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Application of Anthocyanins from Blackcurrant (*Ribes nigrum* L.) Fruit Waste as Renewable Hair Dyes

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ABSTRACT: There is much concern about the toxicological effects of synthetic hair dyes. As an alternative approach, renewable waste blackcurrant (*Ribes nigrum* L.) fruit skins from the fruit pressing industry were extracted using acidified water with a solid-phase purification stage. Anthocyanin colorants were isolated in good yields (2−3% *w*/*w*) and characterized by HPLC. Sorption of anthocyanins onto hair followed a Freundlich isotherm; anthocyanin−anthocyanin aggregation interactions enabled high buildup on the substrate. Sorption energy of cyanidin-3-*O*-glucoside (monosaccharide) > cyanidin-3-*O*-rutinoside (disaccharide), but sorption properties of different anthocyanin glucosides were very similar. Intense blue-colored dyeing on hair could be achieved with $\lambda_{max\text{-}vis}$ at 580 nm, typical of the anionic quinonoid base; it is suggested that hair provides an environment that enables the stabilization of the anionic quinonoid base on adsorption through association with cations in the hair and copigmentation effects. Dyeings were stable to multiple washes.

KEYWORDS: *anthocyanins, glycosylation, hair, keratin, sorption, isotherm, dyes, coloration, colorants, sustainable, natural, cosmetics*

INTRODUCTION

Hair Colorants. Hair coloration is an enormous, high profit, global industry (>$10bn *p.a.*) with the number of people coloring their hair in professional salons and at home still increasing.[1,2] However, a significant risk to people applying colorants and having their hair colored exists, and an alternative natural method involving nontoxic and nonsensitizing components is desirable, in order to minimize any potential hazards to human health. Permanent hair coloration represents 70−80% of the hair coloration market.[3,4] This system requires three main components: (i) an *o*- or *p*-substituted (hydroxy or amino) aromatic amine, often referred to as the primary intermediate; (ii) a coupler, commonly an aromatic compound with electron-donating groups arranged *meta* to each other; and (iii) an oxidant.[1,3] The uncolored precursors (primary intermediate and coupler) diffuse into the hair and undergo oxidation reactions to produce the desired color in situ; these colorants usually last at least 24 washes with shampoo.

However, these precursors are historically derived from coal tar, and there is much concern about the toxicological effects of the components used, particularly the aromatic amines (more specifically anilines) that represent nearly all of the primary intermediates and many of the couplers employed. Many of these anilines are suspect carcinogens, tumorgens, and/or mutagens and may also affect reproduction, although these are the subject of much debate.[5−7] Of particular concern are *p*- and *m*-phenylenediamine and *p*-toluenediamine, which are extremely potent contact allergens.[7−9] However, these harmful compounds are important components of most hair colorant formulations in order to achieve darker shades. Similarly, in semipermanent dye systems, many of the colorants employed are known irritants and sensitizers.[7] Therefore, it is desirable and potentially necessary to use colorants that minimize health hazard, and natural colorants have the potential to achieve this.

Anthocyanins as Colorants. Anthocyanins (**1**) are the largest group of polyphenolic pigments in the plant kingdom; they are nontoxic, water-soluble, and responsible for pink, red, purple, violet, and blue and coloration in fruits, vegetables, and flowers. Their colors are determined by the number of hydroxyl groups (and degree of methylation) and the nature, number, and position of sugar moieties (glycosides) including associated aliphatic or aromatic acids attached to the sugar.[10] The six different aglycons shown in Table 1 are the most common components found in foods, leading to many anthocyanins, due to the diversity of glycosylation.[11−14]

Anthocyanins exhibit a remarkable framework of reactions with varying pH. Extensive detailed studies have led to the understanding of the equilibrium forms[12,15] of the core pyrylium cation, which is vitally important for understanding the physical and chemical properties of anthocyanins, and developing potential applications of these molecules. It is generally agreed that anthocyanins take part in acid−base equilibria in aqueous solutions. A general scheme of reactions (Figure 1) highlights the important equilibrium forms, although their relative importance varies significantly depending on the substitution pattern.[15] In aqueous solution of pH < 3, the anthocyanin is red, and the flavan nucleus exists mainly as the very stable flavylium cation ($\mathbf{AH^+}$). Increasing pH leads to kinetic and thermodynamic competition between two reactions.

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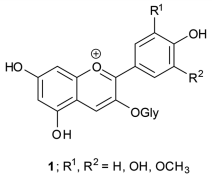
Table 1. Structures and Absorption Maxima for Common Anthocyanins[11]



1; R^1, R^2 = H, OH, OCH_3

Anthocyanin	R^1	R^2	$\lambda_{max\text{-}vis}{}^a$
pelargonidin	H	H	503
cyanidin	OH	H	517
peonidin	OCH_3	H	517
delphinidin	OH	OH	526
petunidin	OCH_3	OH	526
malvidin	OCH_3	OCH_3	529

[a] $\lambda_{max\text{-}vis}$ values shown are for the corresponding 3-*O*-glucoside in water at pH 3.

