

Color Stability of Commercial Anthocyanin-Based Extracts in Relation to the Phenolic Composition. Protective Effects by Intra- and Intermolecular Copigmentation

C. Malien-Aubert,^{†,‡} O. Dangles,[‡] and M. J. Amiot^{*,‡}

CHR-Hansen-SEFCAL, 30760 Saint Julien de Peyrolas, France, and UMR INRA-UAPV, Qualité et Sécurité des Aliments d'Origine Végétale, Site Agroparc, 84914 Avignon Cedex 9, France

Anthocyanin extracts are increasingly used as food ingredients. A current challenge is to maintain their color properties. The stability of some colorants has been studied in sugar and non-sugar drink models at three pH values (3, 4, and 5) under thermal and light conditions simulating rapid food aging. At a given pH, color stability mainly depends on the structures of anthocyanins and of colorless phenolic compounds. Colorants rich in acylated anthocyanins (purple carrot, red radish, and red cabbage) display great stability due to intramolecular copigmentation. The protection of red chromophore is higher for diacylated anthocyanins in red radish and red cabbage. For colorants without acylated anthocyanins (grape-marc, elderberry, black currant, and chokeberry), intermolecular copigmentation plays a key role in color protection. Colorants rich in flavonols and with the highest copigment/pigment ratio show a remarkable stability. By contrast, catechins appear to have a negative effect on red colorants, quickly turning yellowish in drink models. This effect is more pronounced when the pH is increased. Finally, color does not seem to be greatly influenced by the addition of sugar.

Keywords: *Colorants; anthocyanins; flavonols; flavanols; phenolic acid; CIELAB; spectrophotometry; HPLC; diode array detection; color stability; copigment effect*

INTRODUCTION

Synthetic food colorants are steadily replaced by natural ones because of consumer preferences. Anthocyanins (polyphenolic pigments) are natural, water-soluble, nontoxic pigments displaying a variety of colors from orange to blue. Because of their antioxidant properties, they may also have a beneficial influence on human health (Shenoy, 1993). Anthocyanin-rich extracts from fruits and vegetables can be used as food colorants. Until now, grape-marc has constituted the main industrial source of anthocyanin-based colorants, commercially produced in Italy since 1879 (Girard and Mazza, 1998). With an annual world production of ~65 million tons, grape-marc constitutes a very abundant, relatively inexpensive source of anthocyanins (Francis, 1989; Timberlake, 1986; Mazza, 1995). Other fruits such as elderberries, black currants, or chokeberries have also been used as sources of red colorants for food products and acidic beverages. For instance, elderberry is cultivated in Denmark and Poland as a colorant in fruit juices and soups (Brønnum-Hansen et al., 1985).

The main drawback in the use of anthocyanins as food colorants is their low stability. In fact, the color stability of anthocyanins depends on a combination of factors: structure and concentration of anthocyanins, pH, temperature, and presence of complexing agents (phenols, metal ions) (Markakis, 1982). Recent investigations (Davies et al., 1993; Brouillard et al., 1989; Mazza and

Brouillard, 1990) suggest that the molecular complexation of anthocyanins with other phenols—also named copigments—is the main color-stabilizing mechanism in plants. Copigmentation consists of the stacking of the copigment molecule on the planar polarizable nuclei of the anthocyanin-colored forms (flavylium ion, quinonoid forms). Thereby, the nucleophilic attack of water at position 2 of the pyrylium nucleus, which leads to colorless hemiketal and chalcone forms, is at least partially prevented (Figure 1). Consequently, at a given pH, anthocyanin solutions are more intensely colored (hyperchromic effect). In addition, the wavelength of maximum absorbance within the visible spectrum of the anthocyanin–copigment complex is higher than that of the free anthocyanin (Asen et al., 1972; Dangles, 1997). This bathochromic shift probably points to the fact that the copigment molecule interacts with the anthocyanin in its first excited state (electronic transition in the visible range) more strongly than with the ground-state anthocyanin (Alluis et al., 2000). Copigmentation is a widespread phenomenon in nature and can occur in fruit-derived products such as juices and wines (Mazza and Brouillard, 1987). Copigmentation is most efficient when the pigment and copigment moieties are covalently linked via a suitable spacer. Such is the case in anthocyanins, the sugar residues of which are acylated (most often on their primary 6-OH group) by phenolic acids. Within these acylated pigments, copigmentation consists of a conformational folding of the aromatic acyl group(s) on the flavylium nucleus (intramolecular copigmentation) (Dangles et al., 1993). Acylated anthocyanins are particularly promising food colorants because of their greatly improved color stability in the 4–5 pH range, where nonacylated anthocya-

* Author to whom correspondence should be addressed [telephone (33) 4 32 72 24 98; fax (33) 4 32 72 24 92; e-mail amiot@avignon.inra.fr].

