Molar Absorptivity and Color Characteristics of Acylated and Non-Acylated Pelargonidin-Based Anthocyanins

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The effects of glycosylation and acylation on the spectral characteristics, molar absorptivity, and color attributes of purified acylated and non-acylated pelargonidin derivatives were compared. Pigments were obtained from strawberries, radishes, red-fleshed potatoes, and partially hydrolyzed radish pigments. Individual pigments were isolated by using semipreparative HPLC. Spectral and color (CIEL*ch) attributes of purified pigments were measured. Molar absorptivity ranged from 15 600 to 39 590 for pelargonidin-3-glucoside (pg-3-glu) and pg-3-rutinoside-5-glucoside acylated with *p*-coumaric acid, respectively. The presence of cinnamic acid acylation had a considerable impact on spectral and color characteristics, causing a bathochromic shift of λ_{max}. Sugar substitution also played an important role, with a hypsochromic shift caused by the presence of glycosylation. Pg-3,5-diglu and pg-3,5-triglu possessed a higher hue angle (>40°) than the other pg derivatives at pH 1.0, corresponding to the yellow-orange region of the color solid. Acylation with malonic acid did not affect λ_{max} and showed little effect on color characteristics. The solvent system had an effect not only on the molar absorptivity, but also on the visual color characteristic of the pigments.

Keywords: *Pelargonidin derivatives; acylation; molar absorptivity; color*

INTRODUCTION

There is considerable interest in alternative sources of anthocyanin pigments which can serve as colorants with improved functionality, i.e., better stability and more desirable hue. Anthocyanin-based colorants approved for use in the United States, such as grape skin, red cabbage, and black carrot extracts, exhibit a red-purple hue at pH values above 3. Under acidic conditions, the color of non-acylated or monoacylated anthocyanins is determined largely by the substitutions in the B ring of the aglycon (Jackman and Smith, 1996; Mazza and Miniati, 1993). However, anthocyanins bearing the same chromophore can give rise to different colors, depending on physical and chemical factors (Figueiredo et al., 1996a,b). The presence of additional acylation with cinnamic acids produces a bathochromic shift in the λ_{max} of the pigment, with a slight blueing effect (Jackman and Smith, 1996; Dangles et al., 1993; Harborne, 1967).

Anthocyanin structural variation, including sugar substitution, hydroxylation, and methoxylation of the B ring, and the presence of acylating groups have been correlated with pigment stability. However, not much information is available regarding the effect of sugar substitutions on anthocyanin color characteristics.

Measurement of anthocyanin content is critical for both research and industrial applications. In evaluating anthocyanin colorants and their respective sources, it is necessary to measure total pigment content, the qualitative anthocyanin composition, and, in addition, the color characteristics. Molar absorptivities in the solvent of choice are required for accurate measurement of anthocyanin content of identified pigments. Availability of an efficient method for separation of anthocyanins, such as HPLC, combined with a list of absorption coefficients should simplify the quantitative estimation of individual anthocyanins (Francis, 1989). However, through the years there has been a lack of uniformity on the values of absorptivity reported, mainly due to the difficulties of preparing crystalline anthocyanins, free from impurities, in sufficient amounts to allow reliable weighing under optimal conditions (Fuleki and Francis, 1968; Francis, 1982).

Our objective was to determine the role of glycosylation and acylation with both cinnamic and aliphatic organic acids on the molar absorptivity and color characteristics of pelargonidin-based anthocyanins in aqueous or methanolic solutions.

MATERIALS AND METHODS

Sources of Pigments. Anthocyanins were extracted from strawberry (Kerr Concentrates, Inc., Salem, OR), radish, and red-fleshed potato (grown at the OSU Lewis-Brown Horticultural Farm, Corvallis, OR) using acetone and partitioned with chloroform as described by Giusti and Wrolstad (1996).

Pelargonidin-3-glucoside (pg-3-glu) was extracted from strawberries. Four acylated pg derivatives were obtained from radishes: pelargonidin-3-sophoroside-5-glucoside (pg-3-soph-5-glu, also known as raphanusin) acylated with *p*-coumaric acid, raphanusin acylated with ferulic acid, raphanusin acylated with *p*-coumaric and malonic acid, and raphanusin acylated with ferulic and malonic acids. Pelargonidin-3-rutinoside-5-glucoside (pg-3-rut-5-glu) acylated with *p*-coumaric acid was extracted from potatoes. Other pg derivatives, pg-3-soph-5-glu, pg-3-soph, pg-3-glu-5-glu, and pg-5-glu, pg aglycon, were obtained from partial hydrolysis of radish anthocyanins.

