucosylation.[129] The *m*/*z* values are from the $[M + H]^+$ ions.

effect in their coloration. During the degradation of betacyanins, they may undergo deglucosylation, decarboxylation, dehydrogenation, and isomerization processes (Fig. 9).

The first heat-degradation structural studies on betanin from *B. vulgaris*, as well as betacyanins isolated from *H. polyrhizus* (betanin, phyllocactin, and hylocerenin), were performed by Herbach *et al.*[122–124] and Wybraniec *et al.*[125–127] Betanin was proven to be the most stable pigment, while the stability of phyllocactin and hylocerenin was seemingly enhanced due to the formation of red degradation products, exhibiting improved color retention in comparison to their precursors. Betanin degradation proceeded through hydrolytic cleavage, while hylocerenin dominated in decarboxylation and dehydrogenation. The degradation of phyllocactin involves the decarboxylation of the malonic acid moiety and the formation of betanin by demalonylation along with subsequent betanin degradation. After prolonged heating, dehydrogenation at C2–C3 carbons was also noticed.[123–125,127]

Decarboxylated and dehydrogenated betanin derivatives occurring at the highest concentrations in a betacyanin-rich red beetroot extract (RBE) were also characterized (Table 2).[128] The main pigments in the RBE are betanin and its isoform with 17-decarboxy-betanin/-isobetanin, 15-decarboxy-betanin, and small quantities of 2-decarboxy-betanin/-isobetanin as well as 2,17-bidecarboxy-betanin/-isobetanin.[128] Furthermore, mixtures of mono-, bi-, and tridecarboxylated betanins together with their corresponding neo-derivatives obtained from the RBE were identified as the heating degradation products.[129] In addition, new isomeric decarboxylated derivatives, namely, 2,15-bidecarboxy-betanin and 15,17-bidecarboxy-betanin, were detected for the first time. These newly detected derivatives were also formed during heating experiments on red beetroot extract. Furthermore, the presence of 2,15,17-tridecarboxy-betanin was also acknowledged.[129] Presumably, the presence of these additional betanin derivatives results from enhanced decarboxylation, which is an integral process during RBE preparation. It was also reported that the higher concentration of acetic acid during the thermal treatment of RBE contributes to a selective formation of 2-decarboxy-betanin/-isobetanin. The latter pigments are present in the RBE at a very low level. However, according to the report, the most decisive factors are the concentrations of the substrate and acetic acid. Higher RBE concentration favors the formation of 2,17-bidecarboxy-betanin/-isobetanin over 2,15-bidecarboxy-betanin.[129]

An effective method for the separation of newly formed derivatives was also proposed. A bunch of decarboxylated and dehydrogenated betacyanins obtained during mild thermal treatment of *B. vulgaris* juice was subjected to high-performance counter-current chromatographic (HPCCC) preparative fractionations.[130] The mixtures composed of betacyanins with different polarity and physicochemical properties were separated in highly polar solvent systems containing a high concentration of ammonium sulfate and ion-pairs, aqueous-organic solvent systems including ion-pair reagents (trifluoroacetic acid, heptafluorobutyric acid). The application of both the solvent systems enabled the separation and purification of 2-decarboxy-betanin, 2,17-bidecarboxy-betanin, their corresponding isoforms, and neobetanin from a mixture composed mainly of betacyanins, neobetanin, and their decarboxylated derivatives (Table 2), as well as 17-decarboxy-neobetanin, 2,15,17-tridecarboxy-2,3-dehydro-neobetanin, 2,17-bidecarboxy-2,3-dehydro-neobetanin, 2,15,17-tridecarboxy-neobetanin, and 2-decarboxy-neobetanin from another mixture consisting of decarboxy- and dehydrobetacyanins. The results also show that the utilization of heptafluorobutyric acid as an ion-pair reagent is more suitable than a polar solvent system with trifluoroacetic acid because it contributes to the formation of more hydrophobic betacyanin ion-pairs.[130] The separation of betacyanins from a processed *B. vulgaris* juice (betanin, isobetanin, neobetanin, and decarboxylated betacyanins) was also demonstrated in a food-grade, gradient solvent system consisting of sodium chloride, butanol, water, as well as different volumes of phosphoric acid and/or ethanol. The quality of isolation was dependent on the ethanol and acid concentrations with the lowest volume gradient, providing the best separation conditions. Betacyanins were eluted in the organic, upper mobile phase, which has a low salt content compared to the aqueous lower phase.[131]

The first study on the thermal decomposition of gomphrenin pigments present in the fruit juice of *B. alba* was carried out and the chromatographic profiles, as well as fragmentation pathways of decarboxylation and dehydrogenation products of gomphrenin (Table 2), were determined by LC-DAD-ESI-MS/MS and LC-MS-IT-TOF.[16] The short-term treatment of *B. alba* juice revealed the profiles of mono- and bi-decarboxylated gomphrenins analogous to betacyanins from heated beetroot juice.

Prolonged heating of *B. alba* extract acidified with acetic acid led to the formation of dehydrogenated derivatives as a result of gomphrenin derivative oxidation. The presence of neogomphrenin was detected at a low concentration level in the heated juice, which is opposite to RBE rich in neobetanin. In addition, in contrast to the results of the RBE heating experiments,[129] the isomeric 2,15- and 15,17-decarboxylated derivatives were not detected in the heated samples. The above experiments indicated that the chromatographic differences between gomphrenin and betanin derivatives arose from the position of glucosylation in betanidin at carbon atoms C-5 or C-6.[16]

Sawicki and Wiczkowski investigated the impact of boiling and spontaneous fermentation on the betalain profile and content in beetroot. The boiling and fermentation of beetroot decreased the content of the betalains by 51–61% and 61–88%, respectively. Processes occurring during boiling and spontaneous fermentation such as heating, softening, leaching, and acidification together with the matrix effect may be responsible for the changes. In addition, the microbial activity and matrix softening occurring during fermentation caused a release of betalains, which is responsible for the potent antioxidant capacity of the formed beet juice.[132]

Sawicki *et al.* determined the impact of three technological processes (fermentation, boiling, and microwave-vacuuming treatment) and *in vitro* digestion on betalain profiles. The content of betalains in the products was reduced by 42–70%. Microwave-vacuuming treatment has contributed to the degradation of betanin at the lowest level

and may be regarded as the best treatment method. In addition, the content of betalains released from the beetroot products after *in vitro* digestion reached the range of 0.001–0.10%, and the number of compounds identified in the digestion phases was decreased.[133]

3.1.2. Oxidation of betacyanin pigments. Preliminary studies on the enzymatic and non-enzymatic oxidation of betanidin, betanin, neobetanin, as well as decarboxylated betanins along with the research on intermediate and final reaction products, were carried out.[134–136]

Betanidin is the basic structure of all the betacyanins and the only one with the catechol moiety (5,6-dihydroxyl moiety). Studies show that during the oxidation of betanidin, the pigment is most likely converted into three tautomeric quinoid derivatives (Fig. 10). Interconvertions, which occur slowly at pH 4–7 and quickly in a more acidic environment, are likely to result in the formation of dehydrogenated and decarboxylated derivatives. Interconversions between the three tautomeric quinoid forms of oxidized betanidin are based on the proven oxidation pathways of DOPA and dopamine.[137,138]

The initial oxidation of betanidin should generate the transient radical cation, which rapidly loses a proton and forms a neutral phenoxy radical; it is equivalent to the donation of a hydrogen atom by betanidin or to the loss of a proton with further electron transfer. In the next step, the loss of a second proton and electron results in the formation of a two-electron

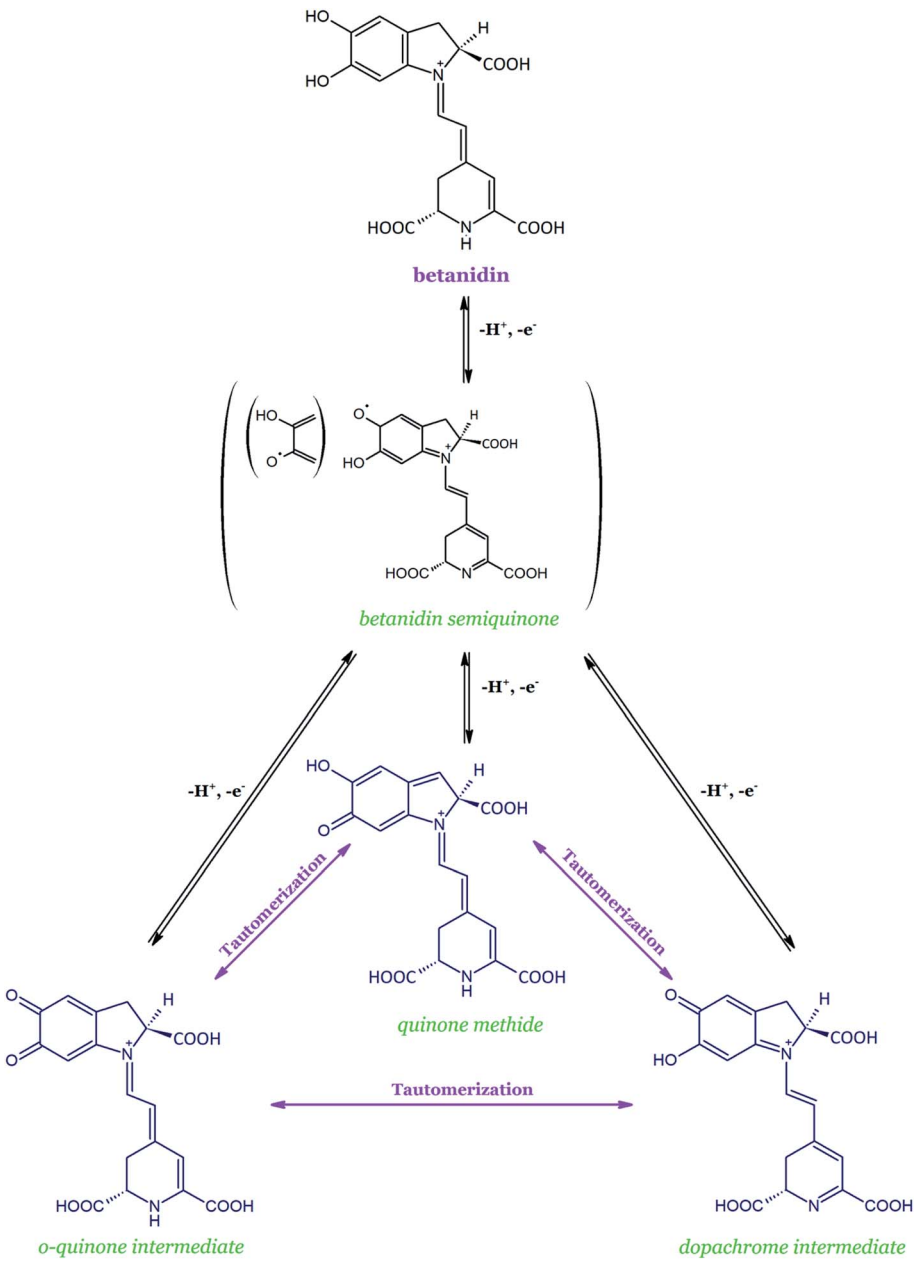


Fig. 10 Scheme of the initial transformations of betanidin during its oxidation and with an indication of the intramolecular conversions between three tautomeric forms based on.[134]