When pH increases, $\mathbf{AH}^+$ undergoes a rapid deprotonation reaction ($pK_{a1} \sim 3.7$) to form the purple quinonoidal base (**A**) as the kinetic product, which leads to formation of the anionic quinonoidal base ($\mathbf{A}^-$) at higher pH ($pK_{a2} \sim 7$) that has a blue color.[16−19] The alternative thermodynamically favored colorless hemiketal (**B**) is relatively slowly formed via hydration above pH 2, at position 2 (pK_h 2−3). Ring opening is also slow compared to deprotonation but typically faster than hydration and can lead to the formation of yellow *E*-chalcone ($\mathbf{C_E}$), although for many common anthocyanins this is a relatively minor component of the equilibrium.; Once formed, $\mathbf{C_E}$ isomerizes to give the *Z*-chalcone ($\mathbf{C_Z}$).[20] This is activated by UV absorption, as is the reverse process, which allows reversion back to the cyclic hemiketal under favorable conditions. Although anthocyanins can take on all the forms in aqueous solutions, it has been reported that the colorless hemiketal dominates most of the 3-substituted anthocyanins at pH > 4.[15] Cabrita et al.[21] demonstrated that 3-glucosides of pelargonidin, peonidin, and malvidin (all of which have only one OH on the B ring) exhibit intense blue color and stability at pH 8−9, which would suggest quinonoidal bases were the predominant species in the mixture. The precise mole fraction of these species and the rate of their interconversion vary with the number and nature of the substituents, which can be particularly complex when multiple anthocyanin core structures are present, as is the case in most extracts.

Anthocyanin copigmentation is a noncovalent complexation that has been shown to be the main mechanism by which certain colors, particularly blue, violet, and red, are stabilized and modulated in flowers, vegetables, and fruit. Pigment−copigment π-stacking is driven primarily by dispersion interactions and the hydrophobic effect, and these forces are by far the dominant noncovalent interactions between nonpolar molecules. Hydrogen bonding may also play a role in stabilizing noncovalent copigmentation complexes, but its role is not sufficiently documented so far and is usually weakened in aqueous media. Stacking interactions may also involve self-association (anthocyanin + anthocyanin) and self-association of acylated anthocyanins.[18] Copigmentation stabilizes anthocyanins against thermal degradation, and these complexes are generally moderately stable, with pigment−copigment binding constants $<10^3$ M^{-1}. Comparison of the pigment−copigment binding constants for the flavylium ion (K_{AHCP+}) and quinonoid bases (K_{ACP}) shows that AH^+ binds slightly more strongly than A or A^-; e.g., for the malvidin-3-*O*-glucoside (Mv3glc)···rutin complex, $K_{AHCP+} \approx 3{,}000$ M^{-1} and $K_{ACP} \approx 1{,}000$ M^{-1} at 25 °C.[18] Copigmentation also helps to stabilize against light induced degradation pathways.[22] The potential for copigmentation and related association effects further justifies the need to have a substantially refined, well understood extract for larger scale potentially commercial applications.

There is a desire across industries to replace synthetic dyes with natural sources of colorants, and application of anthocyanins and their derivatives has attracted great interest

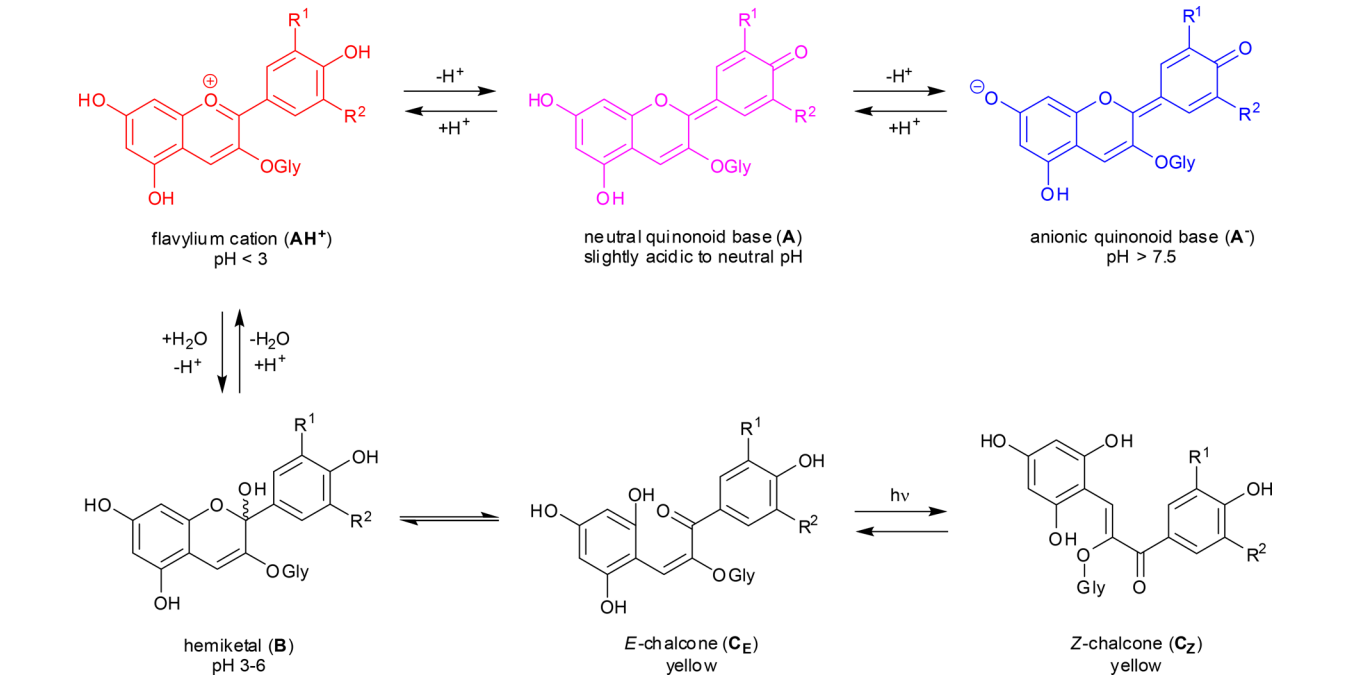


Figure 1. Effect of pH on anthocyanin structure and resultant color.

