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Effect of Glucuronosylation on Anthocyanin Color Stability

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The effect of glucuronosylation on the color stability of anthocyanins was investigated using glucuronosylated anthocyanins isolated from the flower petals of the red daisy (*Bellis perennis*) or obtained by enzymatic in vitro synthesis using heterologously expressed red daisy glucuronosyltransferase *Bp*UGT94B1. Color stability toward light and heat stress was assessed by monitoring CIELAB color coordinates and stability at pH 7.0 by A_{550}. Cyanidin-3-*O*-2′′-*O*-glucuronosylglucoside showed improved color stability in response to light compared to both cyanidin 3-*O*-glucoside and cyanidin 3-*O*-2′′-*O*-diglucoside. A similar increase in color stability was not observed following heat treatment. Glucuronosylation did not increase the stability of anthocyanins at pH 7.0 as determined by A_{550}. To test for a possible effect of glucuronosylation on the color stability of anthocyanins in plant extracts used for food coloration, an elderberry (*Sambucus nigra*) extract was glucuronosylated in vitro. Glucuronosylation of approximately 50% of total anthocyanins proceeded fast and resulted in increased color stability in response to both heat and light. The data show that glucuronosylation may be used to stabilize industrially used extracts of natural colorants.

KEYWORDS: Red daisy; *Bellis perennis*; cyanidin-3-*O*-2′′-*O*-glucuronosylglucoside; cyanidin 3-*O*-glucoside; cyanidin 3-*O*-2′′-*O*-diglucoside; *Bp*UGT94B1; natural colorant stability

INTRODUCTION

Anthocyanins are plant pigments responsible for the pink, red, violet, and blue coloration of many flowers, berries, fruits, and vegetables. Anthocyanins are used by the food industry as natural food colorants and provide a consumer preferred alternative to synthetic colors, augmented by their reported beneficial effects on human health based on their function as antioxidants (*3, 9, 12, 14*). In vitro studies have indicated the activity of anthocyanins as modulators of immune and inflammatory responses and as anticancer agents (*9, 14*). Accordingly, a high content of anthocyanins in the human diet is recommended. In plants, anthocyanins serve important functions, e.g., in plant−insect interactions where they may attract pollinators, function as antifeedants, or protect against UV−B irradiation (*3, 12*). The color tonation and stability of different anthocyanins in food products determine their application range and commercial value. Stability during exposure to high or low pH, light, elevated temperatures, and oxygen are desired properties (*3, 4, 10*).

Anthocyanins are glycosylated anthocyanidins, and chemical diversity arises by modification of the core aglycon (anthocyanidin) and by hydroxylations and methylations as well as by the number and position of attached sugar and acyl groups. The sugars most often found in anthocyanins are glucose, galactose, rhamnose, fructose, and xylose. Sugar moieties may be attached at one or more positions of the aglycone and di and trisaccharides can be formed by different combinations of the monosaccharides. In addition, the sugars may be acylated to different degrees and at varying positions (*15, 18*).

In aqueous solution, the anthocyanins exist in a chemical equilibrium between different forms. The relative distribution between these is determined by the anthocyanin structure and by the characteristics of the medium in which the anthocyanin is embedded. The most intensely colored form of the anthocyanin is the flavylium cation. Several aspects of anthocyanin chemistry influence the distribution between these forms under a given set of conditions and the susceptibility to irreversible degradation and color loss. At pH 7.0, the decrease in A_{550} versus time reflects the slow reversible water addition on the small fraction of flavylium cation in fast equilibrium with the

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neutral and anionic quinoid bases (colored forms) with concomitant formation of colorless hemiacetals and chalcones. High stability is conferred by aromatic acylation of the anthocyanin. The presence of aromatic groups protect the flavylium ion by adopting a folded conformation where stacking of the ring structures occurs (*2*, *6*, *8*, *16*). Glycosidic residues are proposed to play a role as spacers in such folding, assuring the correct positioning of the aromatic rings (*2*, *6*). Acylated anthocyanins show increased resistance to heat, light, and SO_2 (*3*, *17*, *20*). Stabilizing effects of secondary glycosylations seem to depend on the site of glycosylation as well as the type of sugar added (*3*, *7*, *20*, *24*). Stabilization by sugar residues could occur by formation of H-bonds between glycosyl groups and the aglycone (*1*, *11*). Acylation by the nonaromatic malonic acid may also contribute to anthocyanin stability (*6*, *21*, *22*) facilitated by H-bonding between the carboxylate group and the core aglycone (*1*). The idea that the acidic group of malonic acid can contribute to stabilization by H-bond formation or by stabilizing the flavylium cation would suggest a similar effect of the carboxylate group of glucuronic acid. Increased stability of both malonynated and glucuronosylated anthocyanins from flowers of the red daisy (*Bellis perennis*) has indeed been reported (*22*). In addition to the described intramolecular stabilization mechanisms, the presence of other flavonoids can contribute to anthocyanin stability and color tonation (*5*). Such copigments may stabilize intermolecular interactions by aromatic ring stacking and H-bonding with the anthocyanins (*11*, *16*).

