Tracking color and pigment changes in anthocyanin products

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Anthocyanin pigments readily degrade during processing and storage of foodstuffs, which can have a dramatic impact on color quality and may also affect nutritional properties. Total anthocyanin pigment content and indices for polymeric color and browning are easily measured with simple spectrophotometric methods. Once individual pigments are identified, their changes can be monitored by high-performance liquid chromatography (HPLC). Modern color instrumentation has made measurement of CIEL*a*b* indices practical and easy. Indices of lightness (L*), chroma (C*), and hue angle (h°) are particularly useful for tracking color change.

Introduction

The Pacific Northwest of the USA produces a wide range of fruits and vegetables that are processed into many different commodities such as canned and frozen fruits, preserves, fruit juices and wines. Color quality will determine whether these products are acceptable to the consumer, and our laboratory has addressed many projects concerning color quality and color degradation during processing and storage. Some specific examples include color and pigment changes in strawberry preserves (Abers & Wrolstad, 1979), frozen strawberries

(Wrolstad, Skrede, Lea, & Enersen, 1990), fruit juice concentrates (Garzon & Wrolstad, 2002; Rwabahizi & Wrolstad, 1988; Skrede, Wrolstad, & Enerson, 1992) and berry wines (Pilando, Wrolstad, & Heatherbell, 1985; Rommel, Wrolstad, & Heatherbell, 1992). Development of anthocyanin-based natural colorants that have better color and stability properties is another research interest, with investigations on natural colorants derived from radishes (Giusti & Wrolstad, 1996), red-fleshed potatoes (Rodriguez-Saona, Giusti, & Wrolstad, 1999) and black carrots (Stintzing, Stintzing, Carle, Frei, & Wrolstad, 2002). We have adopted, modified, and developed a number of methods for monitoring color and pigment changes that have been effective for several research projects. They would also be suitable for many industrial quality control applications. For more comprehensive reviews on anthocyanin pigment analytical chemistry, the following recent articles can be consulted: (Anderson & Francis, 2004; Kong, Chia, Goh, Chia, & Brouillard, 2003; Rivas-Gonzalo, 2003).

Anthocyanin pigment composition

A generalized structure for anthocyanin pigments is shown in Fig. 1. Linus Pauling (1939) gave an elegant explanation of how the resonating flavylium structure accounts for the pigments' depth and intensity of color. While there are six common anthocyanidins, more than 540 anthocyanin pigments have been identified in nature (Anderson & Francis, 2004), with most of the structural variation coming from glycosidic substitution at the 3 and 5 positions and possible acylation of sugar residues with organic acids. Anthocyanins lend themselves to systematic identification as the component anthocyanidins, sugars and acylating acids can be liberated by acid hydrolysis and subsequently identified by chromatographic procedures. Saponification of acylated anthocyanins will produce the anthocyanin glycosides and acylating acids for subsequent identification. These methods are described in several publications (Durst & Wrolstad, 2001; Hong & Wrolstad, 1990; Wrolstad, Durst, Giusti, & Rodriguez-Saona, 2002). Electrospray (ES-MS), tandem (MS/MS), and liquid chromatography mass spectroscopy (LC-MS) are powerful techniques for identifying anthocyanins from their discrete mass units and fragment ions (Giusti, Rodriguez-Saona, Griffin, & Wrolstad, 1999; Wang, Race, & Shrikhande, 2003). For more complete identification, NMR can be used

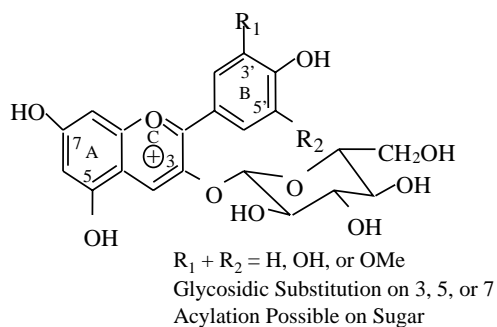Fig. 1. Generalized structure for anthocyanin pigments. Pelargonidin, R_1 and R_2=H; cyanidin, R_1=OH, R_2=H; delphinidin, R_1 and R_2=OH; peonidin, R_1=OMe and R_2=H; petunidin, R_1= Me and R_2=OH; malvidin, R_1 and R_2=OMe.

