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One-Step Synthesis of Betalains Using a Novel Betalamic Acid Derivatized Support

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ABSTRACT: Betalains are plant pigments with high antioxidant and cancer chemopreventive properties used by the food industry as safe colorants. Betalains are restricted to species of the order Caryophyllales, and difficulty in obtaining individual molecules has limited their structural identification and application. This study was designed to develop a betalamic acid derivatized support generated from a primary amine polymer. The novel material presents color properties of a pseudobetaxanthin, and it is stable for at least 6 months. The bond formed can be displaced at mild conditions by the addition of amines in aqueous solutions over a broad pH range and at 25 °C. This releases the betalamic acid while forming the corresponding pigment. This one-step procedure significantly simplifies the process of obtaining semisynthetic betalains, and it is optimized here for the formation of betaxanthins and betacyanins derived from tyramine, dopamine, pyrrolidine, and indoline. The new method makes access to single betalains available to the entire scientific community and could stimulate research and applications in the field.

KEYWORDS: *betalamic acid, derivatization, pigments, support, synthesis*

INTRODUCTION

Betalains are the natural pigments that bestow coloration to plants of the order Caryophyllales. Betalains are yellow (betaxanthins) or violet (betacyanins) conjugates of the structural unit betalamic acid (**1**) with free amines or indoline-derived compounds, respectively (Figure 1).[1] The presence of both types of pigments generates the wide variety of shades ranging from pale yellow to violet including the orange, red, and pink colors characteristic of the flowers, fruits, and roots of betalain-containing plants. Notable among the edible sources are red beet roots (*Beta vulgaris*) and the fruits of cacti belonging to the genus *Opuntia*, but betalains are also present in the ulluco tubers (*Ullucus tuberosus*) or the berries from *Rivina humilis* among others.[2−5] In addition, betalains are used by the food industry to give color to foods as the additive 73.40 in the 21 CFR section of the U.S. Food and Drug Administration (FDA) and under the E-162 code in the European Union.

In recent years, strong bioactive properties have been described for betalains. These include a high antioxidant and free radical scavenging capacity present in all pigments, which is modulated by structural factors.[6,7] The characterized antiradical activity of betalains may explain their proven potential in the chemoprevention of cancer using different cell lines.[8,9] The inhibition of the growth of cancer cells is dose-dependent, and it has been demonstrated with both plant extracts and purified betalains. In addition, experiments with model animals have shown that very low concentrations of dietary pigments inhibit the formation of tumors in vivo, showing a potent cancer chemopreventive activity.[10,11]

Other applications of individual betalains come from their use in dye-sensitized solar cells for solar energy conversion due to their redox capacity to transfer electrons. The use of pure pigments yields energy conversion efficiencies of up to 2.7%, above that of natural photosynthesis.[12,13] Other potential applications of betalains are their use in microscopy as a new probe for live cell imaging, based on their fluorescent properties,[14,15] and as a sensor for colorimetric assays.[16]

The promising bioactive and applied potential of betalains contrasts with the limited number of available tools developed to obtain individual molecules. The synthesis of standards is of importance in food technology research, in pigment identification from natural sources and in bioactivities characterization. Betalamic acid (**1**) is the structural unit of all betalains and can be used as the starting point to obtain betalains through a Schiff condensation reaction with free amine groups (betaxanthins) or indoline-containing structures (betacyanins) (Figure 1).[1] Betalamic acid can be synthesized chemically in a procedure that involves multiple steps and gives low yields.[17] A feasible alternative is to get the acid from the degradation products of natural betanin (betanidin-5-*O*-*β*-glucoside), but the presence of the other degradation product, cyclo-DOPA-glucoside, reverses the reaction to the starting compound.[18,19] To date, the technology for the formation of betalains involves betalamic acid extraction in ethyl acetate and further condensation with amines, which results in very low yields, or a combined procedure for betanin degradation and betalains synthesis, which implies an immediate purification to avoid the reversal of the reaction.[20,21]

A betalamic acid derivatized support is developed here for the first time. It is based on an imine formation reaction among

