**Station Bulletin 624**
Reprinted May 1993

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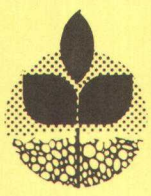

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# Color and Pigment Analyses in Fruit Products

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# COLOR AND PIGMENT ANALYSES IN FRUIT PRODUCTS

Ronald E. Wrolstad

## ABSTRACT

Methods are described for making the following determinations in fruits and processed fruit products: anthocyanin pigment content, color density, polymeric color, browning, turbidity, and anthocyanin degradation index. Tables list the anthocyanins of common fruits, their molecular weights, molar absorbances, and wavelengths of maximum absorption. Thirty-one references are given.

KEY WORDS: color analyses, anthocyanin pigment, browning, fruits

## Introduction

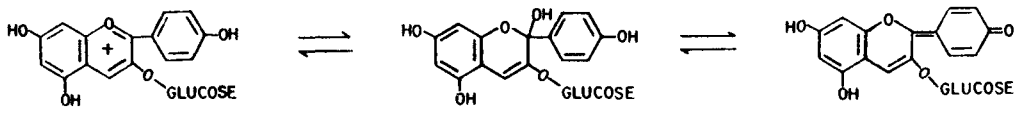
Measurement of pigment content, browning, color density, and haze are analytical problems confronting the food technologist working with fruit concentrates, juices, wines, jams and other processed products. This publication includes some of the methods we have found practical in determining these parameters. We also have included tables which list anthocyanin pigments of fruits likely to be processed in the Northwest and the molar absorption of the common occurring anthocyanin pigments.

We have not included procedures of pigment extraction, isolation, and identification or methods for tristimulus colorimetry. Tristimulus colorimeters, such as the Hunter color-difference meter and the Lovibond tintometer, give the best measurements for visual appearance of a product. This is another important dimension of color measurement, but one which is dependent on sophisticated and expensive instrumentation not likely to be found in many processing plants.

## Determination of anthocyanin pigment content - pH differential method

The structural transformations which pelargonidin-3-glucoside (the major anthocyanin pigment of strawberries) undergoes with change in pH are illustrated in Figure 1. Anthocyanin pigments can be described as being indicators - that is, their hue (shade of color) and intensity (depth of color) change with pH. At pH 1.0, anthocyanins exist in the

highly colored oxonium or flavilium form and at pH 4.5 they are predominately in the colorless carbinol form. The quantitative procedure for determining anthocyanin content which will be described is based on those facts. One aliquot of an aqueous anthocyanin solution is adjusted to pH 1.0 and another to pH 4.5. The difference in absorbance at the wavelength of maximum absorption will be proportional to anthocyanin content. Figure 2 compares the absorbance throughout the visible spectrum (350-700 nm) of purified blackberry anthocyanins in pH 1.0 and 4.5 buffer. These spectra were determined with a scanning spectrophotometer but a similar plot could be obtained with a less sophisticated spectrophotometer by determining the absorbance at every 5 nm and plotting the data. From Figure 2 it can be seen that the wavelength of maximum absorption for anthocyanins is 510 nm.




| OXONIUM SALT (FLAVILIUM CATION) | CARBINOL BASE | QUINOIDAL ANHYDRO BASE |
|---|---|---|
| pH 1 | pH 4-5 | pH 7-8 |
| ORANGE-RED | COLORLESS | BLUE |

Figure 1. Structural transformation of anthocyanins with change in pH.

Determination of anthocyanin content is based on Lambert-Beer's Law: $A = \varepsilon CL$. A stands for absorbance which is measured with a spectrophotometer. Absorbance values are sometimes given in units of Optical Density (O.D.); the two terms are interchangeable. $\varepsilon$ stands for molar absorbance, a physical constant for a molecular species in a given solvent system at a given wavelength. Molar absorbance values for purified pigments taken from the literature can be used, making it unnecessary to determine them. Molar absorbance is also referred to as the molar extinction coefficient. C is the molar concentration and by rearranging the Lambert-Beer's Law equation, $C = A/\varepsilon L$. L is the pathlength in cm and most spectrophotometer cells have a pathlength of 1. Concentration in milligrams per liter can be determined by multiplying by the molecular