for sugar identification and determining the position of sugar attachment and angle of the glycosidic linkages (Giusti, Ghanadan, & Wrolstad, 1998; Anderson & Fossen, 2003). This review will not focus on pigment identification since most anthocyanins in commercially significant food crops and natural colorants have already been identified. The analytical chemist has the somewhat easier task of confirming pigment identities, making peak assignments, and then monitoring their changes by HPLC (Durst & Wrolstad, 2001; Hong & Wrolstad, 1990).

Measurement of total anthocyanins by the pH differential method

Anthocyanins reversibly change color with pH (Fig. 2), which limits their effective use as food colorants for many applications, but also provides an easy and convenient method for measuring total pigment concentration (Giusti & Wrolstad, 2001). The described method is a modification of methods originally described by Fuleki & Francis (1968a, 1968b). Samples are diluted with aqueous pH 1.0 and 4.5 buffers and absorbance measurements are taken at the wavelength of maximum absorbance of the pH 1.0 solution. The difference in absorbance between the two buffer solutions is due to the monomeric anthocyanin pigments. Polymerized anthocyanin pigments and non-enzymic browning pigments do not exhibit reversible behavior with pH, and are thus excluded from the absorbance calculation (Fig. 3). It is customary to calculate total anthocyanins using the molecular weight and molar extinction coefficient of the major anthocyanin in the sample matrix. The number of anthocyanins for which molecular extinction coefficients have been determined is limited, however (Giusti & Wrolstad, 2001). When using this procedure, extinction coefficients that have been determined in aqueous solutions should be used rather than those determined in acidic ethanol or methanol because of solvent effects. This also holds true for wines since the amount of ethanol in a diluted wine sample is insufficient to have a measurable solvent effect (Lee, Durst, & Wrolstad, 2005).

There is a need for a standardized method for determining total anthocyanins in commerce, since products are being marketed on the basis of their pigment content. Our laboratory has completed a collaborative study where 11 collaborators
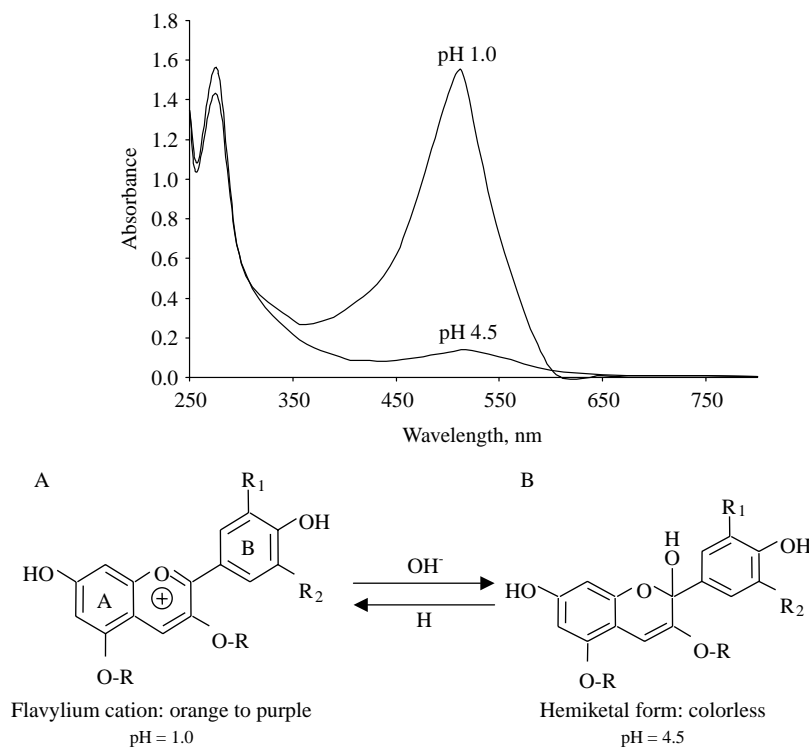