Anthocyanin Semipurification. Semipurification was carried out by using a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (Wrolstad et al., 1990). Anthocyanins (and

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10.1021/jf981271k CCC: $18.00
Published on Web 10/06/1999

Table 1. Absorptivity Coefficients (L cm^{-1} mol^{-1}) of Different Pelargonidin Derivatives

anthocyanin	weight (mg)	conc. mg/L	conc. (mol/L) × 10^{-5}	MW	λ_{max} MeOH	λ_{max} buffer	molar absorptivity MeOH	molar absorptivity buffer	*E* 1% MeOH	*E* 1% buffer
Pg aglycon	10.2	2.5	0.77	325.3	524	505	19780	18420	608	566
Pg-3-glu	21.2	8.56	1.76	487	508	496	17330	15600	356	320
Pg-3-soph-5-glu	24.2	24.2	2.98	810.5	506	497	30700	25370	379	313
Pg-3-soph-5-glu + p-coumaric	8.3	16.6	1.74	956.5	508	506	34890	28720	365	300
Pg-3-soph-5-glu + ferulic	18.1	27.2	2.76	986.5	507	506	29640	24140	300	245
Pg-3-soph-5-glu + p-coumaric & malonic	64.1	25.6	2.46	1042.5	508	508	39780	33020	382	317
Pg-3-soph-5-glu + ferulic & malonic	17.5	35	3.26	1072.5	508	508	39380	31090	367	290
Pg-3-rut-5-glu + p-coumaric	38.5	15.4	1.64	940.9	511	504	39590	32080	421	341

other phenolics) were adsorbed onto the minicolumn; sugars, acids, and other water-soluble compounds were removed with 2 volumes of 0.01% aqueous HCl. Less polar phenolics were removed from the minicolumn by washing with 2 volumes of ethyl acetate (Oszmianski and Lee, 1990), and anthocyanins were subsequently eluted with methanol containing 0.01% HCl (v/v). The methanolic extract was then concentrated using a Büchi rotovapor at 35 °C, and pigments were dissolved in distilled deionized water containing 0.01% HCl.

Alkaline and Acid Hydrolysis of Anthocyanins. Approximately 30 mg of purified pigment was hydrolyzed (saponified) in a screw-cap test tube with 10 mL of 10% aqueous KOH for 8 min at room temperature in the dark, as described by Hong and Wrolstad (1990). The solution was neutralized using 2 N HCl, and the hydrolysate was purified using semipreparative HPLC and a C-18 Sep-Pak cartridge, as previously described.

Fifteen milliliters of 2 N HCl was added to ca. 1 mg of purified saponified pigment in a screw-cap test tube, flushed with nitrogen, and capped. The pigment was hydrolyzed for different times, ranging from 15 to 30 min at 100 °C, then cooled in an ice bath. The hydrolysate was purified using a C-18 Sep-Pak cartridge, as previously described (Hong and Wrolstad, 1986). The partially hydrolyzed pigments were separated using semipreparative HPLC.

Pigment Isolation. Semipurified pigments were isolated using semipreparative HPLC. Individual pg derivatives were collected and further purified by passing them through a C-18 Sep-Pak cartridge as previously described. Pigments were recovered from the cartridge with 90% methanol and 10% acidified methanol (0.01% HCl methanol). The methanol was evaporated in a Büchi rotovapor at 35 °C, and pure methanol was added and evaporated again to facilitate the removal of water remaining in the sample. This procedure was repeated 3 times. The flask containing dried pure anthocyanins was cooled for 1−2 h in a desiccator in the dark, and the weight was recorded.

Pigment isolation was performed in triplicate, but only the extract with the highest purity level was used for further analyses. Purity of isolated pigments was checked using analytical HPLC collecting data at 280, 310, and 520 nm and monitoring spectra of the peaks. The percent area of the isolated pigments in the 280 nm chromatogram was used as an indicator of purity.

High-Performance Liquid Chromatography (HPLC). *Apparatus.* A semipreparative Dynamax Rainin model SD-300 high-performance liquid chromatograph was used, equipped with a Hewlett-Packard 1040A photodiode array detector and a Gateway 2000 P5-90 computer with a Hewlett-Packard HPLC^{2D} ChemStation software. A 1-mL injection loop was used.

Columns and Mobile Phase. A Microsorb C-18 column (5 μm), 250 × 21.4 mm i.d. fitted with a 50 × 21.4 mm i.d. guard module (both from Rainin Instrument Co., Inc., Emeryville, CA) was used. The solvents used were (A) 100% HPLC-grade acetonitrile and (B) 1% phosphoric acid (concentrated), 10% acetic acid (glacial), 5% acetonitrile (v:v:v) in water. Flow rate: 20 mL/min. Solvents and samples were filtered through a 0.45 μm Millipore filter type HA (Millipore Corp., Bedford, MA).