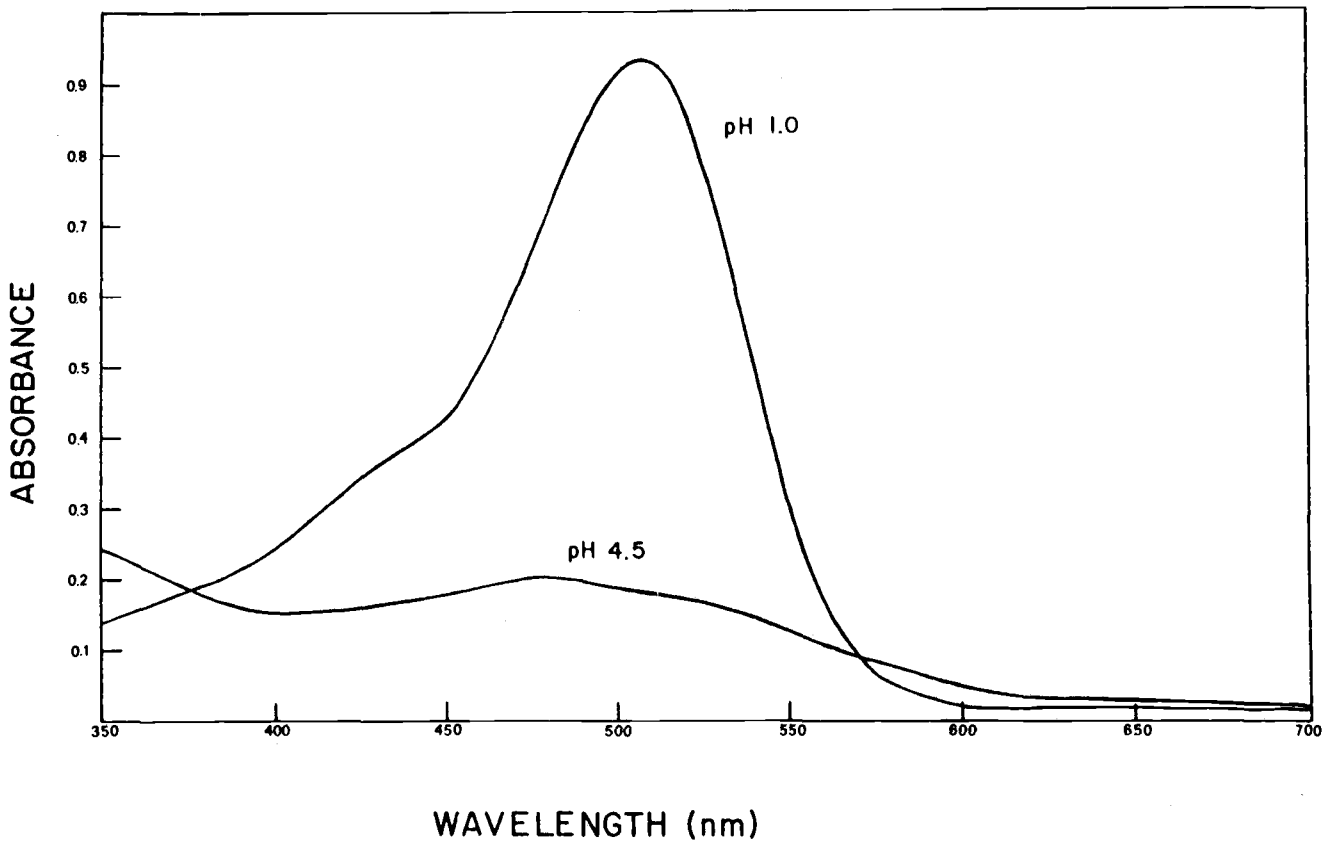



Figure 2. Visible absorption spectra of purified blackberry anthocyanins in pH 1.0 and pH 4.5 buffer

weight (MW) of the pigment.

$$C_{(mg/l)} = \frac{A}{\varepsilon L} \times MW \times 10^3$$

The molecular weights (MW) and molar absorbance ($\varepsilon$) for several of the anthocyanins are listed in Table 1. The pigments when purified are crystallized as chloride salts and often contain water of crystallization. As our major interest is in determining the pigment content in fruits and their processed products, the molecular weights given do not include the chloride ion (MW = 35.5) or water of crystallization. The molar absorbances listed are selected from the literature; there is considerable variation in the values reported for a single pigment by different workers - this is inherent with the difficulties encountered in isolating pure, crystalline material. The literature usually recommends that $\varepsilon$ be from the same solvent system employed in analysis. However, we have found that while the $\lambda$ max varies considerably with different solvent systems, the absorbance of pure pigment solutions in aqueous vs. alcoholic systems differs only to a small degree. For example, we elected to use the value of 29,600 reported by Blundstone and Crean for cyanidin-3-glucoside to 26,900 even though the latter was run in an aqueous system.