Fig. 2. UV–Visible spectra of anthocyanins in pH 1.0 and 4.5 buffers, and the structures of the flavylium cation (A) and hemiketal forms (B). R=H or Glycosidic substituent.

$$\text{Total Anthocyanins (mg/L)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l}$$

$A = (A_{max} - A_{700nm})pH_{1.0} - (A_{max} - A_{700nm})pH_{4.5}$

MW = Molecular Weight

DF = Dilution Factor

ε = molar extinction coefficient, $L \times mol^{-1} \times cm^{-1}$

l = pathlength (1 cm)

Fig. 3. Calculation for determining total monomeric anthocyanin pigment concentration.

representing academic, government and industrial laboratories analyzed 7 fruit juice, beverage, natural colorant and wine samples by this method (Lee, Durst, & Wrolstad, 2005). A cyanidin-3-glucoside standard was also included in the sample set. The samples were shipped frozen to the participants, and they were instructed to refrigerate and analyze the samples promptly. Identity of the individual samples was unknown to the analysts, and they were instructed to make all measurements at 520 nm and calculate pigment content as cyanidin-3-glucoside equivalents. Cyanidin-3-glucoside was selected since it is the most common anthocyanin pigment in nature (Francis, 1989). There was excellent agreement between laboratories. The relative standard deviation for repeatability ranged from 1.06 to 4.16%, and for reproducibility, from 2.69 to 10.12%. Horrat values ranged from 0.30 to 1.33 with less than 2.0 being considered acceptable. The method has been approved as a First Action Official Method (Lee, Durst, & Wrolstad, 2005).

An issue that concerned the Official Methods Board of AOAC was the low recovery of the cyanidin-3-glucoside standard, an average of 60% being reported for the analysts. The test sample consisted of a cyanidin-3-glucoside chloride standard that had been weighed and dissolved in distilled water to a final volume of 1 L. A possible explanation was that while the standard was chromatographically pure, it may have contained impurities. To further investigate this possibility, another sample was purchased from the same company and a third sample from a different company. Percent purity was determined by HPLC (monitoring at 280 and 520 nm), and found to range from 93.8 to 98.9%. H_2O content was measured by placing weighed amounts of the standards in a desiccator in the presence of phosphorus pentoxide (P_2O_5) under vacuum until a constant weight was reached. Moisture content ranged from 3.5 to 10.5%. Hygroscopicity was determined by measuring water uptake of standards in an 83% relative humidity chamber. Hygroscopicity ranged from 10.0 to 22.4%. The molar absorbtivity of the standards were measured at 520 nm, the λ_{max} used in the collaborative

study, and its true λ_{max}, 510 nm. Molar extinction coefficients from $A_{520\ nm}$ were 19,103, 20,526, and 25,076 in contrast to 20,072, 26,672 and 21,606, when measured at $A_{510\ nm}$. Determination of %purity by comparing the extinction coefficients to that used for calculation in the collaborative study, $26,900\ Lcm^{-1}\ mol^{-1}$, gave values ranging from 71.0 to 93.2%. Higher recovery would have been achieved in the collaborative study if measurement had been at 510 nm, the true λ_{max} rather than 520 nm. The method assumes that anthocyanin pigments show zero absorbance at pH 4.5. This is not actually true, and the low absorbance of standards at pH 4.5 (Fig. 2) would contribute to error in the order of 4%. The presence of water and other impurities, measuring absorbance at 510 nm, and the minor contribution of quinoidal and flavylium forms to absorbance at pH 4.5 are believed to account for the low recovery. In addition, 26,900 may not be the 'true' extinction coefficient. The experiments concerning moisture content, purity and hygroscopicity of anthocyanin standards call attention to the importance of taking these properties into consideration when conducting experiments with anthocyanin standards. An alternative method for determining total anthocyanins is to separate the anthocyanins by HPLC, measure the amounts of individual pigments by use of an external standard, and then sum the individual anthocyanins. Chandra, Rana, and Li (2001) used cyanidin-3-glucoside chloride as an external standard in quantitating anthocyanins in botanical supplements and applied molecular weight correction factors for individual peaks that were identified by HPLC-MS. This approach to quantitation is subject to the same concerns regarding purity of external standards. In addition, anthocyanin standards are expensive; the cost of the standards used in our collaborative study ranged from $290–$1,614 (USA)/100 mg.