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over the past few years in the food and cosmetic industries due to their inherent color. Anthocyanins are widely permitted as natural sources as food/beverage colorants within Europe (E163), Japan, the US, and many other countries.[23] Grape skin extract has been used as a colorant for more than 100 years, first being applied to enhance wine color.[24] In the US, "grape-color extract" is obtained as a byproduct in processing Concord grapes (*Vitus labruscana*), but its application is limited by the FDA to nonbeverage food use.[25] There is also increasing interest in the use of anthocyanins as cosmetic colorants. Reports providing toxicological data on anthocyanins support the view that these pigments pose no threat to human health[26] and indeed have been shown to have beneficial effects in many cases,[27−32] thus potentially offering benign alternatives to many current hazardous hair dyes.

Sources and Uses of Anthocyanins. Application and effectiveness of anthocyanins depend on preserving their stability, bioactivity, and bioavailability; however, anthocyanins are quite unstable and susceptible to degradation, particularly in solution by pH, temperature, and light. Practical sources of anthocyanins are limited by overall economic considerations and availability of suitable raw material. Blackcurrant pomace, the focus of this work, is the residue of juice-pressing, typically consisting containing mainly the skins and seeds, and is available in substantial consistent quantities.[33] A total of 15 distinct anthocyanins have been recovered and characterized from blackcurrant, although a combination of only four major components account for >97% of the total content;[34] a composition study on varietals gave the following approximate content: cyanidin-3-*O*-rutinoside (Cy3rut; 33−38%), delphinidin-3-*O*-rutinoside (Dp3rut; 27−34%), cyanidin-3-*O*-glucoside (Cy3glc; 8−10%), and delphinidin-3-*O*-glucoside (Dp3glc; 8−10%).[35] Many other sources of anthocyanins are available,[36−44] and our accompanying work provides an extensive analysis on the typical composition of blackcurrant extracts, including coextracted nonanthocyanin polyphenols.[46]

Applications of Extracted Anthocyanins. Our other article in this series[46] provides a detailed overview of the extraction of anthocyanins from blackcurrants. The purpose of this research was to develop hair colorants with a whole life cycle of sustainable technology from source, through production, to end use and appropriate disposal. The colorants employed in the proposed system, described herein, are anthocyanin-rich extracts isolated from renewable botanical sources (primarily blackcurrant skin waste) as opposed to existing popular hair dyes derived from petroleum products; hence, their use would reduce pressure on fossil fuel sources. The use of colorants from natural sources, in particular biomass, will provide a green alternative to current hazardous resources used and hazardous intermediates produced in their manufacture. It will reduce solvent consumption and waste produced in colorant manufacture, and the colorant itself will be biodegradable, opening the possibility for a fully biodegradable hair formulation. This is also important considering the fact that with current hair coloration techniques, up to 95% of the color applied is waste and ends up in watercourses. In addition, this nonfood use of plant material will promote the use of agricultural crop products in industry.

MATERIALS AND METHODS

Blackcurrant pomace was obtained from GlaxoSmithKline, UK and more recently from A&R House Ltd., UK. The raw fruit grown in the UK had been pressed in production of blackcurrant cordial (Ribena). The crude waste is referred to as pomace, which comprises the fruit skins (ca. 50 wt %), seeds (ca. 45 wt %), and extraneous matter (e.g., berry stalks, ca. 5 wt %). Seeds are separated from this pomace and unwanted stalks removed; the subsequent material received was predominantly dried blackcurrant fruit skins and used without any further modification. Seedless dark-skinned whole table grapes were purchased from Leeds Kirkgate market and pressed using a wine press to remove as much juice as possible; the material used for extraction was the pressed fruit skins. Amberlite XAD7HP was obtained from Rohm & Haas. General purpose chemicals were obtained from Sigma-Aldrich. Delphinidin-3-*O*-glucoside was purchased from Polyphenol AS. Light blonde (bleached) European human hair swatches of uniform color and treatment, bound on one end by wax, each ca. 1.0 g and 15 cm length, were purchased from Banbury Postiche Ltd. The conditioner base formulation comprised water (92.45%), cetyl alcohol (4.0%), ceteareth-20 (0.5%), almond oil (0.5%), liquid paraffin (0.5%), stearamidopropyl dimethylamine (0.9%), silicone fluid (0.5%), coconut extract (0.5%), Phenoxetol NIPA (0.8%), citric acid (0.2%), and perfume (0.05%).

Extraction and Semipurification of Polyphenols. Blackcurrant fruit skins (30 g) were immersed in water (600 mL) acidified 0.01% v/v with conc. HCl and stirred very gently by a magnetic stirrer at room temperature for 2 h. The resulting aqueous extract was subjected to solid-phase extraction (SPE) using a column of Amberlite XAD-7HP (60 g) using acidified water (0.01% v/v conc. HCl) until a colorless eluent was obtained and then acidified with ethanol (0.01% v/v conc. HCl). The collected ethanol fractions were combined and concentrated under vacuum on a rotary evaporator and then subjected to high vacuum to remove trace solvent, yielding a dark violet amorphous solid (660 mg, yield 2.2%), which could be powdered by grinding.[46]

Dark grape fruit skins (30 g) were extracted using the same method as described above for blackcurrant skins. Again, a dark violet amorphous solid resulted (105 mg, yield 0.35%), which could be powdered by grinding.

