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ORIGINAL ARTICLE

Structural implications on color, fluorescence, and antiradical activity in betalains

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Abstract Betalains are water-soluble pigments with high antiradical capacity which bestow bright colors on flowers and fruits of most plants of the order Caryophyllales. They are classified as betacyanins, exhibiting a violet coloration, and betaxanthins, which exhibit yellow coloration. Traditionally, betalains have been defined as condensation products of betalamic acid with different amines and amino acids, but the implication of the pigment structure for their properties has not been investigated. This paper explores different structural features of the betalains, revealing the clues for the switch from yellow to violet color, and the loss of fluorescence. A relevant series of 15 betalain-related compounds (both natural and novel semisynthetic ones) is obtained and characterized by chromatography, UV-vis spectrophotometry, fluorescence, and electrospray ionization mass spectroscopy. Antiradical properties of individual pure compounds in a broad pH range are studied under the $ABTS^{\bullet+}$ radical assay. Relevance of specific bonds is studied, and differences between betaxanthins and betacyanins are used to explore in depth the structure–antiradical activity relationships in betalains.

Keywords Betalain · Color · Fluorescence · Structure–activity relationship · Antiradical · TEAC

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Abbreviations

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
DOPA	Dihydroxyphenylalanine
LDL	Low density lipoproteins
PDA	Photodiode array
TEAC	Trolox equivalent antiradical capacity
TFA	Trifluoroacetic acid

Introduction

Betalains are water-soluble, nitrogen-containing pigments that are present in most plants belonging to the order Caryophyllales (Piattelli 1981; Moreno et al. 2008), where they fulfill the role of anthocyanins in most of Angiosperms (Stafford 1994). They have also been described in the fungal genera *Amanita* (Musso 1979) and *Hygrocybe* (von Ardenne et al. 1974). Betalains are classified as either betacyanins, which exhibit a red-violet coloration, or betaxanthins, which are yellow pigments. Betalains bestow color on flowers of a great variety of plant genera, such as *Mirabilis* (Piattelli et al. 1965), *Glottiphylum* (Impellizzeri et al. 1973), and *Portulaca* (Gandía-Herrero et al. 2005a), but they are also present in bracts (*Bougainvillea*) (Heuer et al. 1994), roots (*Beta vulgaris*) (Schliemann et al. 1999), stems (*Amaranthus*) (Cai et al. 2001), and fruits (*Opuntia*) (Castellanos-Santiago and Yahia 2008).

In addition to their natural colorant properties, which have attracted the interest of the food industry, betalains are antiradical molecules, whose activity has only been described in recent years. The first studies that demonstrated a radical scavenging capacity in betalains were carried out with pigments extracted from beet root (Escribano et al. 1998; Pedreño and Escribano 2001). Betalain activity

was also investigated from other sources (Butera et al. 2002; Pavlov et al. 2002; Cai et al. 2003), and the capacity of betacyanins to inhibit the peroxidation of linoleic acid and the oxidation of LDL was demonstrated (Kanner et al. 2001). The inhibition of skin and liver tumor formation in mice has been demonstrated with very low concentrations of dietary pigments (Kapadia et al. 2003). More recently, protection against the effects of gamma radiation has also been reported in these animals (Lu et al. 2009). In addition, plasma concentrations of betalains after ingestion are sufficient to promote their incorporation into the LDL and red cells, which are then protected from oxidative damage and hemolysis in humans (Tesoriere et al. 2003). The potential of betalains is in contrast to the lack of systematic analyses of these molecules, and it is necessary to identify the structural clues responsible for their biological activities.

Structurally, betaxanthins are defined as immonium condensation products of betalamic acid with amino acids or amines. Traditionally, betacyanins have been defined as condensation products of betalamic acid with *cyclo*-DOPA, usually glycosylated (Piattelli 1981). Glycosylation refers to the multiple possibilities of derivatization of the resulting condensation compound, betanidin (Strack et al. 2003). The simplest and best-known glycosylated derivative of betanidin is betanin (betanidin-5-*O*-*β*-glucoside). Betacyanin definition is independent of the actual biosynthetic mechanism followed in vivo to reach such a cycle attached to the betalamic acid moiety, and there are two plausible pathways proposed in the literature (Piattelli 1981; Gandía-Herrero et al. 2005b). When structures without the characteristic carboxylic group of DOPA were described, they were called descarboxy-betacyanins (Piattelli and Impellizzeri 1970; Kobayashi et al. 2001). In this case, betalamic acid is condensed with descarboxy-*cyclo*-DOPA, which is *cyclo*-dopamine. The new pigments possessed the same chromatographic behavior and analogous spectrophotometrical properties. These were accepted as the only two families of betacyanins, leaving the issue of what the structural unit of betacyanins is unresolved.

When betalamic acid is condensed with *cyclo*-DOPA (or *cyclo*-dopamine) to form betacyanins, there is a connection through a proline like substructure between the resonance system of the betalamic acid moiety and the aromatic system of DOPA. It has been suggested that such a combination is responsible for the particular absorbance properties of betacyanins. However, there has been no experimental approach to the relevance of the different bonds involved in the betacyanin structure.

This paper is aimed at exploring the relations between the betalain pigment structures and the spectroscopic properties they exhibit. Differences between betaxanthins and betacyanins are investigated, and the minimum structure responsible for betacyanin properties is identified. The significance of the different structural features is also studied in terms of structure–antiradical activity relationships.

Materials and methods

Chemicals

All chemicals and reagents were obtained from Sigma (St. Louis, MO, USA). Solvents were from Merck Chemicals Ltd. (Dorset, England). HPLC-grade acetonitrile was purchased from Labscan Ltd. (Dublin, Ireland). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Extraction and semi-synthesis of betalains

The betalains dopaxanthin, betanidin and betanin were extracted from yellow flowers of *Lampranthus productus* (Gandía-Herrero et al. 2005b), violet flowers of *L. productus* (Gandía-Herrero et al. 2007), and red roots of *Beta vulgaris* (Wyler and Dreiding 1957), respectively. The rest of betalains were obtained as immonium condensation products of betalamic acid with the following amines: ethylamine, propylamine, 2-phenylethylamine, *N*-methyl-ethanamine, *N*-methyl-*N*-propylamine, pyrrolidine, aniline, *N*-methyl-aniline, *N*-ethyl-aniline, indoline, (*S*)-phenylalanine, and (*S*)-indoline-2-carboxylic acid. Semi-synthesis was carried out following a previously described method (Wyler et al. 1965; Gandía-Herrero et al. 2006). In short, betanin purified from red beet was used as starting material. Basic hydrolysis (pH 11.4) of 0.2 mM betanin released betalamic acid, which was then condensed with the appropriate amine after reaching pH 5.0. The corresponding betaxanthin was obtained, revealed by a change of color. The whole process was carried out under nitrogen atmosphere. Once synthesis was achieved, a C-18 solid phase extraction step was performed, and an automated system was used for pigment purification.