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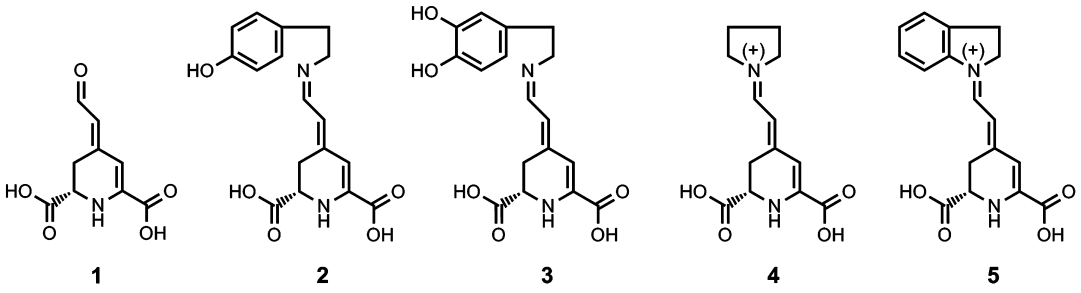

Figure 1. Structures for betalamic acid (**1**), the structural unit of betalains, and the derived pigments tyramine–betaxanthin (**2**), dopamine–betaxanthin (**3**), pyrrolidine–betaxanthin (**4**), and indoline–betacyanin (**5**).

betalamic acid and the amine groups present in the matrix surface, which can be displaced at mild conditions by the addition of amines. The novel material allows a one-step procedure for the formation of betaxanthins and betacyanins, thus significantly simplifying the process of obtaining semisynthetic betalains.

MATERIALS AND METHODS

Chemicals and Reagents. Red beet juice concentrate (B-50-WS) was purchased from CHR Hansen (Madrid, Spain). It is a liquid formulation obtained by squeezing out, concentrating, and pasteurizing the juice of beetroots, *B. vulgaris*. The starting matrix was Lewatit VP OC 1065 (surface area = 50 m^2/g), obtained from Sigma (Madrid, Spain). Solvents were from Merck (Madrid, Spain). HPLC grade acetonitrile was purchased from Labscan Ltd. (Dublin, Ireland). Other chemicals and reagents were obtained from Sigma (Madrid, Spain). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

Development of the Betalamic Acid Support. Red beet juice concentrate was filtered by a 10 kDa ultrafiltration step (QuixStand System, GE Healthcare, Milwaukee, WI, USA). Betanin from this filtered solution was used as starting material. Basic hydrolysis (pH 11.4) of betanin in the presence of the starting matrix released betalamic acid (30 min, at room temperature) (**1**), which was then condensed with the primary amine present in the inorganic matrix after reaching pH 5.0. This bound betalamic acid to the surface of the support, which was then washed three times with 20 mM sodium acetate buffer, pH 5.0. The binding process was carried out in nitrogen atmosphere.

Semisynthesis of Betalains. For standard assays, the betalamic acid derivatized support (0.16 g) was incubated in a final volume of 0.5 mL with different amines, at the specified concentration, in 20 mM sodium acetate buffer, pH 5.0, or in 20 mM sodium phosphate buffer, pH 8.0, for tyramine. The reaction mixture for pyrrolidine–betaxanthin (**4**) and indoline–betacyanin (**5**) syntheses also contained 1.36 M sodium chloride to facilitate the release of the pigments from the support surface into the reaction medium. The formation of the corresponding betaxanthin or betacyanin was revealed by a characteristic deep yellow or violet color, respectively, in the reaction medium. After the indicated reaction time, an aliquot of 100 μL was removed for HPLC analysis. To analyze the formation of betalains with time, the reaction was scaled up to a final volume of 8 mL. The evolution of the reaction was monitored by HPLC analysis of the aliquots extracted at different times. For pH studies the reaction was carried out in 20 mM sodium acetate buffer (pH 3.5–5.5) or in 20 mM sodium phosphate buffer (pH 6.0–7.0). The buffer used for pH values >7.5 was 20 mM sodium borate.

Stability of the Betalamic Acid Support. The stability of the novel support was investigated by studying its capacity to synthesize betaxanthins as a function of storage time. Freshly made betalamic acid support (0.16 g) was incubated with 1.35 mM dopamine in 20 mM sodium acetate buffer, pH 5.0. An aliquot was taken after 40 min of reaction to analyze dopamine–betaxanthin (**3**) formation by HPLC. The betaxanthin concentration value obtained was considered as the starting capacity of synthesis. Then, the dopamine betaxanthin synthesis was performed with the support at different times throughout 6 months. Measurements were performed in triplicate, and mean and standard deviations were plotted. Relative synthetic activity was determined as the percentage of activity remaining after storage with respect to controls. Errors associated with the results provided were calculated on the basis of the residual standard deviations. The stability of the support was also studied by color assessment analysis. Color determinations of the immobilized betalamic acid support were made at 25 °C using a V-650 spectrophotometer equipped with an ISV-722 integrating sphere (Jasco Corp., Tokyo, Japan). The color parameters corresponded to the uniform CIELAB space (L^*, a^*, b^*, C^*, and $h°$) and were directly obtained from the apparatus software Spectra Manager version 2.07.[22]