HPLC Conditions for Anthocyanin Separation and Isolation. Radish anthocyanins and saponified radish anthocyanins were separated by using isocratic conditions at 10% A. The identity of anthocyanins was verified by collecting data at 280, 310, and 520 nm and collecting peak spectra (from 260 to 600 nm) of all peaks at 520 nm.

Molar Absorptivity. Purified dried pigments were dissolved in 10 mL of 100% HPLC-grade methanol. An exact aliquot of that solution was diluted in methanol containing 0.1% HCl and in pH 1.0 buffer (0.2 N KCl), by duplicate, to obtain final concentrations of (1−5) × 10^{-5} mol/L. Spectral characteristics of known dilutions were recorded on a Shimadzu 300 UV spectrophotometer using 1-cm path length quartz cells. For molar absorptivity calculations, the molecular weight used included the weight of a chloride counterion and a water molecule of hydration.

Color Analyses. Color parameters (Hunter CIEL*ch) were obtained with a ColorQuest Hunter colorimeter (HunterLab, Hunter Associates Laboratories Inc., Reston, VA). Solutions containing purified anthocyanins were placed in a 1-cm path length optical glass cell (Hellma, Germany), and CIEL*ch values along with percent haze were measured in duplicate in the total transmission mode using Illuminant C and 10° observer angle.

RESULTS AND DISCUSSION

Molar Absorptivity and Spectral Characteristics. The use of semipreparative HPLC allowed for collection and purification of considerable amounts of individual pelargonidin derivatives, ranging from 8 to 65 mg for pg derivatives extracted from strawberries, radishes, and potatoes. Pigments obtained from the acid hydrolysis of radish anthocyanins were not produced in sufficient amounts to go through all purification steps and obtain reproducible weight measurements. Therefore, only spectral and color characteristics will be reported for pg-5-glu, pg-3-glu-5-glu, and pg-3-soph, and no absorptivity coefficients are reported for them. The purity of isolated pigments was checked by analytical HPLC, monitoring the chromatogram at 520, 310, and 280 nm, as well as spectral characteristics. A high purity of pigments isolated was obtained, and the isolated pigments accounted for more than 95% of the total peak area in the 280 nm chromatogram: 100% for the aglycon, 99−100% for non-acylated pg derivatives, and 96−99% for acylated pg derivatives, with small contamination from the other closely eluting acylated pigments.

The absorptivity is constant for a particular compound and is more commonly expressed as the molar absorptivity at an absorption band maximum (Kemp, 1991). The calculated absorption coefficients (Table 1) were expressed as molar absorptivities and as the absorption of a 1% solution (E 1%).

The solvent system and the presence of sugars and acylating groups played an important role on the absorption coefficients of pg derivatives.

Effect of Solvent System. Spectral characteristics as well as the molar absorptivity were recorded in two

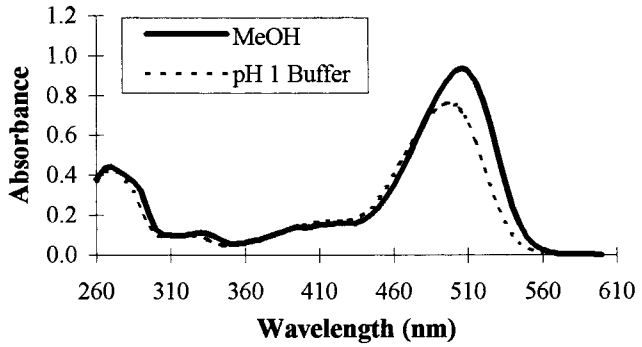

Figure 1. UV−vis spectra of pg-3-soph-5-glu in methanolic (0.1% HCl in MeOH) and aqueous (pH 1.0 buffer) solutions.

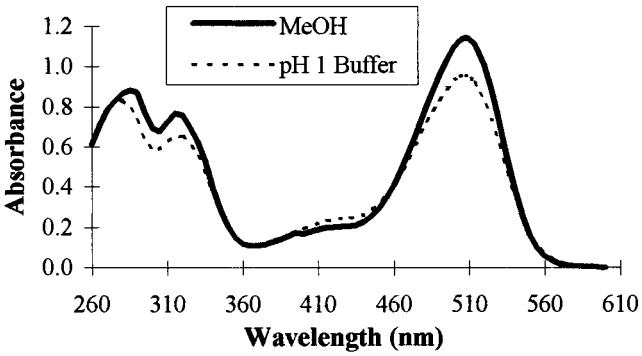

Figure 2. UV−vis spectra of pg-3-soph-5-glu acylated with *p*-coumaric and malonic acids in methanolic (0.1% HCl in MeOH) and aqueous (pH 1.0 buffer) solutions.

different solvent systems. Pigment purity, concentration, and pH were exactly the same in the aqueous (pH 1.0 buffer) and alcoholic (0.1% HCl in methanol) solutions for each individual pigment, permitting analysis of the effect of the solvent system without interference from other confounding effects.