C-18 solid phase extraction

One-mL C-18 cartridges (Waters, Milford, MA, USA) were conditioned with 5 mL of methanol followed by 10 mL of purified water. Salts and buffers from the samples were removed by rinsing the column with water. Betaxanthins were eluted with ethanol and then concentrated to dryness under vacuum at room temperature. The residue was redissolved in water for further use. The yield obtained for the process was in the range of 92–97%. Sample conductivity after desaltation was below 1.0 mS/cm for all samples (pH/C-900 conductivity detector, General Electric Healthcare, Milwaukee, USA).

Purification of betalains

Anionic exchange chromatography of betalains was performed in an Äkta purifier apparatus (General Electric Healthcare, Milwaukee, USA). The equipment was operated via a PC using Unikorn software version 3.00. Elutions were monitored at 280, 480 and 536 nm. The solvents used were 10 mM sodium phosphate buffer, pH 6.0 (solvent A), and 10 mM sodium phosphate buffer, pH 6.0, with 2 M NaCl (solvent B). A 25 × 7 mm, 1-mL Q-Sepharose Fast Flow column (cross-linked agarose with quaternary ammonium as an exchanger group, 90 μm particle size) purchased from General Electric Healthcare was used. After sample injection, the elution process was as follows: 0% B from 0.0 to 2.0 mL; after washing, a linear gradient was performed over 15 mL from 0% B to 26% B, with 1 mL fractions being collected. Injection volume was 1 mL and the flow rate was 1.0 mL/min. Pigment containing fractions were pooled and salts removed through C-18 solid phase extraction as described earlier.

HPLC analysis

A Shimadzu LC-10A apparatus (Kyoto, Japan) equipped with a SPD-M10A PDA detector was used for analytical HPLC separations. Reversed phase chromatography was performed with a 250 × 4.6 mm Luna C-18(2) column packed with 5 μm particles (Phenomenex, Torrance, CA, USA). Gradients were formed with two helium degassed solvents. Solvent A was water with 0.05% TFA, and solvent B was composed of acetonitrile with 0.05% TFA. A linear gradient was performed for 25 min from 0% B to 35% B. The flow rate was 1 mL/min, operated at 25°C. Injection volume was 20 μL.

Elecrospray ionization mass analysis

An Agilent VL 1100 apparatus with LC/MSD Trap (Agilent Technologies, Palo Alto, CA, USA) was used for HPLC–ESI–MS analyses. Elution conditions were as described earlier using a Zorbax SB-C18 (30 × 2.1 mm, 3.5 Rm) column (Agilent Technologies) with a flow rate of 0.3 mL/min. Vaporizer temperature was 350°C, and voltage was maintained at 3.5 kV. The sheath gas was nitrogen, operated at a pressure of 45 psi. Samples were ionized in positive mode. Ion monitoring mode was full scan in the range m/z 50–600. The electron multiplier voltage for detection was 1,350 V.

Absorbance spectroscopy

A Jasco V-630 spectrophotometer (Jasco Corporation, Tokyo, Japan), attached to a Tectron thermostatic bath (JP Selecta, Barcelona, Spain) was used for absorbance spectroscopy. Measurements were made in water at 25°C. For the quantification of betanin and betanidin the molar extinction coefficients at 536 nm, $\varepsilon = 65{,}000$ and 54,000 $M^{-1}\ cm^{-1}$ were taken, respectively (Schwartz and von Elbe 1980). Molar extinction coefficients for the rest of the pigments were determined by an end-point method, carrying out a set of degradation experiments of the betalains. Spectra for solutions of each pigment were recorded with the above instrument and then submitted to basic hydrolysis using ammonia at a final concentration of 1.2 M. The process was monitored spectrophotometrically for 30 min, taking spectra at 2-min intervals, with a scan speed of 2,000 nm/min. The resulting betalamic acid solution was compared with that obtained from betanin solutions of known concentration, and thus the initial pigment concentration and molar extinction coefficient at the corresponding maximum wavelength were calculated. All the pigments yielded betalamic acid under the assay conditions more quickly than betanin. Betalamic acid solutions were stable under the experimental conditions and no appreciable change in its spectral properties could be recorded for 30 min.

Fluorescence spectroscopy

Fluorescence spectroscopy studies were performed in a Hitachi F-4500 fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan), attached to an Ultraterm 200 thermostatic bath (JP Selecta, Barcelona, Spain). Quartz cuvettes were used, and samples were diluted in water, with the final concentration of the pigment being 3 μM. Excitation and emission spectra were recorded at 25°C, and excitation and emission slit was set at 5 nm. Alanine derived betaxanthin at the same concentration was used as a standard for the determination of relative fluorescence intensity values (Gandía-Herrero et al. 2005c). Excitation spectra were recorded in each case through the emission at the maximum emission wavelength. Emission spectra were obtained by exciting at the corresponding maximum wavelength. 3D fluorescence spectra were obtained under the same general conditions in the range of 400–550 nm for excitation, and 450–650 for emission, and intensity was recorded at 5-nm intervals.