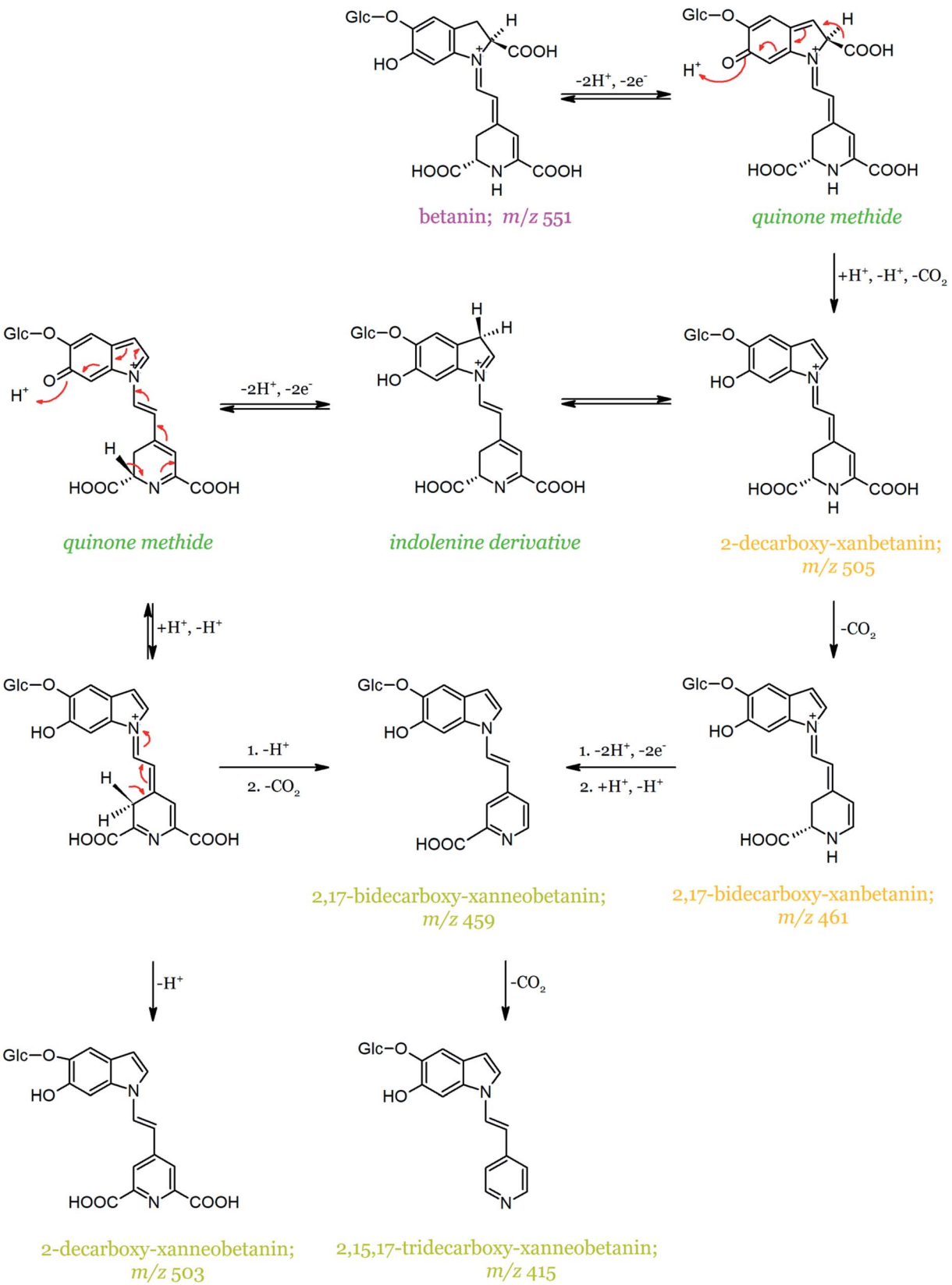


Fig. 11 Proposed mechanism of betanin oxidation proceeding through the quinone methide intermediate based on.[134,136]

oxidized form, *i.e.*, one of the three quinoid tautomers. Presumably, not only the betanidin *o*-quinone with its characteristic absorption spectrum (λ_{max} at 550 and 400 nm) is formed on the first oxidation stage but it is assumed that the two other quinoid derivatives are also possible: dopamine derivative and/or quinone methide. The discussion of the balance between the three tautomeric forms of quinoid remains open.[135] As a result, 2,17-bidecarboxy-xanbetanidin (synonym of 2,17-bidecarboxy-2,3-dehydro-betanidin) and 2-decarboxy-xanbetanidin (synonym of 2-decarboxy-2,3-dehydro-betanidin) are postulated as the principal oxidation products formed (Table 2). The recently proposed xan is derived from the hypsochromic shift toward the yellow color of the resulting compounds.

The further oxidation of 2,17-bidecarboxy-xanbetanidin again involves the formation of one of the two possible quinoid forms as the oxidized intermediates, *e.g.*, the dopachromic derivative of the indolic system. Subsequent rearrangement of the conjugated system of the originated structures into the neo-forms (14,15-dehydrogenated derivatives) is possible which finally leads to 2,17-bidecarboxy-xanneobetanidin (2,17-bidecarboxy-2,3-dehydro-neobetanidin), *i.e.*, a compound doubly dehydrogenated at the positions C-2,3 and C-14,15.[134–136]

In contrast to betanidin oxidation, no compound that could be regarded as the betanin oxidation intermediate (quinone methide) was detected.[139] This could be a consequence of its instability and fast rearrangement combined with decarboxylation. The main compound formed during the first step of betanin oxidation is 2-decarboxy-xanbetanin (Table 2), which exhibits a characteristic protonated molecular ion $[M + H]^+$ at *m*/*z* 505 and an absorption maximum at $\lambda_{max} = 446$ nm (Fig. 11). It is not excluded, however, that the generated 2-decarboxy-xanbetanin rearranges to a more stable structure of 2-decarboxy-neobetanin (synonym of 2-decarboxy-14,15-dehydro-betanin). According to the alternative pathway proposed by Wybraniec *et al.*,[135] the formation of 2-decarboxy-neobetanin can result from a rearrangement of the quinone methide generated from 2-decarboxy-betanin. This alternate pathway does not include the presumably more stable product, 2-decarboxy-xanbetanin. Further, the decarboxylation of 2-decarboxy-xanbetanin presumably results in the formation of 2,17-bidecarboxy-xanbetanin. The most hydrophobic product of betanin degradation/oxidation is 2-decarboxy-xanneobetanin, characterized by a protonated molecular ion at *m*/*z* 503 and maximum absorption at λ_{max} 422 nm, followed by 2,15,17-tridecarboxy-xanneobetanin.

In Fig. 12, the schematic formation of dehydrogenated betanin derivatives is presented along with 2,3-dehydro- and 14,15-dehydro-positions marked on particular pigment structures, together with their abbreviations (xan- and neo-, respectively).[52]

In the case of gomphrenin oxidation by the ABTS cation radical, except for its decarboxylated and dehydrogenated derivatives, the formation of betanidin as well as betanidin derivatives was detected. It is assumed that the rearrangement of dopachromic derivative affects the hydrolysis of the glucosidic bond. This is opposite to betanin, which is not deglucosylated during oxidation in the same reaction conditions. Therefore, it is important to notice that the

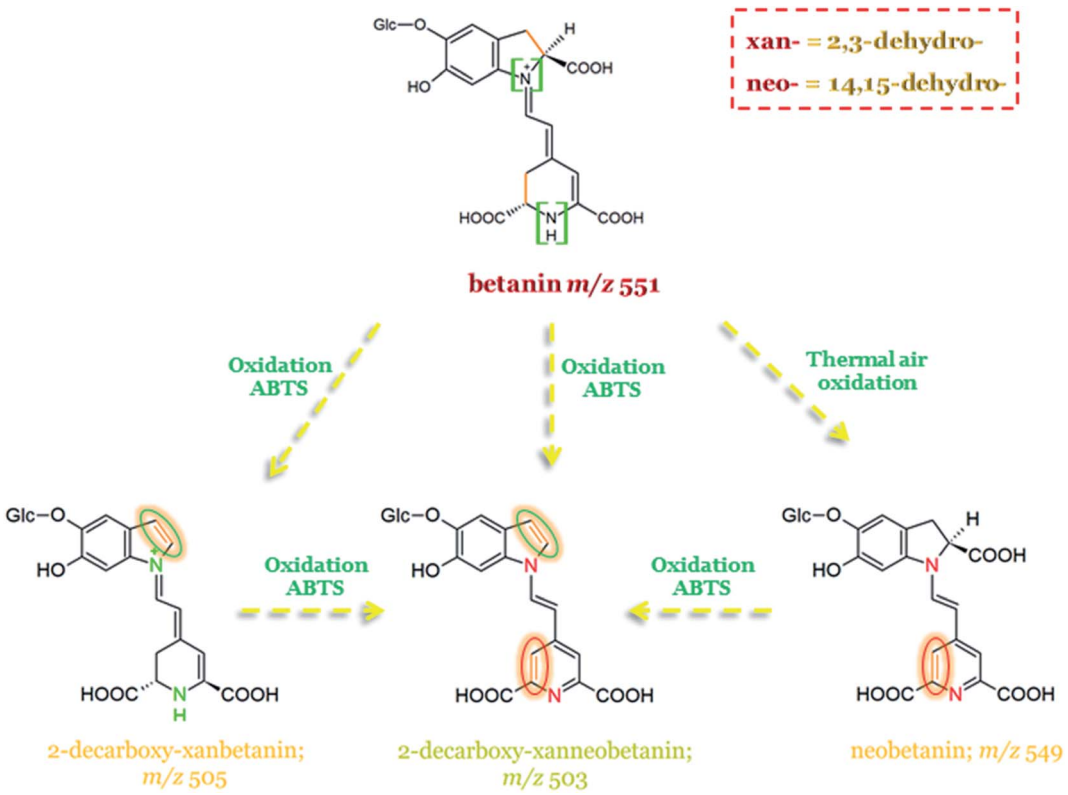


Fig. 12 Possible pathways of betanin oxidation with dehydrogenation positions marked on dehydrogenated derivative structures.[52,136]

position of betanidin glucosylation at carbon C-5 or C-6 has a significant influence on the differences in the reactivity of both the 5-*O*- and 6-*O*-glucosides.[140]