Analytical HPLC. The analytical HPLC system (Agilent 1290 infinity series) was equipped with a diode-array detector (DAD), a binary pump system connected with online degasser and Zorbax Eclipse XDB C18, 150 × 4.6 mm, 5 μm. For the aqueous extract, the binary solvent system consisted of solvent A, acidified water (0.5% TFA), and solvent B, acidified acetonitrile (0.5% v/v TFA). The elution profile consisted of a linear gradient from 5% B to 20% B in the first 20 min, then linear increase to 100% B at 20−23 min followed by isocratic elution (100% B) at 23−24 min, and then linear decrease to 5% B at 24−25 min followed by 5% B isocratic elution at 25−30 min. The flow rate was 1 mL min^{-1}, and the peaks were monitored at 254, 285, 325, 350, and 520 nm. For organic extracts, the binary solvent system consisted of solvent A, acidified water (0.1% v/v TFA), and solvent B, acidified acetonitrile (0.1% v/v TFA). The elution profile consisted of a linear gradient from 5% B to 20% B in the first 30 min, then linear increase to 100% B at 30−33 min followed by isocratic elution (100% B) at 33−34 min, and then linear decrease to 5% B at 34−35 min followed by 5% B isocratic elution at 35−40 min. The flow rate was 1 mL min^{-1}, and aliquots of 20 μL were injected with Micro Autosampler (Agilent 1200 series). The peaks were monitored at 254, 285, 325, 350, and 520 nm.

UV/Visible Spectrophotometry. Dye solutions that were retained after dyeing were measured using a Jasco V-630 spectrophotometer in the visible region of the spectrum (360−700 nm), at 1 nm intervals. The solutions were diluted using distilled water and measured at the wavelength of maximum absorption in the visible region of the spectrum ($\lambda_{max\text{-}vis}$) for the dye. No difference in the shape of the absorption spectrum before and after dyeing was noted. From the UV/vis spectrophotometry data, the equilibrium dye concentration in solution (C_e; mg dm^{-3}) was calculated from absorbance values referenced to calibration plots; the equilibrium concentration of dye in fiber (q_e; mg g^{-1}) was calculated by subtracting C_e from the initial dye concentration in solution at the start of the dyeing process (C_0; mg dm^{-3}), relative to the mass of hair dyed.

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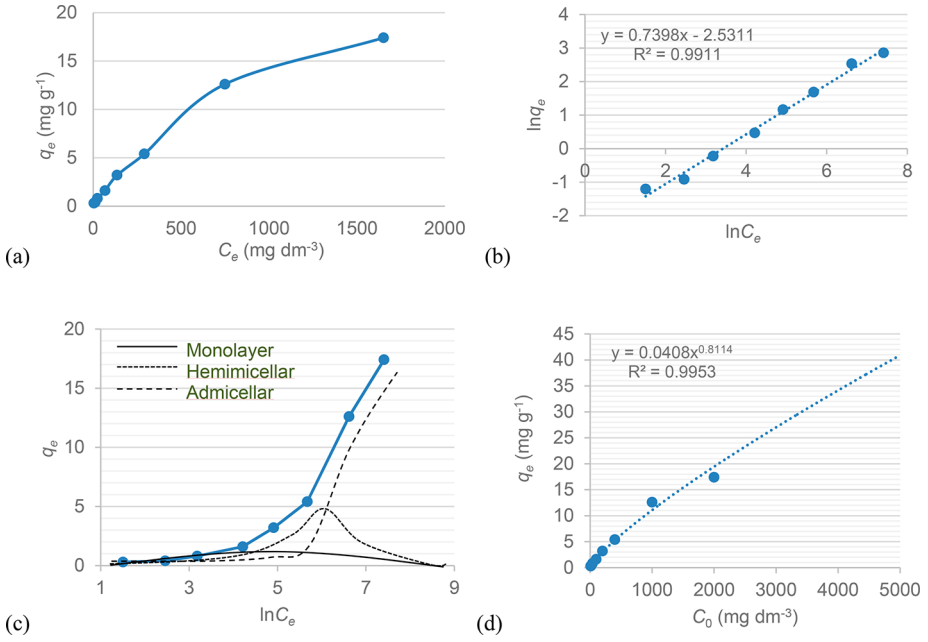

Figure 2. (a) Adsorption isotherm (q_e vs C_e) for anthocyanins extracted from blackcurrant skin on light blonde bleached human hair. (b) Plot of ln q_e vs ln C_e according to the Freundlich sorption isotherm model. (c) Plot of q_e vs ln C_e for total anthocyanins extracted from blackcurrant skin adsorbed onto human hair; regions of monolayer sorption and hemimicellar and admicellar aggregation are indicated. (d) Plot of q_e vs initial concentration (C_0) of anthocyanins extracted from blackcurrant skin applied onto human hair.

Application of Anthocyanins to Human Hair. Prior to dyeing, light blonde (bleached) hair swatches were shampooed using a shampoo obtained from a supermarket and dried using a hair drier.

Aqueous Application. Initial investigations into sorption of anthocyanins onto hair was from aqueous solution. Anthocyanin-rich extracts from either blackcurrant or grape were dissolved in 5 mL of 0.2 M citric acid/sodium citrate buffer (pH 3.0) at 20 °C, and 100 mg prepared hair added to the solution and stirred for 1 h maintaining the same temperature; pH monitoring throughout dyeing showed that the pH was maintained at around pH 3.0 for the duration of the dyeing process. Initial concentrations of the anthocyanin extract used were 0.05%, 0.1%, 0.2%, 0.5%, 1.0%, 2.0%, 5.0%, and 10.0% on mass of (hair) fiber (omf). The resultant hair swatches were removed from their respective dyebaths and rinsed under running warm water. Each swatch was dried with a hair drier on a low temperature setting and then the color measured by reflectance spectrophotometry.

Application from Dye Base Formulation. Subsequent investigations incorporated anthocyanin extracts into typical commercial dye base formulations. Blackcurrant extract was initially dissolved in the minimum volume of solvent (ca. 0.5 mL; 0.2 M citric acid/sodium citrate buffer, pH 3.0) and subsequently incorporated into a 2.5 g formulation base (pH 5.0−5.5) with thorough mixing. The dye paste was then applied to preshampooed, wetted bleached hair swatches (1 g) by hand with gentle massaging and combing and left to stand at room temperature for 45 min. After standing, the swatch was rinsed under running warm water for 2−3 min with combing to ensure complete removal of dye paste, subsequently conditioned and rinsed before drying with a hair dryer. Each swatch was dried with a hair drier on a low temperature setting and then the color measured by reflectance spectrophotometry.

