The Role of Phenolic Hydroxy Groups in the Free Radical Scavenging Activity of Betalains

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Received February 26, 2009

Free radical scavenging compounds play important roles as health-protecting factors. Betalains are natural water-soluble pigments present in most plant families belonging to the order Caryophyllales. They are the subject of increasing attention following the discovery of their antiradical capacity, but a systematic analysis of the structural features involved in the activity is necessary. In this paper, both natural and previously unconsidered betaxanthins were obtained in order to study the role of phenolic hydroxy groups in the high free radical scavenging activity of betalains. Pigments were characterized spectrophotometrically, chromatographically, and by ESI-MS, and their antiradical and antioxidant properties were studied under the $ABTS^{\bullet+}$ radical and FRAP assays. A high intrinsic activity is described that is not linked to the presence of hydroxy groups or aromaticity in the pigment structure. In addition, the presence of phenolic hydroxy groups implies an enhancement of the antiradical activity, reaching a TEAC value in the $ABTS^{\bullet+}$ assay of 5.8 ± 0.2 for the pure compound with two hydroxy groups.

Epidemiological studies have demonstrated that reduced cancer risk is associated with frequent consumption of vegetables and fruits in general. Dietary polyphenols, found in higher plants, are well recognized for their antioxidative properties, and numerous studies have demonstrated their inhibition of carcinogenesis.[1] Antioxidant or free radical scavenging activity of polyphenols prevents the formation of reactive oxygen and nitrogen species, which play important roles in carcinogenesis.

Betalains are water-soluble, nitrogen-containing pigments of growing interest in the food industry. They are present in most plants belonging to the order Caryophyllales,[2,3] where they replace anthocyanins,[4] and can be classified as either betacyanins or betaxanthins. Betacyanins contain a *cyclo*-DOPA residue (usually glycosilated) and exhibit a violet coloration. Betaxanthins contain different amino acid or amine side chains and exhibit yellow-orange coloration. Betaxanthins are known to give color to flowers of a great variety of plant genera, but they are also present in edible sources like beet root (*Beta vulgaris*)[5] and cactus pear (*Opuntia*).[6]

Knowledge of the biological activities of betalains is still some distance from that of other pigment families such as chlorophylls, anthocyanins, or carotenoids.[7−9] However, the activity of betalains has been investigated in some recent papers. The first studies that demonstrated a radical scavenging capacity in betalains were carried out with pigments extracted from beet root.[10,11] Additional work extended the information available to other betalains.[12−14] Kanner et al.[15] demonstrated the capacity of betanin and betanidin to inhibit the peroxidation of linoleic acid and the oxidation of LDL (low-density lipoproteins) at lower concentrations than other known antioxidants such as α-tocopherol or catechin.

Recently, the inhibition of skin and liver tumor formation in mice has been demonstrated with very low concentrations of dietary betanin.[16] In addition, plasma concentrations of these compounds after ingestion are sufficient to promote their incorporation into the LDL and red blood cells, which are then protected from oxidative damage and hemolysis in humans.[17]

The available information regarding the biological activity of pure pigments is scarce. Betanin, the most widely distributed betalain,[18] has been studied in some depth, and its exceptionally high free radical scavenging capacity has been linked to its electron donation ability.[19] However, a systematic analysis of the structural features involved in the activity of betalains is necessary.

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For phenolic compounds, there is a general agreement that the more hydroxy groups present, the greater the antiradical activity.[20] In the case of flavonoids, the lack of any hydroxy group (as in the case of *trans*-chalcone, flavone, flavanone, and isoflavone) implies no radical scavenging capacity at all.[21]

In the present study, selected structures of betaxanthins were synthesized in order to obtain a series relevant for comparative purposes on aromaticity and the presence of hydroxy groups. Their individual radical scavenging capacities and antioxidant properties are characterized in an attempt to unveil the role of phenolic hydroxy groups in the antiradical activity of betalains.