To measure absorbance, the juice, concentrate, or wine needs to be diluted with buffer. The order of dilution must be such that the sample at pH 1.0 will have an absorbance less than 1.0 and preferably in the range of 0.4-0.6. The dilution strength should be the same for both 1 and 4.5 samples. In our analyses of blackberry wines, we dilute 10 ml of wine to 50 ml. This gives us a dilution factor of 5. The diluted samples should be clear and not contain haze or sediment. Any sediment should be removed by centrifuging or filtering the sample. If the sample is free of haze, the absorbance at 700 nm should be 0. Turbidity (haze) can be corrected for by measuring the absorbance at 700 nm and subtracting this from the absorbance at the wavelength of maximum adsorption (510-540). (Daravingas and Cain, 1968).

We can illustrate determination of anthocyanin content by reviewing our calculation of pigment content of an Oregon blackberry wine.

TABLE 1. ANTHOCYANINS OF COMMON FRUITS - MOLECULAR WEIGHTS (MW), MOLAR ABSORBANCE (ε), WAVELENGTH OF MAXIMUM ABSORPTION (λ)

| PIGMENT | MW* | LOGε | ε | SOLVENT | λ | REFERENCE |
|---|---|---|---|---|---|---|
| PGD-3-glu (callistephin) | 433.2 | 4.35 | 22,400 | 1%HCl/$H_2O$ | 520 | Goodwin, p. 541 |
| | | 4.50 | 31,600 | 1%HCl/MeOH | 516 | Goodwin, p. 541 |
| Cyd-3-gal (Idaein) | 445.2 | 4.48 | 30,200 | 1%HCl/MeOH | 530 | Goodwin, p. 541 |
| | | 4.62 | 41,700 | HCl/ETOH | 535 | Fuleki & Francis |
| | | 4.49 | 30,900 | HCl/ETOH | 535 | Fuleki & Francis |
| Cyd-3-rut | 595.2 | 4.46 | 28,800 | 1%HCl/$H_2O$ | 541 | Goodwin, p. 541 |
| Cyd-3-glu (Chrysanthemin or Asterin) | 445.2 | 4.47 | 29,600 | 0.01%HCl/MeOH | 528 | Blundstone & Crean, |
| | | 4.43 | 26,900 | Aqueous Buffer pH 1 | 510 | Jurd & Aseh |
| Dpd-3-glu (Myrtillin) | 465.2 | 2.90 | 795 | 1%HCl/MeOH | 543 | Fuleki & Francis |
| Cyd-3-soph | 611.2 | | | | | |
| Cyd-3-($2^G$-xylrut) | 727.2 | | | | | |
| Cyd-3-($2^G$-glurut) | 757.2 | | | | | |
| Mvd-3-glu (Oenin) | 493.5 | 4.44 | 28,000 | $10^{-1}$N HCl | 520 | Niketic-Aleksic & Hrazdina |
| Mvd-3,5-diglu (Malvin) | 655.5 | 4.57 | 37,700 | $10^{-1}$N HCl | 520 | Niketic-Aleksic & Hrazdina |

* Molecular weights do not include the chloride ion or water crystallization.