[†] CHR-Hansen-SEFCAL.

[‡] UMR INRA-UAPV.

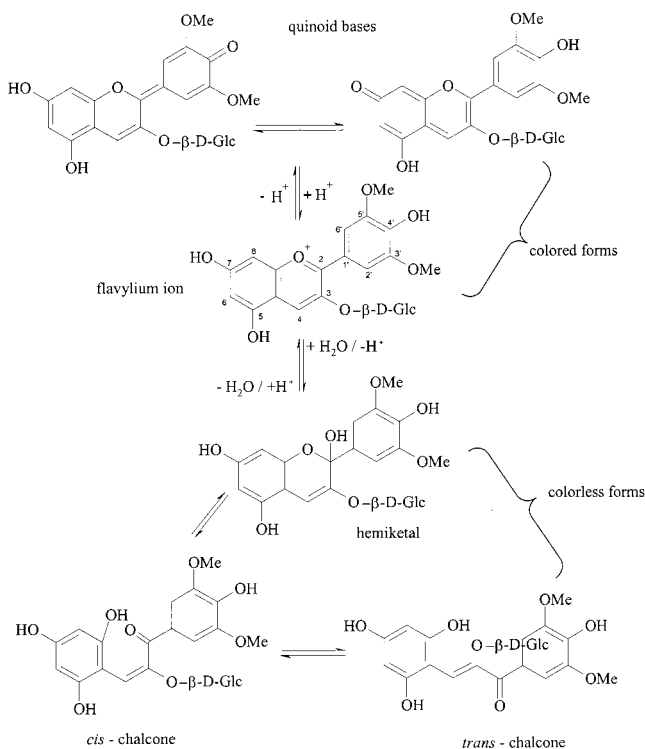


Figure 1. Structural transformations of flavylium ions (malvidin 3-glucoside or oenin) in mildly acidic aqueous solutions.

nins are nearly colorless. In the food industry, likely sources of acylated anthocyanins are *Tradescantia pallida*, *Zebrina pendula*, radish, purple carrot, and red cabbage.

The aim of this work is to describe the phenolic composition of anthocyanin-rich extracts and its influence on color stability. The stability of colorants will be investigated at different pH values (3–5) under heating or light irradiation, in sugar and nonsugar drink models. Comparison will be made between colorants containing nonacylated anthocyanins and acylated ones. Specific attention is given to grape-marc colorants. The role of various phenolic molecules or classes in the stability of anthocyanin-based colorants is discussed, and some protection mechanisms by intermolecular and intramolecular copigmentation are proposed.

MATERIALS AND METHODS

Phenolic Composition. Colorants were obtained from commercially available sources. Liquid extracts from four different grape-marcs (*Vitis vinifera*), elderberry (*Sambucus nigra*), and purple carrot (*Daucus carota*), as well as extracts from red cabbage (*Brassica oleracea*), red radish (*Raphanus sativus*), and black currant (*Ribes nigrum*) in powder form were provided by CHR-Hansen (Saint Julien de Peyrolas, France). Chokeberry (*Aronia melanocarpa*) liquid extract was provided by Wild Co. (Germany). Each colorant was accurately weighed (0.5 g) and dissolved in 0.3% aqueous HCl (25 mL). Three extractions were carried out with ethyl acetate. The organic phases were collected, subsequently filtered through a Whatman 1PS filter, and concentrated under vacuum at 38 °C. The dry residue mainly containing the colorless phenolic copigments was dissolved in methanol (3 mL) and stored at –10 °C. The anthocyanin fraction (aqueous phase) was stored at 4 °C. Two repetitions were performed for each colorant.

Determination of the phenolic composition was performed using an HPLC linked to a diode array detector (Hewlett-Packard Vectra 436/33N). Anthocyanins were separated on a 5 μm Lichrosorb RP18 (Alltech) column (250 × 4.6 mm i.d.)

thermostated at 35 °C. 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.