It was reported that metal cations, *e.g.*, copper, aluminum, tin, and iron, accelerate the degradation of betanin even at trace amounts. It is worth noting that the release of metals from food

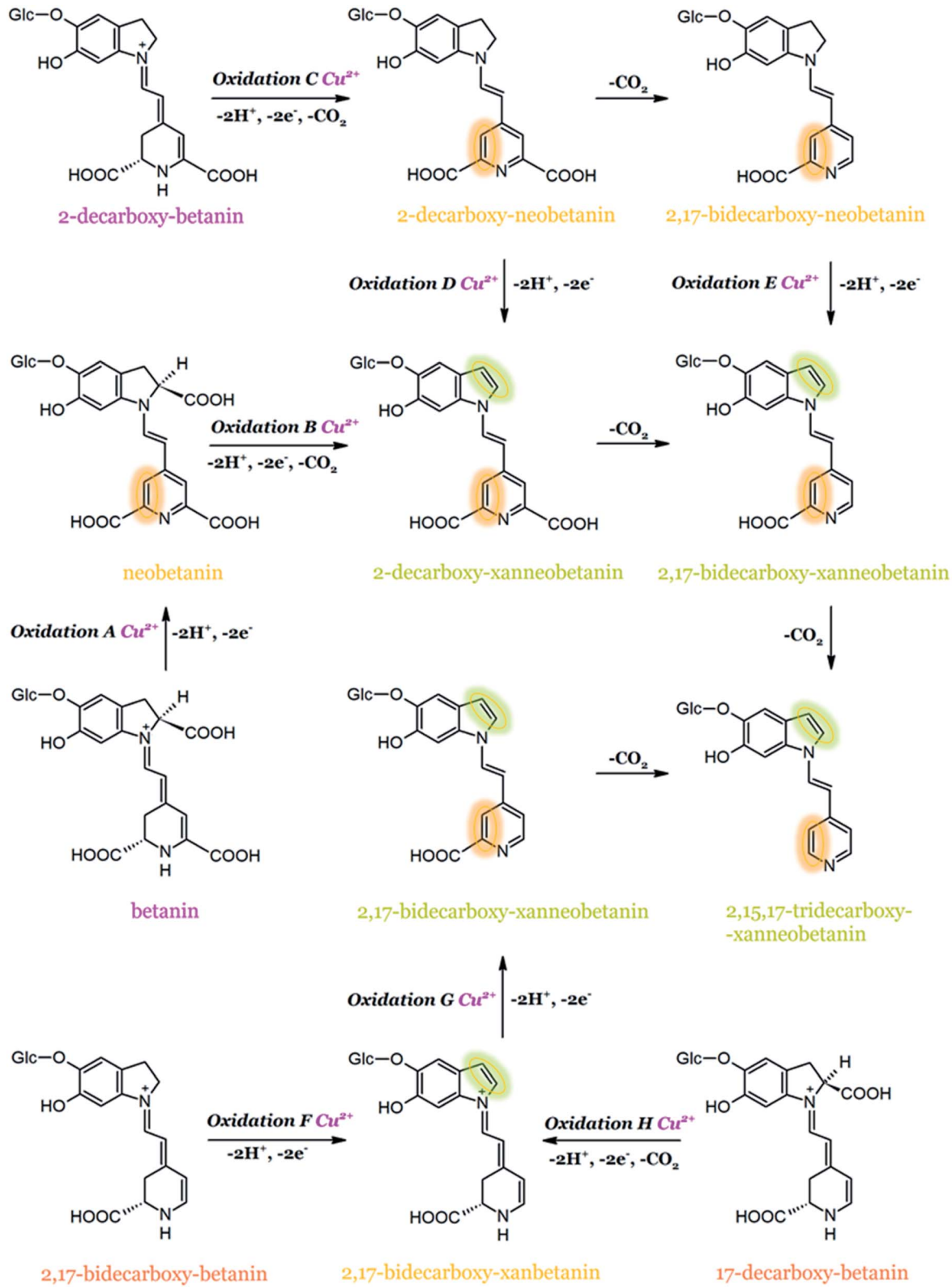


Fig. 13 Proposed general oxidation mechanism for betanin and its derivatives, superimposing the effect of Cu^{2+}-catalyzed oxidation with an indication of marked 2,3- and 14,15-dehydrogenation positions based on.[52]

packaging materials may also catalyze the unwanted betacyanin degradation.[141] The results of high-resolution mass spectrometry experiments (HRMS) and NMR structural studies on key dehydrogenation products generated during the oxidation of betanin, neobetanin, and decarboxylated derivatives by ABTS cation radical as well as by Cu^{2+} complexation confirmed that Cu^{2+} catalyzed oxidation of betanin and 2-decarboxy-betanin leads to the formation of neo-derivatives (14,15-dehydrogenated betanins). It has been proven for the first time that betanin oxidation is possible in the dihydropyridine ring and this can be achieved presumably by omitting the stage of quinone methide formation in the dihydroindole system (Fig. 13). The additional results obtained for Cu^{2+}-catalyzed oxidation of 17-decarboxy-betanin and 2,17-bidecarboxy-betanin suggest the possibility of formation of xan-derivatives, similar to the results obtained for oxidation by the ABTS cation radical. In this case, the factor determining the oxidation direction is presumably the absence of the carboxyl moiety at carbon C-17, clearly changing the geometry of the complex formed with Cu^{2+}. In addition, the exclusive effect of 2,3-dehydrogenation in 17-decarboxy-betanin is probably supported by the concurrent occurrence of decarboxylation at carbon C-2.[52]

3.1.3 Conjugation of oxidized betacyanin pigments with sulfhydryl scavengers. A well-developed approach for the direct detection of reactive, short-lived intermediates generated during the oxidation processes is to utilize trapping agents capable of forming stable adducts with the reactive species. The adducts may be detected and characterized using LC-MS/MS and NMR techniques.[142] There are many reports on the formation of adducts between nucleophilic thiols and a variety of compounds.[143–148] Research on the conjugation of quinones generated during betacyanin oxidation in the presence of thiols seems essential and may broaden the knowledge on betacyanin functions in biological systems.

The first studies on the possibility of conjugation of betacyanin reactive quinoids with sulfhydryl scavengers such as glutathione (GSH), cysteine (Cys), *N*-acetylcysteine (NAC), cysteamine (CSH), and DL-ditiothreitol (DTT) were performed recently (Table 2, Fig. 14).[140,149] The formation of glutathionylated quinoids, following betanidin and gomphrenin oxidation, confirmed the presence of quinoid forms in the products of pigment oxidation. The chemical structure of glutathionylated betanidin was confirmed by NMR studies. It was also reported that glutathione reacts with quinones at the C-4 carbon of the betanidin ring. On the contrary, the conjugation of the betanin quinoid intermediate (quinone methide) with glutathione was not observed, presumably because of a steric hindrance at the C-7 carbon of betanin, which presumably confirms that the quinoid form generated during betanin oxidation is a quinone methide.[140]

In order to determine the possibility of attaching thiols to the C-7 carbon of the betanin quinoid, additional research was performed using low molecular weight thiols (Cys, CSH, NAC, DTT). Furthermore, since the conjugation of betacyanins with the thiol groups may generate new molecules with the modified chemical and biological properties, the conjugation reactions with quinones derived from betanidin and gomphrenin were also performed (Fig. 14). The chemical structure of *N*-acetylcysteinyl-gomphrenin, generated with the highest yield, was determined by the NMR method. The dopachrome derivative from gomphrenin oxidation undergoes conjugation at the C-4 carbon atom. In addition, cysteine conjugate with the oxidized betanin derivative was produced for the first time. The results suggest that the formation of quinone methide during the oxidation of betanin and the production of dopachrome derivative during the oxidation of gomphrenin is highly possible.[149]

The results confirm so far that gomphrenin is able to form conjugates with a higher yield than betanin but the ability to form stable conjugates with dehydrogenated betanin derivatives produced during the oxidation of betanin or its intermediate conjugates may also favor this pigment as a valuable food protection component.[149] From the perspective of the food industry, this aspect appears to be relevant as the biological activity of gomphrenin and betanin in combination with the possibility of adduct formation between the thiols and betacyanin quinoids may contribute to the increased quality and durability of food products such as meat.[146] From another perspective, adduct research can significantly contribute to the development of detection methods for betacyanin quinoids formed in the complex food matrices as well as to research the bioavailability and distribution of betacyanins in humans. The sulfhydryl part of the molecules may modulate their ability to penetrate the biological membranes as well as their absorption and metabolism. Many attempts have been made in the food chemistry to inhibit the formation of oxidative cross-linking proteins, affecting the quality, water content, and red color of meat products. In this respect, CS-conjugates of betacyanins may play a significant role in blocking the thiol groups in food proteins, contributing to improving the food quality.[150–152]

Whether betacyanins act as effective antioxidants without adverse pro-oxidative effects depends on how quickly their oxidized derivatives gradually degrade through decarboxylation and dehydrogenation as well as through other transformations, including hydrolysis and the final polymerization of betalamic acid and *cyclo*-DOPA to inactive forms.[134,136] So far, no research on the toxicity and stability of quinones produced from betacyanins has been conducted. The literature data are lacking on the pro-oxidative properties of betalains. In addition, there are questions whether betalains are susceptible to undesired metabolic activation and if the blocking of the sites prone to the formation of reactive forms in molecules is required for betalain application as potential therapeutic agents.