The position and intensity of an absorption band may shift when the spectrum is recorded in different solvents. The use of a solvent with increased polarity may cause bathochromic or hypsochromic changes in absorption bands, and these changes are dependent on the nature of the chemical group and the molecular orbitals involved (Kemp, 1991). Absorption coefficients obtained for pg derivatives were consistently higher in acidified methanol than in the pH 1.0 buffer solutions, on the order of 20% higher in all cases except for the aglycon where the difference was only 7%.

Solvent effects were dependent on the pigment chemical structure. Non-acylated pigments showed the highest solvent effect on spectral characteristics, with a drastic shift not only on the dimension of the molar absorptivity but also with a marked change in the λ_{max}. In acidified methanol, absorption at the 490−520 nm band was substantially higher than in aqueous solution. Methanol also caused a bathochromic shift of about 10 nm in λ_{max} as compared to the same pigment in buffer solution (Figure 1). All acylated pigments showed increased absorptivity in all major absorption bands, the 280, 320, and 500 nm regions (Figure 2), when they were dissolved in acidified methanol. However, a decrease in absorption was observed in the 400−440 nm region, decreasing the $A_{400-440}/A_{max}$ ratio. Only a small bathochromic shift was observed on the 280 nm band due to the solvent system. No shifts were observed on λ_{max} for the other bands, and this was consistent for all acylated derivatives (Table 1 and Figure 2).

The effects of the solvent system on anthocyanins will determine the quaternary structure of the molecule, and it is believed to have a strong impact on the color of the primary, secondary, or tertiary structures (Brouillard and Dangles, 1993).

Effect of Glycosylation and Acylation. The lowest molar absorptivity coefficients (Table 1) obtained corresponded to the pg aglycon and pg-3-glu, with values for methanol and buffer systems that were almost one-half of those obtained with the other pg derivatives. However, it is important to highlight that the absorptivity of the aglycon was almost twice as much as that of the other pigments when they were compared on a weight basis instead of a molar one. Other molar absorptivity coefficient values reported in the literature are presented in Table 2.

Glycosylation at the 3 and 5 positions of the anthocyanidin molecule has an important impact on the spectral characteristics, and extensive research has been published in that area (Harborne, 1967; Hong and Wrolstad, 1990; Giusti and Wrolstad, 1996). Anthocyanins with glycosidic substitutions at position 3 only exhibit a ratio of absorbance at 400−440 nm to the absorbance at the visible λ_{max} that is almost twice as much as that of anthocyanins with glycosidic substitution at position 5 or both positions 3 and 5 (Table 3).

Inconsistent information is available with respect to the role of sugars on anthocyanin absorptivity and color characteristics. The nature of the glycosyl group has been reported (Jackman and Smith, 1996) as having no apparent influence in anthocyanin coloration, although increased glycosyl substitution and/or substitution at the C-7 hydroxyl is believed to increase color intensity. It has also been reported (Figueiredo et al., 1996a,b; Elhabiri et al., 1995) that molecules possessing a disaccharide as a substituent group in position 3 of the chromophore exhibit a large drop in their absorptivity. Cyanidin-3,5-triglycoside, delphinidin-3-gentiobioside, and cyanidin-3-rutinoside showed a marked hypochromic effect when compared to the corresponding 3-glucosides and 3,5-diglucosides with a similar substitution pattern (Figueiredo et al., 1996a,b; Elhabiri et al., 1995).

In this study, we found a slight drop in the molar absorptivity when one sugar was present, as compared to the aglycon. The addition of more glucose units to the molecule seemed to have a hyperchromic effect of the pigment, since pg-3-soph-5-glu showed a molar absorptivity substantially higher than the corresponding monoglucoside.

When comparing the values obtained with other values reported in the literature (Table 2), we must consider that most of the values available dated from the 1970s or before and that the analytical techniques have advanced throughout the years. Difficulties of preparing crystalline anthocyanin, free from impurities, in sufficient quantities to allow reliable weighing under optimal conditions (Fuleki and Francis, 1968; Francis, 1982) have limited the consistency of the reported data. The more common effect of the presence of impurities in the sample would be an underestimation of the absorptivity coefficient due to the presence of compounds that contribute to the weight but not to the color. Impurities, however, may also have a hyperchromic effect on anthocyanins due to copigmentation of anthocyanins with other noncolored phenolic compounds (Lenoble et al., 1996; Mazza and Brouillard, 1990; Goto and Kondo, 1991), causing an overestimation of the