Colorless phenols were eluted on a 5 μm Alltima C18 (Alltech) column (150 × 4.6 mm i.d.) with a combination of 2% aqueous acetic acid (A) and methanol (B) (flow rate = 0.8 mL/min, 35 °C). The elution conditions were as follows: from 0 to 7% B over 5 min, 7% B over 13 min, from 7 to 10% B over 2 min, 10% B over 3 min, from 10 to 14% B over 2 min, 14% B over 5 min, from 14 to 20% B over 2 min, 20% B over 3 min, from 20 to 45% B over 25 min, 45% B over 15 min, from 45 to 100% B over 5 min, 100% B over 20 min. UV-visible spectra were recorded from 250 to 600 nm. This analytical method was adapted from the HPLC method described by Gao and Mazza (1994). Quantification was performed by external calibration at 520 nm for anthocyanins, at 280 nm for flavanols (monomers and oligomers), and at 320 nm for phenolic esters and flavonols. Results were expressed in milligrams per gram of colorant (liquid or powder) in equivalents of cyanin (Extrasynthèse) for anthocyanins (acylated and nonacylated) and in equivalents of (+)-catechin (Sigma) for flavanols. Concerning phenolic acids, hydroxybenzoic, hydroxycinnamic, and dihydroxycinnamic acids and esters were quantified using gallic acid (Prolabo), *p*-coumaric acid (Extrasynthèse), and caffeic acid (Sigma), respectively. Finally, glycosides of kaempferol and quercetin (flavonols) were determined using kaempferol 3-glucoside (Extrasynthèse) and quercetin 3-rutinoside (Sigma), respectively. HPLC quantification was twice carried out. The variability including extraction and HPLC quantification was <4% for all of the colorants studied.

Thermal and Photochemical Stability. Color stability was studied in sugar drink (100 g/L sucrose, 500 g/L citric acid) and nonsugar drink models at three pH values (3, 4, and 5) using McIlvaine buffers. Colorant concentrations were adjusted so as to develop an initial absorbance at 520 nm of 1.0 (±0.1). For thermal stability, colorant solutions (10 mL) in flask tubes closed with screw caps were placed in a hot air steam cabinet at 50 °C. Each tube was used for one spectral measurement only so as to minimize the contact with oxygen. For photochemical stability, colorant solutions (10 mL) in flask tubes, filled to the top and closed with screw caps, were placed under visible light (Mazda lamp, type MA 400W, E8) in a room at 32 °C, especially used to study food aging under light (CTCPA in Avignon, France).

Spectral and Colorimetric Measurements. Absorbance at 520 nm and brown index (BI) (expressed as the absorbance ratio at 430 nm by that at 520 nm) were estimated using a UV-vis Varian spectrophotometer (Cary 1E). Results were the average of three measurements. The variability was <2% for all of the colorants studied. The L^* , a^* , and b^* values (CIE 1976) were determined using a spectrophotometer (Minolta CR 1000, color space CIELAB). The colorimetric coordinates of the colorant solutions were computed in the CIELAB scale in a CIE D65/10° illuminant/observer condition. In this scale, each color is numerically specified by a unique set of three cylindrical coordinates: L^* for the light, C^* for the metric chroma (the correlate of saturation), and H^* for the hue angle (the correlate of chromatic tonality). C^* and H^* were calculated by the transformation of the a^* and b^* Cartesian coordinates into polar ones according to the following equations: $C^* = (a^{*2} + b^{*2})^{1/2}$ and $H^* = \tan^{-1}(b^*/a^*)$. On the chromatic circle, H^* values are stepped from 0 to 360° (magenta red) across a continuously fading hue circle, the other reference values of which are 90° (yellow), 180° (bluish green), and 270° (or –90°) (blue).

RESULTS

Phenolic Composition and Coloring Properties of Extracts from Different Sources. Great differences in phenolic composition were found among the seven colorants (Table 1). Red cabbage and red radish were both rich in total phenolic compounds (435 and

Table 1. Phenolic Composition of Natural Colorants Expressed as Milligrams per Gram of Colorant and Color Data (Chroma C^* and Hue Angle H^p) at pH 4 in a Nonsugar Drink Model

phenolic subgroup sources	acylated anthocyanins	nonacylated anthocyanins	phenolic acids and esters	monomeric catechins	oligomeric catechins	flavonols	color data	
							C^*	H^p
red cabbage	141.9	61.5	105.4	69.4	54.2	2.8	20.4	-17.0
red radish	116.1	0.0	116.5	0.0	0.0	0.0	25.7	0.3
purple carrot	6.4	2.4	17.4	0.0	0.0	0.0	25.6	2.3
elderberry	0.0	37.5	19.6	5.5	10.4	14.6	16.2	23.3
black currant	0.0	21.8	13.2	3.5	6.7	7.3	18.1	18.2
chokeberry	0.0	6.3	19.9	0.0	1.0	3.6	20.1	19.8
grape-marc	0.0	0.3	3.7	0.7	1.6	7.5	16.3	8.5

233 mg/g, respectively), 50% of which were anthocyanins (acylated and nonacylated). In red radish, only acylated anthocyanins and phenolic acids or esters were found. These two phenolic groups were also mainly present in purple carrot. The major difference between purple carrot and red radish was the structure of their anthocyanins, although purple carrot also displayed lower anthocyanin concentrations. For purple carrot, the main pigments derived from cyanidin and for red radish from pelargonidin, as reported by Harborne and Grayer (1988) and Fuleki (1969). Cyanidin derivatives, previously described by Hrazdina et al. (1977) and Idaka (1987), were also found in red cabbage. Beside phenolic acids or their esters, red cabbage also contained flavanols as monomers (69 mg/g of colorant) and as oligomers (54 mg/g of colorant), representing 28% of the total phenolic composition. In red cabbage, flavonols were minor compounds, whereas no flavonol was detected in red radish and purple carrot.