Results and Discussion

Semisynthesis of Selected Betaxanthins. Selected pigments were designed to explore the effect of the presence of phenolic hydroxy groups on the free radical scavenging activity of betalains. Figure 1 shows the structures for the pigments used in this study. Two of them are the naturally occurring tyramine-betaxanthin and dopamine-betaxanthin, also known by the trivial names miraxanthin III (**4**) and miraxanthin V (**5**), respectively, and have been described in the species *Mirabilis jalapa*,[22,23] *Beta vulgaris*,[5,24,25] *Portulaca grandiflora*,[26,27] *Celosia argentea*,[28] and *Portulaca oleracea*.[29] 2-Phenylethylamine-derived betaxanthin **2** was found in trace amounts in some *Opuntia* cultivars.[30] Compounds **1** and **3** are derived from the amines 1-aminopropane and 3-hydroxyphenethylamine, respectively, to obtain structurally related betaxanthins not previously described. A series of betaxanthins is thus obtained with the presence of the aromatic ring and with hydroxy groups on it.

The newly synthesized structures possess UV−visible spectra analogous to those described for all betaxanthins.[31,32] Table 1 shows the values obtained for absorbance maximum wavelengths, both in water and in the MeCN gradient used for the HPLC analyses. A single peak in the HPLC analysis with PDA detection was obtained for each pigment (recordings are shown in Figure 2). The elution order of aromatic betaxanthins in the MeCN gradient is related to the presence of hydroxy groups, thus causing the least polar compound **2** to be eluted last. Different positions of the hydroxy groups of compounds **3** and **4** caused a shift of 0.92 min, with compound **3** eluting later than compound **4**. Compound **1** eluted first, with a retention time of 14.73 min.

ESIMS was used to determine the molecular masses of the synthesized betaxanthins. The mass values are listed in Table 1. In all cases, values were as expected for the corresponding protonated molecular ions $[M + H]^+$ of the betaxanthins, thus confirming the

10.1021/np900131r CCC: $40.75
Published on Web 05/20/2009

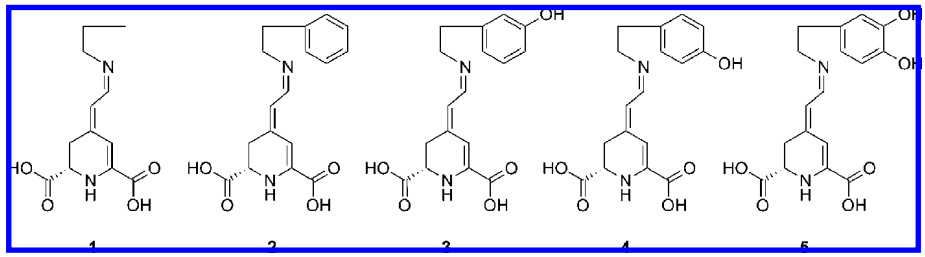

Figure 1. Structures for the betaxanthins. Structures **4** and **5** correspond to naturally occurring miraxanthins III and V, respectively. Compound **2** has been found on certain cacti, and compounds **1** and **3** are novel pigments.

Table 1. Spectroscopic and Chromatographic Characteristics of Betaxanthins

	amine	λ_{max} (nm)	t_R (min)	PDA-λ_{max} (nm)	$[M + H]^+$ (*m/z*)	daughter ions (*m/z*)
1	1-aminopropane	469	14.73	452	253	209, 179
2	2-phenylethylamine	473	22.30	458	315	271, 223
3	3-hydroxyphenethylamine	473	18.16	456	331	285, 239
4	4-hydroxyphenethylamine	473	17.24	456	331	285, 239
5	3,4-dihydroxyphenethylamine	473	15.28	456	347	301, 255

proposed structures for the pigments. For the isomeric **3** and **4**, not only was the same parent ion observed (331 *m/z*) but also the same fragmentation pattern, with the main daughter ions *m/z* 285 and 239 being detected. These two fragments may correspond to the loss of one and two carboxylic groups, respectively.

Free Radical Scavenging Activity of Betaxanthins. The free radical scavenging activity of a molecule can be evaluated by its effect on stable colored solutions of radical $ABTS^{\bullet+}$.[33] The assay is based on monitoring the decrease in the absorbance of the radical solution. Figure 3A shows the recordings obtained for the activity of the five betaxanthins. As can be seen, there is a decrease in the concentration of the radical with time after the addition of the corresponding pigment.

The activities of the betaxanthins were compared with that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a potent antiradical, water-soluble derivative of vitamin E. Increasing concentrations of each betaxanthin or Trolox were added to the $ABTS^{\bullet+}$ radical mixture, causing a linear response in the removal of the radical. Figure 3B shows the effects of increasing the concentrations of the betaxanthins and Trolox in radical solutions at pH 7.0. As can be seen, all betaxanthins exhibit a high free radical scavenging capacity at much lower concentrations than Trolox.