TABLE 2. ANTHOCYANINS OF COMMON FRUITS

| FRUIT | ANTHOCYANINS* | REFERENCES |
|---|---|---|
| **Berries** | | |
| Blackberry (*Rubus ursinus*, *Rubus laciniatus*, *Rubus procerus*, Marion) | Cyanidin-3-glucoside<br>Cyd-3-rut | Barritt & Torre, 1973 |
| Blueberry (*Vaccinium angustifolium*) | Delphinidin-3-glucoside<br>Mvd-3-glu, Pet-3-glu, Pnd-3-glu, Cyd-3-glu, Dpd-3-gal, Mvd-3-gal, Pet-3-gal, Pnd-3-gal, Cyd-3-gal, Dpd-3-arab, Mvd-3-arab, Pet-3-arab, Pnd-3-arab, Cyd-3-arab | Francis, Harborne & Barker, 1966 |
| Boysenberry | Cyanidin-3-sophoroside<br>Cyd-3-glu, Cyd-3-rut, Cyd-3-glurut | Barritt & Toore, 1973 |
| Cranberry (*Vaccinium macrocarpum*) | Cyanidin-3-galactoside<br>Pnd-3-gal, Cyd-3-arab, Pnd-3-arab | Zapsalis & Francis, 1965 |
| Currant, black (*Ribes nigrum*) | Cyanidin-3-rutinoside<br>Dpd-3-rut, Cyd-3-glu, Dpd-3-rut | Chandler & Harper, 1962 |
| Currant, red (*Ribes patraeum*) | Cyanidin-3-($2^G$-xylosylrutinoside)<br>Cyd-2-($2^G$-glurut), Cyd-3-rut, Cyd-3-sam, Cyd-3-soph, Cyd-3-glu | Oydvin, 1973 |
| Elderberry (*Sambucus nigra*) | Cyanidin-3-glucoside<br>Cyanidin-3-sambubioside<br>Cyd-3-sam-5-glu | Van Buren, 1970<br>Barritt & Torre, 1973 |
| Loganberry | Cyanidin-3-($2^G$-glucosyl rutinoside)<br>Cyd-3-glu, Cyd-3-rut, Cyd-3-soph | Nybom, 1968 |
| Raspberry, black (*Rubus leucodermis*, *Rubus occidentalis*) | Cyanidin-3-xylosylrutinoside<br>Cyd-3-rut, Cyd-3-sam, Cyd-3-glu | Barritt & Torre, 1973;<br>Nybom, 1968 |
| Raspberry, red (*Rubus idaes*) | Cyanidin-3-sophoroside<br>Cyd-3-glu, Cyd-3-glurut, Cyd-3-rut, Cyd-3,5-diglu, Pgd-3-glu, Pgd-3-soph, Pgd-3-glurut, Pgd-3-rut | Barritt & Toore, 1975 |
| Strawberry (*Fragaria x ananassa*) | Pelargonidin-3-glucoside<br>Cyd-3-glu | Robinson & Robinson, 1932; Lukton, Chichester & MacKinney, 1955 |
| **Tree Fruits** | | |
| Apple (*Malus pumila*) | Cyanidin-3-galactoside<br>Cyd-3-glu, Cyd-3-arab, Cyd-3-xyl | Timberlake & Bridle, 1971 |
| Cherry, sweet (*Prunus avium* L. var. Bing) | Cyanidin-3-rutinoside<br>Cyd-3-glu, Pnd-3-rut(tr), Pnd-3-glu(tr) | Lynn & Luh, 1964 |

| FRUIT | ANTHOCYANINS | REFERENCES |
|---|---|---|
| Cherry, sour (Prunus cerasus L. var. Montmorency) | Cyanidin-3-2$^{G}$-glucosylrutinoside<br>Cyanidin-3-rutinoside<br>Cyd-3-soph, Cyd-3-glu<br>Pnd-3-rut(tr) | Dekazos, 1970;<br>Fisher & vonElbe. 1970 |
| Peach (Prunus persica) | Cyanidin-3-glucoside | Hsia, Luh, & Chichester, 1965 |
| Plum (Prunus domestica) | Cyanidin-3-glucoside, Cyd-3-rut<br>Pnd-3-rut, Pnd-3-glu | Van Buren, 1970 |
| Grapes | | |
| Concord (Vitis labrusca var. Concord) | Delphinidin-3-glucoside<br>Cyd-3-glu, Ptd-3-glu, Mvd-3-glu, Pnd-3-glu, P-Coumaric acid ester of Dpd-3-glu, and Cyd-3-glu<br>Dpd-3,5-diglu, Cyd-3,5-digly | Ingalsbe, Neubert, & Carter, 1963; Shewfelt, 1966; Singleton & Esau, 1969 |
| Vitis vinifera | Malvidin-3-glucoside, P-coumaroyl-mvd-3-glu, Pnd-3-glu, Ptd-3-glu, dpd-3-glu, Cyd-3-glu | Singleton & Esau, 1969; Koppen & Basson, 1966; Van Buren, et al, 1970; Rankine, Kepner & Webb, 1968 |
| Miscellaneous | | |
| Rhubarb (Rheum rhaponticum) | Cyanidin-3-glucoside<br>Cyd-3-rut | Wrolstad & Heatherbell, 1968 |

* The major pigment is spelled out; the remaining pigments are abbreviated and an attempt has been made to place them in decreasing order. The abbreviations are: Pgd, pelargonidin; cyd, cyanidin; Dpd, delphinidin; Pnd, peonidin; Ptd, petunidin; Mvd, malvidin; glu, glucose; gal, galactose; xyl, xylose; arab, arabinose; gly, glycose; rut, rutinose (rhaαl→6 glu); soph, sophorose (gluβl→2 glu); sam, sambubiose (xylβl→2 glu).