3.1.4 Chlorination of betacyanins. The literature reports suggest that betacyanins may be regarded as potent scavengers of inflammatory factors such as hypochlorous acid (HOCl). In 2007, Allegra *et al.* reported that betanin and indicaxanthin are effective in the scavenging of hypochlorous acid.[77] In addition, both the betalains reduce the myeloperoxidase activity and oxidation, and are therefore regarded as the two key components of the anti-inflammatory processes, leading to the reduction of hypochlorous acid generation.[153] In 2016, Wybraniec *et al.*[154] reported that in the reaction between betanin/betanidin/neobetanin and sodium hypochlorite or

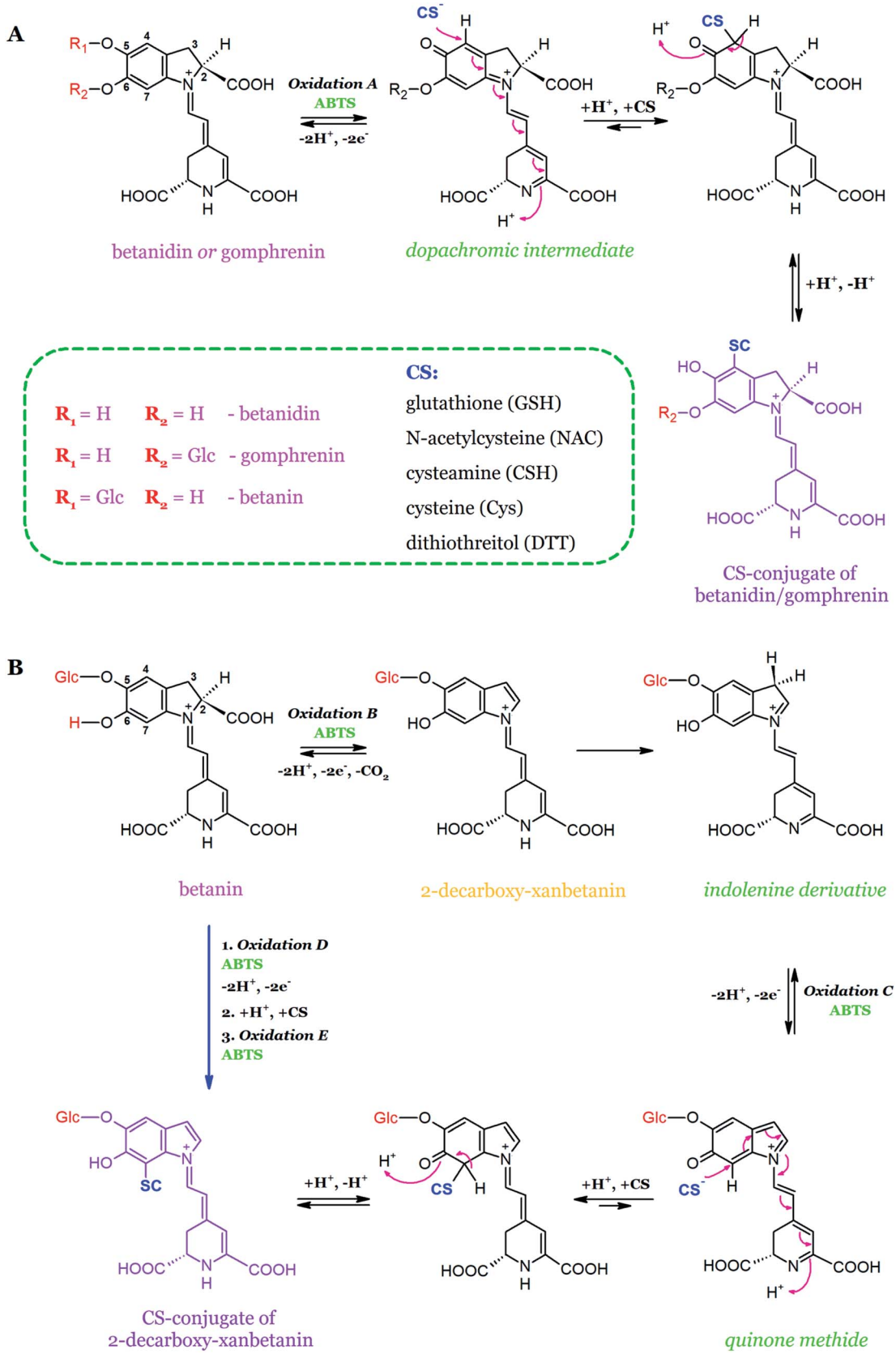


Fig. 14 Schematic representation of the conjugation mechanism of betanidin and gomphrenin (A) with sulfhydryl radical scavengers as well as the oxidation of betanin to 2-decarboxy-xanbetanin (B) through the quinone methide intermediate with the following conjugation based on.[149]

myeloperoxidase (MPO)/H_2O_2/Cl^- systems, the chlorination of betanin and betanidin took place within the aglycone unit. In addition, the possible reaction with another potent chlorinating agent, Cl_2O, which co-exists with HOCl in equilibrium, especially in acidic conditions, was suggested according to a similar mechanism.

For neobetanin, no chlorinated products in the reaction mixtures were detected. The fact that the pyridinic ring within the neobetanin structure cannot be chlorinated to form the detectable pigment products indicates that only the unsaturated bond is attacked, preferably at C-18, due to its partial negative charge.[154] Monochloro-betanin and monochloro-betanidin were formed with the highest yield at pH 3–5 while at higher Cl^- concentrations, the efficiency was dramatically decreased, suggesting that the generated Cl_2 is not the chlorinating agent in the presence of sodium hypochlorite. The low activity of Cl_2 during betanin and betanidin chlorination compared to HOCl and/or Cl_2O was explained by a special position (C-18) of the attack by HOCl and/or Cl_2O. In the case of the MPO/H_2O_2/Cl^- system, the highest efficiency of monochloro-betanin/-betanidin generation was observed at pH 5.

In another study,[155] additional chlorination experiments were extended to decarboxylated betacyanins (Table 2). The comparison of the chromatographic profiles of the chlorinated decarboxylated betanins and betanidins generated under the activity of hypochlorous acid revealed two different directions of retention changes in relation to the corresponding precursors. The chlorination of betacyanins decarboxylated at C-17 (17-, 2,17-bi-, and 2,15,17-tri-decarboxy derivatives) resulted in higher retention times in comparison to the corresponding substrates. In contrast, the non-decarboxylated betacyanins as well as their 2- and 15-decarboxy-derivatives exhibited lower chromatographic retention after chlorination.[155]

The identification of the chlorinated betacyanins was completed by a further study on the chlorination mechanism and the position of the electrophilic substitution in betacyanins by high-resolution mass spectrometry and further structural analyses by NMR techniques,[156] which confirmed that the chlorination position in betanin and decarboxylated betanins occurs within the dihydropyridinic moiety at the C-18 carbon.[156]

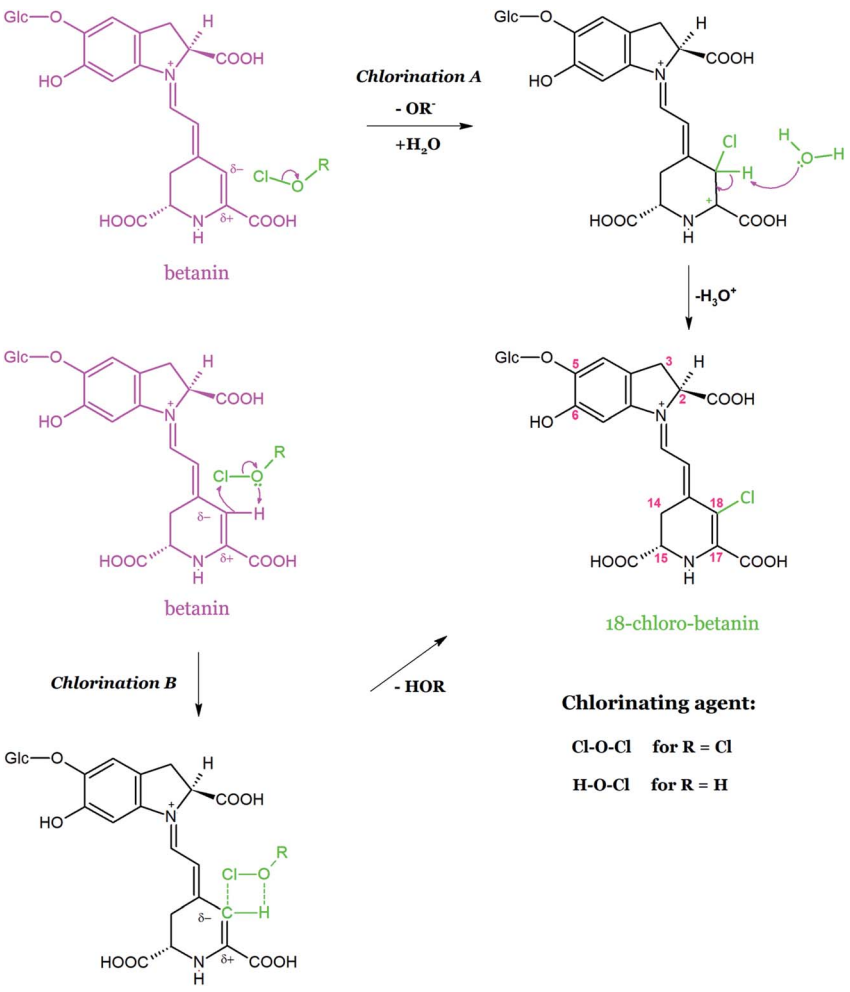


Fig. 15 Proposed mechanism of betanin chlorination based on two versions of Cl^+ ion attack on C-18 carbon. Chlorination version B presents a cyclic intermediate formed during the electrophilic attack of the whole HOCl or Cl_2O with the stabilizing effect of the hydrogen bonding.