In this study, the color stability of glucuronosylated anthocyanins was studied with respect to heat and light stress. Additionally, a stabilizing effect of glucuronosylation at pH 7.0 was studied. Stability trials were performed on glucuronosylated anthocyanins purified from flower petals of the red daisy as well as obtained by enzymatic synthesis using a glucuronosyltransferase *Bp*UGT94B1 from the red daisy for in vitro glucuronosylation of commercially available cyanidin 3-*O*-glucoside.

We selected red daisy for the study as the flower petals are a rich source of glucuronosylated anthocyanins, and the flower is commercially available. In addition, the gene encoding the enzyme catalyzing the glucuronosylation, *Bp*UGT94B1, has been isolated and sequenced. This opened the possibility to obtain the glucuronosylated anthocyanin by in vitro glucuronosylation of cyanidin 3-*O*-glucoside using the recombinant enzyme. The color stability of the glucuronosylated anthocyanins was compared to that of the corresponding glucosylated anthocyanins. Color stability was quantified using black carrot (*Daucus carota*) extracts known to have high color stability as a reference. Using an extract from elderberry (*Sambucus nigra*), we demonstrate that glucuronosylation can be used to stabilize commercial products already in use as food colorants.

MATERIALS AND METHODS

Commercially Purchased Anthocyanins. Cyanidin 3-*O*-glucoside (Kuromanin) was purchased from Polyphenols (Hanavejen 4−6, 4327 Sandnes, Norway). Delphinidin 3-*O*-glucoside (Myrtillin) and cyanidin 3-*O*-diglucoside (keracyanin) were from Extrasyntese (Z.I Lyon Nord B.P62, 69726 Genay Cedex, France). All anthocyanins were obtained as chloride salts. Black carrot (*Daucus carota*) extract (color fruit 3WS) and elderberry (*Sambucus nigra*) powder (WS-P-13 614026) were obtained from Chr. Hansen A/S (Bøge alle 10−12, 2970 Hørsholm, Denmark).

Extraction and Isolation of Anthocyanins from the Petals of Red Daisies (*Bellis perennis*). Petals (freshly harvested or freeze-dried) from red daisies were extracted overnight using 1 mM HCl in MeOH completely covering the sample (~10 mg petals/mL). The anthocyanin extract was concentrated by evaporation (40 °C, reduced pressure) and resuspended in 1 mM HCl (pH 3). Phenolic acids were removed by EtOAc extraction. Anthocyanins in the aqueous phase were purified by column chromatography (XAD16HP adsorbent resin, 3.5 mm diameter, 300 mL bed volume) using EtOH as eluent (2 bed volumes of each of following concentrations: 15, 20. 30, 35, 40, 50, and 80%). Flow rate was 2 bed volumes per hour. Fractions (1 bed volume) were collected and the anthocyanin content analyzed by HPLC. Fractions with identical anthocyanin content were combined and concentrated under vacuum. The major red daisy anthocyanin cyanidin 3-*O*-2″*O*-glucuronosyl-6″*O*-malonylglucoside was further purified by size exclusion chromatography (Toyopearl TSK HW40s resin, 300 × 26 mm column, equilibrated in MeOH) using 50% MeOH/50% 2 mM HCl (pH 3.0) as eluent (flow rate: 0.625 mL/min). Fractions (4.0 mL) were collected, and the maximum absorbance of each fraction was recorded. Fractions containing the major red daisy anthocyanin cyanidin-3-*O*-2″-*O*-glucuronosyl-6″-*O*-malonylglucoside (purity >78% as determined by LC-MS) were combined.

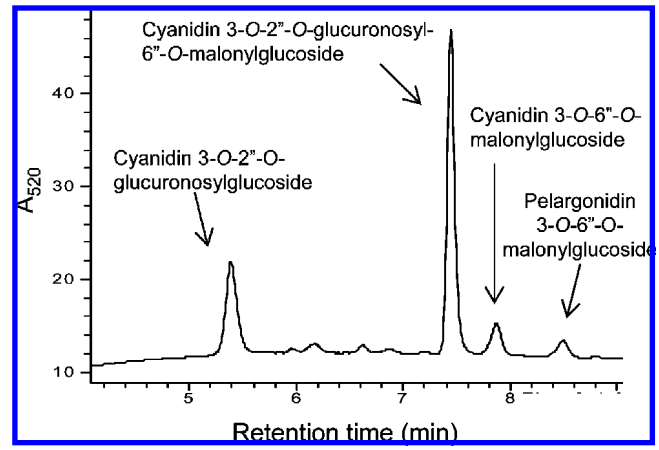

Figure 1. Determination of anthocyanin composition of red daisy petals by HPLC-MS.

HPLC Analysis of Anthocyanin Content in Petals from Red Daisies. HPLC analysis of total extracts and anthocyanin containing fractions obtained from petals of the red daisy were performed on an 1100 BRAND apparatus hyphenated to a diode array detector (Hewlett-Packard) and fitted with a Lichrosorb RP-18 5U column (250 × 4,6 mm, flow rate: 0.8 mL/min, 35 °C thermostatted, Alltech, France). Formic acid−water (10/90) (A) and formic acid−water−acetonitrile (10/60/30) (B) were used as eluents. A linear gradient from 20% B to 85% B (flow rate: 0.8 mL/min) was established over 55 min.

Analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies) hyphenated to a HCT*plus* ion trap mass spectrometer (Bruker Daltonics) and fitted with a Zorbax SB-C18 RRHT column (2.1 × 50 mm, 1.8 μM (Agilent), protected by a Phenomenex Gemini C18 security guard cartridge; flow rate, 0.2 mL min^{-1}). The mobile phases were A, 2% (v/v) formic acid in water; B, 0.1% (v/v) formic acid in acetonitrile. The gradient program was 0 to 8 min, linear gradient 5% to 25% (v/v) B; 8 to 9 min, linear gradient 25% to 100% B; 9 to 10.5 min 100%B followed by equlibration at 5%B for 3.5 min.

Recombinant *Bp*UGT94B1: Cloning, Heterologous Expression, and His-Purification. For the cloning of the glucuronosyltransferase *UGT94B1* (*23*), mRNA was prepared from red daisy petals using a MicropolyA purist kit. cDNA was prepared from the isolated mRNA and used as template for PCR with the following *UGT94B1* specific primers: 5′gcatggtaccttaattattcatttcac3′ and 5′gctactcgagatggattcaaaaatcg3′ introducing Kpn1 and Xho1 sites at the 5′- and 3′-ends, respectively. The construct was cloned first into the pCR-blunt II-TOPO vector (Invitrogen) and then transferred into pET30a+ (Novagen) adding an N-terminal His-tag.

PET30a+ plasmids were transformed into the *E. coli* XJa DE30 autolysis strain (Zymo Research). Cultures were inoculated and grown in LB media (overnight, 28 °C) with kanamycin (30 μg/mL). Expression of recombinant enzyme was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 mM) and cell lysis prepared by the

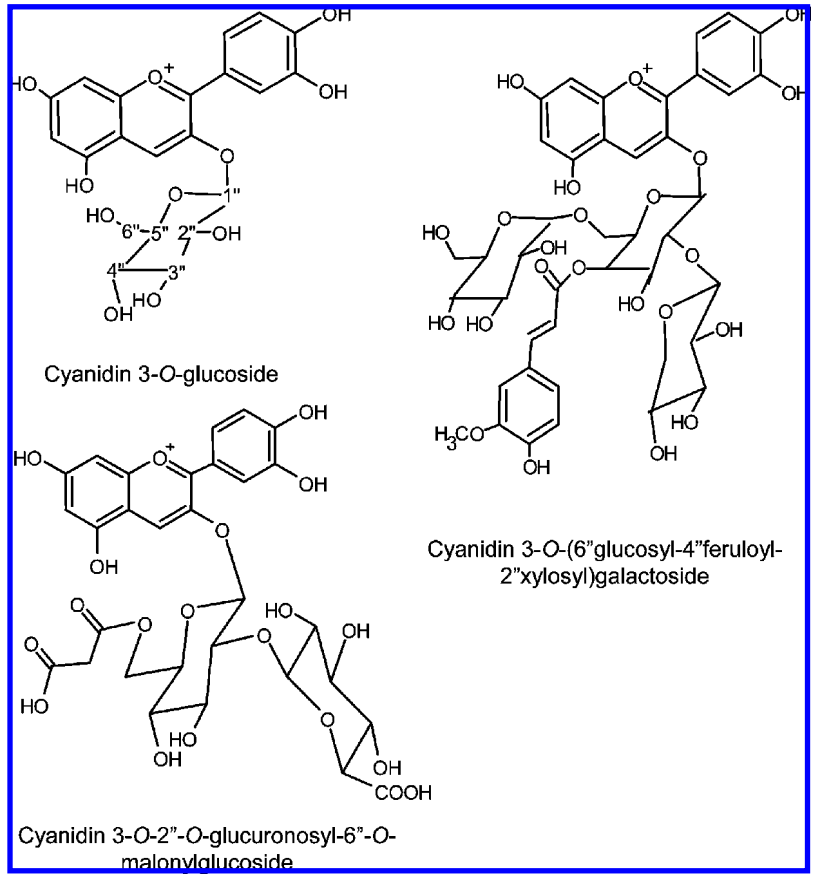

Figure 2. Chemical structures of cyanidin 3-*O*-glucoside, cyanidin 3-*O*-2′′-*O*-glucuronosyl-6′′-*O*-malonylglucoside (the major anthocyanin in red daisy petals) and cyanidin 3-*O*-(6′′-glucosyl-4′′-feruloyl-2′′-xylosyl)galactoside (the major anthocyanin in black carrots.)

addition of arabinose (3 mM, for expression of lambda lysosyme) to overnight cultures (20 °C). Cells were harvested, resuspended in GT buffer (10 mM Tris/5 mM $CaCl_2$/1 mM dithiothreitol (DTT) with added protease inhibitor (Roche complete tablets)) and disrupted by freezing. The cleared cell lysate was incubated (2 h, 4 °C, gentle rotation) with Sigma HIS-Select column material. Column material was washed 3 times with buffer A (20 mM sodium phosphate/500 mM NaCl/20 mM immidazole pH 7.4) and His-tagged protein eluted with buffer A containing 250 mM immidazole. Purified enzymes were kept at −20 °C in glycerol stocks (50%).