Two 10 ml aliquots were each diluted to 50 mls with pH 1.0 and 4.5 buffers. The wavelength of maximum absorption was 510 nm. The absorbance at 510 nm of the pH 1.0 solution was 0.62 and the pH 4.5 solution was 0.32. The sample appeared to be free from haze and the absorbance at 700 nm was found to be 0. Thus, to calculate the difference in absorbance between the two samples we proceeded as follows:

$$\text{Absorbance} = (A_{510nm}\ \text{pH 1.0} - A_{700nm}\ \text{pH 1.0}) - (A_{510nm}\ \text{pH 4.5} - A_{700nm}\ \text{pH 4.5}).$$
$$= (0.62 - 0) - (0.32 - 0)$$
$$= 0.62 - 0.32$$
$$= 0.30$$

From Table 2 we see that cyanidin-3-glucoside is the major anthocyanin of blackberries and in Table 1 we see that its molar absorbance is 29,600 and its molecular weight 445. The dilution factor would be 5; these values can be substituted into the previously described equation:

$$\text{Concentration (mg/l)} = \frac{A}{\varepsilon L} \times 10^3 \times MW \times \text{Dilution Factor}$$
$$= \frac{0.30}{(29,600)\ (1)} \times 10^3 \times 445 \times 5$$
$$= 22.5\ \text{mg/l}$$

A check should be run to see if the assay deviates from Lambert-Beers' Law. This can be done by varying the dilution strength so there is a series of samples of different concentration whose optical density will be in the range of 0 to 1.0. A plot of absorbance vs. concentration should give a straight line which passes through the origin. Often at higher concentrations there will be a deviation from linearity. The non-linear portion may still be used as a calibration curve, but one needs to be aware that he is working close to the limits of the system. Figure 3 illustrates that when we applied the assay to varying concentrations of purified plum anthocyanins, Lambert-Beer's Law was being followed.

It should be emphasized that the pH differential method is a measure of the monomeric anthocyanin pigments and the results may not seem to be correlated with the color intensity of the juice or wine samples as they are judged visually. This is because polymeric anthocyanins and brown pigments arising from enzymic browning, maillard