According to an electrophilic mechanism based upon the leaving group ability from Cl^+ in HOCl ($-OH^-$) and in Cl_2O ($-OCl^-$), the reaction paths between betanin and HOCl/Cl_2O were proposed (Fig. 15).[157] The mechanism was supported by the NMR elucidation results as well as by the inactivity of neobetanin toward chlorination.[154] Chlorination version A shows the position of Cl^+ attack on C-18 carbon within the betanin structure. The failure in the chlorination of betanin with Cl_2, which is an active oxidizing agent, confirmed that Cl^+ leaving from HOCl or Cl_2O can be the chlorinating factor. Also, the hypothesis that H_2OCl^+ may be regarded as a chlorinating factor carrying Cl^+ is intriguing but has not been confirmed for decades.[158]

In the chlorination version B, a cyclic intermediate[159] is formed during the electrophilic attack of the whole HOCl or Cl_2O molecules as a result of the polarization of Cl–O bond along with the stabilizing effect of hydrogen bonding. The oxidation of C-18 carbon in betanin did not take place because of its negative charge, making the nucleophilic attack of oxygen[159] impossible.

Since fluorescent probes have been promising analytical tools for the rapid and specific detection of HOCl/OCl^-, the HCSe and HCS probes were tested in the determination of the betalains' anti-hypochlorite activity. The comparison of the *in vitro* anti-hypochlorite activities of a betalain-rich red beetroot extract (RBE) with its pure betalainic pigments revealed that the extract had the highest anti-hypochlorite activity, far exceeding the activity of all of the betalainic derivatives and selected reference antioxidants.[160] In addition, the pilot clinical studies showed that the short-term treatment with RBE improved the function and comfort of knee joints in individuals with knee distress resulting from increased hypochlorous acid formation.[76]

3.1.5 The acyl migration phenomenon. The acyl migration phenomenon in betacyanins isolated from *Mammillaria* and *Hylocereus* fruits was discovered by Wybraniec *et al.*[11,18,36,56] The driving force behind the research on the acyl migration effect was the fact that 4′-*O*-malonyl betanin isolated from the cacti fruits of *Hylocereus* species isomerized to 6′-*O*-malonyl betanin (phyllocactin) (Fig. 16). It was proved that acyl migration is intramolecular; hence, hydrolysis and re-esterification mechanisms are not relevant in that process.[161] For that reason, free carboxylic groups in the acyl moieties present in hylocerenin, phyllocactin, and their derivatives are not involved in the rearrangement. The acyl migration mechanism is based on the interaction of the adjacent group with an *ortho* acid ester being an intermediate. In many cases, the distance between the acyl migrating group and the free hydroxyl is appropriate for creating of a cyclic intermediate.[161–163] The formation of the six-membered cyclic intermediate between the glucosidic *O*-6′ and *O*-4′ hydroxyls

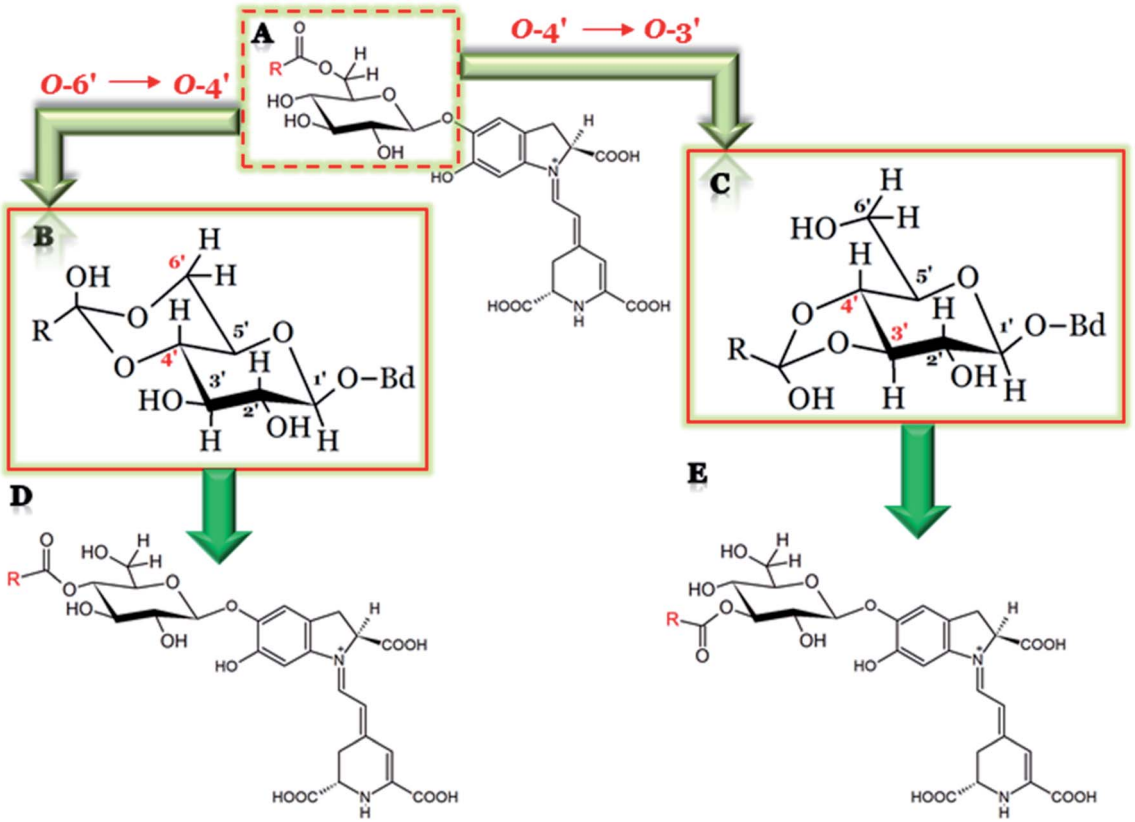


Fig. 16 The general chemical structure of 6′-*O*-acylated betacyanin submitted to acyl migration (A) with an established six-membered cyclic intermediate structure formed during acyl migration between the glucosidic *O*-6′ and *O*-4′ oxygens in betacyanins (B); a five-membered cyclic *ortho* ester formed during the acyl migration between *O*-4′ and *O*-3′ (C) as well as the final products of acyl migration (D) and (E) (Bd = the rest of betanidin; R = a fragment of an acyl moiety).

may be responsible for the intramolecular rearrangement in phyllocactin. The significant factor that provides this phenomenon is the alkaline reaction environment; however, the effect is also observed in acidic conditions.

A series of new isomers of various 6′-*O*-acylated betacyanins as well as decarboxylated betacyanins formed by intramolecular pH-dependent acyl migration between adjacent hydroxy groups on polyhydroxy compounds in aqueous solutions was chromatographically characterized.[18] The migration rate was markedly accelerated under alkaline conditions at pH 10.5, however, always favoring the 6′-*O*-position. The phenomenon of acyl migration was less apparent at pH below 7.0. Partial rearrangement may result in the formation of 3′-*O*- and 4′-*O*-acylated compounds. In malonylated betacyanins and 17-decarboxy-betacyanins, the 3′-*O*-acylated forms were presumably the most polar isomers and were eluted first in RP-HPLC, whereas the 4′-*O*-forms were characterized by retention higher than the 6′-*O* forms. In contrast, in 2-decarboxy- and 2,17-bidecarboxy-betacyanins, both 3′-*O*- and 4′-*O*-acyl-betacyanins were eluted before the 6′-*O*-acylated forms. The study on the acyl migration effect in 4′-*O*-malonyl-betanin revealed a strong tendency to reverse acyl migration (4′ → 6′) and also partial rearrangement (4′ → 3′), leading to the monoester regioisomeric distribution in the proportion 87 : 7 : 6 [%] in relation to 6′-*O*-, 4′-*O*-, and 3′-*O*-, respectively.

A new malonyl betacyanin, 6′-*O*-malonyl-amaranthin (celoscristatin), as well as 4′-*O*-malonyl-amaranthin, formed as a result of the malonyl group migration in celoscristatin, were also identified in *Celosia cristata* Linn. callus culture.[29]

3.1.6 NMR elucidation of betacyanin pigments. First NMR structural investigations on betacyanins performed at low pH resulted in the quick degradation of the substances, preventing longer in-depth structural analysis of the betacyanins.[53] Nevertheless, key experiments were performed, *e.g.*, confirming the *E*/*Z*-stereoisomerism at one of the partial double bonds (C-11,12) in betanidin/isobetanidin.[50] As a consequence, only the ^{13}C NMR spectrum of neobetanin was properly registered due to the stability of this dehydrogenated derivative in a highly acidic environment in contrast to other betacyanins. In Fig. 17, an overview of the solvents used for NMR analysis of particular