Among the colorants studied, four sources (elderberry, black currant, chokeberry, and grape-marc) were devoid of acylated anthocyanins. Elderberry was the richest source of both anthocyanins (37.5 mg/g) and colorless phenols (50.1 mg/g). In contrast, grape-marc was characterized by the lowest phenolic content and the presence of malvidin (in the form of different glycosides) as previously reported by Bakker and Timberlake (1985). Taking into account the very low content in grape-marc, and because grape-marc is a byproduct of wine, no acylated anthocyanins were detected. Cyanidin (conjugated with different sugars) was the major aglycon in elderberry, chokeberry, and black currant. These results are in good agreement with those found by Brønnum-Hansen and Hansen (1983), Oszmianski and Sapis (1988), and Koeppen and Hermann (1977). Finally, delphinidin glycosides were identified in black currant as reported by Koeppen and Hermann (1977).

Considering the color data (Table 1), colorants rich in acylated anthocyanins were characterized by the highest C^* and the lowest H^p . Red cabbage had a negative value for H^p corresponding to a bluish hue. For all colorants rich in acylated anthocyanins (purple carrot, red radish, and red cabbage) hue angle decreased when the pH increased, confirming a bluing effect for these colorants (data not shown). By contrast, H^p was stable or even slightly higher (yellowish tonality) when the pH was raised in colorants containing only nonacylated anthocyanins.

Color Stability. Tables 2 and 3 show spectrophotometric data obtained in sugar and nonsugar drink models at three pH values submitted to storage at 50 °C. Results were expressed in terms of λ_{\max} and BI at time zero (t_0) and after 72 h. They were very similar in sugar and nonsugar drink models. At t_0 , the wavelength of maximum absorbance in the visible range (λ_{\max}) spread from 515 nm (chokeberry at pH 3) to 549 nm

Table 2. Spectral Characteristics of the Nonacylated Colorants during Storage at 50 °C in Two Drink Models at Three pH Values

colorant	pH	time (h)	sugar solution		nonsugar solution	
			λ_{\max}	BI	λ_{\max}	BI
grape-marc	3	0	526	0.57	528	0.61
		72	514	0.90	456	1.07
	4	0	531	0.67	527	0.65
		72	442	1.09	438	1.15
	5	0	530	0.66	529	0.64
		72	445	1.19	445	1.29
elderberry	3	0	518	0.65	517	0.65
		72	505	0.94	509	0.88
	4	0	523	0.81	522	0.81
		72	444	1.18	437	1.20
	5	0	528	0.76	529	0.78
		72	454	1.30	451	1.32
black currant	3	0	521	0.57	521	0.58
		72	450	1.06	438	1.07
	4	0	527	0.71	524	0.69
		72	449	1.29	436	1.41
	5	0	527	0.72	528	0.64
		72	452	1.34	451	1.56
chokeberry	3	0	515	0.47	514	0.47
		72	491	1.04	496	0.95
	4	0	516	0.56	516	0.56
		72	446	1.29	446	1.25
	5	0	519	0.61	518	0.59
		72	455	1.33	445	1.42

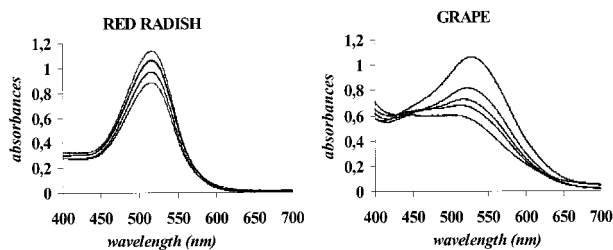
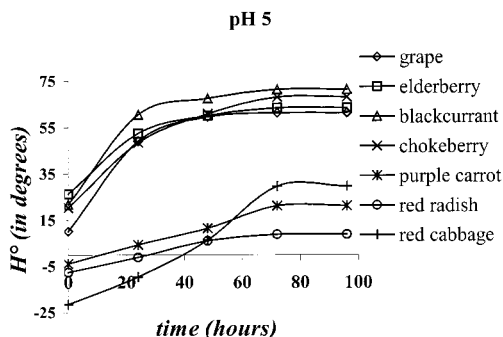
Table 3. Spectral Characteristics of the Acylated Colorants during Storage at 50 °C in Two Drink Models at Three pH Values

colorant	pH	time (h)	sugar solution		nonsugar solution	
			λ_{\max}	BI	λ_{\max}	BI
purple carrot	3	0	526	0.38	523	0.37
		72	524	0.41	525	0.41
	4	0	530	0.44	527	0.43
		72	527	0.54	527	0.55
	5	0	534	0.47	533	0.48
		72	531	0.67	531	0.70
red radish	3	0	517	0.29	516	0.27
		72	516	0.29	516	0.29
	4	0	522	0.34	523	0.30
		72	527	0.40	522	0.37
	5	0	529	0.36	530	0.32
		72	527	0.51	531	0.50
red cabbage	3	0	534	0.34	534	0.32
		72	534	0.38	532	0.37
	4	0	546	0.48	545	0.48
		72	544	0.64	543	0.67
	5	0	549	0.47	547	0.44
		72	548	0.84	544	0.97