Free radical scavenging activity

Antiradical capacity of betaxanthins was evaluated by following their effect on stable free radical $ABTS^{\bullet+}$. Decolorizing activity on $ABTS^{\bullet+}$ solutions was monitored spectrophotometrically at $\lambda = 414$ nm (Escribano et al. 1998). $ABTS^{\bullet+}$ radical was prepared from 2 mM ABTS through peroxidase activity (88 units/L commercial horseradish

peroxidase type VI, obtained from Sigma) in the presence of hydrogen peroxide (45 μM), in 12 mM sodium acetate buffer, pH 5.0. The reactive was then diluted by 2/3 through the addition of samples, and the reactions were carried out in 53 mM sodium phosphate buffer, pH 7.0. Other conditions are specified in the text. Measurements of 96-well plates were performed after 24-h incubations at 20°C in a Synergy HT plate reader (Bio-Tek Instruments, Winooski, USA). All experiments were performed in triplicate and mean values and standard deviations were plotted. Final volume was 300 μL (calculated path length = 0.87 cm). Detector linearity under the assay conditions was confirmed ($r = 0.999$). In each case, errors associated with the results provided were calculated on the basis of the residual standard deviations.

Results and discussion

Semi-synthesis and characterization of selected pigments

The absence of systematic analyses on the different properties of betalains, and their relation with the pigments structures is justified by the lack of a suitable procedure to obtain pure pigments. The application of an improved procedure for the semi-synthesis and purification of betaxanthins (Gandía-Herrero et al. 2006) based on the Schiff base synthesis (Wyler et al. 1965) allowed us to work with both natural and previously unconsidered pigments. A relevant series of structurally related pigments was obtained in order to clarify the role of different structural features in betalain properties.

Figure 1 shows the compounds obtained and purified for the present study. Pigments were selected in order to explore the role of additional electronic resonance through aromaticity in the betalain pigments and the effects of cyclization, carboxylation, and quaternary ammonium charge. A combined procedure for semi-synthesis and purification was applied to obtain both betaxanthins and betacyanins. Compounds 13, 14, and 15 were extracted and purified from natural sources. All pigments were analyzed by HPLC using a PDA detector to confirm purity of the samples obtained. Retention times are shown in Table 1. Elution order was related with polarity, with N-ethyl-aniline derived betalain (compound 9) eluting the last. In the series of aromatic compounds, betanin (compound 15) was the first to elute due to the effect of glucosylation.

Electrospray ionization mass spectrometry (ESI–MS) was applied to the characterization of the compounds. The mass values determined for the parent ions of all compounds are listed in Table 1. In all cases, values were as expected for the corresponding protonated molecular ions $[M + H]^+$ of the betalains, thus confirming the proposed structures for the purified pigments. For molecules with a quaternary ammonium substructure (compounds 4, 5, 6, 8, 9, 10, 12, 14, and 15), the mass obtained corresponds to the charged form, as shown in Fig. 1. In all cases a daughter ion corresponding to $[M + H]^+$–44 *m/z* was detected, accounting for the loss of a carboxylic group. For compound 15, the main daughter ion (389 *m/z*) corresponded to compound 14. The use of *Beta vulgaris* as the source of betanin and the cleavage of a unit of glucose ($[M + H]^+$–162 *m/z*) confirms the *O*-glucosidic nature of the bond (Cuyckens and Claeys 2004; Gandia-Herrero et al. 2008).

Color differences between betaxanthins and betacyanins

Since they are pigments the most important feature of betalains is color. Colorant properties are linked to the electron resonance system supported by both nitrogen atoms of the molecule. A full range of color, from yellow to violet, was obtained with the molecules used in the study (Fig. 2a). Maximum wavelengths obtained ranged from 467 to 542 nm (Table 2). For the betalains with the simplest structural features around the nitrogen atom (structures 1, 2, 3, 4, 5, 6, 11, and 13), the maximum wavelength is restricted to the range 467–478 nm, so they are yellow. Those molecules involving aromaticity in resonance with the nitrogen atoms present a bathochromic shift with maximum wavelengths of 507 nm (structure 7), 492 nm (structure 8), and 489 nm (structure 9), and appear orange in color. Compounds with an analogous aromatic resonance system, but structurally hindered by an extra intramolecular cycle (structures 10, 12, 14, and 15) possess wavelengths ranging from 524 to 542 nm. This extra bathochromic effect makes them violet. Table 2 lists the spectroscopic properties determined for all the pigments, including spectrum width at an absorbance value half of that at the corresponding maximum. This parameter shows how yellow pigments spectra are sharper than those for the orange and violet betalains. A positive linear relation between the maximum wavelength and the calculated width in the range studied can be found ($r = 0.9558$). Spectra with the characteristic shapes for the different groups of pigments identified above are shown in Fig. 2b. In all cases a secondary peak in the UV region accompanies the main absorbance peak in the visible region.

In addition, molar absorption coefficients were calculated for all the semi-synthetic betalains. A degradation procedure based on the basic hydrolysis of betalains to betalamic acid was applied, using betanin solutions of known concentration as reference ($65{,}000\ M^{-1}\ cm^{-1}$) (Schwartz and von Elbe 1980; Trezzini and Zrÿd 1991). Values are high for all the molecules, and varied from 46,000 to 76,000 $M^{-1}\ cm^{-1}$, as shown in Table 2. Molecules with maximum absorbance wavelengths between 467