Indices for polymeric color and browning

Anthocyanin pigments are labile compounds that will undergo a number of degradative reactions. Their stability is highly variable depending on their structure and the composition of the matrix in which they exist (Wrolstad, 2000; Delgado-Vargas, & Paredes-López, 2002). Increased glycosidic substitution, and in particular, acylation of sugar residues with cinnamic acids, will increase pigment stability. Polyphenoloxidase, peroxidase, and glycosidase enzymes can have a devastating effect on anthocyanins. These enzymes may be native to the plant tissue, or their source may be from mold contamination. Another possible source is side activities of commercial enzymes used as processing aids (Wrolstad, Wightman, & Durst, 1994). Glycosidase enzymes will act directly on anthocyanins, but the action of polyphenoloxidase and peroxidase is indirect. Presence of ascorbic acid will accelerate anthocyanin degradation (Skrede, Wrolstad, & Enerson, 1992). Anthocyanins will condense with other phenolic compounds to form colored polymeric pigments. This reaction can be accelerated by the presence of acetaldehyde. Light exposure
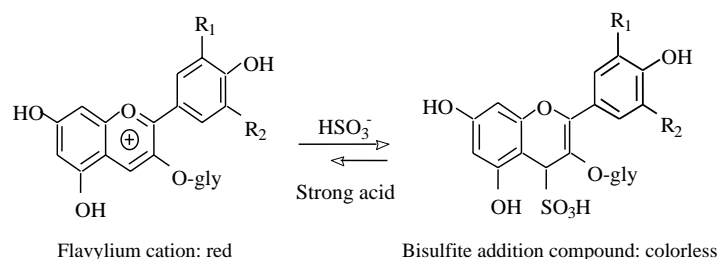
Fig. 4. Reaction of anthocyanin pigments with bisulfite to form colorless anthocyanin-sulfonic acid adducts.

will promote pigment destruction while reduced water activity will enhance stability. Anthocyanin pigments in dried forms can exhibit remarkable stability.

Polymeric anthocyanin pigments will not show the pronounced reversible change in color with pH change (Fig. 2) that is characteristic of monomeric anthocyanins. Monomeric anthocyanins will combine with bisulfite at the pH of most foods and beverages to form a colorless sulfonic acid addition adduct (Fig. 4). Berké, Chéz, Vercauteren, and Deffieux (1998) using ^{1}H, ^{13}C and ^{33}S NMR spectroscopies established that the position of the sulfonate adduct was the C-4 position. Polymeric anthocyanins will not undergo this reaction, as the 4-position is not available, being covalently linked to another phenolic compound. T.C. Somers of Australia's Wine Research Institute developed a simple spectrophotometric procedure for measuring polymeric color and browning in wines (Somers & Evans, 1974). We have applied this method to a wide range of anthocyanin food products and colorants and found it to be extremely useful for monitoring the development of polymeric color during processing and storage (Giusti & Wrolstad, 2001). Pyranoanthocyanins or Vitisin A type pigments (Fig. 5) have only become known in the past decade (Bakker & Timberlake, 1997; Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998; Fulcrand, Aatanasova, Salas, & Cheynier, 2004). They are derived from reaction of anthocyanins at the C-4 position with pyruvic acid and

other compounds to form cyclo addition products. These pigments will not be bleached with bisulfite since the C-4 position is blocked; hence they will be measured as 'polymeric color' with this assay.