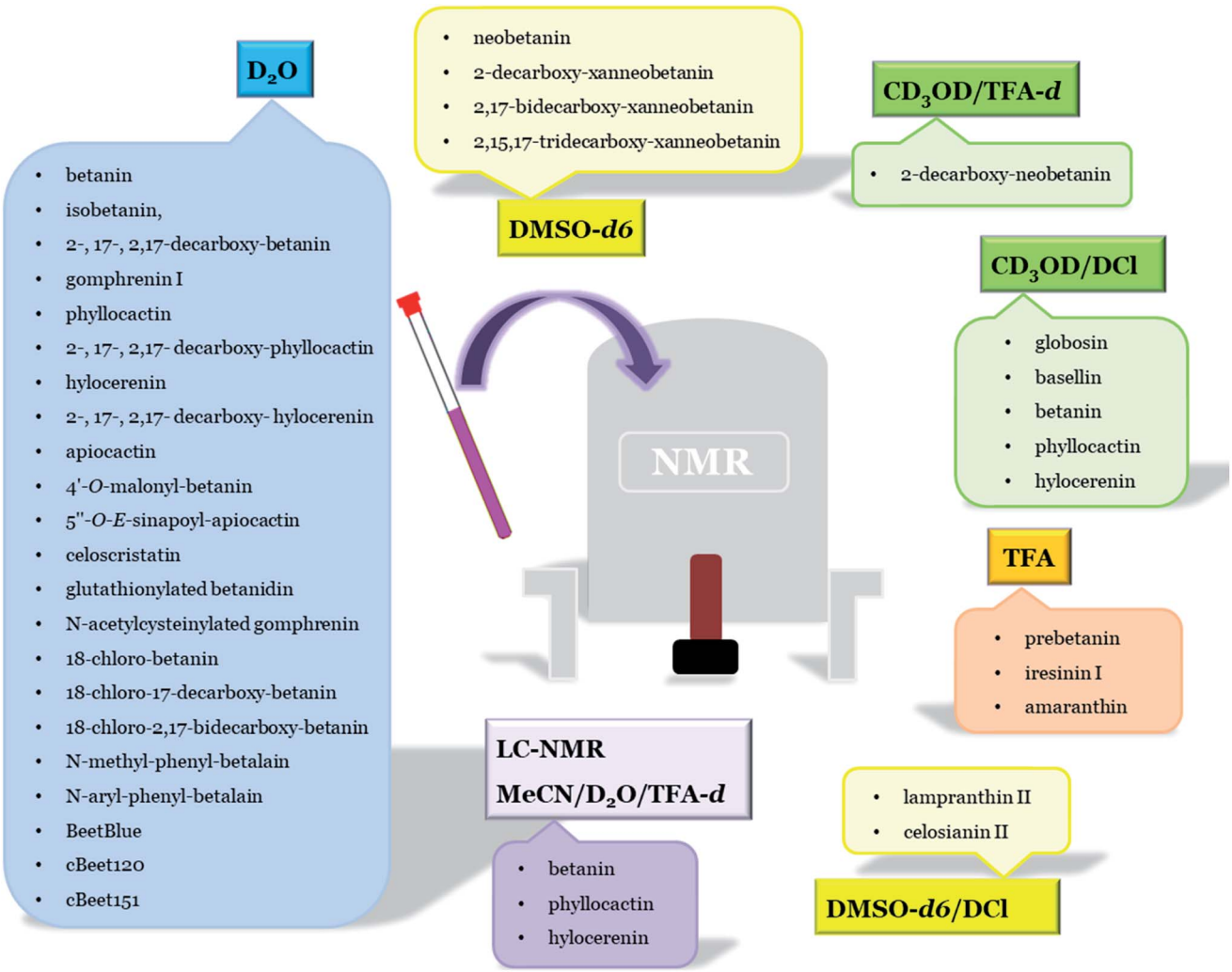


Fig. 17 The summary of the solvents used for NMR spectra registration of particular betacyanins.

betacyanin pigments is shown. Data acquisition was improved by changing the solvent system for NMR analysis. D_2O was found to be the most suitable solvent, which afforded the best long-term stability required for NOESY, HSQC, and HMQC experiments by preventing betacyanin hydrolysis. 1D (^{1}H, 1D TOCSY) and 2D NMR (COSY, TROESY, HSQC, HMQC) measurements for betanin, isobetanin, phyllocactin, and hylocerenin isolated from red-purple pitaya (*H. polyrhizus*) were performed in D_2O.[164] For the confirmation of the presence of malonyl moiety exchangeable protons, additional ^{1}H, HSQC, and HMQC experiments for phyllocactin were carried out in a mixture of H_2O and D_2O (90/10, v/v), slightly acidified with 20 mL of 0.05% aqueous TFA solution (v/v).[164]

The key products formed after betanin decomposition were identified and their structures were confirmed by NMR analysis. The structural elucidation of 2-, 17-, and 2,17-decarboxy-betacyanins generated during the decarboxylation of betanin, phyllocactin, and hylocerenin isolated from *H. polyrhizus* fruits was performed by Wybraniec *et al.*[127] A variety of coupled spin systems of all the analyzed compounds were successfully distinguished and assigned by ^{1}H NMR, ^{1}D TOCSY, gCOSY, and NOESY spectra in D_2O. Due to gHSQC correlations, the ^{13}C chemical shifts for all the carbons directly bound to the protons were unambiguously assigned, while the gHMBC (the ^{1}H–^{13}C long-range) correlations enabled the establishment of the chemical shifts of the quaternary carbons. In addition, the first ^{1}H NMR spectra of 2-decarboxy-hylocerenin together with all the studied 17-decarboxy- and 2,17-bidecarboxy-betacyanins were received. Furthermore, the ^{13}C NMR spectra of the decarboxylated betacyanins were obtained for the first time.[127]

The NMR spectra of amaranthin and iresinin I were recorded in trifluoroacetic acid.[61] The structures of the two other acylated betacyanins and lampranthin II from the petals of *Lampranthus peersii* and *L. sociorum* as well as celosianin (previously named as celosianin II) from the cell suspension cultures of *Chenopodium rubrum* were elucidated as 6′-*O*-*E*-feruloyl-betanin and 2″-*O*-*E*-feruloyl-β-(1″,2′)-glucuronosyl-betanin, respectively, by ^{1}H NMR. All 1D (normal and NOE difference) and 2D spectra (COSY) were registered in acidic DMSO-*d6* containing traces of DCl.[58] A low field shift of the protons H-6′A and H-6′B of the glucosylic moiety in lampranthin II caused by acylation indicated an attachment of the feruloyl moiety at the C-6′ carbon. For celosianin II, the NOE difference spectra indicated that the β-glucuronosyl moiety was bound to C-2′ of the β-glucosyl moiety. The presence of the feruloyl moiety attached at C-2″ of β-glucuronosyl was evident from the low field shift of H-2″.[58]

In addition to betanin and phyllocactin in *H. polyrhizus* fruits, another characteristic pigment, hylocerenin [6′-*O*-(3″-hydroxy-3″-methyl-glutaryl)-betanin], was also characterized by 1D and 2D (COSY) NMR techniques in deuterated methanol (CD_3OD) containing traces of deuterium chloride (DCl).[56] Other unknown pigments, 2′-*O*-β-apiofuranosyl-betanin and 2′-*O*-β-apiofuranosyl-phyllocactin, were also elucidated by ^{1}H and ^{13}C NMR techniques in non-acidified D_2O.[11] The structure of a new malonyl derivative, celoscristatin (6′-*O*-malonyl-amaranthin), isolated from *C. cristata* callus culture was confirmed by NMR analysis in D_2O.[29]

In order to determine the oxidation mechanism of betanin, structures of tentatively identified key dehydrogenation products of betanin, decarboxylated betanin, and neobetanin oxidation by the ABTS cation radical were elucidated by NMR.[52] The ^{1}H and 2D (COSY, TOCSY, HSQC, HMBC, and NOESY) measurements were performed in DMSO-*d6* for all the studied compounds except for 2-decarboxy-neobetanin, which was analyzed in CD_3OD acidified with 0.01% deuterated TFA. The structures of five neo- and xanneo-derivatives, namely, neobetanin, 2-decarboxy-neobetanin, 2-decarboxy-xanneobetanin, 2,17-bidecarboxy-xanneobetanin, and 2,15,17-tridecarboxy-xanneobetanin, were confirmed.[52]

The structures of gomphrenin as well as its acylated derivatives, globosin and basellin, (gomphrenin I, II, and III, respectively) were elucidated by NMR spectroscopy by Heuer *et al.*[24] The ^{1}H and 2D COSY NMR spectra were recorded in CD_3OD containing traces of DCl. The presence of acyl (4-coumaroyl- and feruloyl-) moieties at the C-6′ carbon in globosin and basellin, respectively, was indicated by a low field shift of the corresponding protons H-6′A and H-6′B. Non-typical ^{1}H chemical shift differences between gomphrenin I and globosin and basellin suggested intramolecular stacking occurring in the latter two compounds.[24] In another attempt, gomphrenin was identified by means of ^{1}H, selective NOESY, and TOCSY as well as 2D NMR (COSY, NOESY, HSQC, and HMBC) techniques in D_2O.[69] By NOESY-correlation spectral analysis, it was demonstrated that the glucopyranosyl group is linked to the phenolic group at the C-6 carbon of gomphrenin, which differentiated it from betanin.

The structures of glutathionylated conjugate of betanidin[140] and *N*-acetylcysteinylated gomphrenin[149] were established by NMR analysis, confirming the conjugation position at the C-4 carbon, thus indicating the presence of a dopachromic intermediate during gomphrenin oxidation. All ^{1}H and 2D NMR (COSY, HSQC, HMBC, TOCSY, and NOESY) analyses were accomplished in non-acidified D_2O. The attachment position of the glutathionyl moiety was primarily indicated by the strong long-range correlation of the H-12a/b′ protons to quaternary C-4 and C-9 carbons as well as a weak correlation to C-8. The lack of the H-4 signal in the ^{1}H spectrum unambiguously confirmed this position.[140,149] In the case of *N*-acetylcysteinylated gomphrenin, the attachment position of the *N*-acetyl-cysteinyl moiety was initially suggested by a strong, long-range correlation of H-6a/b″ protons to quaternary carbon C-4. In addition, the lack of the H-4 signal in the 1H spectrum definitely confirmed this position. Furthermore, the CS-conjugation position in gomphrenin was supported by the downfield chemical shift of the H-6a/b″ proton signal compared to the signal obtained for free *N*-acetyl-cysteine.[149]

In order to prove the position of chlorination in betanin and its decarboxylated derivatives, structural studies were also performed.[156] The structure elucidation of 18-chloro-betanin, 18-chloro-17-decarboxy-betanin, and 18-chloro-2,17-bidecarboxy-betanin confirmed that the chlorination position in betanin occurs within the dihydropyridine moiety at carbon C-18. The results of ^{1}H NMR, COSY, and TOCSY analyses obtained for 18-chloro-betanin in non-acidified D_2O enabled to distinguish and characterize several diagnostic, conjugated spin systems

characteristic for betanin (H-2, H-3ab; H-11, H-12; H-14ab, H-15) in D_2O. The dihydroindolic system was indicated by the HSQC correlations of H-2, H-3ab, H-4, and H-7 with their respective carbons. In the dihydropyridine system, the correlations of H-14ab and H-15 with their respective carbons in the HSQC spectra were visible.[156]

The structural studies of the newly semi-synthesized pseudo-natural pigments based on betanin hydrolysis were also reported. The structures of various compounds semi-synthesized by coupling betalamic acid with *N*-methyl anilines, *N*-aryl anilines, and blue solid named BeetBlue as well as 7-amino-4-methylcoumarin- and 7-amino-4-trifluoromethylcoumarin-betacyanins (cBeet120 and cBeet151) were accomplished by NMR analysis in D_2O.[165–167]