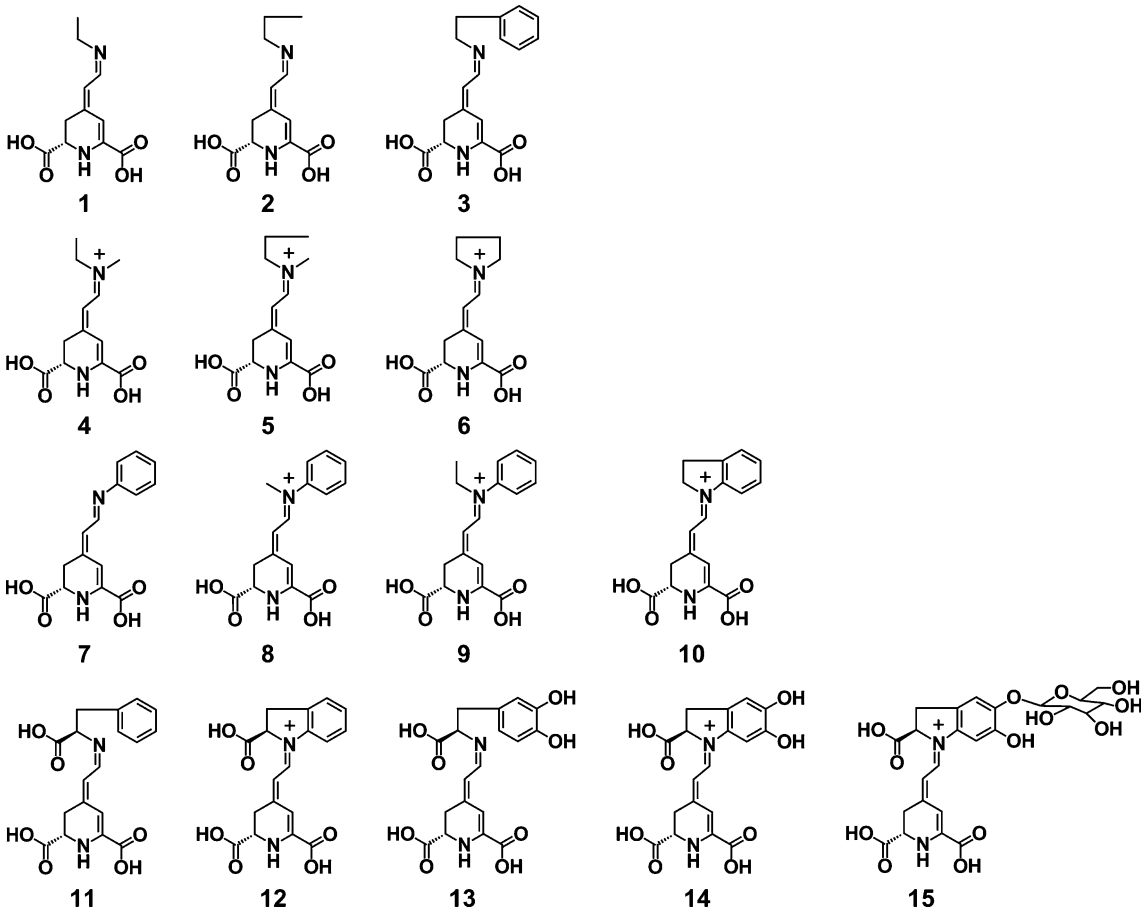

Fig. 1 Structures of betalain pigments

and 478 nm, possess a molar absorption coefficient restricted to the range 46,000–50,000 M^{-1} cm^{-1}. In contrast, those pigments of violet color have the highest molar absorption coefficient values, except for compound 14 (betanidin). For the compound derived from (*S*)-indoline-2-carboxylic acid (compound 12) a molar absorption coefficient of 76,000 M^{-1} cm^{-1} was obtained. This is the betalain with the highest absorbance ever reported.

For pigments 7, 8, and 9, the coefficient values were between those of the yellow and violet pigments. Figure 2c shows how the maximum wavelengths and the values for the molar absorption coefficients are related, and it classifies the betalains graphically. Three different groups are displayed according to their spectroscopic properties: Betaxanthins, with restricted values for the variables presented, betacyanins with the highest values in absorbance and wavelengths, and compounds 7, 8 and 9, with intermediate properties.

As can be seen, the inclusion into the yellow betaxanthin group is independent of the nature of the amine condensed to the betalamic acid moiety, insofar as there is no aromaticity in resonance with the nitrogen atoms. Thus, condensation can be performed with primary and secondary amines and aminoacids. As a result, there are compounds with betaxanthin properties involving cycles (6), aromaticity (3, 11, 13), and with a positive charge on the nitrogen group (4, 5, 6). The data obtained in this study support the use of the mean value of $\varepsilon = 48{,}000$ M^{-1} cm^{-1} at 480 nm for all betaxanthins (Schliemann et al. 2001). Previous estimations of molar absorption coefficients for other betaxanthins were similar to the present data (Trezzini and Zrÿd 1991).

On the other hand, molecules with betacyanin properties contain an aromatic system in resonance with the betalamic acid moiety, plus a second cycle fused in an indoline manner. Compound 10 can be considered the simplest pigment with betacyanin properties, and thus the betacyanin defining structure. It is likely that the five bonded ring causes a structural distortion of the planarity in the resonance system and it may be responsible for the characteristics of the derived pigments (compounds 10, 12, 14, and 15). This is more evident when considering the properties of compound 7. In this case, the resonance system is unrestricted, and likely to be fully planar. Even in the absence of a second

Table 1 Chromatographic and mass spectroscopic data for betalains used in this study

Amine		Trivial name	Rt (min)	PDA-λ_m (nm)[a]	$[M + H]^+$ (m/z)[b]	Main daughter ion (m/z)	References
1	Ethylamine	–	11.71	452	239	195	
2	Propylamine	–	14.60	453	253	209	Gandía-Herrero et al. (2009)
3	2-Phenylethylamine	–	22.11	460	315	271	Castellanos-Santiago and Yahia (2008)
4	*N*-Methyl-ethanamine	–	12.82	458	253	209	
5	*N*-Methyl-*N*-propylamine	–	15.83	459	267	223	
6	Pyrrolidine	–	13.87	459	265	221	
7	Aniline	–	19.91	507	287	241	
8	*N*-Methyl-aniline	–	20.30	487	301	255	
9	*N*-Ethyl-aniline	–	22.63	486	315	269	
10	Indoline	–	22.10	523	313	269	
11	(*S*)-Phenylalanine	–	20.08	472	359	315	Stintzing et al. (2002)
12	(*S*)-Indoline-2-carboxylic acid	–	19.17	523	357	313	
13	(*S*)-dihydroxy-phenylalanine (DOPA)	Dopaxanthin	13.33	472	391	347	Impellizzeri et al. (1973)
14	(*S*)-*cyclo*-dihydroxy-phenylalanine (*cyclo*-DOPA)	Betanidin	14.11	542	389	343	Kanner et al. (2001)
15	(*S*)-*cyclo*-dihydroxy-phenylalanine-glucoside (*cyclo*-DOPA-glucoside)	Betanin	11.38	535	551	389	Gliszczyńska-Świgło et al. (2006)

Amines from which the pigments are derived and selected previous references, where available, are also shown

[a] Wavelength corresponding to the maximum absorbance in the HPLC gradient, measured through a PDA detector

[b] Protonated molecular ion obtained for each pigment

cycle, the introduction of a methyl or an ethyl residue at the nitrogen level may cause a minor distortion and this may be behind the different properties of compounds 8, and 9, respectively, with respect to compound 7.