In Vitro Synthesis and Isolation of Cyanidin 3-*O*-2′′-*O*-Glucuronosylglucoside. Cyanidin 3-*O*-2′′-*O*-*β*-glucuronosylglucoside was synthesized from cyanidin 3-*O*-glucoside in a reaction mixture (total volume: 75 mL) containing 2 mM cyanidin 3-*O*-glucoside, 2.5 mM UDP-glucuronic acid, 10% isolated recombinant *Bp*UGT94B1 in glycerol stock (1 mg/mL) (see above), 100 mM Tris (pH 7.0), 10 mM $CaCl_2$, and calf intestine alkaline phosphatase (0.15 U/mL). Product formation was measured at regular time intervals by HPLC analysis. At the end of the incubation period (6 h, 30 °C, gentle rotation), the entire reaction mixture was applied directly onto a Reverse Phase C18 column (150 mm × 30 mm, Thermoelectron). Elution using acetonitrile (0−30%)/0.05% trifluoro acetic acid (TFA) afforded fractions (20 mL) containing pure cyanide-3-*O*-2′′-*O*-glucuronosylglucoside. These fractions were combined and the solvent removed by freeze-drying.

In Vitro Glucuronosylation of an Elderberry Pigment Extract. Elderberry anthocyanins were glucuronosylated in a reaction mixture (total volume: 75 mL) containing elderberry powder WS-P-13 (800 mg), 1.5 mM UDP-glucuronic acid, 10% purified *Bp*UGT94B1 in glycerol stock (1 mg/mL) and calf intestine alkaline phosphatase (0.15 units/mL), $CaCl_2$ (10 mM), and Tris (100 mM, pH 6.8). In control experiments, *Bp*UGT94B1 was replaced by 50% glycerol as in the enzyme stock. At the end of the incubation period (6 h 30 °C, gentle rotation), the reaction mixture was stabilized by the addition of HCOOH (0.3%). Aliquots made at different time points as well as the final preparation were analyzed by LC-MS and compared with control samples. Solvents were removed by freeze-drying as required.

Light and Heat Stability Tests of Anthocyanins. Color stability in response to heat and light stress was tested for the following anthocyanins/anthocyanin containing extracts: black carrot extract, *Bp*UGT94B1- and control-treated elderberry extract, red daisy purified anthocyanin fractions cyanidin 3-*O*-2′′*O*-glucuronosyl-6′′*O*-malonylglucoside and cyanidin 3-*O*-2′′*O*-glucuronosylglucoside, and in vitro glucuronosylated purified cyanidin 3-O-glucoside, cyanidin 3-*O*-glucoside and cyanidin 3-*O*-diglucoside. Color stability was tested in a standard soft drink solution used at Chr. Hansen to evaluate the stability of colored beverages exposed to light and elevated temperatures (*16*). The soft drink solution was prepared in distilled water with 43% sucrose, 0.09% potassium sorbate, and 0.07% sodium benzoate. The solution was diluted with distilled water to obtain 10 °Brix. Final pH was adjusted with citric acid to pH 3.0. A highly stable black carrot extract (ColorFruit carrot 3WS colorant) was used as reference in all experiments (Chr. Hansen). Colorant concentrations were adjusted so as to develop an initial lightness (L*) of 70.0 ± 0.3 using a Minolta CT310 colorimeter. Each colored solution was transferred to an IWAKI flask (70 mL) and exposed either to accelerated light in a controlled light cabinet (SUNTEST CPS+, 450 W/m^2, 25 °C, 6 h) or to elevated temperature (50 °C, darkness, 7 days with one measurement per day, this corresponds to 1 month typical storage conditions in a supermarket (Chr. Hansen internal measurement protocol)). Colorimetric parameters L*a*b* (CIE 1976) were directly measured on the IWAKI flasks during the treatments using a DataColor SpectraFlash SF450 spectrophotometer (color space CIELAB). The colorimetric coordinates of the colorant solutions were computed in the CIELAB scale in a CIE D65/10° illuminant/observer condition. Color change was calculated as $DEab^* = (DL^{*2} + Da^{*2} + Db^{*2})^{1/2}$. All stability trials were repeated at least twice either in independent experiments or by running two flasks of