Dyed Hair Wash Fastness Studies. One wash cycle comprised application of shampoo massaged into the dyed hair by hand for 1 min; rinse under running warm water for 1 min; combing; second application of shampoo; second rinse; application of conditioner massaged into the hair by hand for 1 min; third rinse; and drying with a domestic hair dryer with combing. A total of 6 wash cycles were conducted for each sample (12 individual shampoings).

Hair Color Measurement. Dyed hair samples were measured using a Datacolor SF600 Spectraflash reflectance spectrophotometer connected to a personal computer using DCI Color Tools software. Reflectance measurements were recorded for each hair sample using an average of 4 measurements as taken across the visible spectrum (360−700 nm). From reflectance values (R) at a specified wavelength (λ) of the dyeings, the color strength (K/S) of the sample was calculated using the Kubelka−Munk equation (eq 1):

$$\frac{K}{S} = \frac{(1 - R_\lambda)^2}{2R_\lambda} \quad (1)$$

where K is the absorption coefficient, and S is the scattering coefficient.[46]

RESULTS AND DISCUSSION

Extraction and Purification. In our accompanying work,[45] colorants were developed based on extracts from renewable botanical sources, specifically waste blackcurrant (*Ribes nigrum* L.) fruit skins from the fruit pressing industry. An extraction process was developed using acidified water in combination with a solid-phase purification stage. Anthocyanins were extracted from blackcurrant skin waste material in good yields (ca. 2% w/w based on dry weight of raw material) and extracts extensively characterized by HPLC, mass spectrometry, IR, NMR, and UV−vis spectroscopy.[45] HPLC confirmed the presence of four anthocyanins: Dp3rut (45%), Cy3rut (31%), Dp3glc (16%), and Cy3glc (8%). The post-SPE sample was shown to contain monomeric anthocyanins (54.7%) and polymeric anthocyanins (18%), myricetin-3-*O*-rutinoside (3.1%), quercetin-3-*O*-rutinoside (3.2%), myricetin-3-*O*-glucosides (3.1%), quercetin-3-*O*-glucoside (2%), myricetin (2.5%) quercetin (3.2%), caffeic acid (3%), *p*-coumaric acid (5%), nigrumin-*p*-coumarate (1%), and nigrumin ferulate (0.5%).[45] This extract was used in further hair coloration studies described herein.

For comparative sorption isotherm work herein, dark grape skins were also extracted using the same process described for blackcurrants.[45] Anthocyanins were extracted from pressed grape skin, but yields were significantly lower (0.35% w/w) in

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comparison with that of blackcurrant. Extracts were characterized by HPLC, which confirmed the presence of five anthocyanins: Cy3glc (8%), Dp3glc (7.3%), Mv3glc (65.3%), peonidin-3-*O*-glucoside (Pn3glc; 6.0%), and petunidin-3-*O*-glucoside (Pt3glc; 15.6%). It is notable that all anthocyanins extracted from grape skins are glucosides, which is in agreement with previous observations.[47]

Adsorption Isotherm Studies. To investigate the sorption properties of the dyes on hair, application of blackcurrant skin anthocyanin extract in aqueous solution was developed. The dye exhaustion from the dyebath onto the hair fiber was measured by recording either a UV−vis spectrum or a HPLC chromatogram of the dyebath before and after the dyeing procedure. Results obtained were applied to Langmuir[48,49] and Freundlich[50,51] isotherm models, and it was observed that sorption showed the greatest correlation to a Freundlich isotherm (Figure 2; R^2 = 0.987); Langmuir correlation: R^2 = 0.877.

The Freundlich isotherm is commonly used to describe the adsorption characteristics for heterogeneous surfaces. These data often fit the empirical equation proposed by Freundlich (eq 2):[50,52]

$$q_e = K_F C_e^{1/n_F} \tag{2}$$

where q_e is the equilibrium concentration of sorbate on the sorbent (solid-phase) (mg g^{-1}), C_e is the equilibrium sorbate concentration in solution (mg dm^{-3}), K_F is the Freundlich constant (dm^3 g^{-1}), and $1/n_F$ is the heterogeneity factor. The capacity constant K_F and the affinity constant n_F are empirical constants dependent on several environmental factors. A linear form of the Freundlich isotherm can be obtained by taking logarithms of eq 2 (eq 3):

$$\ln q_e = \ln K_F + \frac{1}{n_F} \ln C_e \tag{3}$$

Therefore, a plot of ln q_e versus ln C_e should yield a straight line of intercept value ln K_F and slope $1/n_F$ if the isotherm obtained experimentally observes the Freundlich expression. K_F is an approximate indicator of adsorption capacity, while $1/n_F$ is a function of the strength of adsorption in the adsorption process. If n_F = 1, then the partition between the two phases are independent of the concentration. If the value of $1/n_F$ is below one, it indicates a normal adsorption; conversely, if $1/n_F$ is above one, this indicates cooperative adsorption. This expression reduces to a linear adsorption isotherm when $1/n_F$ = 1. If n_F lies between 1 and 10, this indicates a favorable sorption process.[52] The Freundlich isotherm is another form of the Langmuir approach for adsorption on an "amorphous" surface where the amount of adsorbed material is the summation of adsorption on all sites. The Freundlich isotherm is derived by assuming an exponential decay energy distribution function inserted into the Langmuir equation. It describes reversible adsorption and is not restricted to the formation of the monolayer.