Structural considerations on betalains fluorescence

Fluorescence properties of all the betalains were investigated. Excitation spectra were obtained by following the emission at the maximum emission wavelength determined in a previous measurement. Emission spectra were obtained by exciting at the corresponding maximum wavelength. Maximum wavelengths are presented in Table 2. As can be seen, betaxanthins present maximum excitation wavelengths between 471 and 474 nm, while emission maxima are found in the range 548–551 nm. For those betacyanins where fluorescence could be detected, compounds 10 and 12, maximum excitation wavelengths were 521 and 529 nm, respectively, and emission maxima occurred at 570 and 575 nm, respectively. Compounds 7, 8, and 9 present intermediate fluorescence characteristics with maximum excitation wavelengths between 494 and 513 nm, and maximum emission wavelengths between 553 and 560 nm. Figure 3 shows both excitation and emission spectra for characteristic betalain molecules of the different groups. Separation between both spectra maxima (Stokes shift) is shown in Table 2, and it was found to be higher for betaxanthins. A linear relation can be found in the decrease of the Stokes shift with increasing excitation wavelengths ($r = 0.9807$).

The width for the spectra at half the maximum intensity is also given (Table 2). Shapes of the spectra obtained in all cases respond to the same pattern for each identified group, and are shown in Fig. 3. For simple betaxanthins, spectra widths range between 22 and 33 nm for excitation, and between 21 and 23 nm for emission. Those betaxanthins containing an aromatic ring present excitation and emission widths of 38–48 nm and 25–31 nm, respectively. For betacyanin compound 13, spectra widths were calculated as 38 nm for excitation, and 25 nm for emission. For compounds 8 and 9, excitation and emission widths were similar (20–24 nm and 21 nm respectively), while for compound 7, widths are considerably higher (41 nm for excitation, and 25 nm for emission).

The influence of structural factors on the fluorescence intensity of betalains can be detected from the molecules studied. The presence of a carboxylic group in the pigment structures leads to an enhancement of the fluorescence displayed by pigments 11 and 12 with respect to their decarboxylated analogous molecules 3 and 10, respectively. This may be related to the electron density withdrawing effect of the group, as was also identified in the case of carboxylated tyrosine and DOPA derived betaxanthins with respect to tyramine and dopamine derived pigments (Gandía-Herrero

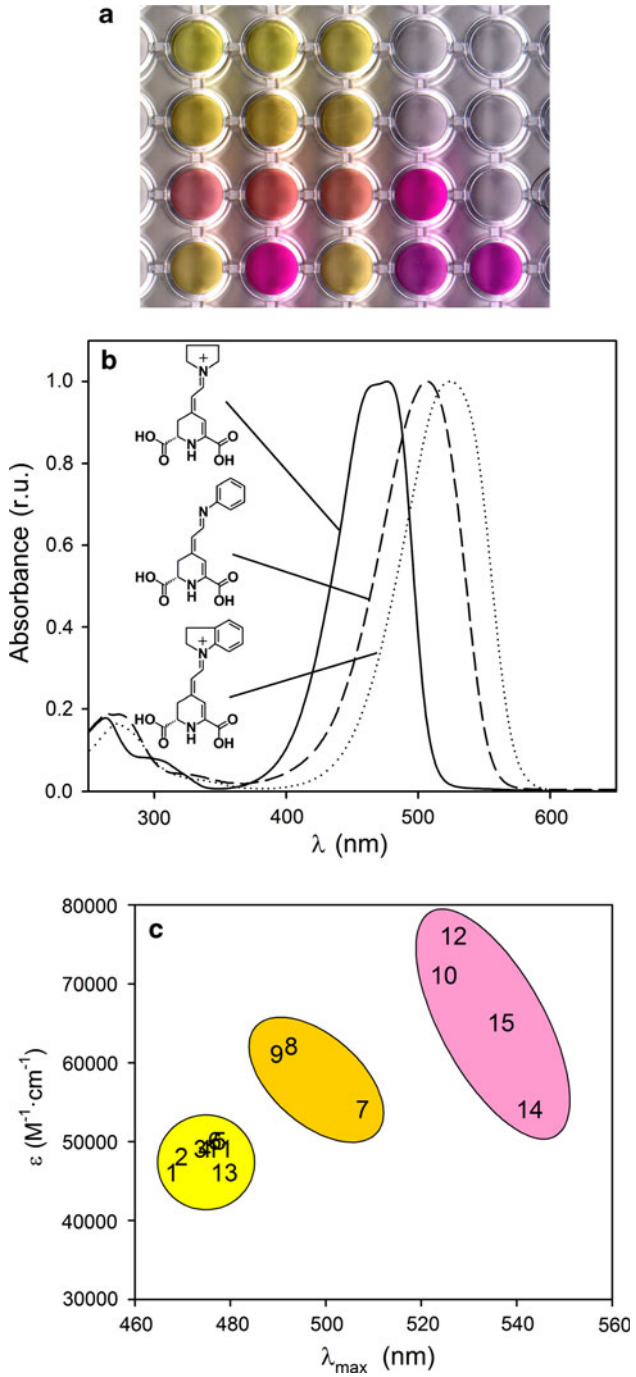

Fig. 2 Absorbance properties of betalains. **a** Plate picture showing the color obtained for the different compounds. Each well contained 300 µL of pure betalain 3.3 µM. b Absorbance spectra for pure betalains 6 (*solid line*), 7 (*dashed line*), and 10 (*dotted line*). **c** Relationship between molar absorption coefficients ($M^{-1}\,cm^{-1}$) and absorbance maximum wavelengths (nm). Pigment distribution in **a** and numbers in **c** correspond to those of Fig. 1

et al. 2005c). The effect of electron density drawing groups in betalains fluorescence is the opposite, and fluorescence intensity is reduced by the presence of hydroxyl groups in molecules 13 and 14 with respect to 11 and 12, respectively. The presence of an aromatic system connected to the electron resonance system supported by the nitrogen atoms implies a marked decrease in the fluorescence intensity. This can be seen in the case of molecules 3 and 9, where the only difference lies in the bond that connects the aromatic ring to the rest of the structure. Among the betalains presenting aromaticity in conexion with the electron resonance system, only molecule 7, likely to be fully planar, presents a fluorescence intensity value close to betaxanthins. With an analogous aromatic resonance system, betacyanin fluorescence intensity is lowered under the effect of the closure of the indoline ring. The effect of this bond is observed in the comparison of molecules 3–10, and 11–12. No detectable fluorescence was found for betacyanin molecules 14 and 15.