Spectroscopy. A V-630 spectrophotometer (Jasco Corp., Tokyo, Japan), attached to a Tectron thermostatic bath (JP Selecta, Barcelona, Spain), was used for absorbance spectroscopy. For the quantitation of betalains, pigment concentration was evaluated by taking a molar extinction coefficient of ε = 48000/M/cm at 480 nm for betaxanthins and ε = 71000/M/cm at 524 nm for indoline–betacyanin.[7,23] Measurements were made in water at 25 °C.

HPLC Analysis. A Shimadzu LC-20AD apparatus (Kyoto, Japan) equipped with an SPD-M20A 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, USA). Gradients were formed with two solvents, A and B. Solvent A was H_2O with 0.05% trifluoroacetic acid (TFA); solvent B was acetonitrile with 0.05% TFA. A linear gradient was performed from 5 to 35% B for 35 min. The flow rate was 1 mL/min, operated at 25 °C. Injection volume was 20 μL.[24] All assays were performed in triplicate, and mean and standard deviations were plotted. Errors associated with the results provided were calculated on the basis of the residual standard deviations.

Scanning Electron Microscopy (SEM). Particle morphology was evaluated by SEM. Powders were attached to pieces of double-sided adhesive tape mounted on SEM stubs, coated with gold under vacuum using a SEM coating system by Bio-Rad Polaron Division and examined with a JSM-6100 (JEOL, Tokyo, Japan) scanning electron microscope operated at 15 kV. Images were analyzed using the open source image analysis software ImageJ (National Institutes of Health, NIH, Bethesda, MD, USA). Briefly, images were calibrated and, after that, an interactive measurement of the spheres was carried out. Micrographs were obtained with an Inca Oxford Image capture system (Oxford Instruments, Abingdon, UK).

RESULTS AND DISCUSSION

Development of a Betalamic Acid Derivatized Support. Betalamic acid (**1**) is the structural and bioactive unit of plant pigments betalains and the starting point for their formation both in vivo and in vitro. The production of individual compounds implies a purification of the acid, which leads to multistep procedures with low yields.[17,20,21] The aim of this work is the development of a novel support derivatized

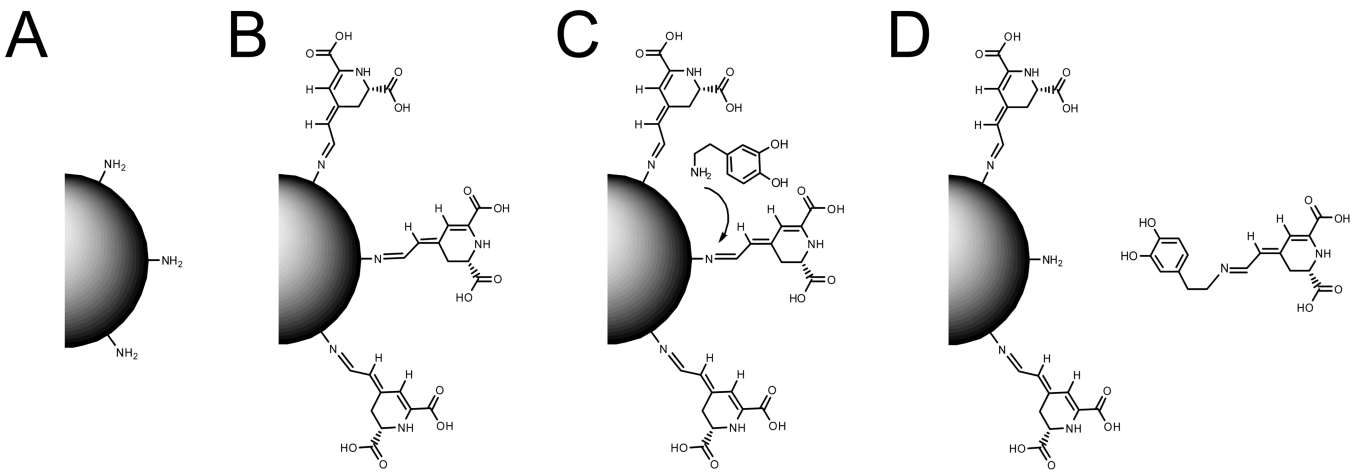

Figure 2. Schematic overview for the betalamic acid derivatized support and the surface reaction leading to pigment synthesis, exemplified for dopamine-derived betaxanthin (**3**): (A) starting material containing primary amine groups; (B) betalamic acid derivatized support; (C) amine attack to betalamic acid; (D) synthesis and concomitant release of the pigment.

with betalamic acid suitable for the synthesis of betalains. The basis of the proposed process is summarized in Figure 2.