Measurement of color by the CIEL*a*b* system

To investigate color quality in a systematic way it is necessary to objectively measure color, as well as pigment concentration. In this context, color denotes the visual appearance of the product whereas pigments or colorants are the chemical compounds that impart the observed color. Special color measuring instruments are available, and their ruggedness, stability, portability, sensitivity and ease of use has vastly improved in recent years. The CIEL*a*b* system (International Commission on Illumination, Vienna) has been embraced by the USA food industry for measuring color of food products. While this system does not necessarily give an accurate definition of color, it is very effective for measuring color differences and tracking color changes during processing and storage. Color indices derived from CIEL*a*b* measurements are increasingly being reported in natural colorant research articles, but in many instances they are applied inappropriately. Instruments will measure L* which is a measure of 'lightness' and two coordinates a* and b*. Positive values of a* are in the direction of redness and negative values in the direction of the complement green. Positive values of b* are the vector for 'yellowness', and negative for 'blueness'. A very prevalent error is to use the a* value as a measure of the amount of 'redness' or 'greenness', and b* values as a measure of 'yellowness' or 'blueness'. Samples with identical a* values may exhibit colors ranging from purple
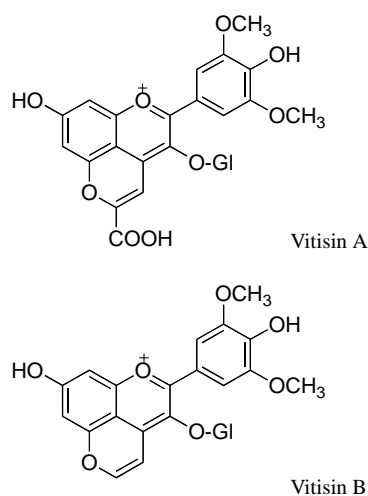

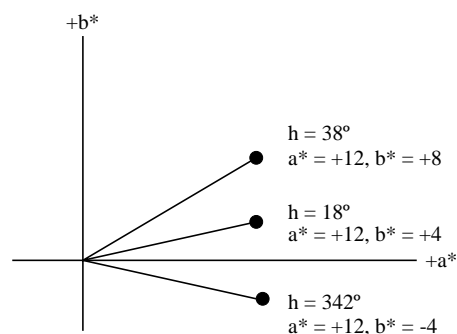Fig. 5. Structure of Vitisn A and Vitisin B, examples of pyranoanthocyanins.

Fig. 6. Hue angle of 3 solutions varying from orange-red to purple.

to red to orange. Fig. 6 plots three samples having identical a* values that range in hue from orange–red to purple. Color is three dimensional, and better descriptions of color are obtained by using the L*Ch system where L* = lightness with 100 = absolute white and 0 = absolute black. Hue angle is derived from the two coordinates a* and b* and determined as arctan b*/a*. Hue angle is expressed on a 360° grid where 0° = bluish–red, 90° = yellow, 180° = green, and 270° = blue. This system avoids the use of negative numbers and differences in hue angle of 1° are readily discernible by the human eye. Chroma is a measure of intensity or saturation and calculated as $(a* + b*)^{1/2}$. A red colored sample of different dilution strengths going from pink to red will have the same hue angle but increasing chroma values. A confounding phenomena regarding chroma, is that it will increase with pigment concentration to a maximum, and then decrease as the color darkens. Thus a pink and a dark red color can have identical values for chroma.

Concluding remarks

We have used the above indices for measuring the color and stability properties of natural colorants, as well as for monitoring color and pigment changes of many different foods during processing and storage. Our investigation on the use of radish anthocyanin extract for coloring maraschino cherries is a particularly good example of the effectiveness of these methods (Giusti & Wrolstad, 1996). These methods should also find useful application in industrial quality control.

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