(red cabbage at pH 5), which corresponds to a hue ranging from orange to purple. The initial BI was below 0.81 (elderberry, pH 4–5) for all of the colorants studied. After 72 h of storage at 50 °C, the colorants devoid of acylated anthocyanins (typically, grape) displayed λ_{\max}

Table 4. Industrial Characteristics and Phenolic Composition in Milligrams per Gram of Different Grape-marc Anthocyanin-Based Colorants in 1997 and 1998

grape	industrial characteristics				anthocyanic pigments				colorless copigments					ratio copigment/pigment	
	pH	Brix (%)	BI	SO ₂ (ppm)	glycosides of malvidin		glycosides of cyanidin	total	phenolic acids and esters	flavanols		flavonols	total		
					oenin	others				monomeric	oligomeric				
1997															
a	3.31	19	0.42	320	1.9	1.8	0.0	3.7	4.6	6.9	0.0	3.6	15.1	4.0	
b	3.25	35	0.56	1574	0.9	0.8	0.0	1.7	8.6	6.4	7.5	31.1	53.6	30.9	
c	3.53	24	0.48	979	1.8	3.2	0.0	5.0	9.3	16.1	14.0	14.8	54.2	10.7	
d	1.56	31	0.44	3224	6.4	6.0	0.0	12.4	10.2	2.6	0.0	20.9	33.7	2.7	
1998															
a	3.28	18	0.40	598	1.3	2.1	0.5	3.9	1.0	0.8	2.0	1.3	5.2	1.3	
b	3.66	34	0.58	1553	0.1	0.1	0.1	0.3	3.7	0.7	1.6	7.5	13.5	50.2	
c	3.71	30	0.45	942	0.8	0.7	0.7	2.2	2.5	0.7	2.7	0.8	6.7	3.1	
d	1.95	31	0.51	1792	0.5	1.1	0.4	2.0	10.6	1.7	3.0	5.1	20.5	9.9	

**Figure 2.** Changes in the UV-visible spectrum of red radish and grape in sugar drink models at pH 3 during storage (0, 24, 48, 72, or 96 h) at 50 °C (for the red radish the spectra recorded at 0 and 48 h were blended).**Figure 3.** Evolution of the hue angle (H°) during storage at 50 °C of pH 5 in nonsugar drink models for 96 h.

values <500 nm and BI values >1 (Table 2), thus giving yellowish shades. By contrast, colorants containing acylated anthocyanins (typically, red radish) were relatively stable at all pH values and displayed constant λ_{\max} values and BI values <1 (Table 3). Figure 2 clearly shows that the color of red radish extracts is much more resistant to heating than that of grape extracts. In addition, grape-marc extracts quickly turn yellow during the first day of storage at 50 °C and pH 5, whereas the hue angle of red radish extracts slowly increases and remains in the red area under 15° after 96 h of storage (Figure 3).

Variation of Phenolic Composition within the Same Source: Grape-marc. The phenolic content and some industrial characteristics (pH, dry matter, BI) of four grape-marc-based colorants were analyzed over two seasons of production (Table 4). For all of the grape-marc extracts studied, pH, dry matter, and BI were similar from one year to another; the SO₂ concentration was found to depend on wine processing and industrial colorant extractions. However, the concentrations of anthocyanins and other phenols were lower in 1998 than in 1997 (Table 4); these results are related to lower

Table 5. Evolution of Maximal Wavelengths, BI, and Absorbance at 520 nm at pH 3.0

grape		sugar solution			nonsugar solution		
		λ_{\max}	BI	% $A_{520\text{nm}}$	λ_{\max}	BI	% $A_{520\text{nm}}$
a	t_0	522	0.41	100	520	0.40	100
a	$t_{63\text{h}}$	521	0.57	60	518	0.51	65
b	t_0	529	0.56	100	530	0.57	100
b	$t_{63\text{h}}$	518	0.75	80	519	0.70	80
c	t_0	522	0.49	100	521	0.46	100
c	$t_{63\text{h}}$	515	0.75	68	516	0.65	74
d	t_0	528	0.52	100	528	0.54	100
d	$t_{63\text{h}}$	518	0.75	74	519	0.69	75