First, betalamic acid is obtained in the presence of a resin that contains free primary amine groups. Given the reactivity of the aldehyde group of the acid, it was proposed that an imine would be formed with the amine of the support, thus causing the immobilization of betalamic acid to the resin. Betalamic acid was obtained by basic hydrolysis of purified betanin, the main pigment present in red beet root (*B. vulgaris*) extracts by the addition of ammonia.[21] Hydrolysis was carried out in the presence of the easily available cross-linked polystyrene resin VP OC 1065 (Lewatit), which contains primary amine groups, at room temperature and under nitrogen atmosphere. Under these experimental conditions betalamic acid was released from betanin. Neutralization with acetic acid was carried out after hydrolysis to promote the reaction of betalamic acid with the amines through a Schiff condensation reaction. This involved a change of color of the resin spheres from pale yellow to deep yellow, indicating the derivatization with betalamic acid. The resin was washed with 20 mM sodium acetate buffer, pH 5.0, until the washing solution was colorless. It was then allowed to dry at room temperature.

The binding reaction of betalamic acid to the matrix was confirmed by UV−vis spectrophotometry using an integrating sphere. This allowed the analysis of the color changes in the solid matrix. Figure 3A shows the spectrum obtained for the resin after betalamic acid reaction. The maximum wavelength was determined as λ_{max} 486 nm. Both the shape and maximum wavelength corresponded to the typical results of a betaxanthin, the yellow betalains formed by condensation of betalamic acid with simple amines.[7,23] Thus, the reaction between betalamic acid and the amine groups of the resin generated a solid support with the spectroscopic properties of a pseudobetaxanthin. The novel support was further characterized by SEM to analyze the effect of the treatment on the particle surface. Figure 3B shows a SEM microphotograph of the resin after betalamic acid immobilization. Particle analysis and sphericity were analogous to the results obtained for the resin without betalamic acid, indicating that the morphology of the resin was not altered by the immobilization process. Analysis of the particle morphology shows that the resin spheres possess a mean particle diameter of 0.59 ± 0.08 mm. SEM analysis also

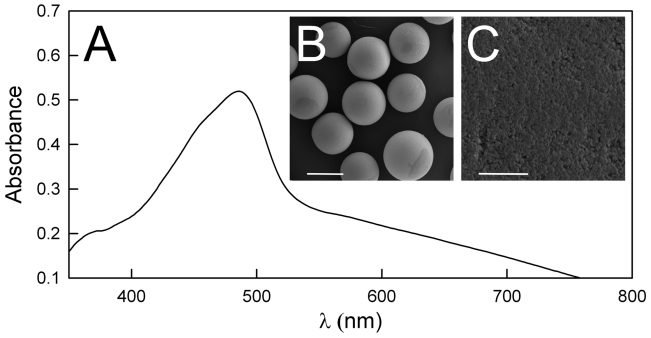

Figure 3. Spectroscopic and microscopic characterization of the betalamic acid derivatized support: (A) UV−vis spectrum for the solid matrix showing the characteristic spectrum for betaxanthins (spectrum was obtained with an integrating sphere after the subtraction of the spectrum for the basal absorbance of the starting matrix); (B) SEM image obtained for the spheres that constitute the novel matrix (scale bar = 500 μm); (C) closer SEM view showing the surface of the spheres (scale bar = 5 μm).

shows that the surface porosity of the spheres was not affected by the process (Figure 3C).

Thus, a matrix derivatized with betalamic acid was obtained for the first time. It is proposed that this support could be used for betalains synthesis simply by adding the selected amine. The reaction of betalamic with the amine would lead to the release of the pigment, as shown graphically in Figure 2.