Compound **5** possesses the highest radical scavenging activity of the betaxanthins assayed. This corresponds with the results described for other families of compounds where the presence of the catechol substructure in the molecule was identified as a particularly active motive.[21] The TEAC (Trolox equivalent antiradical capacity) value of the diphenolic betaxanthin, as calculated from the slopes of Figure 3B, is 5.8 ± 0.2, which is higher than that obtained for the most powerful antioxidant constituent of green tea, epigallocatechin gallate (TEAC = 4.8).[34,35] Both monophenolic betaxanthins were also very active, with nearly identical TEAC values, calculated as 4.0 ± 0.1 (compound **3**) and 4.0 ± 0.2 (compound **4**). Compound **2** contains the aromatic ring with no hydroxy groups in the structure and presented a TEAC value of

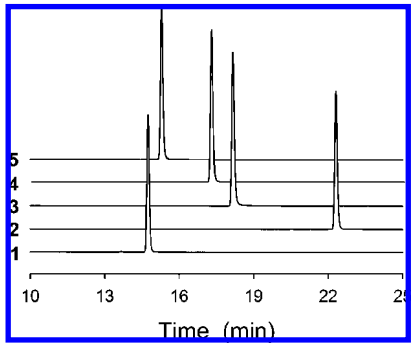

Figure 2. HPLC chromatographic profiles for individual pigment analysis of purified samples. A 20 μL amount of pure pigment solutions was injected with a concentration of 50 μM. Absorbance was detected at λ = 480 nm. Full scale is 0.2 absorbance units.

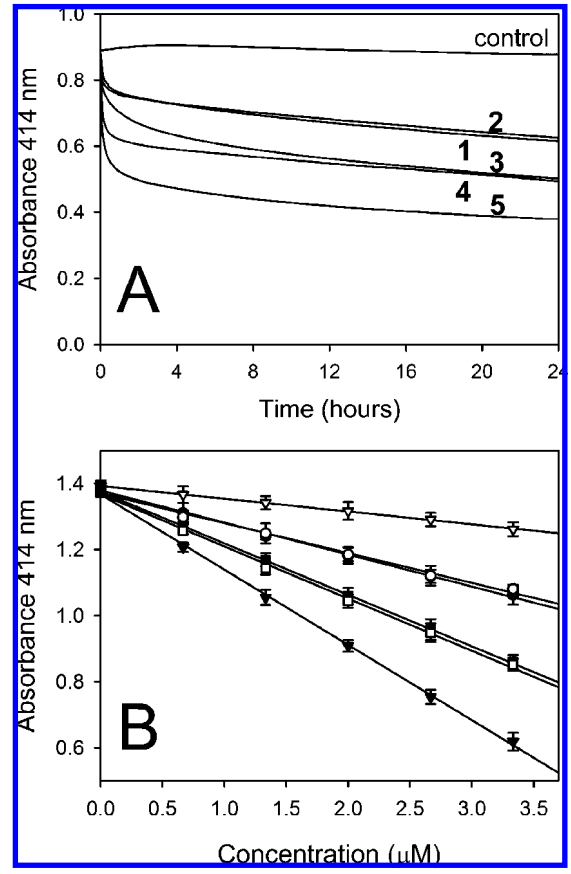

Figure 3. (A) $ABTS^{\bullet+}$ depletion recordings after addition of different betaxanthins. Final concentration of betaxanthins was 1.6 μM and that of the $ABTS^{\bullet+}$ radical was 29 μM. (B) $ABTS^{\bullet+}$ radical depletion dependence on betaxanthin concentration compared to Trolox. $ABTS^{\bullet+}$ was used at a concentration of 57 μM. The reaction was monitored spectrophotometrically at λ = 414 nm, at pH 7.0. In B symbols are as follows: ●, compound **1**; ○, compound **2**; ■, compound **3**; □, compound **4**; ▼, compound **5**; ▽, Trolox.