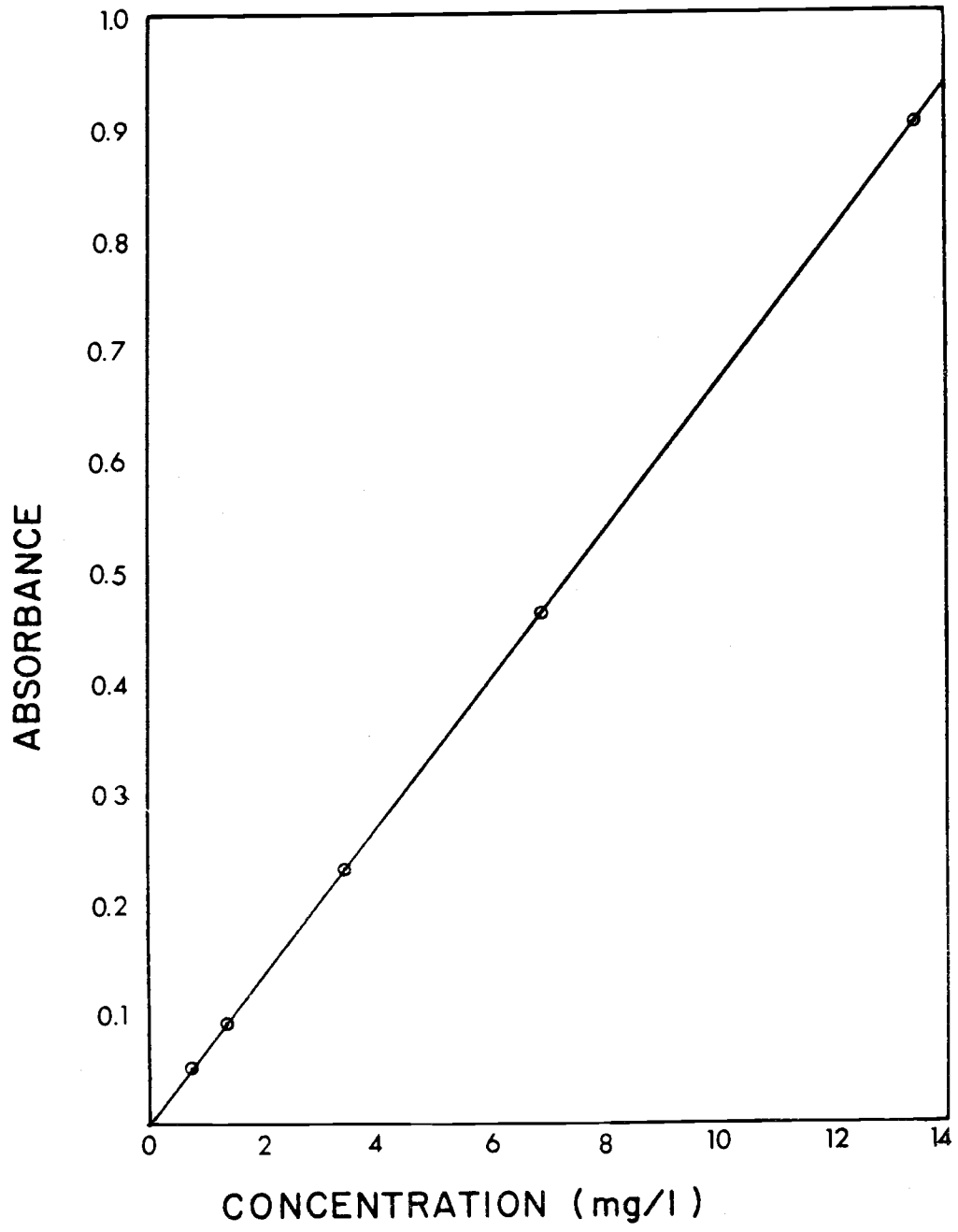



Figure 3. Relationship between absorbance and concentration of purified plum anthocyanins

browning, and anthocyanin degradation also contribute to the color intensity. Some workers (Niketic'-Aleksic' and Hrazdina, 1972) have reported anthocyanin content where absorbance was measured only at pH 1, and there was no subtraction of the absorbance at pH 4.5. Results determined in this manner would be similar to those of the pH differential method for fresh fruit products where presence of brown polymeric pigments was insignificant. In products where browning and anthocyanin degradation occurred, a better correlation with color intensity would be obtained but it would not be as accurate a measurement of anthocyanin content, per se.

Fuleki and Francis (1968) in studying the anthocyanin degradation of cranberry juice utilized both methods of anthocyanin determination to formulate a degradation index. The formula was:

$$\text{DI (Degradation Index)} = \frac{\text{Anthocyanin content by single pH method}}{\text{Anthocyanin content by pH differential}}$$

Table 2 lists the anthocyanin pigments of common fruits. The major pigment provides a key in selecting the appropriate molar absorbtivity. It is preferred to calculate anthocyanin concentration in terms of the major pigment. In some cases, however, the molar absorbtivity may not be known or may be questionable. To determine the anthocyanin content of blueberries, the total anthocyanin should be calculated as malvidin-3-glucoside rather than delphinidin-3-glucoside; while delphinidin-3-glucoside is the major pigment, the reported molar absorbance is so low its accuracy should be suspect. In reporting results, it is important that the molar absorbance used in calculating pigment concentration be given; this is useful to other workers who want to compare results.

The anthocyanins of wine grapes deserve special comment. The red wine grapes most popular with Oregon viticulturists are Cabernet Sauvigon, Pinot noir, Zinfandel, Gamay Beaujolais, Gamay, and Grenache; all of these are cultivars of *Vitis vinifera* and all contain malvidin-3-glucoside as their major pigment (Van Buren, *et al.*, 1970; Rankine, Kepner and Webb, 1958). Pinot noir and Gamay Beaujolais do not contain the acylated pigments. This difference from other *Vitis vinifera* has been used to detect fraud in varietal wines (Singleton and Esau, 1969). *Vitis vinifera* are free of diglucosides and this chemical evidence has

been used to support classification of a variety as Vitis vinifera (Koeppen and Basson, 1966). Van Buren et al. examined the pigments of 151 varieties of hybrid grapes and found malvidin monoglucoside and malvidin diglucoside to be the dominant pigments. Niketic'-Aleksic' and Hrazdina (1972) recommended that pigment content of juices and wines of Vitis labruscu, V. vipara, V. rupestris, and V. rotundifolia be calculated as malvidin-3,5-diglucoside and that those from Vitis vinifera be expressed as malvidin-3-glucoside.