4 Chemistry of betacyanins and their semi-synthetic derivatives based on betanin hydrolysis

4.1 Hydrolysis of betanin

Betanin is susceptible to acid- and base-catalyzed hydrolysis, leading to the formation of colorless *cyclo*-DOPA-5-*O*-β-D-glucoside and fairly stable bright yellow betalamic acid (Fig. 18). Recently, new light was shed on betanin hydrolysis mechanism.[169] The impact of temperature and pH on the rate of betanin hydrolysis was examined. The mechanism of betanin decomposition was studied in the phosphate solution at pH ranging from 2 to 11 and temperature of 60–85 °C. It was postulated that the fully protonated betanin form is reactive toward the nucleophilic attack of water and the intramolecular hydrogen bond between the carboxylic group at C-2 carbon and N-1 nitrogen stabilizes the transition state, leading to the hydrated betanin. The N-1 nitrogen atom was expected to be protonated in the acidic media and to form a leaving group. The protonation of the N-16 nitrogen atom enhances the electrophilicity of C-11, C-13, and C-17 carbons and catalyzes betanin hydrolysis, finally contributing to the opening of the 2,6-dicarboxy-1,2,3,4-tetrahydropyridine ring of betalamic acid. It was indicated that the hydrogen phosphate ion may act as a general base at pH 8.2, even at a low buffer concentration. The formation of *cyclo*-DOPA and 2,6-dicarboxy-4-methylpyridine as the products of betanin degradation in an alkaline environment at 130 °C identified by IR spectroscopy was reported in the pioneering work of Wyler and Dreiding on betanin purification by crystallization.[47,170] In contrast to that study, 2,6-dicarboxy-4-methylpyridine was not detected.[169] However, the presence of *cyclo*-DOPA, betalamic acid, and decarboxylation/oxidation products was observed.

Based on theoretical calculations, the double protonation of the nitrogen atom within the tetrahydropyridine ring cannot be excluded from what was observed for the amino dicarboxylic acids of simpler structures.[169]

Next to hydrolysis, betacyanins are also sensitive to water-catalyzed isomerization. A study on the hydrolytic stability of betanin Bn, indicaxanthin BtP, and an artificial betalainic coumarin BtC soluble hydrogen bond donating 2,2,2-trifluoroethanol (TFE) was conducted.[171] The hydrolytic stability in water was as follows: BtP > Bn > BtC. Improved hydrolytic stability of the abovementioned betalains in hydroalcoholic solutions was noticed with an increase in the TFE co-solvent. The hydrolysis rate of betalainic coumarin in water was 700-fold greater than in TFE, whereas the use of TFE as a co-solvent reduced the hydrolysis of betanin and indicaxanthin by 20 and 100-times, respectively. The proportion of water and TFE in the co-solvent mixture determined the hydrogen-bond donating capacity, polarity, and ionization power. However, the most important factor of the enhanced hydrolytic stability of the betacyanins is probably due to the low nucleophilicity of TFE. In addition, the fluorescence quantum yields of betaxanthins were also increased with the presence of TFE.[171]

4.2 One step semi-synthesis of betalains from betalamic acid-derivatized support

So far, all the proposed methods of the *de novo* synthesis of betalains achieve very low efficiency; therefore, the best way to obtain individual betalain derivatives is to isolate them from the biological material and purify. However, this makes the whole process highly time-consuming due to the plant matrix that is rich in various compounds, especially polysaccharides. The process of betalain formation was simplified using a novel betalamic acid-derivatized support (Fig. 19).[172] Betalamic acid was produced during betanin hydrolysis from *B. vulgaris* extract in the presence of cross-linked polystyrene resin at room temperature and nitrogen atmosphere. After the release of betalamic acid and neutralization with acetic acid, the reaction of imine formation between the aldehyde group of betalamic acid and the free primary amine groups present in the matrix

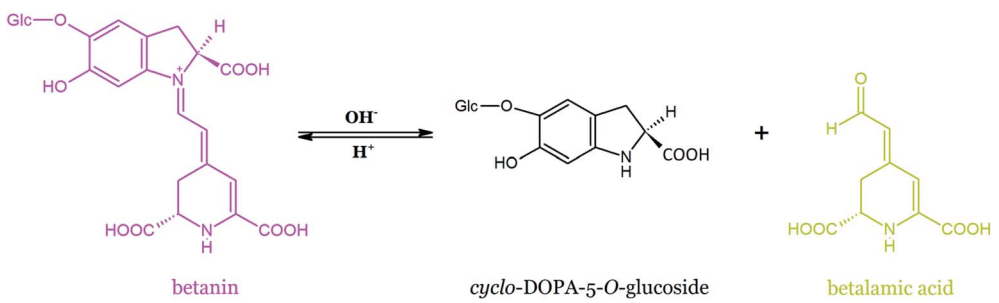


Fig. 18 Hydrolysis of betanin leading to the formation of betalamic acid and *cyclo*-DOPA-5-*O*-β-D-glucoside.

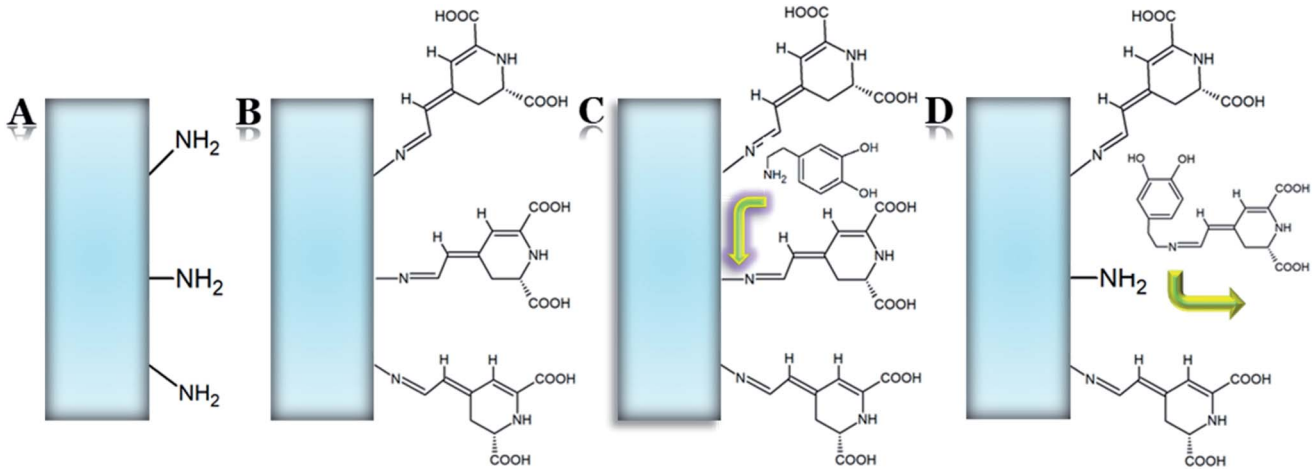


Fig. 19 Betalamic acid-derivatized support and the surface reaction leading to pigment isolation, based on dopamine-derived betaxanthin: (A) starting material containing primary amine groups; (B) betalamic acid-derivatized support; (C) amine attack to betalamic acid; (D) synthesis and concomitant release of miraxanthin V.[172]

surface was performed, resulting in the immobilization of betalamic acid in the resin. The derivatization of the resin with betalamic acid was indicated by the resin color change from pale to intense yellow. After washing and drying at room temperature, the novel solid support was generated and characterized by SEM. The material exhibited the spectroscopic properties of a pseudobetaxanthin. Then, the resin from the betalamic acid-derivatized support was tested toward the production of betalains. As model amines, individual dopamine, tyramine, indoline, and pyrrolidine were added to the support turning the color of the solutions from colorless into yellow in the case of dopamine, tyramine, and pyrrolidine, as well as into purple in the case of indoline. Then, a Schiff's condensation between the amine and betalamic acid anchored in the support provided the dopamine-, tyramine-, pyrrolidine-, and indoline-betaxanthins.[172]

4.3 Semi-synthetic betalain pigments with extended conjugated systems

4.3.1 Semi-synthesis of blue pigments from betanin. Naturally-occurring blue pigments are rare, especially among plants. A metal-free photostable blue pigment was designed by extending the π-system of betalains (Table 2).[166] The dye was semi-synthesized from betalamic acid, which may be obtained from the hydrolysis of red beetroot juice or by the enzymatic oxidative cleavage of *S*-DOPA. The 1,11-diazaundecamethinium chromophore of the dye is formed by the irreversible dehydrative C–C coupling of betalamic acid with the carbon nucleophile 2,4-dimethylpyrrole in acidified ethyl acetate (Fig. 20). The reaction lasted less than 30 min and it was performed at room temperature under air condition. The 1,11-diazaundecamethinium chromophore induces a redshift of the absorption and fluorescence spectra in water in relation to standard betanin and indicaxanthin. The product was purified by flash gel permeation chromatography with water used as eluent. This dye exhibits high solubility in polar solvents, *e.g.*, water, and does not produce singlet oxygen upon photoexcitation.[166]

4.3.2 Semi-synthesis of a pseudo-natural betalain-nitrone OxiBeet pigment. A prototypical betalain-nitrone pigment (OxiBeet) based on the *N*-oxide 1,7-diazaheptamethinium scaffold was semi-synthesized for the first time by Capistrano Pinheiro *et al.* (Table 2).[173] Betalamic acid was produced by the hydrolysis of the betalains present in red beetroot juice and coupled with *N*-phenylhydroxylamine in acidified water, giving rise to OxiBeet pigment (Fig. 21). The resulting product was

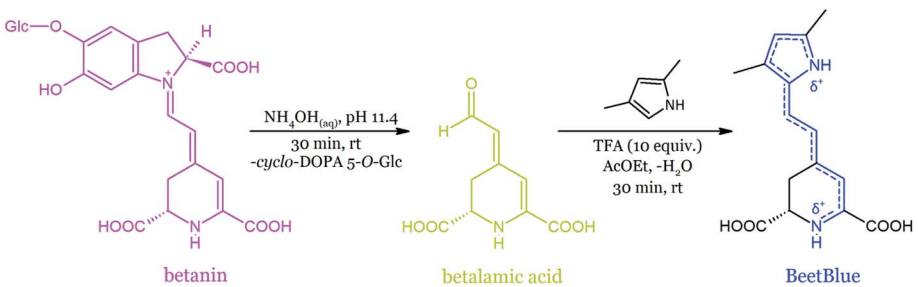


Fig. 20 Semi-synthesis of a blue dye from betanin derived from beetroot juice.[166]

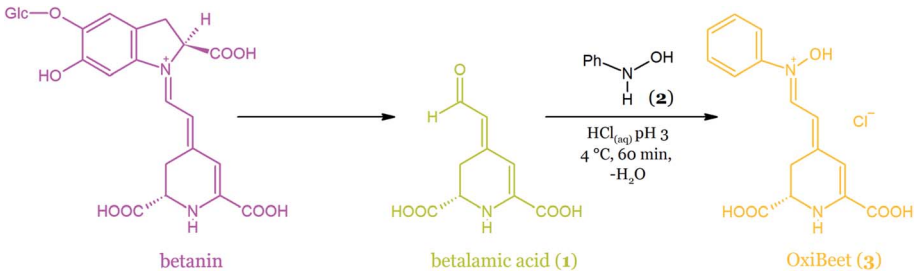


Fig. 21 Semi-synthesis of OxiBeet pigment (3) by the acid-catalyzed coupling of betalamic acid (1) and *N*-phenylhydroxylamine 2 in water.

purified and isolated with 50% yield, which is twice as high as the values reported for other betalains.