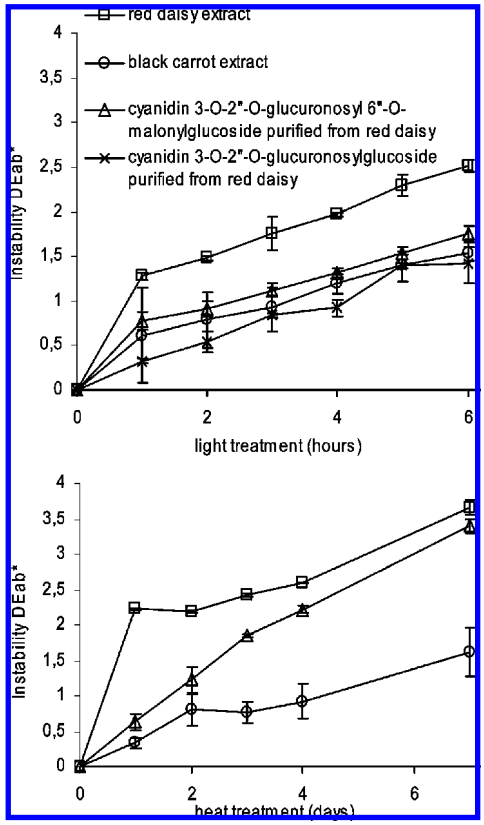

Figure 3. Light (**top**) and heat (**bottom**) stability of red daisy and black carrot anthocyanins.

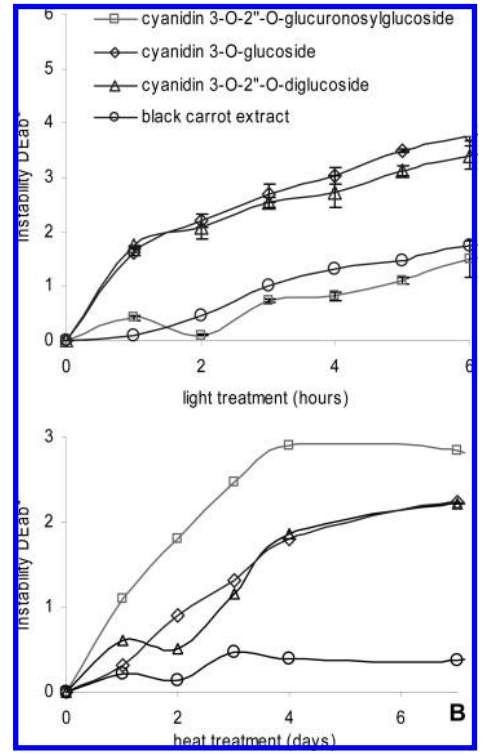

Figure 4. Light (**top**) and heat (**bottom**) stability of glucuronosylated and non-glucuronosylated cyanidin derivatives compared to that of the black carrot extract.

the same sample in parallel. For each of the stability trials, the results of a representative experiment is shown. For experiments where two flasks of each were run in parallel, standard deviations are indicated.

Stability Tests of Anthocyanins. Stability of red daisy extracts at pH 7.0 was tested in phosphate buffer at room temperature. Anthocyanin extracts (1% v/v) were added to provide an absorbance A_{550} of 0.5−0.8 at time 0. Samples were kept at daylight and room temperature, and the A_{550} was measured regularly over 4 h.

RESULTS AND DISCUSSION

Heat and Light Stability of Red Daisy (*Bellis perennis*) Anthocyanins. The anthocyanin composition of red daisy petals was determined by HPLC/-MS analysis (**Figure 1**) and by comparing the fragmentation pattern of the observed molecular ions with earlier reported anthocyanin composition in red daisy petals (*25*). The major anthocyanin constituted 65% of total anthocyanins, had a molecular ion at 711 *m*/*z*, and was identified as cyanidin 3-*O*-2″-*O*-glucuronosyl-6″-*O*-malonylglucoside (**Figure 2**). A minor peak constituting 20% of total anthocyanins and with a molecular ion at 611 *m*/*z* was identified as cyanidin-3-*O*-2″-*O*-glucuronosylglucoside. The last 15% of total anthocyanins were identified as cyanidin-3-*O*-6″-*O*-malonylglucoside and pelargonidin 3-*O*-6″-*O*-malonylglucoside. Apart from slight differences in relative amounts, this composition was in accordance with earlier reported data on anthocyanins in red daisies (*22*, *25*). The two major anthocyanins cyanidin 3-*O*-2″-*O*-glucuronosyl-6″-*O*-malonylglucoside and cyanidin 3-*O*-2″-*O*-glucuronosylglucoside were isolated from extracts of red daisy petals by preparative HPLC chromatography. In the purified fractions, they constituted >80% of total anthocyanins and >60% of total flavonoids.

To investigate the color stability of glucuronosylated anthocyanins from red daisies, color stability of total red daisy extract and of the isolated anthocyanin fractions was determined during heat and light treatment. The color stability of black carrot extract (*Daucus carota*) was determined in parallel as a reference for high color stability. The major anthocyanin of the black carrot extract is cyanidin 3-*O*-(6″-glucosyl-4″-feruloyl-2″-xylosylgalactoside (**Figure 2**). This anthocyanin harbors 3 sugar moieties of which one is acylated by ferulic acid. The acylation with a phenylpropanoic acid confers a very high stability to the black carrot anthocyanin (*16*, *20*, *24*). The major anthocyanin in red daisies (**Figure 2**) is also acylated but by the aliphatic malonic acid, which does not allow the same stabilizing interactions as an aromatic acylation.