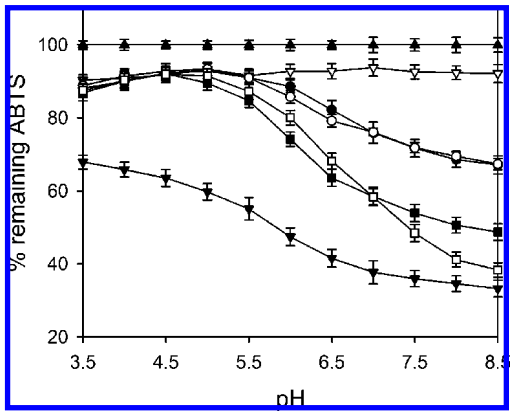

Figure 4. Dependence of betaxanthin free radical scavenging activity on pH. $ABTS^{\bullet+}$ radical initial concentration was 57 μM. Betaxanthins were added at a final concentration of 3.2 μM. Symbols are as follows: ●, compound **1**; ○, compound **2**; ■, compound **3**; □, compound **4**; ▼, compound **5**; ▽, Trolox; ▲, water control.

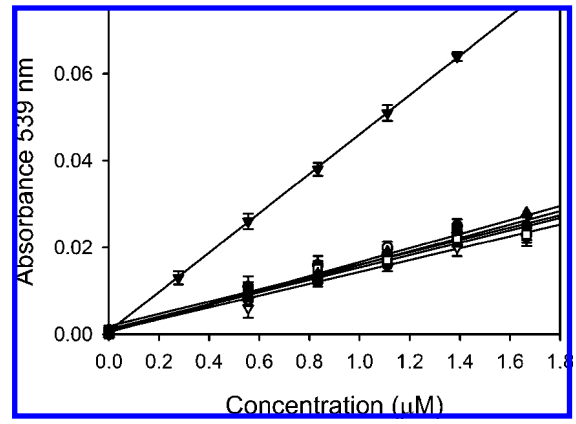

Figure 5. Antioxidant activity of betaxanthins measured by ferric reducing ability and standard curve. The effect was evaluated spectrophotometrically at λ = 593 nm. Initial concentration of Fe(III) was 1.48 mM. Symbols are as follows: ●, compound **1**; ○, compound **2**; ■, compound **3**; □, compound **4**; ▼, compound **5**; ▽, Trolox; ▲, Fe(II) standard curve.

2.3 ± 0.1. This is in strong contrast to studies where the lack of hydroxy groups is linked to no radical scavenging capacity at all[21] and is of particular relevance if the TEAC value is compared to potent known antiradical compounds, such as resveratrol (TEAC = 2.7).[36] Furthermore, compound **1** lacks an aromatic ring system and presents an analogous TEAC value of 2.5 ± 0.1.

Thus, in betaxanthins, there is an "intrinsic activity" not linked to the presence of hydroxy groups or aromaticity in the pigment structure, which might be associated with the common electronic resonance system supported between the two nitrogen atoms, and be general to all betalains. In other studies, free radical scavenging activity has been reported for betaxanthins derived from glutamic acid,[10] proline,[12] and tryptophan.[14]

However, the presence of a phenolic hydroxy group in betaxanthins implies a significant enhancement of their radical scavenging capacity. The increase of 1.6 units in the calculated TEAC value for the pure pigments points in this direction. The presence of two phenolic hydroxy groups implies an increase of 3.4 units in the TEAC value determined.

Effect of pH on the Free Radical Scavenging Activity of Betalains. The pH was varied in the range 3.5 to 8.5 in the assay medium in order to evaluate its effect on the activity of betaxanthins. Sodium acetate was used as buffer for pH values ranging from 3.5 to 5.5, and sodium phosphate for 5.5 to 8.5. No difference was obtained for the activity measured for both buffers at pH 5.5.

Figure 4 shows how pH values above 5.5 indicate an appreciable increase in the free radical scavenging activity measured for all the pigments assayed, in contrast to acidic values. At pH values below 5.5, there is a basal activity. Behavior for compounds **1** through **4** in this range is the same, removing around 10% of the initial radical (activity analogous to that found for Trolox). For compound **5**, the activity displayed at acidic pH values is higher (removal of 30% of the initial radical). In this case, at pH values above 4.5, the capacity to remove ABTS radical from the medium shows a remarkable increase, and a plateau seems to be reached above pH 7.0. Betaxanthins **1** and **2** follow the same trend at pH values above 5.5, and a similar trend is observed for the monophenolic betaxanthins **3** and **4**.