Thermodynamic data such as adsorption energy ($-\Delta G^0$) can be obtained from K_F (eq 4), where K is constant in terms of dm^3 mol^{-1}.

$$-\Delta G^\circ = RT \ln K \tag{4}$$

Anthocyanins extracted from blackcurrant waste material adsorb onto light blonde bleached human hair, as shown in the isotherm in Figure 2a. A plot of ln q_e vs ln C_e reveals that the mechanism is consistent with the Freundlich isotherm (Figure 2b; R^2 = 0.991), wherein K_F = 0.08 dm^3 g^{-1}, and n_F = 1.35 (favorable adsorption). On the basis of the molecular weight of the four anthocyanins present in blackcurrant extract and their relative abundance, an average molecular weight for the blackcurrant extract is calculated as 571.8 g mol^{-1}; accordingly, ΔG^0 = −9.10 kJ mol^{-1}. Dyeings obtained were intensely blue colored, and a surprising level of buildup was achieved at the highest concentrations, giving very deep dyeings (a detailed investigation into dyeing properties in formulation is provided later).

In addition to this isotherm plot, the data may also be represented in a number of depictions in order to elucidate further information regarding the interaction between dye and fiber. When sorbate molecules have the potential for electrostatic and dispersive interactions, dense packing of adsorbed molecules into hemimicelle and admicelle aggregates is possible and is manifest as an s-shaped isotherm for a plot of q_e versus ln C_e.[53] Hemimicelle (monolayered aggregation) formation may be attributed to the side-to-side interactions of anthocyanins contributing to ordering on the surface, and admicelle (bilayered aggregation) formation can be attributed to various stacking interactions possible for anthocyanins, which may actually exceed a bilayer and occur in multilayers. As can be seen in Figure 2c, sorption follows a low gradient at the lowest concentrations (1 < ln C_e < 3.5), which is consistent with monolayer buildup of anthocyanins on the substrate, wherein q_e < 1.2 mg g^{-1}. At higher concentrations (3.5 < ln C_e < 5.8; 1.2 < q_e < 5.0 mg g^{-1}), an increase in the gradient is observed, which is typical of sideways stacking of dye molecules (hemimicellar). Beyond this region, the gradient of the curve increases dramatically (5.8 < ln C_e < 7.5; 5.0 < q_e < 17.5 mg g^{-1}), which represents multilevel stacking of the dye molecules (admicellar), effectively where color buildup occurs. These results are again consistent with the Freundlich isotherm, which has characteristic unlimited adsorption,[50,52] facilitated herein by anthocyanin−anthocyanin aggregation interactions, enabling high buildup of dye on the substrate and resulting in intense coloration of the hair.

Further support for a Freundlich isotherm and demonstration of unlimited adsorption is obtained by plotting q_e vs initial concentration (C_0) of anthocyanins applied onto hair and extrapolating the data (Figure 2d), where it is observed that the most appropriate trendline fit for the data is a power curve (R^2 = 0.995), which fits directly with the Freundlich equation (eq 2),[50,52] which has the form $y = ax^b$. By applying the values for K_F and n_F obtained from Figure 2b to eq 2, the Freundlich equation for the isotherm obtained is q_e = 0.08 $C_e^{0.741}$, which corresponds very favorably with the equation obtained for the data trendline in Figure 2d: q_e = 0.04 $C_0^{0.811}$. These observations support the unlimited adsorption nature of the data obtained, and accordingly, there is no theoretical substrate saturation point ($q_{e\,max}$); hence, sorbate−sorbate interactions are highly likely.

The adsorption study was repeated using HPLC chromatograms to assign concentrations rather than UV−vis absorbance. All experimental parameters were maintained and quantitative HPLC analysis performed using Cy3glc chloride (**2**) and Cy3rut chloride (**3**) as standards. The results of the HPLC analysis were used to assign concentrations in order to gain more accurate information regarding individual anthocyanin components of the extract. It can be seen from Figure 3 and Table 2 that although both Cy3glc and Cy3rut adsorb onto the

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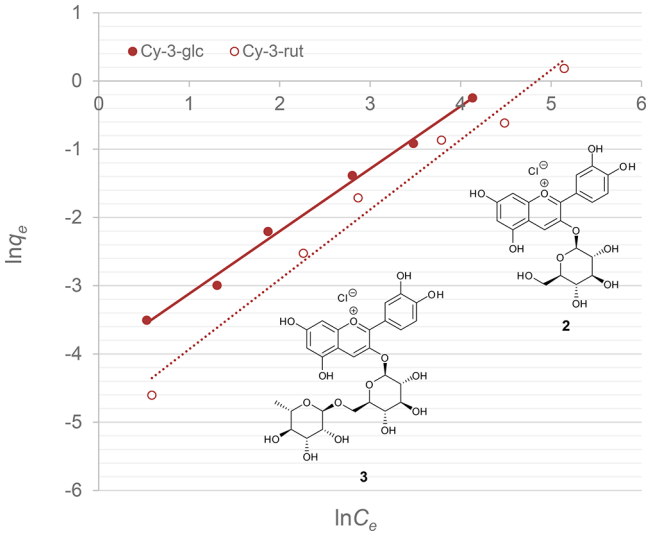

Figure 3. Comparative sorption isotherm for Cy3glc (**2**) and Cy3rut (**3**) anthocyanins extracted from blackcurrant skin adsorbed onto human hair.