Stability trials were conducted by diluting the anthocyanins into a soft drink solution that was then exposed to heat or light irradiation. Color stability is expressed as DEab* values where an increase in DEab* value represents decreased stability. When the red daisy and black carrot extracts as well as isolated anthocyanins from red daisies were introduced into the soft drink test solution (pH 3.0) and subjected to light irradiation for 6 h, the changes in DEab* values differed with less than one unit indicating similar color stability (**Figure 3A**). The two purified red daisy anthocyanins cyanidin 3-*O*-2″-*O*-glucuronosyl-6″-*O*-malonylglucoside and cyanidin 3-*O*-2″-*O*-glucuronosylglucoside differ by the presence of a malonate residue, and we concluded that at the experimental conditions used, this does not alter color stability in response to light. The purified red daisy anthocyanins exhibited as high stability during light treatment as the black carrot extract (**Figure 3A**) despite the fact that the red daisy anthocyanins are not modified by aromatic acylation (**Figure 2**). The results of the light stability trials demonstrated a high color stability of the red daisy anthocyanins during light treatment that was most likely conferred by the presence of the glucuronic acid residue.

Color stability in response to heat was measured for red daisy and black carrot extracts as well as cyanidin 3-*O*-2″-*O*-glucuronosyl-6″-*O*-malonylglucoside isolated from red daisies.

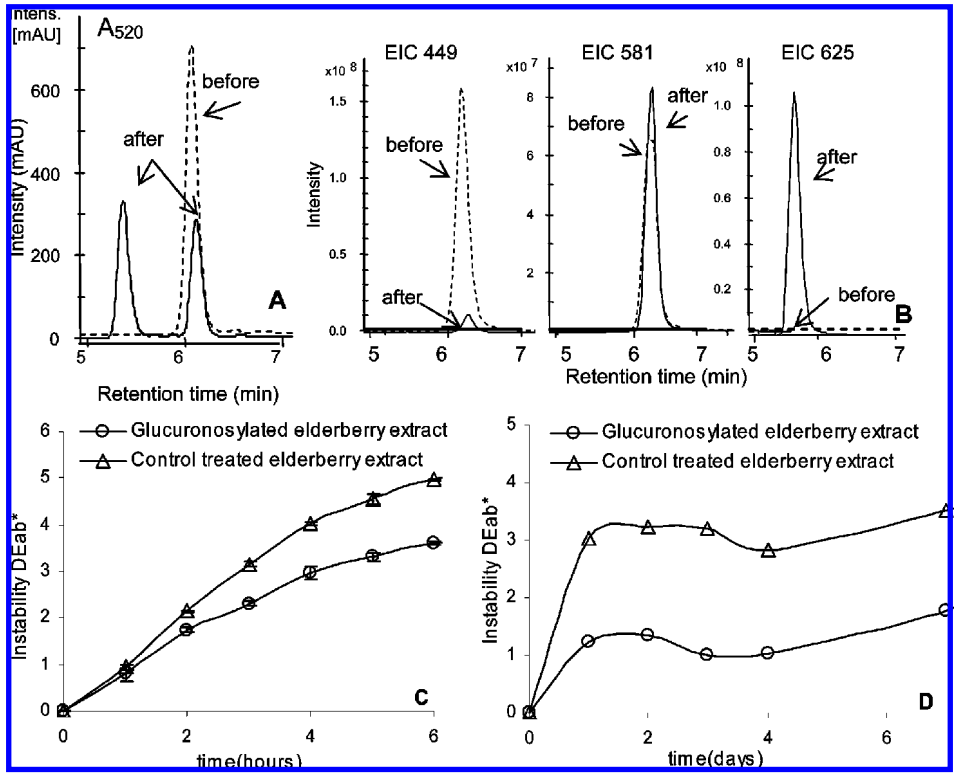

Figure 5. HPLC analysis of elderberry anthocyanins before (dotted line) and after (solid line) enzymatic treatment monitored as A_{520} (panel **A**) and extracted ion chromatograms of detected molecules (panels **B**). Light (panel **C**) and heat (panel **D**) stability trials of glucuronosylated and control treated elderberry anthocyanins monitored as color loss (DEab*).