The presence of phenolic hydroxy groups enhances the radical scavenging activities of betaxanthins **3** and **4**, in relation to the activities of betaxanthins **1** and **2** at pH values above 5.5. The diphenolic nature of compound **5** makes it the most active betaxanthin in the entire range of pH assayed. Only compound **4** presents analogous activity at the most basic pH. Interestingly, these two structures are the physiological betaxanthins miraxanthins III and V.

The pH dependence on the measured activity suggests the existence of a protonation equilibrium in the pigment molecules. A similar pH effect is found in all the betaxanthins, with an increase in the activity at higher pH values, regardless of the nature of the condensation molecule. Thus, it must be due to the common electronic resonance system and be related to the deprotonation of the NH group present in all betalains (Figure 1). In the same manner, for flavonoids, deprotonation generates a phenolate anion, which is a better electron donor and a more active antioxidant.[37,38]

Antioxidant Activity of Betaxanthins. The antioxidant activity of betaxanthins was characterized by their capacity to directly reduce Fe(III) to Fe(II). The FRAP assay was used,[39] monitoring the reduction reaction spectrophotometrically at λ = 593 nm. Solutions of known concentrations of iron Fe(II) ($FeSO_4$) were used to set a calibration curve. Figure 5 shows the signals obtained for the ferric reduction by the different betaxanthins assayed and Trolox, compared to the signal obtained for the calibration curve. A similar trend is observed for compounds **1** through **4**, and it is analogous to the signal obtained for Trolox and the ferrous calibration. However, in the case of compound **5**, there is a much higher reduction of Fe(III) to Fe(II). The presence of the two phenolic hydroxy groups in the betaxanthin derived from 3,4-dihydroxyphenethylamine is linked with a higher activity than that of the other pigments.

The antioxidant capacity for each pigment can be evaluated from the ratio between the slopes as the amount of Fe(II) produced per mg of pigment. The results obtained are as follows: 3.7 ± 0.5 μmol Fe(II)/mg (compound **1**); 2.8 ± 0.4 μmol Fe(II)/mg (compound **2**); 2.7 ± 0.2 μmol Fe(II)/mg (compound **3**); 2.7 ± 0.2 μmol Fe(II)/mg (compound **4**); 8.0 ± 0.3 μmol Fe(II)/mg (compound **5**).

For comparative purposes, the Trolox molar equivalence in the reduction of Fe(III) for each pigment was also determined. For betaxanthin molecule **1**, it is 1.1 ± 0.2; for **2**, it is 1.1 ± 0.2; for **3**, it is 1.1 ± 0.1; for **4**, it is 1.1 ± 0.1; for **5**, it is 3.3 ± 0.2. The non-hydroxylated betaxanthins assayed presented a ferric reducing activity identical to that of the vitamin E analogue Trolox. This result supports the aforementioned observation of an activity not linked to the presence of hydroxy groups or aromaticity in the pigment structure. For compounds **3** and **4**, the presence of one hydroxy group does not confer an increased activity with respect to the compounds lacking it, and the same activity is observed. However the FRAP assay is able to show an enhancing effect linked

to the catechol substructure of compound **5**, which is shown to be 3 times more potent than Trolox. Taking into account that the FRAP assay is performed at pH 3.6, these observations are related to the results obtained with $ABTS^{\bullet+}$ at lower pH values (Figure 4).

Betaxanthins present high antioxidant and free radical scavenging activities not linked to the presence of phenolic hydroxy groups. This may be general to all betalains, which contain a similar electronic resonance system. In addition to their intrinsic activity, a clear enhancing effect on the scavenging of the free radical $ABTS^{\bullet+}$ and on the antioxidant power of betaxanthins has been demonstrated to be linked to the presence of one or two phenolic hydroxy groups in their structure.