Table 2. Comparative Freundlich Isotherm Data for Anthocyanins Extracted from Blackcurrant Skin Adsorbed onto Human Hair

anthocyanin	M_w (g mol^{-1})	K_F (dm^3 g^{-1})	n_F	ΔG^0 (kJ mol^{-1})	R^2
Cy3glc	449.4	0.018	1.09	−5.06	0.994
Cy3rut	595.5	0.007	0.98	−3.49	0.980

hair fiber, sorption is most favorable for Cy3glc, which displays a greater adsorption energy in comparison with that of Cy3rut. The structural difference between these two compounds lies in the sugar substitution; glucoside is a monosaccharide unit, whereas rutinoside is a disaccharide unit. In terms of sorption, this difference is significant as compounds of lower molecular size are favored due to steric hindrance of larger molecules, with smaller glucosides more likely to form close packing associated with the formation of aggregates (hemimicellar and admicellar). Additionally, the disaccharide moiety of Cy3rut will confer greater water solubility in comparison with that of the monosaccharide Cy3glc; greater water solubility can decrease adsorption energy as the sorbate molecule has greater preference for the solvent phase in comparison with that of a less water-soluble sorbate molecule, which is likely to have greater affinity for the more hydrophobic sorbent. The same trends were observed when comparing Dp3glc with Dp3rut, where the monosaccharide was preferentially adsorbed over the disaccharide.

In order to investigate the sorption properties of different anthocyanins, the adsorption study was conducted with dark-skinned grape skin extract prepared using an analogous process. This extract was used because the anthocyanins identified in the grape extract were all glucosides, namely, Cy3glc, Dp3glc, Mv3glc, Pn3glc, and Pt3glc; accordingly, any differences in sorption would be exclusively related to differences in anthocyanin substitution rather that glycosylation. All experimental parameters were maintained, and standards were used for calibration of HPLC chromatograms. It can be seen from Figure 4 that anthocyanin glucosides adsorb onto the hair fiber. Freundlich isotherm data (Table 3) demonstrated that sorption properties were very similar; n_F values had a narrow range from

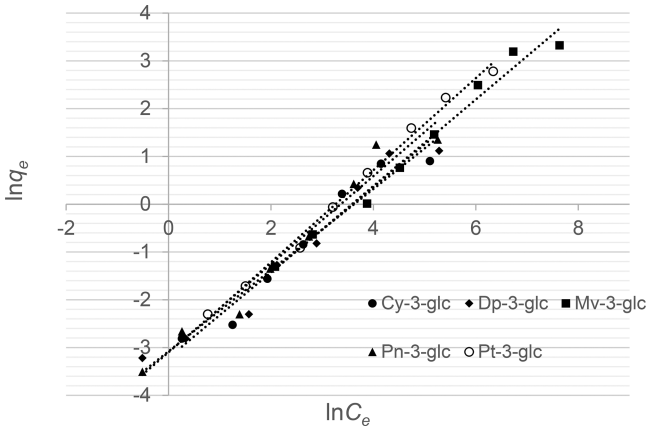

Figure 4. Comparative sorption isotherm for anthocyanin glucosides extracted from grape skin.

Table 3. Comparative Freundlich Isotherm Data for Anthocyanin Glucosides Extracted from Grape Skin Adsorbed Onto Human Hair

anthocyanin	M_w (g mol^{-1})	K_F (dm^3 g^{-1})	n_F	ΔG^0 (kJ mol^{-1})	R^2
Cy3glc	449.4	0.040	1.12	−7.05	0.959
Dp3glc	465.4	0.046	1.18	−7.47	0.960
Mv3glc	493.4	0.039	1.10	−7.21	0.982
Pn3glc	463.4	0.045	1.08	−7.41	0.964
Pt3glc	479.4	0.043	1.04	−7.39	0.993

1.04 to 1.18. ΔG^0 values for the five anthocyanin glucosides differed by only 0.42 kJ mol^{-1}. It is suggested that differences in anthocyanin substitution on the B ring have a minimal effect on sorption properties onto hair and that sorption differences observed herein are primarily related to glycosylation.

It is noted that there are differences in the Freundlich isotherm data for Cy3glc from blackcurrant versus those from grapes; while n_F values are very similar, ΔG^0 is −5.06 kJ ml^{-1} for blackcurrant and −7.05 kJ ml^{-1} for grapes. It is unclear why these differences are observed, but it is possible that other components in the extract (other flavonoids and hydroxycinnamic acids), which differ between blackcurrant and grape extracts, interact with the anthocyanins and influence sorption energy.

Application of Anthocyanins in Formulation as Hair Dyes. Extracted anthocyanins from blackcurrant skin were tested as dyes for human hair initial experiments due to the consistency observed in anthocyanin profile from batch to batch extraction of the raw material. For translation to a real hair dye system, the extracted colorants were incorporated into a dye base formulation. The dye base system did not allow direct analysis by UV−vis or HPLC, but the visible color of the resultant hair dyeing was analyzed by reflectance color measurement. It was found that intense blue-colored dyeings with high color strength on hair could be achieved with the blackcurrant anthocyanin extract. What was particularly interesting from dyeings with anthocyanins extracted from blackcurrant skin was that the final color on hair was blue (Figure 5) with a $\lambda_{max\text{-}vis}$ from color strength measurements (K/S) at 580 nm, typical of the blue anionic quinonoid base (A^-). At pH 3, aqueous solutions of cyanidin and delphinidin have $\lambda_{max\text{-}vis}$ values at 517 and 526 nm, respectively, and the color of the dyes in formulation was purple, typical of the neutral

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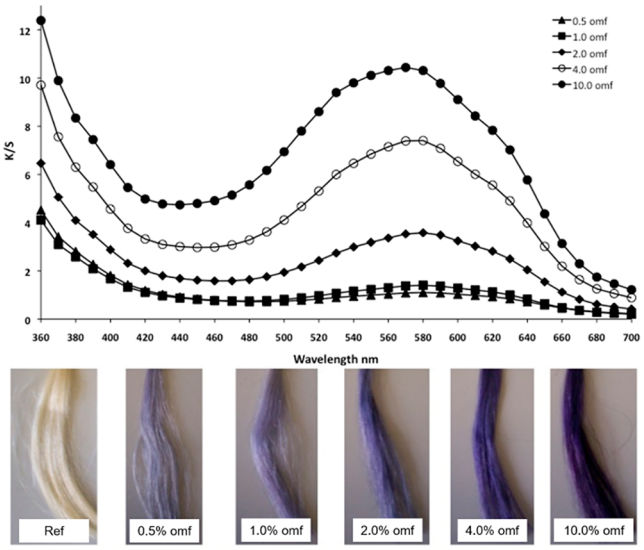