In order to cover the entire range of possible wavelengths for both excitation and emission, three-dimensional fluorescence spectra were recorded for all the molecules. Derived excitation-emission matrix fluorescence spectroscopic data are graphically provided in Fig. 4. The results provide a full characterization of the fluorescence properties of betalains, and show fluorescence peaks centered at the corresponding maximum excitation and emission wavelengths. The same behavior is found for molecules 1, 2, 3, 4, 5, 6, 11, and 13 (betaxanthins), and peak shape and position are nearly the same for molecules 10 and 12 (betacyanins). Although molecules 7, 8, and 9 present intermediate properties, compound 9 is closer to betacyanins in spectral shape and position. In addition, compounds 8 and 9 present secondary fluorescence peaks that are centered at excitation-emission wavelengths of 470–545 nm and 470–550 nm, respectively.

Structure–activity relationships in betalain antiradical capacity

Despite the increasing evidence for the potent biological activities of betalains related to their antiradical or antioxidant capacity (Moreno et al. 2008; Lu et al. 2009), the available information on individual pure pigments is scarce. Betanin (compound 15) was the first to be studied in depth, and its exceptionally high free radical scavenging capacity was linked to its electron donation ability (Gliszczyńska-Świgło et al. 2006). More recently, the effect of phenolic hydroxyl groups on the free radical scavenging activity of betalains was evaluated (Gandía-Herrero et al. 2009), revealing the existence of an intrinsic radical scavenging activity characteristic of betalains which was not linked to the presence of a phenolic ring. These data are in contrast to the information available for other molecules such as flavonoids (Cai et al. 2006). How this intrinsic free radical scavenging activity is modulated remains unexplored, and a systematic analysis of the activity of betalains is necessary.

Table 2 Absorbance and fluorescence spectroscopy data obtained for betalains

	Absorbance			Fluorescence					
	λ_m (nm)	ε_m (M^{-1} cm^{-1})	Width (nm)[a]	$\lambda_{m\ Exc}$ (nm)	$\lambda_{m\ Ems}$ (nm)	Stokes shift (nm)	Relativ. Flu. Int. (%)[b]	Exc. width (nm)[a]	Ems. width (nm)[a]
1	467	46,000	60	472	548	76	25.3	22	23
2	469	48,000	60	472	548	76	28.3	22	23
3	473	49,000	59	473	551	78	104.4	41	28
4	474	49,000	62	472	548	76	16.3	27	21
5	477	50,000	62	472	550	78	30.9	33	23
6	476	50,000	64	471	549	78	8.2	27	21
7	507	54,000	72	513	560	47	28.7	41	25
8	492	62,000	66	494	553	59	1.5	24	21
9	489	61,000	63	494	554	60	0.7	20	21
10	524	71,000	74	521	570	49	0.4	30	–
11	477	49,000	58	471	549	78	182.9	48	31
12	526	76,000	74	529	575	46	8.2	36	23
13	478	46,000	61	474	550	76	84.0	38	25
14	542	54,000	90	–	–	–	–	–	–
15	536	65,000	84	–	–	–	–	–	–

Compounds numbering corresponds to the structures shown in Fig. 1

[a] Spectrum width was calculated at an absorbance/fluorescence intensity value half of that at the corresponding maximum

[b] Relative fluorescence intensity values are calculated in relation to Ala-derived betaxanthin

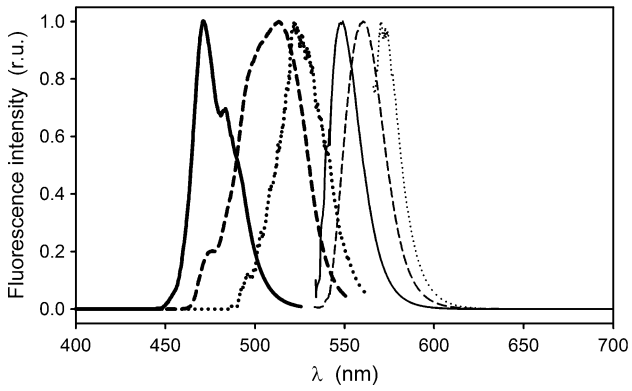

Fig. 3 Excitation (*thick lines*) and emission (*thin lines*) fluorescence spectra for betalain compounds 6 (*solid line*), 7 (*dashed line*), and 10 (*dotted line*). Spectra were obtained in water at 25°C

The individual free radical scavenging capacity for all the molecules purified in this study was characterized in order to shed light on the effect of different structural features.

The scavenging activities of the different betalains were evaluated according to their effect on stable colored solutions of the radical $ABTS^{\bullet+}$ (Re et al. 1999). The assay is based on monitoring the decrease in the absorbance of the radical solution, and it is compared with the decrease due to an equal concentration of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a potent antiradical, water-soluble derivative of vitamin E. Accurate pigment concentration determination was reached through the molar absorption values determined earlier (Table 2). Figure 5 shows the TEAC values obtained at pH 7 for the betalains under study. As can be seen, in all cases there is a high activity—above that found for Trolox.

As regards the intensity of their antiradical activity, betalains can be classified in groups that possess structural similarities. Thus, for betalains without aromatic resonance, charge or hydroxy groups (compounds 1, 2, 3, and 11), the calculated mean TEAC value is 2.4 ± 0.1. In the case of betaxanthins with charge and no aromatic resonance (compounds 4, 5, and 6), the mean TEAC value is 1.8 ± 0.1. For compounds with an aromatic ring in resonance with the electronic system supported by the nitrogen atoms (compounds 7, 8, and 9), the mean TEAC value obtained is 2.8 ± 0.4. If such resonance is combined with a second cycle fused in an indoline manner (betacyanins) the TEAC value raises to 4.1 ± 0.3 (compound 10) and 3.9 ± 0.3 (compound 12). As expected, the presence of phenolic hydroxy groups, in both betaxanthin 13 and betacyanin 14 implies a significant enhancement of the antiradical activity (Gandía-Herrero et al. 2009) with respect to their dehydroxylated analogs. The increase is smaller for compound 15, where glucosylation blocks one of the two hydroxy groups.