Experimental Section

General Experimental Procedures. A Shimadzu LC-10A apparatus (Kyoto, Japan) equipped with a SPD-M10A photodiode array detector (PDA) 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). Gradients were formed with two He degassed solvents. Solvent A was H_2O with 0.05% TFA, and solvent B was composed of MeCN with 0.05% TFA. A linear gradient was performed over 25 min from 0% B to 35% B. The flow rate was 1 mL min^{-1}, operated at 25 °C. Injection volume was 20 μL. An Agilent VL 1100 apparatus with a LC/MSD trap (Agilent Technologies, Palo Alto, CA) was used for ESIMS analyses. Elution conditions were as described above using a Zorbax SB-C18 (30 × 2.1 mm, 3.5 μm) 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. For detection the electron multiplier voltage was 1350 V. A Jasco V-630 spectrophotometer (Jasco Corporation, Tokyo, Japan) attached to a Tectron thermostatic bath (JP Selecta, Barcelona, Spain) was used for absorbance spectroscopy. For the quantification of betalains, pigment concentration was evaluated taking a molar extinction coefficient of ε = 48 000 M^{-1} cm^{-1} at 480 nm for betaxanthins[5,31] and ε = 65 000 M^{-1} cm^{-1} at 536 nm for betanin.[41] Measurements were made in water at 25 °C. 3-Hydroxyphenethylamine was purchased from Fluorochem Ltd. (Old Glossop, England). Other chemicals and reagents were obtained from Sigma (St. Louis, MO). 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).

Synthesis of Betaxanthins. Betaxanthins were obtained as immonium condensation products of betalamic acid with the following amines: 1-aminopropane, 2-phenylethylamine, 3-hydroxyphenethylamine, 4-hydroxyphenethylamine (tyramine), and 3,4-dihydroxyphenethylamine (dopamine). Syntheses were carried out following a method described previously by Gandía-Herrero et al.[32] and by Wyler et al.[40] 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 characteristic deep yellow color (betaxanthin maximum wavelength is around λ_m = 480 nm). The whole process was carried out under nitrogen atmosphere to avoid the oxidation of substrates and products. Once synthesis was achieved, a C-18 solid-phase extraction step was performed, and an automated system was used for pigment purification. One milliliter C-18 cartridges (Waters, Milford, MA) were conditioned with 5 mL of MeOH followed by 10 mL of purified water. Salts and buffers from the samples were removed by rinsing the column with H_2O. Betaxanthins were eluted with acetone and then concentrated to dryness under vacuum. The residue was redissolved in water for further use or stored at −80 °C.

Purification of Betaxanthins. Anionic exchange chromatography of synthetic betaxanthins was performed in an Äkta purifier apparatus (General Electric Healthcare, Milwaukee, WI). 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 (10.0 mL for compound E); 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^{-1}.

Free Radical Scavenging Activity. The antiradical capacity of betaxanthins was evaluated by following their effect on stable free radical $ABTS^{\bullet+}$ [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]. Decolorizing activity on $ABTS^{\bullet+}$ solutions was monitored spectrophotometrically at λ = 414 nm.[10] $ABTS^{\bullet+}$ 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 H_2O_2 (45 μM), in 12 mM NaOAc buffer, pH 5.0. The reactive was then diluted by 2/3 with the addition of samples, carrying out the reactions 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, VT). 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). Data analysis was carried out by using linear regression fitting using SigmaPlot Scientific Graphing for Windows version 8.0 (2001; SPSS, Chicago, IL). In each case, errors associated with the results provided were calculated on the basis of the residual standard deviation around the regression line. For continuous recordings, the above spectrophotometer was used with thermostats and tap-covered 1 cm path length cuvettes.

Antioxidant Capacity. The antioxidant activity of betaxanthins was characterized by means of the reduction of Fe(III) to Fe(II). The method described by Benzie and Strain[39] to evaluate the ferric reducing antioxidant power (FRAP) was used. Briefly, $FeCl_3$ solutions at a final concentration of 1.48 mM, in 223 mM sodium acetate buffer, pH 3.6 were used. Fe(III) reduction to Fe(II) was observed through the addition of the reagent 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) at a final concentration of 741 μM, which is able to yield a colored complex with Fe(II). The reduction reaction was monitored spectrophotometrically at λ = 593 nm. All measurements were performed in triplicate, and mean values and standard deviations were plotted. Data analysis was carried out by using linear regression fitting using SigmaPlot Scientific Graphing for Windows version 8.0. In each case, errors associated with the results provided were calculated on the basis of the residual standard deviation around the regression line.

Acknowledgment. This work was supported by MEC (Spain) and FEDER (Project AGL2007-65907) and by Programa de Ayudas a Grupos de Excelencia de la Región de Murcia, de la Fundación Séneca, Agencia de Ciencia y Tecnología de la Región de Murcia (Plan Regional de Ciencia y Tecnología 2007/2010).

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NP900131R