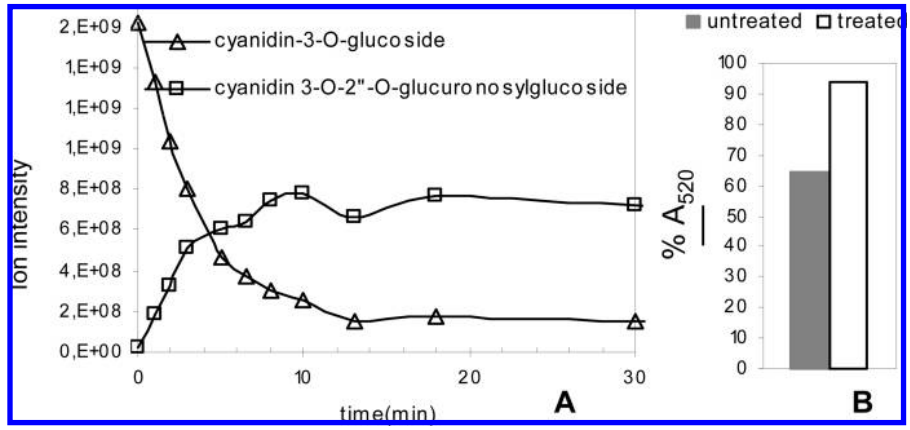

Figure 6. Stabilizing effect of in vitro glucuronosylation of elderberry extract using *Bp*UGT94B1. Panel **A**: Conversion of cyanidin 3-*O*-glucoside into glucuronosylated product. Panel **B**: Comparison of the stability of the glucuronosylated and untreated elderberry extract (4 h, 30 °C, pH 7.0).

The black carrot extract showed higher color stability than the red daisy extract or purified cyanidin 3-*O*-2″-*O*-glucuronosyl-6″-*O*-malonylglucoside (**Figure 3B**). The red daisy anthocyanins thus showed light stability as high as the carrot extract, while heat stability was lower; differences in the stability to heat and light have previously been reported for other anthocyanins (*3*, *13*).

Heat and Light Stability of in Vitro Glucuronosylated Anthocyanins. In the red daisy extract as well as in the purified fractions of individual red daisy anthocyanins, other flavonoids are present. To obtain a more precise assessment of the effect of glucuronosylation on the color stability of anthocyanins, stability trials using isolated pure glucuronosylated anthocyanins were performed and compared with the stability of cyanidin 3-*O*-glucoside and cyanidin 3-*O*-diglucoside. Cyanidin 3-*O*-2″-*O*-glucuronosylglucoside was obtained by *Bp*UGT94B1 catalyzed in vitro glucuronosylation of cyanidin 3-*O*-glucoside in the presence of UDP-glucuronic acid. *Bp*UGT94B1 is the glucuronosyltransferase responsible of the in vivo glucuronosylation of cyanidin 3-*O*-glucoside in red daisies. Color stability during exposure to high light and elevated temperatures was determined using the pH 3.0 soft drink experimental system as described above using a black carrot extract as reference for high color stability. The light stability of the glucuronosylated anthocyanin was high, indicated by stability similar to that of the black carrot extract (**Figure 4A**). The glucuronosylated anthocyanin also showed higher color stability in light compared to the glucosylated anthocyanins as experimentally monitored by decreases in DEab* of 3.7 and 3.5 for the cyanidin-3-*O*-mono- and diglucosides, respectively, compared to 1.5 for cyanidin 3-*O*-2″-*O* glucuronosylglucoside (**Figure 4A**). The experimental setup with pure anthocyanins thus confirmed the color stabilizing effect of glucuronosylation toward light stress. The presence of an additional glucose residue did not alter the

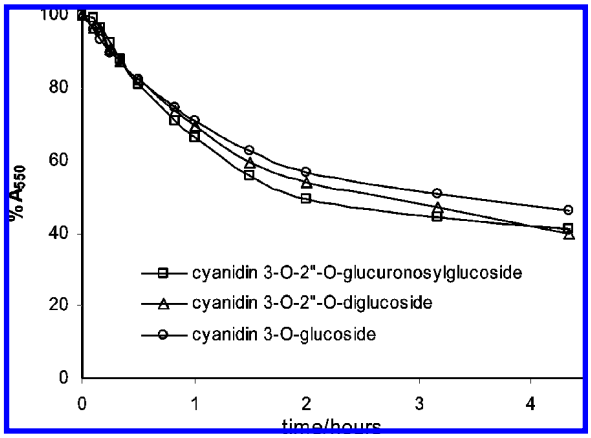

Figure 7. pH stability of cyanidin derivatives following dilution of the anthocyanins into buffer at pH 7 at time 0.

light stability characteristics observed by the similar stability exhibited by the cyanidin 3-*O*-mono- and diglucoside (DEab* values of 3.7 and 3.5, respectively **Figure 4A**). During heat treatment, the glucuronosylated anthocyanin did not show high color stability with a DEab* value of 2.8 compared to 0.5 for the highly color stable black carrot extract (**Figure 4B**). The glucuronosylated anthocyanin also exhibited slightly decreased color stability during heat stress when compared to the two glucosylated anthocyanins, but the difference in DEab* was less than 1 (**Figure 4B**). Again the cyanidin 3-*O*-mono and -diglucoside showed similar instability with DEab* values of 2.3 for both (**Figure 4B**). In agreement with the results obtained using anthocyanin extracts and purified anthocyanins from red daisies, the results using pure glucuronosylated and non-glucuronosylated anthocyanins demonstrated that glucuronosylation improves the color stability of cyaninin-3-*O*-glucoside during light treatment, whereas heat stability remains the same.