Semisynthesis of Betalains. To test the concept of easy synthesis of betalains through the novel betalamic acid derivatized support, the resin was used for the production of different pigments. Four amines were selected as models to demonstrate this application: tyramine, dopamine, pyrrolidine, and indoline. The choice of tyramine and dopamine was based on the bioactive potential of their corresponding betaxanthins. Tyramine−betaxanthin (**2**) and dopamine−betaxanthin (**3**) possess high antioxidant and free radical scavenging activities due to the presence of phenolic hydroxyl groups (Figure 1).[25] They are natural pigments, also known by the trivial names miraxanthin III, and miraxanthin V, respectively, which were originally described in flowers of *Mirabilis jalapa* but are also present in the species *Celosia argentea* and *B. vulgaris* among others.[26−28] Structurally, these betaxanthins are derived from

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primary amines and possess open structures. In contrast, pyrrolidine is a secondary amine, and the pigment derived (**4**) possesses a ring attached to the betalamic acid moiety while maintaining properties of a betaxanthin (Figure 1). Indoline is also a secondary amine and possesses the same ring of pyrrolidine plus an additional aromatic ring (Figure 1). This additional electron resonance system promotes a bathochromic shift in the absorbance spectrum, which makes the derived betalain violet. The indoline-derived pigment (**5**) is the simplest molecule with betacyanin properties and, thus, the betacyanin defining structure.[7]

When colorless solutions containing the individual model amines were added to the novel support, the solutions turned yellow in the case of tyramine, dopamine, and pyrrolidine and purple in the case of indoline. In contact with the support, the amines yielded the corresponding pigments through a Schiff condensation reaction with the aldehyde group of betalamic acid, thus releasing the synthesized betalain as postulated in Figure 2C. All pigments were characterized by HPLC-DAD, confirming the expected identity and the absence of additional coloring molecules. Retention times for tyramine-derived (**2**) and dopamine-derived (**3**) betaxanthins were 18.3 and 16.5 min, respectively, whereas for pyrrolidine-derived (**4**) and indoline-derived (**5**) betalains retention times were 15.2 and 23.6 min, respectively. The optimal experimental conditions for the novel synthesis on support were investigated by measuring the pigment generation by HPLC-DAD analysis of the solutions. Figure 4 shows the evolution of the synthesis reaction with time for each amine assayed at 25 °C. The temperature of 4 °C was also assayed in this study under the same conditions (data not shown). At this temperature the concentration of product obtained was lower than at 25 °C in all cases. Analysis of the time courses obtained at 25 °C determined the optimal times of reaction at this temperature, which varied depending on the amine used. The highest pyrrolidine-derived pigment (**4**) concentration was reached after only 4 min of reaction. For the rest of the compounds the estimated optimum reaction times were 20, 40, and 60 min for indoline-derived (**5**), dopamine-derived (**3**), and tyramine-derived (**2**) betalains, respectively. This variation may result from the different affinities of betalamic acid toward the amine groups of the support and the amines added in solution. This apparent affinity was greater for pyrrolidine. The existence of a maximum in all of the time courses presented indicates the existence of two processes taking place at the surface of the spheres. One is the synthesis and concomitant release of the pigments as discussed, causing the appearance of betalains in the medium immediately after the addition of the amines. The other is responsible for withdrawing the pigments obtained from the medium after their formation, so causing the decrease of concentration after synthesis in the solution. Pigment stability in the medium was confirmed by repetitive analysis of the pigment concentration after separation from the matrix, with no appreciable loss of the betalains (results not shown). Pigment concentration decreased only after the maximum times described above in the presence of the matrix, thus indicating an adsorption phenomenon. The nature of the adsorption process might be unspecific, but an electrostatic contribution was investigated. The addition of sodium chloride to the reaction mixture in the case of the pyrrolidine-derived betaxanthin (**4**) and the indoline-derived betacyanin (**5**) mitigated the electrostatic contribution of the positive charge of the pigments and facilitated the release of the resulting