Determination of Color Density, Polymeric Color, and Anthocyanin Color - Potassium Meta Bisulfite Method

T. C. Somers of the Australian Wine Research Institute has developed some useful procedures for measuring color parameters of wines which can be applied to other anthocyanin-containing products. His methods are advantageous because color density, polymeric color, percent of contribution by tannin, and anthocyanin color can be calculated from a few absorbance readings. We have applied his procedure to blackberry and plum wines in the following manner:

1. Two hundred microliters (0.2 ml) of 20 percent potassium metabisulfite is added to a 3.0 ml wine sample and 200 microliters of $H_2O$ is added to a second 3.0 ml control sample. (It may be necessary to dilute the wine with distilled water so the absorbance at 420 and 520 is below 1.0.)
2. The visible absorption spectrum of each solution is recorded from 700 to 350 nm. (We use a double-beam spectrophotometer with distilled water in the reference cell.)
3. The absorbance at 420, at the $\lambda$ max (within the range of 500-540 nm), and at 700 nm is recorded.

Color Density

The color density can be determined by summing the absorbance of the control sample at 420 nm and at the anthocyanin $\lambda$ max (for many products this will be 520 nm). Turbidity can be corrected for by subtracting any absorbance at 700 nm. If the sample was diluted, the sum is multiplied by the dilution factor. With blackberry wine, we

diluted 5 mls of wine with distilled water to a 10 ml volume, giving us a dilution factor of 2.

Color density = $[(A_{420nm} - A_{700nm}) + (A_{420nm} - A_{700nm})]$ X dilution factor

Figure 4 shows the visible spectrum for a two-fold dilution of blackberry wine. The absorbance at 510 nm is 0.68, at 410 nm, 0.41, and at 700 nm, 0.

Color density = [(0.68 - 0) + (0.41 - 0)] X 2

Color density = 2.18

Polymeric Color

Somers has shown (1971) that the polymeric tannin pigments are resistant to bisulfite bleaching. A measure of polymeric color can be calculated by applying the same procedure as used in determining color density to the bisulfite treated sample.

Polymeric color = $[(A_{520nm} - A_{700\ nm}) + (A_{420\ nm} - A_{700\ nm})]$ X dilution factor

The spectrum of bisulfite bleached blackberry wine is also shown in Figure 4. Absorbance at 520 nm is 0.03, at 420 nm, 0.11, and at 700 nm, 0.

Polymeric color = [(0.03 - 0) + (0.11 - 0)] X 2

Polymeric color = 0.28

The percent contribution of "tannin" (non-monomeric anthocyanin color) to total color can be determined from color density and polymeric color.

$$\% \text{ contribution of tannin} = \frac{\text{polymeric color}}{\text{color density}}$$

For our blackberry wine sample:

$$\% \text{ contribution of tannin} = \frac{0.28}{2.18} = 12.8\%$$

The ability of bisulfite to bleach anthocyanins is pH dependent (Jurd, 1964); therefore, determine the pH of the sample. Somer's procedure will be applicable to fruit products in the normal pH range of 3.0-4.5 but will not apply to products acidified to a low pH. We follow the practice of making these determinations at the natural pH and do not adjust all samples to a common pH value.

While Figure 4 shows the difference between the spectra of blackberry wine and bisulfite-bleached blackberry wine in an explicit manner,