This bio-based molecule exhibits low reduction potential and even higher radical scavenging activity than typical antioxidants such as ascorbic acid and gallic acid. Furthermore, it is non-cytotoxic toward human hepatic cell line HepaRG up to 1 mM concentrations. This pseudo-natural product is resistant to hydrolysis under neutral and slightly alkaline conditions and it may also be converted into a persistent *N*-oxide 1,7-diazaheptamethinium radical cation in an aqueous solutions *via* autoxidation. These findings open up new perspectives for utilizing betalain pigments and betalain derivatives as therapeutics against various human diseases.

4.3.3 Semi-synthesis of *N*-methyl phenyl-betaxanthin and *N*-aryl phenyl-betaxanthin pigments. The semi-synthesis of *N*-methyl phenyl-betaxanthin (common name: mepBeets) and *N*-aryl phenyl-betaxanthin (dipBeets) pigments (Table 2) was described by Pioli *et al.* and the influence of the structure on the betalain derivatives' hydrolytic stability and electronic properties were compared.[165] Eight compounds were formed by the derivatization of betalamic acid and *N*-methyl-anilines as well as *N*-aryl-anilines in ethyl acetate in the presence of *p*-toluenesulfonic acid used as a catalyst (Fig. 22). The stabilization of the positive charge density at the nitrogen atom N-9 by the *N*-methyl group reduces the rate of hydrolysis by decreasing charge delocalization through the 1,7-diazaheptamethinium system as compared to the non-methylated phenyl-betaxanthin. On the contrary, the *N*-aryl moiety increases charge delocalization while improving the hydrolytic stability. The electron-withdrawing substituents within the aryl moiety increase the fluorescence quantum yields of semi-synthesized betaxanthins, accelerate hydrolysis, and lower the anodic potential compared to the corresponding unsubstituted betalains. On the other hand, the presence of electron-donating substituents improves their hydrolytic stability. The fluorescence of *N*-aryl phenyl-betaxanthins is strongly influenced by the polarity and viscosity of the medium which enables the design of the "turn-on" fluorescent (bio)sensors.[165]

4.3.4 Semi-synthesis of artificial coumarinic betalains. Betalains may be modified to produce water-soluble two-photon fluorophores.[167] Coumarinic betalains cBeet120 and cBeet151 (Table 2) were obtained in the reaction between betalamic acid and aminocoumarins in acidified ethyl acetate (Fig. 23). Betalamic acid underwent Schiff condensation (aldimine formation) with 7-amino-4-methylcoumarin c120 and 7-amino-4-trifluoromethylcoumarin c151 in the presence of *p*-toluenesulfonic acid used as a catalyst. The coupling of the betalamic acid chromophore to the fluorescent hydrophobic chromophore *via* the 1,7-diazeheptamethine π-bridge results in a D–π–A molecular arrangement. Coumarinic betalain containing

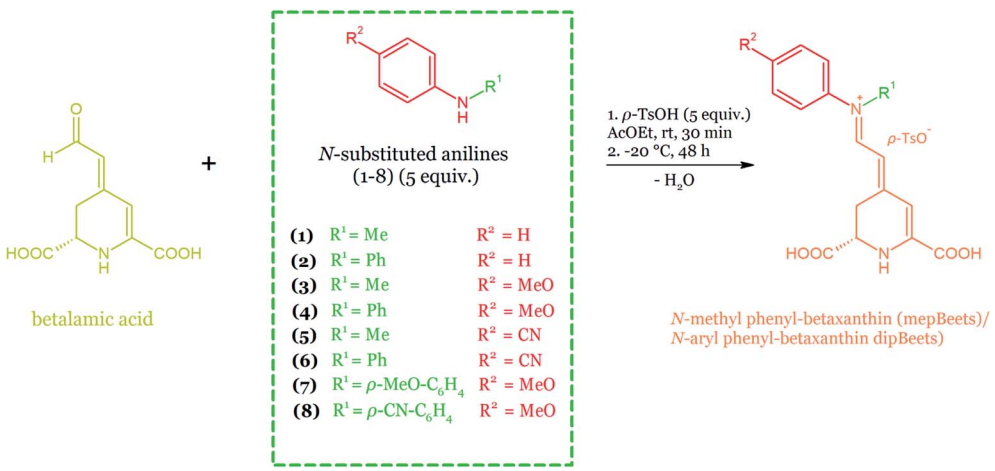


Fig. 22 Semi-synthesis of the mepBeets and the dipBeets *via* the acid-catalyzed coupling of betalamic acid (1) with *N*-substituted anilines (2) in ethyl acetate.[165]

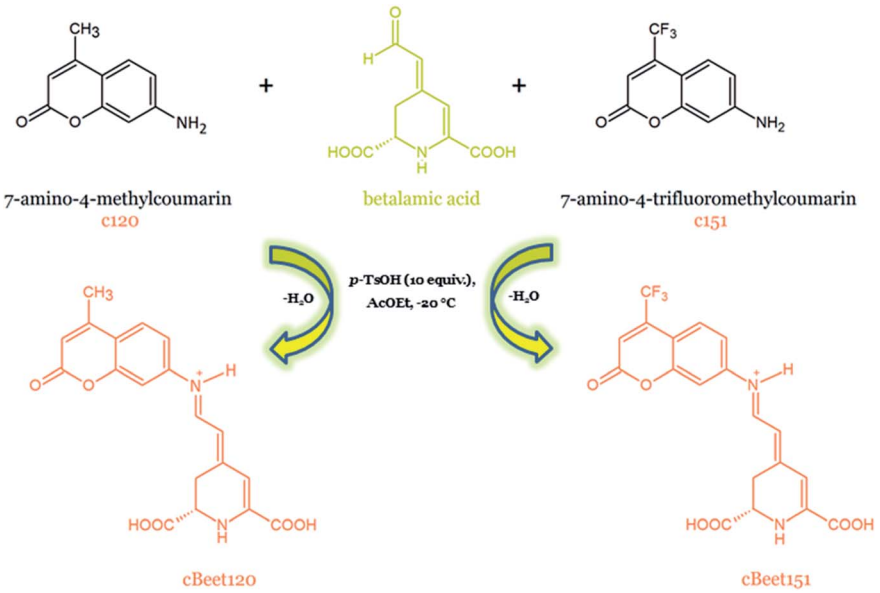


Fig. 23 Semi-synthesis of artificial coumarinic betalain pigments with an extended conjugated system.[167]

a trifluoromethyl electron-withdrawing substituent within the coumarin moiety is more fluorescent than the one with a methyl group but it has a lower two-photon absorption cross-section, which is attributed to the destabilization of the charge transfer character of the excited state affecting the transition dipole moment.[167]

In another study, an artificial coumarinic betalain (BtC) semi-synthesized *via* the aldimine coupling of 7-amino-4-methylcoumarin to betalamic acid was used for the fabrication of the fluorescent probe for the live-cell imaging of erythrocytes infected with *Plasmodium falciparum* responsible for malaria disease in humans.[168] In order to test the capacity of BtC for labeling living *Plasmodium*-infected red blood cells, erythrocytes with parasites were incubated with BtC and examined by fluorescence microscopy. BtC was accumulated within the infected cells selectively and fluorescence imaging microscopy has shown that only the parasite was stained. On the contrary, no stain was observed in the infected erythrocytes incubated with an indicaxanthin (control probe) and uninfected cells.[168]

5 Conclusions

During the last two decades, the research on betalains, more specifically on betacyanins, has experienced significant acceleration due to their health-protective properties as well as applications in the food industry. Before 2000, there were no reports on any pro-health activities of betacyanins; from 2020 onwards, this is the main direction of projects and research on betacyanins. Furthermore, studies on the oxidation mechanism of betacyanins are highly relevant because pigments with different chemical structures seem to have different influence on their oxidation pathways. Considering the fairly simple semi-synthetic methods for additional betacyanin derivatives summarized in this review as well as the first promising results of various studies, the perspectives for further investigations on the bioactivity of betacyanins are inexhaustible. For the time being, the main obstacle that limits the research is the lack of highly efficient and inexpensive methods of betacyanin isolation and purification as well as methods of *de novo* synthesis. The constantly growing number of potential applications of betalains as natural colorants of food, fabrics, and dietary supplements but also their utilization in sensors, photoprotectors, and solar cells set promising directions of the investigations on these still not fully-explored plant pigments in the near future. In spite of the most recognizable betanin-based betacyanins present in a variety of betalainic plant sources, gomphrenin-like pigments also represent a promising group of potentially more stable and more active compounds at once. There are still many unexplored plants with presumably new interesting betacyanin profiles. The chemical structures of only 31 betalain pigments isolated from the plant sources were definitively identified by the NMR method, which is relatively small in comparison to the other groups of plant pigments. Furthermore, a huge group of over 187 betacyanins (including pigments from their richest source, *B. glabra*) detected by LC-MS techniques still awaits the final confirmation of their structures. Particular attention should also be paid to the chemical modifications of betacyanins that result in the generation of an increasing number of new derivatives with completely unknown bioactivities. The aspects discussed in this review show that the field of betalain chemistry is a goldmine and ideas for further research are still limitless.

6 Conflicts of interest

The authors declare no competing financial interest.

7 Acknowledgements

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