All these data support the existence of a strong intrinsic antiradical activity in betalains and allow some conclusions to be drawn on structure–antiradical activity relationships.

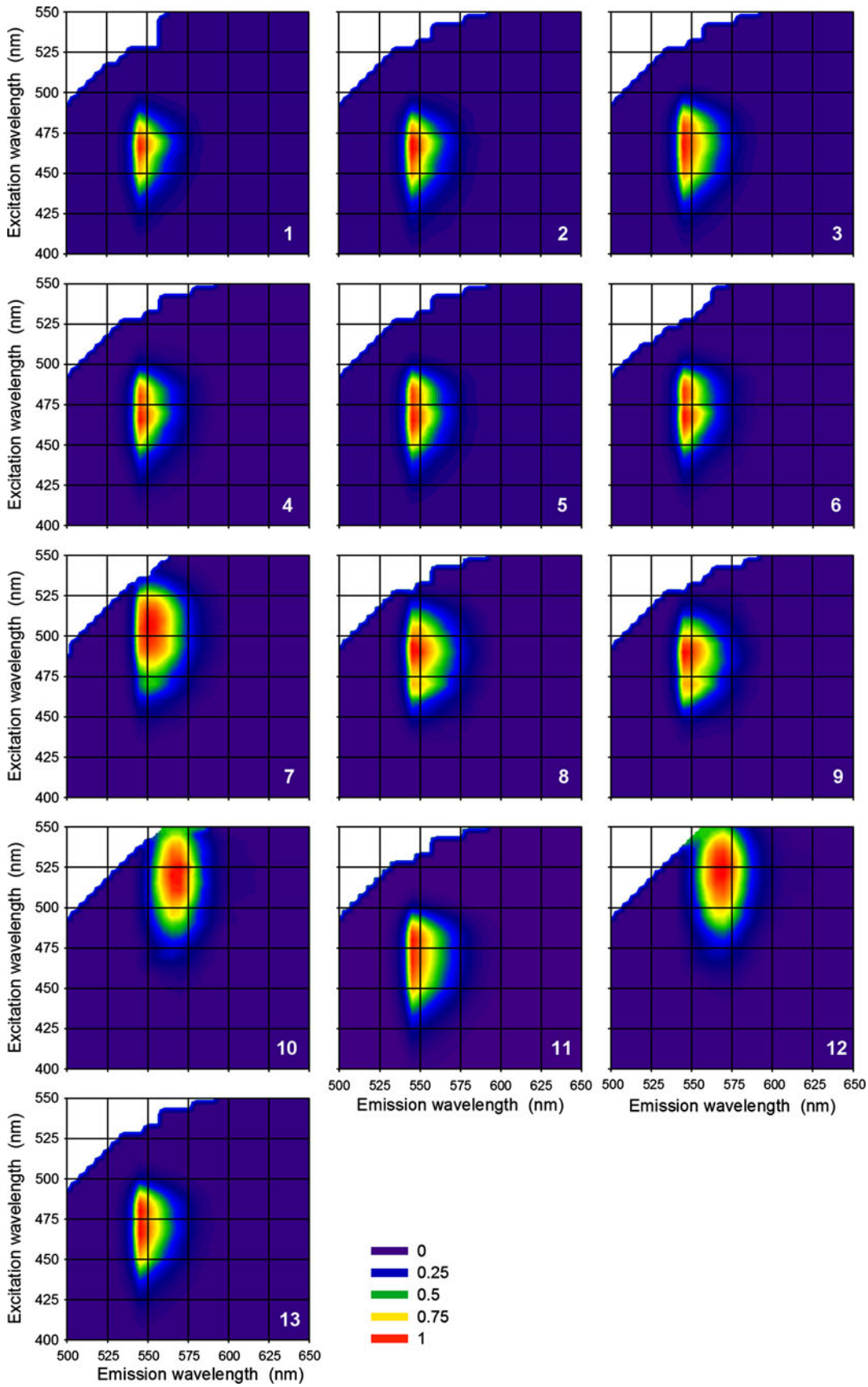

Fig. 4 Contour plots of 3D fluorescence spectra for all betalains under study. No signal could be detected for compounds 14 and 15. All spectra were obtained in water at 25°C, at a pigment concentration of 3 μM. For comparison purposes, relative units are used

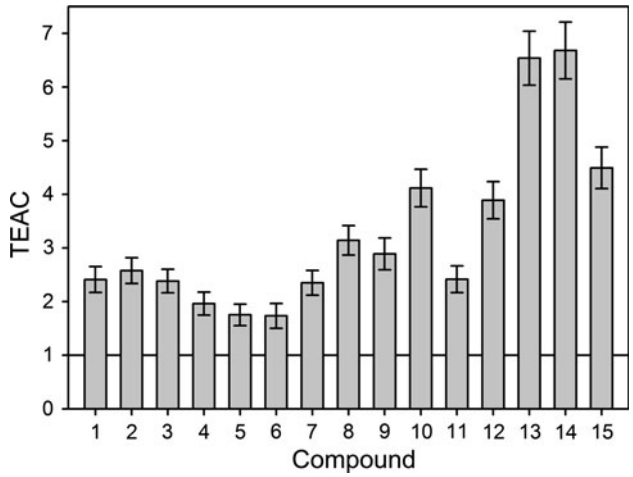

Fig. 5 TEAC values determined for individual betalain pigments under the $ABTS^{\bullet+}$ radical assay at pH 7.0