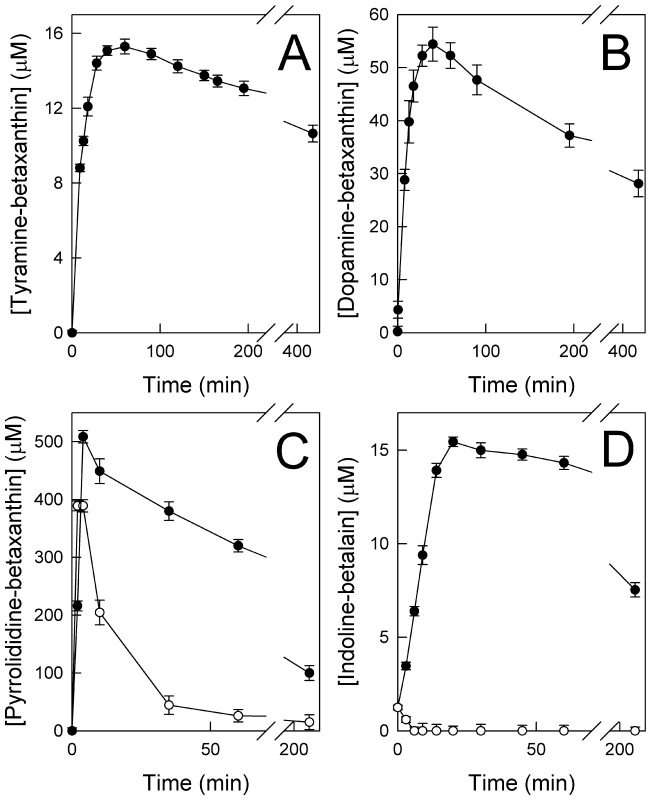

Figure 4. Time course for the evolution of pigment formation in reaction media containing the betalamic acid derivatized support at 25 °C and different amines; (A) tyramine, 0.85 M; (B) dopamine, 1.35 M; (C) pyrrolidine, 0.6 M; (D) indoline, at saturating concentration. In panels C and D, results are shown in the presence (●) and in the absence (○) of 1.36 M NaCl. Experimental conditions are detailed under Materials and Methods.

betalains from the resin surface into the solution (Figure 4C,D). This increased the amount of pigment obtained from the reaction on the novel material. An optimal concentration of 1.36 M NaCl was determined, with values below this causing a lower pigment release.

The effect of pH was also investigated in the pigment synthesis from the derivatized support. Figure 5 shows the results obtained when the pH of the amine-containing solution was varied from 3.5 to 9.0. Only in the case of dopamine was the range limited to 6.5 to avoid the catecholic substructure oxidation at higher pH values. Tyramine−betaxanthin (**2**) synthesis reached a maximum at pH 8.0 (Figure 5A), which was then selected for standard assays with this amine. However, as can be seen for the rest of amines, the pH variation did not significantly affect the synthesis. A pH of 5.0 was preferred to more alkaline values because of the higher stability of the compounds.[29,30]

Betalain synthesis was also dependent on the amine concentration. As shown in Figure 6, an increase in the concentrations of the amines resulted in a general increase in the formation and release of the derived betalains to the solution. However, the substrate concentration effect depended on the nature of each amine. Increasing concentrations of tyramine (Figure 6A) and dopamine (Figure 6B) led to a higher level of the derived betaxanthins in the solution. However, their concentrations could not be raised further because of the relatively low solubility of these compounds. In the case of pyrrolidine (Figure 6C), betaxanthin production

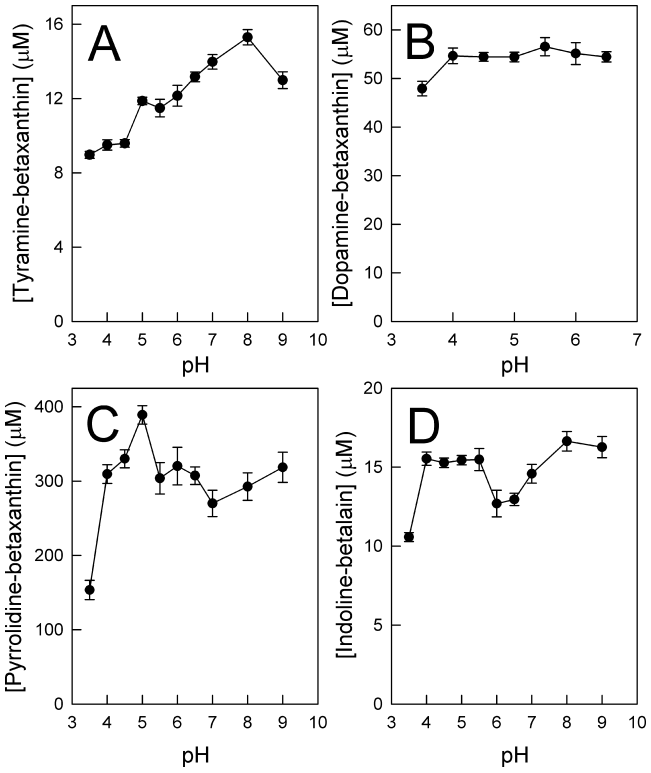

Figure 5. pH effect on the synthesis of betalains using the betalamic acid derivatized support and different amines: (A) tyramine, 0.85 M; (B) dopamine, 1.35 M; (C) pyrrolidine, 0.6 M; (D) indoline, at saturating concentration. The reaction was carried out in 20 mM sodium acetate buffer (pH 3.5−5.5), 20 mM sodium phosphate buffer (pH 6.0−7.0), or 20 mM sodium borate buffer (pH 8.0−9.0).

grew with increasing concentrations of the starting amine up to a maximum at 0.6 M. The scarce solubility of indoline in aqueous media prevented the study of the concentration effect, and this amine was always added to the reaction mixture at saturating concentration under the assay conditions.