Table 2. Reported Molar Absorptivity of Anthocyanins[a]

anthocyanin	MW	solvent system	λ_{max} (nm)	ϵ mol	ref
Pg	324.5[b]	0.1% HCl in EtOH	504.5	17800	Schou, 1927
Pg-3-glu	486.5[b]	1% HCl in H_2O	496	27300	Jorgensen and Geissman, 1955
	433			36600	Wrolstad et al., 1970
		1% HCl	513	22390	Swain, 1965
		1% HCl in MeOH	516	31620	Swain, 1965
Pg-3,5-diglu		HCl in MeOH	510	32360	Swain, 1965
Pg-3-soph-5-glu		aqueous pH 0.8	498	18000−20000	Dangles et al., 1993
caffeoyl derivatives of Pg-3-soph-5-glu		aqueous pH 0.8	498	18000−20000	Dangles et al., 1993
Pg-3-(dicaffeoylglu)-soph-5-glu		aqueous pH 0.8	512	28000	Dangles et al., 1993
Cy	340.5[b]	0.1% HCl in EtOH	510.5	24600	Schou, 1927
	340.5[b]	0.1% HCl in EtOH	547	34700	Ribereau-Gayon, 1959
Cy-3-glu		aqueous buffer pH 1	510	26900	Jurd and Asen, 1966[c]
	449	0.1 N HCl	520	25740	McClure, 1967
		10% EtOH pH 1.5	512	18800	Heredia et al., 1998
	502.5[b]	1% HCl in MeOH	530	34300	Siegelman and Hendricks, 1958
Cy-3-gal	502.5[b]	0.1% HCl in MeOH	530	34300	Siegelman and Hendricks, 1958
	502.5[b]	0.1 N HCl:EtOH (15:85)	535	44900	Sakamura and Francis, 1961
	502.5[b]	0.1 N HCl:EtOH (15:85)	535	46200	Zapsalis and Francis, 1965
	502.5[b]	0.1 N HCl:EtOH (15:85)	535	46230	Fuleki and Francis, 1968
		HCl in MeOH	530	30200	Swain, 1965
Cy-3-ara	472.5[b]	0.1 N HCl:EtOH (15:85)	538	44400	Zapsalis and Francis, 1965
	472.5[b]	0.1 N HCl:EtOH (15:85)	535	44460	Fuleki and Francis, 1968
Cy-3,5-diglu		0.1 N HCl	520	30175	Niketic-Aleksic and Hrazdina, 1972
		methanolic HCl	508.5	35000	Brouillard and El Hague Chahine, 1980
Cy-3-rut		aqueous pH 0.9	510	7000	Figueiredo et al., 1996a
		1% HCl	523	28840	Swain, 1965
Cy-3-soph-5-glu		methanolic HCl	524	37150	Hrazdina et al., 1977
Cy-3-soph-5-glu + malonic		methanolic HCl	528	32360	Hrazdina et al., 1977
Cy-3-soph-5-glu + sinapic		methanolic HCl	528	37150	Hrazdina et al., 1977
Cy-3-soph-5-glu + di-sinapic		methanolic HCl	530	38020	Hrazdina et al., 1977
Cy-3-soph-5-glu + ferulic		methanolic HCl	528	32360	Hrazdina et al., 1977
Cy-3-soph-5-glu + di-ferulic		methanolic HCl	530	34670	Hrazdina et al., 1977
Cy-3-soph-5-glu + p-coumaric		methanolic HCl	526	38020	Hrazdina et al., 1977
Cy-3-soph-5-glu + di-p-coumaric		methanolic HCl	528	32360	Hrazdina et al., 1977
Cy-3-sam-5-glu		aqueous pH 0.9	522	3600	Figueiredo et al., 1996a
Cy-3-sam-5-glu + sinapic + ferulic		aqueous pH 0.9	528	15100	Figueiredo et al., 1996a
Cy-3-sam-5-glu + sinapic + p-coum + malonic		aqueous pH 0.9	536	19000	Figueiredo et al., 1996a
Cy-3-sam-5-glu + sinapic + caffeic + malonic		aqueous pH 0.9	538	21200	Figueiredo et al., 1996a
Cy-3-sam-5-glu + sinapic + ferulic + malonic		aqueous pH 0.9	538	20100	Figueiredo et al., 1996a
Pn	354.5[b]	0.1% HCl in EtOH	511	37200	Schou, 1927
	354.5[b]	0.1 N HCl:EtOH (15:85)	532	40800	Sakamura et al., 1961
Pn-3-glu	516.5[b]	0.1% HCl in MeOH	536	11300	Somers, 1966
		10% EtOH pH 1.5	512	14100	Heredia et al., 1998
Pn-3-gal	516.5[b]	0.1 N HCl:EtOH (15:85)	532	48400	Sakamura et al., 1961
	516.5[b]	0.1 N HCl:EtOH (15:85)	532	48400	Zapsalis and Francis, 1965
	516.5[b]	0.1 N HCl:EtOH (15:85)	531	48340	Fuleki and Francis, 1968
Pn-3-ara	486.5[b]	0.1 N HCl:EtOH (15:85)	532	46100	Zapsalis and Francis, 1965
	486.5[b]	0.1 N HCl:EtOH (15:85)	532	46070	Fuleki and Francis, 1968
Pn-3,5-diglu		0.1 N HCl	520	36654	Niketic-Aleksic and Hrazdina, 1972
Dp	356.5[b]	0.1% HCl in EtOH	522.5	34700	Schou, 1927
Dp-3-glu	518.5[b]	1% HCl in MeOH	543	29000	Asen et al., 1959
		10% EtOH pH 1.5	520	23700	Heredia et al., 1998
Pt-3-glu	532.5[b]	0.1% HCl in MeOH	546	12900	Somers, 1966
		10% EtOH pH 1.5	520	18900	Heredia et al., 1998
Pt-3,5-diglu		0.1 N HCl	520	33040	Niketic-Aleksic and Hrazdina, 1972
		HCl in MeOH	535	23440	Swain, 1965
Mv	400.5[b]	0.1% HCl in EtOH	520	37200	Schou, 1927
	400.5[b]	0.1% HCl in EtOH	557	36200	Ribereau-Gayon, 1959
Mv-3-glu	562.5[b]	0.1% HCl in MeOH	546	13900	Somers, 1966
	562.5[b]	0.1% HCl in MeOH	538	29500	Koeppen and Basson, 1966
	529	0.1 N HCl	520	28000	Niketic-Aleksic and Hrazdina, 1972
	529	methanol pH 1.0	535	36400	Metivier et al., 1980
		10% EtOH pH 1.5	520	20200	Heredia et al., 1998
Mv-3,5-diglu	724.5[b]	0.1% HCl in EtOH	519	10700	Schou, 1927
	724.5[b]	0.1% HCl in EtOH	545	10300	Ribereau-Gayon, 1959
		0.1 N HCl	520	37700	Niketic-Aleksic and Hrazdina, 1972
Mv-3-glu + p-coum	718.5[b]	0.1% HCl in MeOH	536	30200	Koeppen et al., 1966