monthly solar radiation values in 1998 (mean variation between 1998 and 1997 was -297 dJ/cm²/month). They are in agreement with those recently published by Mazza et al. (1999). Most remarkably, the phenolic composition greatly varied within the same source. For all of the grape-marc extracts, the major anthocyanin was oenin (malvidin 3-glucoside), the pigment of *Vitis vinifera*, which represented between 25% (grape d in 1998) and 53% (grape b in 1997) of the total anthocyanin content. For grape b, the anthocyanin concentration was very low compared to those of the other phenolic compounds (2–3% of total phenols). By contrast, grape a in 1998 was characterized by similar concentrations of anthocyanins and other phenols. Consequently, very different copigment/pigment ratios were estimated. Grape b can be distinguished by the highest copigment/pigment ratio ($r = 30.9$ in 1997 and 50.2 in 1998) and the highest concentration of flavonols (~55% of the total concentration in colorless phenols), which are potent anthocyanin copigments. Flavonols were also the main copigments in grape d, whereas grapes a and c were richer in flavanols.

Influence of pH and Sugar Concentration on the Color Stability of Grape-marc Extracts. For a given grape-marc extract, the wavelength of maximum absorbance in the visible spectrum in sugar drink models was similar to that in nonsugar drink models. The decrease in the visible absorbance at 520 nm during storage at pH 3.0 and 50 °C is roughly the same in both of the models studied. The BI appears to be more sensitive to the presence of sugar, its increase in sugar drink models during storage being ~10% stronger in nonsugar drink models (Table 5). The λ_{\max} and the decrease in visible absorbance during storage are both strongly pH-dependent. With time, the grape anthocyanins turned yellow at the three pH values investigated. The gradual loss of color in the red region (520 nm) was

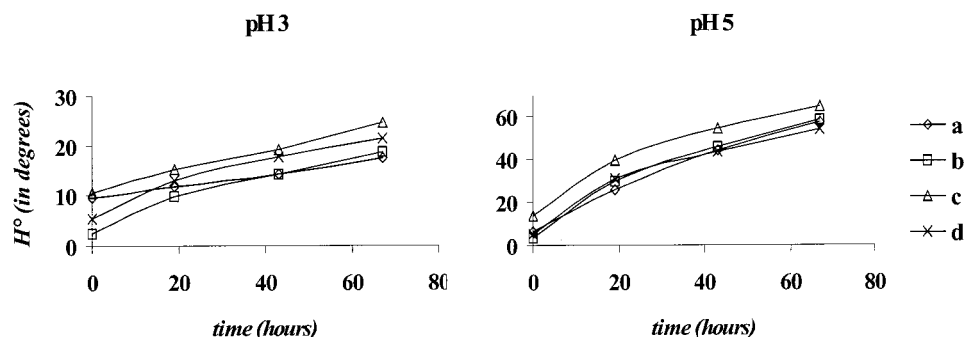


Figure 4. Evolution of the hue angle of grape colorants during storage at 50 °C in pH 3 and 5 of nonsugar drink models.

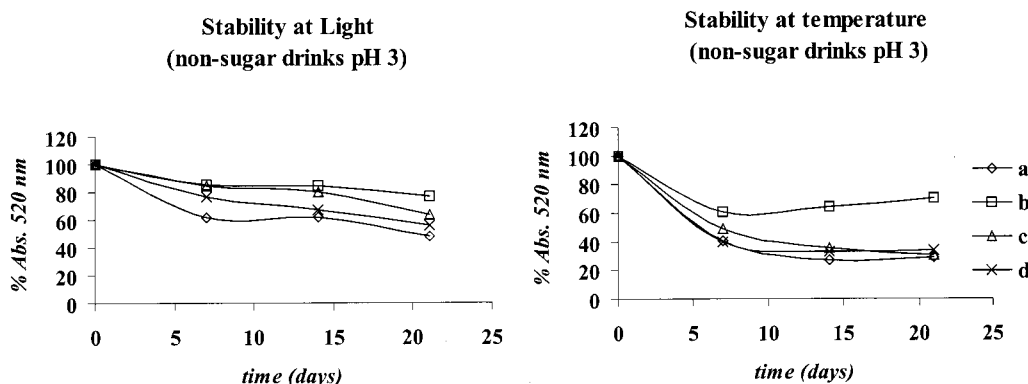


Figure 5. Effect of light and temperature on the absorbance at 520 nm of grape colorants in nonsugar drink models at pH 3.

accompanied by an increase in BI and consequently in H° (Figure 4). The greater changes occurred at pH 5. At pH 3 and after 21 days of storage, the colorant coming from grape-marc b displayed the lowest decrease of absorbance at 520 nm under all of the conditions tested [irradiation (<10%), heating (<30%) with or without sugar]. For grape-marcs a, c, and d, the loss was ~60% for heating and ranged from 30 to 50% for irradiation (Figure 5).