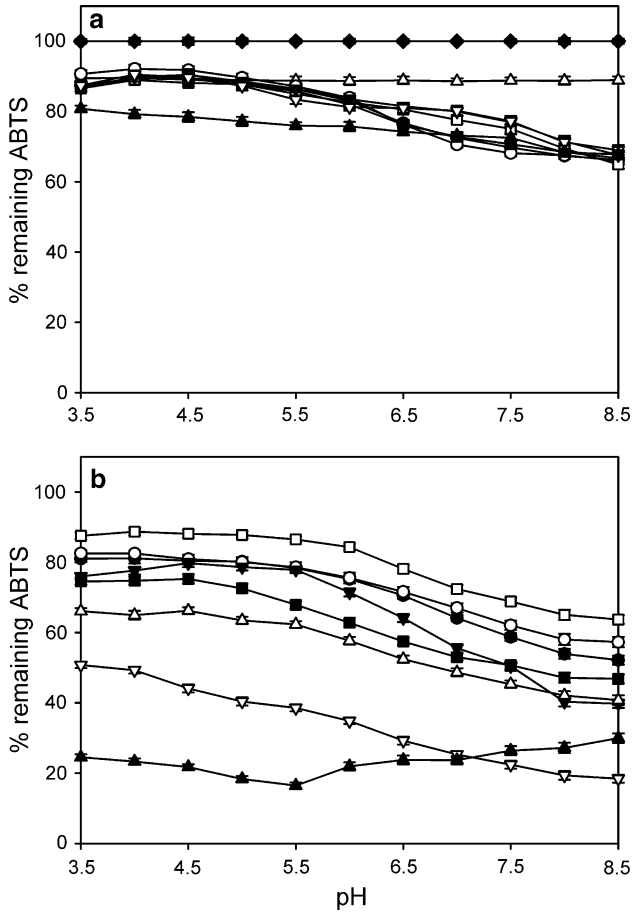

Fig. 6 Betalains' free radical scavenging activity dependence on pH. $ABTS^{\bullet+}$ radical initial concentration was 57 μM. Compounds were added at a final concentration of 3.3 μM. For better visualization, figure is divided in two panels. In **a** symbols are as follows: *filled circle* compound 1, *open circle* compound 2, *filled square* compound 3, *open square* compound 4, *filled inverted triangle* compound 5, *open inverted triangle* compound 6, *filled triangle* compound 7, *open triangle* Trolox, and *filled diamond* water control. In **b** symbols are: *filled circle* compound 8; *open circle*, compound 9, *filled square*, compound 10, *open square* compound 11, *filled inverted triangle* compound 12, *open inverted triangle* compound 13, *filled triangle* compound 14, and *open triangle* compound 15

Betalain activity can be quantified for the simplest pigments to be around TEAC values of 2.4. The presence of a charge in betaxanthins derived from secondary amines seems to reduce this value. However, the connection of the betalain-characteristic electronic resonance system with an aromatic ring produces an enhancement of the antiradical activity, increasing the TEAC value by around 0.4. If this is done to form indoline-like substructures, the enhancement is higher. The bond that converts betaxanthins 3 and 11 into betacyanins 10 and 12, respectively, implies an increase in the TEAC value of 1.6 units. Known effects of hydroxy groups should be added to this characteristic activity of betalains. There is no effect related to the carboxylation of the pigments.

In order to evaluate the effect of pH on the antiradical activity of all betalains, it was varied in the range 3.5–8.5 in the assay medium. Sodium acetate was used as buffer for pH values ranging from 3.5 to 5.5, and sodium phosphate for 5.5–8.5. No difference was observed for the activity measured for both buffers at pH 5.5. Figure 6 shows how pH values above 5.5 indicate a general increase in the free radical scavenging activity measured for all the betalains. At pH values below 5.5, a basal activity can be found. Behavior for compounds 1–6 and 11 in this range is the same, exhibiting an activity analogous to that found for Trolox, with the removal of around 10% of the initial free radical. For the other compounds, the activity displayed at acidic pH values is higher. Aromatic compounds 7, 8, and 9 remove around 20% of the initial radical at acidic pH values, and removal is higher for all the betacyanins. In all cases the pH curve is similar, and only two molecules seem to behave differently: compound 7 (Fig. 6a), in which pH has a limited effect, and compound 14 (Fig. 6b). In this case, the behavior at more acidic pH values is analogous to the other compounds, but at pH values above 5.5 there is a decrease in the scavenging of the radical, instead of the expected enhancement. This might be due to the lability of the molecule in neutral and basic environments. Kinetic studies on its chemical stability have shown how the value for the first-order degradation constant increases at pH values above 6.0 (Gandía-Herrero et al. 2007).

Carboxylated betalains

The betalamic acid moiety of betalains presents two carboxylic groups, with one of them supported by a chiral carbon. The presence of another carboxylic group in the amine moiety, as in pigments 11–15, introduces an extra chiral center that makes betalains diasteroisomeric structures. As

shown earlier, the presence of this carboxylic group enhances fluorescence intensity of betalains due to its electron density withdrawing effect. It is also responsible for a bathochromic shift in the absorbance properties of carboxylated betalains, increasing the maximum wavelength of their visible spectra. This is the case of the betaxanthin 11 with respect to 3, and the betacyanin 12 with respect to 10 (Table 2).

By using carboxylated betalains, derived from amine moieties of unique (*S*) configuration and under the HPLC system capable of isomer separation described, the effect of indoline ring closure on isomers retention times can be analysed. Table 1 shows the retention times for the form (2*S*/*S*) of betalains 11–15. (2*S*/*S*) is the main natural configuration for the pigments, but they can also be found as the (2*S*/*R*) forms (Gandía-Herrero et al. 2005b; Sasaki et al. 2009). For these isomers the retention times obtained are 19.54 min (11), 20.04 min (12), 12.65 min (13), 15.03 min (14), and 12.06 min (15). Retention time seems to be independent of the presence of the ring, and it is not affected by its closure for the pair of molecules 3 and 10, while it is shortened in the case of the pair 11 and 12, and enlarged for the pair 13 and 14. However, elution order follows a trend based on the configuration of the ring. For those diasteroisomeric betalains with the closed cycle, the elution order for the isomers is (2*S*/*S*) earlier than (2*S*/*R*), while for the analogous molecules studied with an open cycle the order is (2*S*/*S*) later than (2*S*/*R*). The effect of the indoline ring closure on the elution order supports previous observations on the chromatographic behavior of betalains (Wybraniec 2007).

The results of this work show the implications of betalain pigment structure in their displayed color, fluorescence, and chromatographic behavior, and establish the structure–activity relationships in relation to the free radical scavenging capacity. This not only explains the different properties of the pigments, but may also allow the identification of potentially interesting molecules and natural sources.

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