[a] E mol, molar absorptivity; Pg, pelargonidin; Cy, cyadinin; Pn, peonidin; Dp, delphinidin; Pt, petunidin; Mv, malvidin; glu, glucoside; soph, sophoroside; gal, galactoside; ara, arabinoside; rut, rutinoside; sam, sambubioside. [b] MW includes the weight of one chloride counterion and a water molecule of hydration. [c] Calculated from data reported by Jurd and Asen (1966).

absorptivity coefficients. It is interesting that most of the values reported more recently (Brouillard and El Hache Chahine, 1980; Dangles et al., 1993; Figueiredo et al., 1996a) are more conservative (35 000 or lower) than many of the values reported in the 1960s, >40 000. The values we report for pg aglycon are very comparable to those reported by Schou in 1927 (Table 2). However, the absorptivity coefficients obtained for pg-3-glu were

Table 3. Color Attributes of Purified Non-Acylated Pg Derivatives

anthocyanin	λ_{max}	λ_{max} 400−440	$A_{440-440}/A_{max}$	L^*	hue	chroma	haze
Pg aglycon	513	423	0.38	81.4	18.8	35.7	0.9
Pg-5-glu	509	407	0.18	83.6	26.8	35.9	1.2
Pg-3-glu	503	429	0.45	83.6	38.0	34.5	0.9
Pg-3-soph	503	427	0.46	82.9	37.4	35.8	1.1
Pg-3-glu-5-glu	500	417	0.2	85.1	41.3	35.4	1.0
Pg-3-soph-5-glu	500	417	0.22	85.0	40.7	35.3	1.0

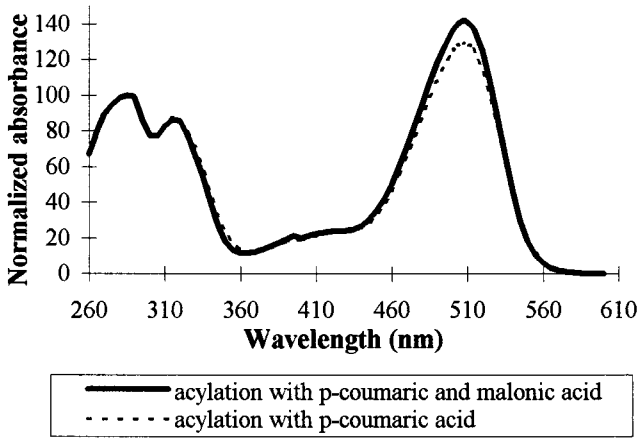

Figure 3. Effect of acylation with malonic acid on spectral characteristics of acylated pg-3-soph-5-glu.

lower than values previously reported (Jorgensen et al., 1955; Swain, 1965), while the molar absorptivity of pg-3-soph-5-glu in aqueous solution (25 370) was higher than the previously reported one (Dangles et al., 1993).