Figure 5. (Above) Color strength (K/S) across the visible spectrum for hair dyed with blackcurrant anthocyanin extract in formulation. (Below) Photographs of corresponding dyeings on hair tresses. Corresponding values for the total anthocyanin concentration for each dyeing are 0.5% omf (2.7 mg anthocyanins per g hair); 1.0% omf (5.5 mg g^{-1}); 2.0% omf (10.9 mg g^{-1}); 4.0% omf (21.9 mg g^{-1}); and 10.0% omf (54.7 mg g^{-1}), based on the blackcurrant extract comprising 54.7% total monomeric anthocyanins.

quinonoid base (A). It is known that A^- is formed at pH 6.0−7.5,[12,15] yet formulation pH was 5.0−5.5, and the color of the formulation was red-purple; accordingly, it is suggested that the hair provides a neutralization environment that enables the anthocyanins to convert to A^- on adsorption.

Nature makes very few stable blue chromophores; some flowers, such as cornflower (*Centaurea cyanus* L.) and *Delphinium* spp., form blue pigments in their petals but usually require the formation of anthocyanin−metal (typically Mg and Al) coordination complexes to achieve this.[12] There are exceptional cases where unusually high vacuolar pH may be sufficient, but these are typically polyacylated anthocyanins,[17] as in the case of the petals of the blue morning glory (*Ipomoea tricolor* cv. Heavenly Blue), which contains the tricaffeoylated "heavenly blue anthocyanin" that attains a vacuolar pH of 7.7 at the flowering stage.[54]

It is demonstrated herein that formation of a stable blue color supported on a substrate without metal coordination with nonacylated anthocyanins is possible; there are only trace concentrations of metal in the hair used in dyeing experiments, and they are insufficient to form coordination complexes with the levels of anthocyanins deposited onto the hair, which even at the highest concentrations applied remain blue.

It is possible that the anionic quinonoid base (A^-) is stabilized by electrostatic interaction with counterions in the substrate, most likely protonated primary amino functions ($-NH_3^+$); this is likely due to the presence of a significant proportion of cationic amino acid residues in human hair that could become involved in this interaction, with lysine (218 μmol g^{-1} dry hair), arginine (499 μmol g^{-1}), and histidine (64 μmol g^{-1}) residues all present.[55,56] Note that the concentration of these amino acid residues increases only slightly (+2 to +6%) upon bleaching.[55]

In addition, although the extract is rich in anthocyanins and many undesired compounds have been removed, some noncolored flavonoids remain; hence, copigmentation may also play a role in the color formed as it is known that this effect can make anthocyanins bluer through intermolecular hydrogen bonding between flavonoid catechol moieties and carbonyl groups of the anthocyanin neutral quinonoid base (A).[12,15] Self-association of anthocyanins may also influence the bathochromic shift in the color observed, and sorption isotherms suggest significant interaction through the formation of hemimicelles and admicelles. Self-association protects the flavylium cation (AH^+) against hydration (like copigmentation) and favors its conversion into the quinonoid bases (A), suggesting that self-association of neutral quinonoid bases is stronger than that of flavylium cations, which is not unexpected because the self-association of flavylium cation is impeded by electrostatic repulsion.[14] Accordingly, sorption of the quinonoid bases is more favorable than the flavylium cation. The ability to form stable blue colors from nature for cosmetic application is significant for applications where blue is the desired hue or when blue is a component in a trichromatic mixture to achieve a certain shade.

In aqueous solution, the anionic quinonoid base (A^-) is unstable and degrades over time.[12] However, it may be observed from wash fastness testing (Table 4) that on the hair this form is stable to multiple washes and does not change $\lambda_{max\text{-}vis}$. It is also observed that the dye is highly stable on the hair, with only 10% of the dye washing off after 12 shampoos. This would certainly place this anthocyanin extract hair dye formulation in the category of semipermanent as the results clearly demonstrate that the dyed hair sample exhibited very good fastness to washing across the study comprising a total of 12 applications of shampoo. Electrostatic association of the anionic quinonoid base with protonated primary amino functions in the hair may contribute to the wash high fastness levels observed.

Table 4. Color Change during Wash Fastness Testing for Hair Dyed with 10% omf Blackcurrant Anthocyanin Extract

washes	$\lambda_{max\text{-}vis}$ (nm)	K/S	% change
0	580	11.67	--
3	580	11.17	4.3
6	580	10.46	10.4

The observations of the high buildup of the anthocyanins on the hair fiber and the impressive stability to washing are in agreement with previous work on anthocyanin−protein interactions. Plundrich et al.[57] demonstrated that anthocyanins from blackcurrant and muscadine grape sources were successfully adsorbed and highly concentrated into protein-rich carrier matrices (hemp and soy protein isolates) and incorporated into topical oil-in-water formulations; they observed that the protein-binding stabilized bioactivity. Roopchand et al.[58] made a complex between anthocyanins extracted from Concord grape pomace and soy protein isolate, and found that anthocyanins were stable after a 16-week incubation at 37 °C but that anthocyanins levels were reduced by up to 60% in dried grape pomace extract. Considering that hair fiber is a proteinaceous substrate, these stabilizing anthocyanin−protein interactions may be also responsible for the sorption and stability of the adsorbed anthocyanins to degradation.

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Notes

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