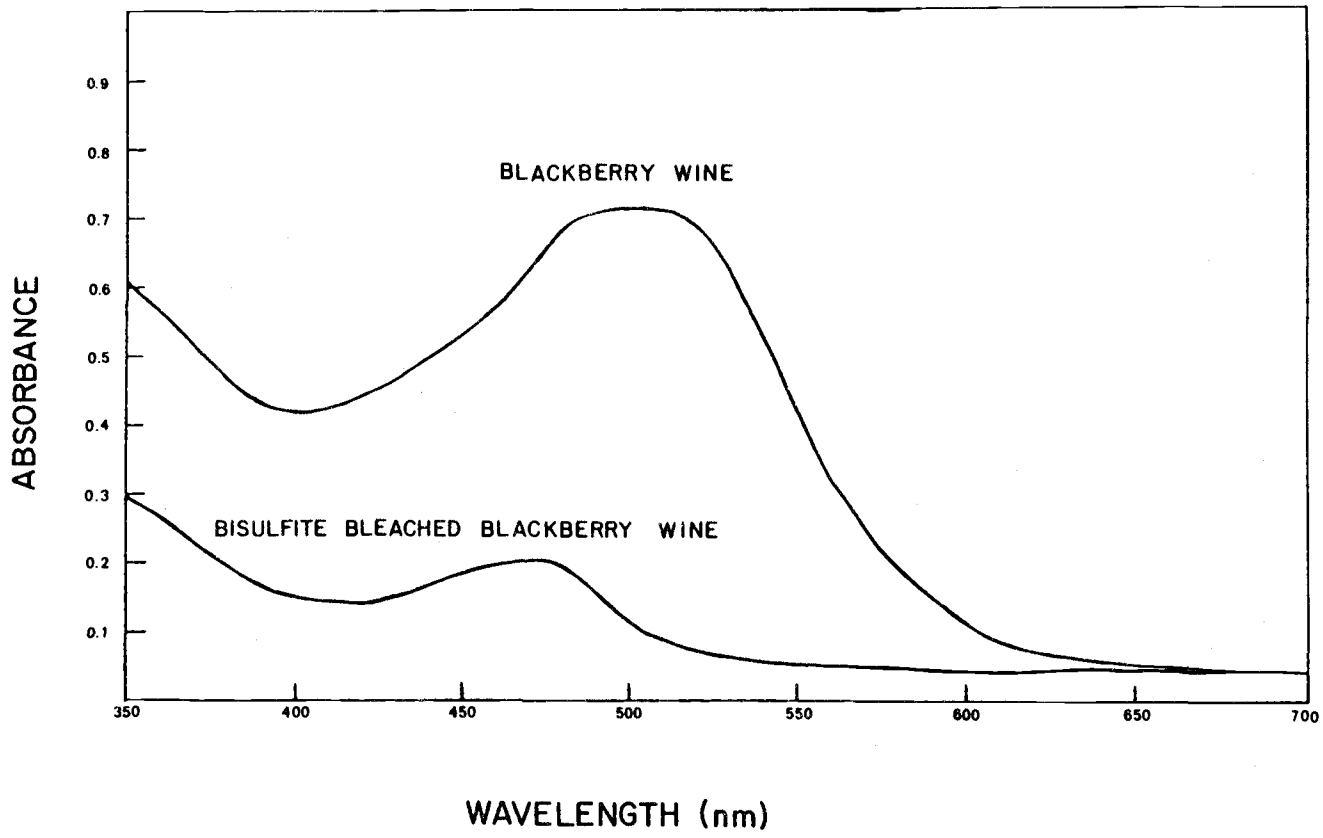



Figure 4. Visible absorption spectra of blackberrry wine and bisulfite bleached blackberry wine

scanning spectrophotometry is not essential to determine color density, polymeric color, etc. A single beam spectrophotometer can be used to determine the absorbance values at 420 and 520 nm.

Anthocyanin Color

The difference in absorbance at the anthocyanin λ max between the control and the bleached sample will be related to anthocyanin pigment content. By again referring to our blackberry wine sample, the absorbance at 520 nm of the diluted wine and the bisulfite treated samples were 0.68 and 0.03, respectively. This difference (0.65) multiplied by the dilution factor (2) would equal 1.30. This number is related to pigment content but is not an actual quantitative measurement. If the absorbance value (0.65) were substituted in the formula for determination of anthocyanin content (Part 1 - pH differential method), a low quantitative value would be obtained as the molar absorbance values in Table 1 are for pH 1 where 100 percent of the anthocyanin is in the oxonium salt form.

Other Spectrophotometric Measurements - Turbidity, Browning, Anthocyanin-Degradation Index

The absorbance at 700 nm can be used as a measure of turbidity or haze. This is a measurement of the scattering of light by the suspended solid particles. Increased haze will result in higher absorbance readings; calibration curves for turbidity measurement are usually not linear, however.

Browning is often expressed in terms of absorbance units at the shorter wavelengths of the visible spectrum; 420, 440, 450, and 490 nm have all been used. We make our measurements at 420 nm as this is a widely adopted practice.

Another method of measuring anthocyanin degradation would be to determine the ratio of the absorbance at the anthocyanin λ max to the absorbance at 420. Samples in which a great deal of browning occurred would tend to have a low numerical value and fresh fruits should have a high number.

## Preparation of Reagents

### Buffers for Determination of Anthocyanin Content

pH 4.5 buffer: 400 ml of 1 M sodium acetate (136 g/l)
+ 240 ml of 1 N HCl (83.0 ml conc. HCl/l)
+ 360 ml distilled water

pH 1.0 buffer: 125 ml of 0.2N KCl (14.9 g/l)
+ 385 ml of 0.2N HCl

The pH of the buffers was adjusted as required to obtain final pH values of 1.0 and 4.5.

### Bisulfite solution for determination of polymeric color, 20 percent potassium metabisulphite solution

Two g of $K_2S_2O_5$ is made up to 10 ml with distilled $H_2O$. (We follow the practice of making this reagent up daily; otherwise, it develops a yellow color which contributes to absorbance readings.)

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