It has been reported as a general characteristic that an increase in the number of substitutions is accompanied by an increase in the molar absorptivity of the flavylium cation form when compared to their parent mono- and diglucosides (Figueiredo et al., 1996b). Our results showed a general hyperchromic effect of acylation of pg derivatives as compared to the parent pg-3-soph-5-glu, in particular, when 2 acylating groups were present. In the case of pg-3-rut-5-glu acylated with *p*-coumaric acid, we also obtained a relatively large molar absorptivity (Table 1) but the molar absorptivity of the parent non-acylated compound was not calculated. When comparing the effect of the different acylating groups, *p*-coumaric and malonic acids seemed to contribute to a greater extent to the absorptivity in the visible band than ferulic acid. The effect of malonic acid addition on spectral characteristic is shown in Figure 3. The absorbance was normalized at the 280 nm band to compare the effect of malonic acid acylation on the visible range. No shift in λ_{max} was observed as a result of the presence of malonic acid, the main spectral difference being the higher absorption of the major visible band. A similar effect was obtained with additional acylation with malonic acid on the ferulic acid acylated pg derivative.

Color Characteristics. For comparison of color characteristics of the same pigment in different solvent, the weight and molar concentrations were exactly the same. However, when making comparisons among the different pigments, we need to consider that they were prepared under different concentrations. The color of non-acylated pg derivatives presented in Table 3 uses a uniform level of chroma since no quantitation of those pigments was possible due to the low amounts available. Color characteristics presented in Table 4 use similar molar concentrations, between 1.5×10^{-5} and 2.7×10^{-5} mol/L, which correspond to concentrations ranging from 5 to 29 mg/L. An additional data point is included for a higher pigment concentration of pg-3-soph-5-glu only to illustrate the effect of pigment concentration on color characteristics. It is also important to mention that extensive work is available on spectral characteristics of anthocyanins, and color inferences have been made based on these characteristics. However, little has been reported on how color parameters correlate to those characteristics.

Effect of Solvent System. The solvent system had a clear impact on color characteristics of all pg derivatives. In most cases, the effect of the methanolic solution was not only hyperchromic, with a higher chroma, but also produced lower hue values (Table 4). Solvent effect was more marked on non-acylated pigments and on more diluted solutions. No sensory analysis was performed, but visual observations were recorded, and the differences registered by ColorQuest on non-acylated pg derivatives were clearly evidenced on the visual appearance of the solutions. We also found solvent effects on the color characteristics of acylated pigments (Table 4), but they were not large enough to produce noticeable visual differences.

Effect of Glycosylation and Acylation. For comparisons of the effect of glycosidic substitution on the color of pg derivatives, we used dilutions of the different pigments at the same level of chroma. These pigments were obtained from partial hydrolysis of acylated pg-3-soph-5-glu, and due to the limited amounts purified, they were only analyzed in one solvent, the mobile phase from HPLC (15% acetonitrile, 10% acetic acid, and 1% phosphoric acid in water).

Table 3 shows the increasing trend in hue obtained with an increasing number of glucose units. The aglycon was the pigment with the lowest hue, in the red region. The position of glycosylation also had an impact on hue, when compared at the same level of chroma: pg-3-glu and pg-3-di-glu (pg-3-soph) showed lower hue than the corresponding pg-5-glu and pg-3,5-di-glu, respectively. Pg derivatives with glycosidic substitution at both the 3 and 5 positions of the pyrylium ring showed the highest hue level, with a yellowish appearance.

The ability of flavonoids to associate with numerous biological molecules is of theoretical and practical importance. Anthocyanins are know to form molecular complexes with other anthocyanins (self-association), with other colorless compounds (intermolecular copigmentation), and, in the case of acylated anthocyanins, intramolecular copigmentation (Brouillard and Dangles, 1993). Anthocyanin copigmentation accounts for the diversity of colors that are produced by anthocyanin-containing solutions as well as for color stabilization. Anthocyanin-3-glucosides have been reported to exhibit weaker self-association as compared to the 3,5-diglucosides, pointing out the importance of the residue at the 5-position in self-association. Hoshino and co-workers (1981) reported bathochromic and hypsochromic shifts in the visible absorption band with various anthocyanins and suggested vertical stacking in a right- or left-handed screw axis.