DISCUSSION

Depending on the sources naturally at hand, the commercially available red colorants investigated in this work display great differences in color intensity, tonality, and stability. At a given pH, such differences mainly result from the anthocyanin structure (B-ring substitution, sugar moieties, and phenolic acyl groups), the copigment distribution (phenolic acids, flavonols, and flavanols), and the copigment/pigment molar ratio.

Anthocyanin Structure. When the number of OH and OMe groups increases within the B-ring, the color typically evolves from orange to purple. Among the colorants studied, four different anthocyanidins (aglycons) were identified: pelargonidin (radish), cyanidin (chokeberry, red cabbage, purple carrot, elderberry, and black currant), malvidin (grape-marc), and delphinidin (black currant). In black currant, two main anthocyanins—cyanidin 3-rutinoside and delphinidin 3-rutinoside—in almost identical concentrations were shown to represent ~60% of total anthocyanin content. The fact that cyanidin is linked to different sugar moieties in elderberry, black currant, and chokeberry can slightly affect the resulting color. More important, purple carrot, red cabbage, and red radish all contain acylated anthocyanins. In red cabbage and red radish, a sophorose moiety acylated at O-6 of the glucose unit is linked to the O-3 position of the aglycon (Baublis et al., 1994;

Rodriguez-Saona et al., 1999). The hue angles of red cabbage, red radish, and purple carrot decrease with increasing pH, a typical effect of acylation on tonality (Jackman and Smith, 1992).

Sugar and pH Effects. Addition of sugar in the drink models had no significant effect on the thermal and photochemical stability of the pigments at pH 3.0 (Duhard et al., 1997). The increase in BI in sugar drink models may be due to the formation of brown products via the Maillard reaction (Shenoy, 1993). Wrolstad et al. (1990) has shown that high sugar concentration (>20%) had a protective effect on strawberry anthocyanins, presumably by lowering water activity. This protective effect does not operate here or is counterbalanced by sugar degradation.

At pH <2, anthocyanins exist primarily in the form of the red-orange flavylium cation (Brouillard and Delaporte, 1977). At pH >2, water addition on the pyrylium nucleus gives colorless hemiketal and chalcone forms (thermodynamic products, overall equilibrium constant K_h), whereas competing fast deprotonations of the flavylium's most acidic OH groups engender purple quinonoid forms (kinetic products, equilibrium constant K_a). At equilibrium, the relative amounts of colored and colorless forms vary with both pH and anthocyanin structure (Brouillard, 1982). For malvidin 3-glucoside (the main anthocyanin in grapes), the following values for pK_h and pK_a were reported at 25 °C and 0.2 M ionic strength (Brouillard and Delaporte, 1977): $pK_h = 2.55$, $pK_a = 4.25$. Hence, at pH 3, 4, and 5 the molar ratio between colorless forms and colored forms at equilibrium is ca. 3, 18, and 42, respectively. The situation is even worse with 3,5-diglucosides, which are especially prone to water addition on the pyrylium ring. For instance, the pK_h value for malvidin (malvidin 3,5-diglucoside) is ~2 (Brouillard et al., 1978). In the absence of color-stabilizing mechanisms, colored forms

are thus very minor components in mildly acidic aqueous solutions. As a consequence, vivid natural colors expressed by anthocyanins must be tightly controlled by copigmentation and metal complexation (in the case of anthocyanins having a 1,2-dihydroxybenzene substitution on ring B).

Intermolecular Copigmentation. Considering the lack of acylated anthocyanins in grape-marc extracts, intermolecular copigmentation is proposed to play the major role in the stabilization of these colorants. Copigmentation must be more efficient in the plant extracts displaying higher copigment/pigment molar ratios even though it is not evidenced by a color intensification because all extract solutions are normalized to an initial visible absorbance maximum of ~ 1 for comparison purposes. However, the influence of copigmentation on color stability is very significant and, for instance, grape-marc b, which displays the highest copigment/pigment molar ratio, is the most thermally stable grape extract. Indeed, the color of grape-marc b remained stable over 21 days under heating or light irradiation at pH 3, whereas the other grape-marc colorants turned yellow. Remarkably, grape b has a high concentration of flavonols, which typically rank among the most efficient anthocyanin copigments. These observations strongly suggest that intermolecular copigmentation plays a key role in the prevention of bleaching during light and heat treatment. The reversible conversion of flavylium ions into chalcone forms has been shown to be a key step in the overall mechanism of anthocyanin thermal degradation (Furtado et al., 1993). Chalcones then undergo scission of their enone moiety (through a nonelucidated mechanism) to yield 2,4,6-trihydroxybenzaldehyde and a benzoic acid derivative derived from ring B. Photodegradation (irradiation at 313 nm) is faster and yields the same products. Although a direct photodegradation of the flavylium ions may also operate, chalcones are still likely intermediates in this mechanism. Hence, copigmentation that lowers the molar ratio between the colorless forms and colored forms via selective complexation of the colored forms must efficiently compete with the first step of anthocyanin degradation (formation of the colorless forms). Moreover, copigments from the phenolic acids and flavonol subgroups, which strongly absorb damaging UV radiations, must provide additional protection against photodegradation of the colored forms. Such a protection has been already evidenced by Sweeny et al. (1981) and Santhanam et al. (1984) with flavylium ions and flavonoid sulfonates. Thermal degradation becomes faster when the pH is raised from 3 to 5, in agreement with the simultaneous increase in the concentration of vulnerable colorless forms. Under temperature, the dissociation of the complex between flavylium cation and copigment is increased (Brouillard et al., 1989, 1991).