From the above results it can be concluded that the optimization of the parameters which affect the novel one-step method for the easy synthesis of betalains should be made for each individual compound. Once optimized, the method allows a routine process to obtain individual compounds that significantly simplifies the previous methodology.[20,21] Betalamic acid obtained from red beet roots was previously used to obtain small amounts of betalain standards for pigment identification by its addition to amines after a process of hydrolysis and immediate partitioning steps with ethyl acetate followed by concentration and re-extraction prior to the synthesis reaction.[20] The combination of the hydrolysis and imine formation reactions in one merged procedure allowed higher amounts of betalains to be obtained but still required immediate purification steps with anionic exchange chromatography to avoid reversal of the reaction.[21] The methodology described here avoids the immediate purification steps and yields stable betalains in minutes, thus simplifying the process to obtain individual pigments. Figure 7 shows the use of the novel betalamic acid derivatized support as a matrix in chromatographic cartridges to speed the recovery of the pigments. The result is shown after the addition of the selected amines at the corresponding optimum concentration, pH, and reaction times determined above.

Stability of the Betalamic Acid Derivatized Support. The stability of betalamic acid after the resin derivatization was investigated by studying its capacity to synthesize betaxanthins as a function of storage time. As a control, a freshly prepared derivatized support was used to obtain dopamine−betaxanthin (**3**) as described above. The amount of betaxanthin obtained was considered to be the starting capacity of synthesis of the support. The synthesis reaction was repeated with the support at different times over 6 months. The support was stored at 4 °C in the absence of light. The results obtained for dopamine−betaxanthin (Figure 8) indicate that the resin retained approximately 50% of its potential after 6 months of storage. The loss of synthetic activity was pronounced during the first month, and from then on it remained practically stable in the production of the betaxanthin.

In addition, the stability of the derivatized support was evaluated by color assessment analysis of the resin. The changes in the appearance of the resin were quantitated by the results of the color CIELAB parameters, determined by spectrophotometry using an integrating sphere. Data were collected immediately after derivatization with betalamic acid. These results obtained for the freshly made support were compared to those found after 6 months of storage (Table 1). As can be seen, the a^* and b^* values decreased, indicating a loss in the red

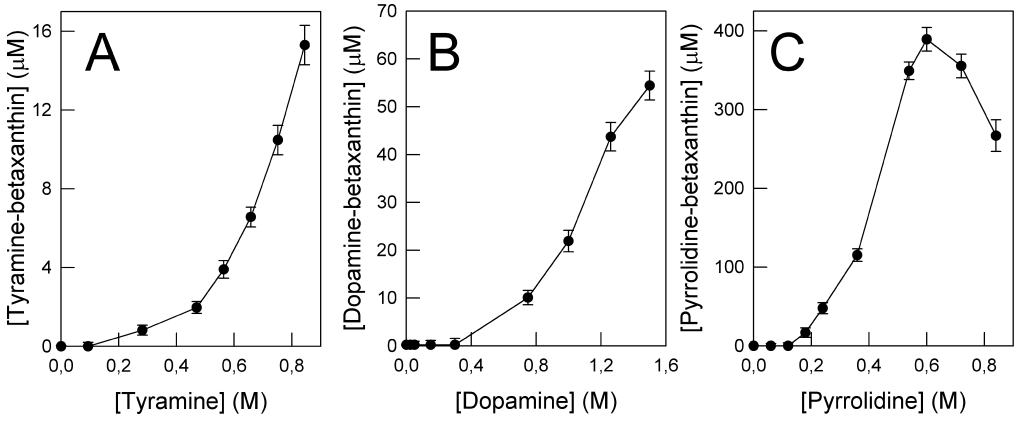

Figure 6. Effect of amine concentration on the formation of the betaxanthins derived from (A) tyramine, (B) dopamine, and (C) pyrrolidine. The reaction medium contained the derivatized support and the amine, at the specified concentration, in 20 mM sodium phosphate buffer, pH 8.0 (tyramine), or in 20 mM sodium acetate buffer, pH 5.0 (dopamine and pyrrolidine).