A comparison between pg-3-soph-5-glu acylated with *p*-coumaric acid and pg-3-rut-5-glu acylated with *p*-coumaric acid illustrates the effect of the nature of the glycosidic substitution and position of acylation. Both pigments are triglycosylated, with a disaccharide at

Table 4. Color Attributes of Purified Pg Derivatives

anthocyanin	conc. mg/L	conc. (mol/L) × 10^{-5}	MW	MeOH			buffer		
				L^*	hue	chroma	L^*	hue	chroma
Pg aglycon	5.0	1.54	271.8	87.1	357.3	22.1	90.0	22.7	16.7
Pg-3-glu	8.56	1.76	433.2	88.1	17.6	20.2	90.3	44.0	17.6
Pg-3-soph-5-glu	15	1.85	757	91.8	15.5	13.5	90.0	41.0	20.3
	24.2	2.98	757	78.4	39.2	53.4	81.8	56.0	53.5
Pg-3-soph-5-glu + p-coumaric	16.6	1.73	903	85.3	16.4	30.3	86.4	23.3	26.8
Pg-3-soph-5-glu + ferulic	27.2	2.76	933	82.8	19.5	36.5	83.4	24.1	33.7
Pg-3-soph-5-glu + p-coumaric & malonic	25.6	2.46	989	83.4	18.3	35.4	86.8	21.7	25.9
Pg-3-soph-5-glu + ferulic & malonic	29	2.70	1019	82.6	20.5	37.0	83.1	22.1	33.9
Pg-3-rut-5-glu + p-coumaric	18.3	1.94	887.4	87.2	11.0	25.7	87.3	23.1	24.8

position 3 of the pyrylium ring, and both are acylated with *p*-coumaric acid. However, the difference in the nature of the sugar (sophorose vs rutinose) and a difference in the position where the cinnamic acid is attached to the sugar are enough to impart different color characteristics to the molecule. Pg-3-rut-5-glu showed lower chroma in both solvent systems, even though the molar absorptivity was higher. It also showed lower hue in methanolic solution.

The bathochromic shift caused by cinnamic acid acylation translated to higher chroma for a similar molar concentration of pigment (Table 4) in the two different solvent systems evaluated. Hue angle was clearly decreased by acylation in the aqueous buffer solutions, going from the orange-yellow hue to a more reddish color, consistent with the bathochromic shift at the visible band. Lightness of the samples decreased with addition of cinnamic acid acylation to the anthocyanin pigment, also consistent with the increased absorptivity obtained. The addition of a malonic acid acylation had little effect in chroma and lightness. However, the addition of malonic acid acylation had an opposite effect on the hue angle in the different solvent systems, increasing the hue in the methanolic solution and decreasing it in the aqueous buffer solution, which results in closer hue values between the two solvent systems. Therefore, pigments acylated with malonic acid did not show much of a solvent effect on color attributes.

Several studies (Brouillard, 1981; Goto, 1987; Goto and Kondo, 1991; Figueiredo et al., 1996a,b) have suggested that intramolecular copigmentation or interaction within anthocyanins may play an important role in the increased stability of acylated anthocyanins. The flexible saccharide chains can act as linkers, allowing the folding of the acyl aromatic rings over the planar pyrylium ring. Formation of a sandwich-type complex has been proposed for anthocyanins with two cinnamic acid acylating groups. This stacking phenomenon exerts a protective effect on anthocyanins but also contributes to the color stabilization of the system. Cinnamic acid residues stack parallel with the anthocyanidin nucleus protecting the chromophore against water nucleophilic attack (Brouillard and Dangles, 1993). Copigmentation has also been reported to have an impact on the anthocyanin spectral characteristics (Mazza and Brouillard, 1990), causing a hyperchromic and bathochromic effect, increasing the color intensity as well as λ_{max} of the pigment. No clear mechanism has been proposed for the intramolecular association of aliphatic acid acylation, but the zwitterionic character of the pigments would make likely an ionic attraction between the positively charged portion of the oxonium form and the negatively charged free carboxylic group from malonic acid.

CONCLUSIONS

Small differences in anthocyanin chemical structure can have a critical impact on color and tinctorial strength of anthocyanin extracts. The nature and position of the glycosylation and acylation played a role in spectral and color characteristics. Findings also showed that the environment where anthocyanins are dissolved need to be considered when quantifying and characterizing anthocyanins.

ABBREVIATIONS USED

pg, pelargonidin; cy, cyanidin; glu, glucoside; gly, glycoside; rut, rutinoside; soph, sophoroside; HPLC, high-performance liquid chromatography.

ACKNOWLEDGMENT

We thank Robert Durst for technical assistance. This is technical paper no. 11425 from the Oregon Agricultural Experiment Station.

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Received for review November 23, 1998. Revised manuscript received August 19, 1999. Accepted August 31, 1999. This work was supported by Grant No. 94-37500-0808 from the USDA NRI Competitive Grants Program and by the Oregon Sweet Cherry Commission and the Northwest Cherry Briners Association.

JF981271K