The less stable grape-marc extract is grape a (in 1997). Remarkably, this colorant displays the highest catechin content. Catechin and malvidin 3-glucoside (and other flavylium ions) are known to slowly form yellow xanthylium pigments with absorption maxima in the range of 420–460 nm (Santos-Buelga et al., 1995; Escribano-Bailon et al., 1996). The initial step is a nucleophilic attack of the electron-rich C-6 and/or C-8 atoms of catechin on the electron-deficient C-4 atom of the anthocyanin. Formation of xanthylium pigments could be responsible for the strong increase in the

absorbance at 430 nm observed with grape-marcs in a nonsugar drink models at pH 3.

Intramolecular Copigmentation. The color of extracts devoid of acylated anthocyanins (grape-marc, elderberry, black currant, and chokeberry) is highly sensitive to heating, especially at pH 4 and 5. Under higher thermal conditions simulating rapid food aging, the BI increase is concomitant with a hypsochromic shift in the visible absorption band of 50–80 nm. By contrast, the color of extracts rich in acylated anthocyanins (purple carrot, red radish, and red cabbage) is highly stable at pH 3, and the wavelength of the visible absorption maximum in these colorants is almost unaffected by heating at the three pH values investigated. Such differences in stability could be related to intramolecular copigmentation. Indeed, aromatic acyl groups of acylated anthocyanins are known to stack on the flavylium nucleus and thereby protect the pyrylium ring from the nucleophilic addition of water, which leads to the colorless forms (Brouillard, 1983). Consequently, the concentration of colorless forms at a given pH is lower than for the corresponding nonacylated pigments. Because colorless forms (chalcones) are primarily involved in anthocyanin degradation, intramolecular copigmentation must efficiently protect anthocyanins from degradation as already reported (Goto et al., 1982). In addition, the difference in color stability observed between purple carrot, red cabbage, and red radish could be related to the different acylated anthocyanins present in these colorants. For instance, purple carrot colorant fades more rapidly than red radish and red cabbage colorants. Remarkably, the latter colorants contain diacylated anthocyanins, the aromatic acyl groups of which can simultaneously stack on both faces of the chromophore (sandwich-type complex), thereby offering optimal protection (Brouillard, 1982; Dangles et al., 1993). Such a protection mechanism has been proposed by Rodriguez-Saona et al. (1999) for red radish. By contrast, the monoacylated anthocyanin present in purple carrot has only one face of its pyrylium ring protected against water addition. Intermolecular copigmentation by chlorogenic acid (5-caffeoylquinic acid) is stronger with flavylium ions than with the quinonoids they form upon deprotonation (typical $pK_a \sim 4$) (Brouillard et al., 1991; Dangles and Brouillard, 1992). This may also be true for intramolecular copigmentation involving cinnamic acid residues. Hence, the folded self-protected conformations of acylated anthocyanins may be more stable in the pH range at which flavylium ions were predominant (pH < 4). This would be consistent with the remarkable stability of the colorants containing acylated anthocyanins at pH 3 (Shi et al., 1992; Baublis et al., 1994). In addition to intramolecular copigmentation, intermolecular copigmentation with phenolic acids may also take part in color stabilization. For instance, the monoacylated anthocyanins of purple carrot display flavylium nuclei still available on one face for stacking interactions with copigments. Intermolecular copigmentation with phenolic acids and intramolecular copigmentation may cooperatively act in such extracts to stabilize the color at low pH.

ACKNOWLEDGMENT

We thank Max Tacchini for technical assistance and Ralph Beisson for help in proofreading the manuscript.

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Received for review June 28, 2000. Revised manuscript received October 11, 2000. Accepted October 12, 2000. This work was financially supported by the Association Nationale de la Recherche Technique in France and by CHR-Hansen-SEFCAL.

JF0007910