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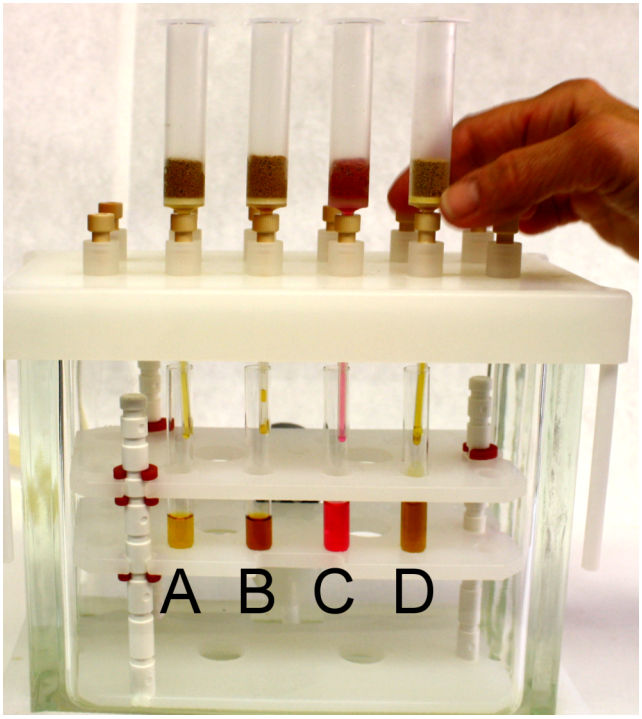

Figure 7. Application of the betalamic acid derivatized support to the synthesis of betalains in chromatographic cartridges. Solutions containing the individual pigments are obtained after the synthesis of (A) tyramine−betaxanthin (**2**), (B) dopamine−betaxanthin (**3**), (C) indoline-derived betacyanin (**5**), and (D) pyrrolidine−betaxanthin (**4**) under the optimized conditions.

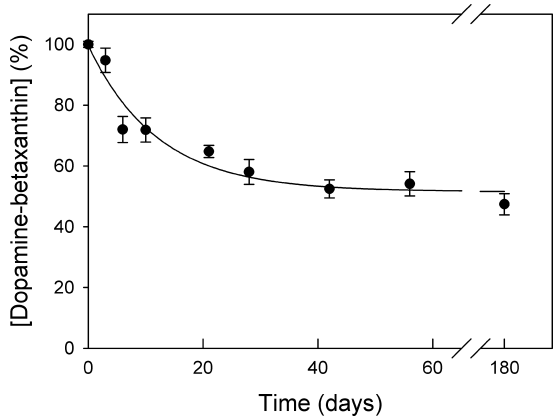

Figure 8. Effect of storage time on the capacity of the betalamic acid derivatized support to yield dopamine−betaxanthin (**3**). The optimized conditions were used for multiple assays during the indicated time.

Table 1. Color Analysis (CIELAB Parameters) for the Betalamic Acid Derivatized Support before and after a Period of 6 Months of Storage

	initial	6 months
a^*	7.18 ± 0.36	5.32 ± 0.11
b^*	32.52 ± 1.47	30.12 ± 1.01
L^*	63.25 ± 1.20	64.91 ± 1.05
$h°$	77.55 ± 0.08	79.98 ± 0.47
C^*	33.31 ± 1.51	30.59 ± 0.98
ΔE^*		3.46 ± 0.23

and yellow colors, respectively. This is probably due to a limited degradation of the pseudobetaxanthin formed, which possesses a maximum wavelength of λ_{max} 486 nm. This contributed to the change in hue angle ($h°$) and chroma (C^*). The L^* parameter was moderately increased, indicating that the color of the resin became lighter after the storage time. In conclusion, the color (a^*, b^*, $h°$), its intensity (C^*), and the lightness (L^*) of the derivatized support changed slightly after 6 months of storage, as confirmed by the value of ΔE^*. This parameter accounts for the color variation calculated with the differences between L^*, a^*, and b^* of the sample stored and the initial conditions. As can be seen, it was only slightly varied, indicating the stability of the betalamic acid resin.

The present work develops for the first time a support derivatized with the structural unit of betalains, betalamic acid. The novel material containing this natural molecule presents color properties of a pseudobetaxanthin and allows a procedure for the easy synthesis of individual betalains. The methodology is based on the addition of amines, which provokes the production of the corresponding derived pigments with the concomitant release from the matrix. The procedure is described in detail and provides a one-step method for the synthesis of betalains, thus considerably simplifying the previous methodologies. This tool opens up new perspectives in the research on betalains and may benefit current and future applications of the pigments.

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Notes

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