Heat and Light Stability of a Glucuronosylated Elderberry Extract. To assess whether glucuronosylation would also increase the color stability of a commercially available anthocyanin extract, stability trials were performed using an elderberry (*Sambucus nigra*) extract. HPLC-MS analysis of the elderberry extract detected two anthocyanins both eluting at 6 min (**Figure 5A**), with molecular ions *m*/*z* 449 and 581, respectively (**Figure 5B**). Both molecules fragmented into a molecular ion of *m*/*z* 287 corresponding to a cyanidin aglycone. The two anthocyanins were identified as cyanidin 3-*O*-glucoside and cyanidin 3-*O*-2″-*O*-xylosylglucoside, in agreement with previous studies (*18*). Cyanidin 3-*O*-glucoside constituted 60−70% of total anthocyanins in the elderberry powder, while cyanidin 3-*O*-2″-*O*-xylosylglucoside constituted 30−40% of total anthocyanins. The elderberry extract was subjected to glucuronosylation by addition of UDP-glucuronic acid and incubation with heterologously expressed *Bp*UGT94B1. The extent of glucuronosylation was monitored by LC-MS. Almost 100% of the initial content of cyanidin 3-*O*-glucoside was glucuronosylated (**Figure 5A** and **B**). The stability tests (**Figure 5C** and **D**) showed that the glucuronosylated elderberry extract exhibited increased color stability toward light irradiation as well as toward heat treatment in comparison to the untreated control extract (DEab* values decreased by 1.5 and 2.0, respectively). The improved stability to light was as expected from the results obtained with the anthocyanins in red daisies (**Figure 3A** and **Figure 4A**), whereas improved stability of the glucuronosylated elderberry preparation in response to heat was not observed in red daisies. This difference may be explained by the presence of other flavonoids in the elderberry extract acting as copigments and able to further stabilize the glucuronosylated anthocyanins.

The glucuronosylation process for the stabilization of the elderberry extracts involved enzymatic treatment for 6 h at pH 6.8 and 30 °C. During incubation, a significant proportion of the elderberry anthocyanins and probably also other flavonoids decomposed. The improved stability of the glucuronosylated extract as presented in **Figure 5C** and **D** was achieved by comparison to an elderberry extract, which was likewise kept at 6 h at pH 6.8 and 30 °C. To test weather the glucuronysylation reaction could be optimized to prevent concomitant anthocyanin degradation, a time course experiment was performed. This showed that a 10 min incubation period was sufficient to achieve 90% conversion of the cyanidin 3-*O*-glucoside in the elderberry extract into cyanidin 3-*O*-2″-*O*-glucuronosylglucoside (**Figure 6A**). Upon incubation of this enzymatically treated elderberry extract and the same amount of untreated elderberry extract for 4 h at pH 7.0 and 30 °C, the loss of total anthocyanins from the glucuronosylated extract as monitored by A_{550} was 6%, whereas 36% was lost from the untreated extract (**Figure 6B**). This demonstrates the potential of glucuronosylation to stabilize commercially sold anthocyanin containing extracts. A 3D structure of *Bp*UGT94B1 obtained by homology modeling has recently been published (*19*). This identified amino acid residues essential for catalytic activity and defined specificity to UDP-glucuronic acid. This opens a new avenue for the use of this type of enzymes to stabilize anthocyanins by glucuronosylation.

pH Stability. Stability of anthocyanins purified from red daisy petals as determined by the measurement of A_{550} has previously been reported to be higher for glucuronosylated anthocyanins as compared to non-glucuronosylated anthocyanins at pH 7.0. This was observed by a slower decrease in A_{550} at pH 7.0 for cyanidin 3-*O*-2″-*O*-glucuronosylglucoside and cyanidin 3-*O*-2″-*O*-glucuronosyl-6″-*O*-malonylglucoside as compared to cyanidin-3-*O*-6″-*O*-malonylglucoside and cyanidin-3-*O*-glucoside (*22*). Using identical experimental procedures, we tested the stability at pH 7.0 of in vitro glucuronosylated anthocyanin in comparison to that of cyanidin 3-*O*-glucoside and cyanidin 3-*O*-2″-*O*-diglucoside. (**Figure 7**). No difference in pH stability between the three cyanidin derivatives was observed with a 50−55% decrease in A_{550} following 3 h of incubation at pH 7.0 (**Figure 7**). In the pH stability study reporting increased stability by glucuronosylation of red daisy anthocyanins (*22*), stability trials were performed using anthocyanins purified from red daisy petals. The presence of copigments in those anthocyanin preparations may explain the different pH stability effects observed. This interpretation would be in accordance with the increased stability at pH 7.0 observed in the present study for the glucuronosylated elderberry extract (**Figure 6B**).

Summary. This study was performed to determine the effect of glucuronosylation on color stability of anthocyanins. The experiments demonstrated that glucuronosylation of cyanidin 3-*O*-glucoside increased the stability of both the pure anthocyanin (purified from plant extract or obtained by in vitro glucuronosylation) and plant extracts in response to light irradiation. Changes in heat stability were less conclusive. No difference in heat stability was observed between monoglucosylated, diglucosylated, and glucuronosylated cyanidin derivatives, whereas the glucuronosylated elderberry extract showed increased heat stability. We conclude that the light stability of commercially used plant extracts such as the elderberry extract may be increased by enzymatic glucuronosylation.

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