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Measuring Phenolics in the Winery

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Abstract: Phenolics are a widely recognized, fundamental quality component of grapes and wines. They are responsible for key wine properties, including color, bitterness, astringency, and longevity. Although many methods are available for measuring grape and wine phenolics, few are suited for rapid, routine analyses in a winery laboratory setting. All current published methods that are within the economic realm of routine winery use require a spectrophotometer. This review concentrates on the spectrophotometric methods designed to measure different phenolic components in grapes and wines, including tri-stimulus, total phenolics, protein precipitable tannins, and color components.

Key words: phenolics, wine, anthocyanins, tannins, analysis, color

Phenolics are responsible for many important properties of wine including color, bitterness, astringency and antioxidant capacity. Phenolics are viewed as a parameter beyond sugar and acid by which to judge the quality of grapes, fermenting musts, and finished wines. It is widely recognized that phenolics are a fundamental component of wine quality. However, measuring phenolics in the winery is not a widespread practice. While most wineries are not equipped with the necessary personnel or equipment, some wineries perform in-house measurements of the phenolic content of musts and wines and some contract with external analytical laboratories. The most common instruments used to measure phenolics in the winery are spectrophotometers because of their ease of use and relatively low cost. Outside laboratories and large wineries often use high-performance liquid chromatography (HPLC) and infrared (IR) spectroscopy. Our goal is to discuss the techniques available for wineries to measure phenolics in white and red wines using a spectrophotometer.

Color Methodologies

One of the most important contributions of phenolics to wine is color. Red and white wine coloration comes from a small percentage (0.5%) of the components present in wine. It is estimated that 99.5% of the components in wine are transparent in the visible light spectrum (Somers 1998).

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Red wine color. Anthocyanins are the main source of pigmentation in red wine. A phenomenon known as copigmentation occurs in young red wines. Copigmentation, the enhancement of color due to formation of complexes between anthocyanins and colorless cofactors (Levengood and Boulton 2004), is a phenomenon that occurs in young red wines. Some cofactors can also cause wine to take on a bluish appearance, which is known as a bathochromic shift or "blue shift" (Boulton 2001).

When grapes are crushed, anthocyanins are able to react with many other must components, such as acetaldehyde, tannins, keto acids, and cinnamates. These anthocyanin-derived pigments were initially described by Somers as polymeric pigments (Somers 1971, Remy et al. 2000, Bakker and Timberlake 1997, Schwarz and Winterhalter 2004). The amount of polymeric pigments increases as the wine ages (Somers 1971). Polymeric pigments are the source of "stable color" in red wine because they are more resistant to bisulfite bleaching and their color is not as pH-dependent as anthocyanins (Somers 1971). It is now recognized that not all anthocyanin-derived pigments included in Somers' initial definition of polymeric pigments are polymers and not all of the new anthocyanin adducts are colored or as stable as the original compounds (Remy et al. 2000). Compounds such as vitisins, pinotins, and portosins have been isolated from wines which contain bisulfite and which are pH stable, but these compounds are not polymeric in nature (Bakker and Timberlake 1997, Schwarz and Winterhalter 2004, de Freitas and Mateus 2004). Copigmentation has only been demonstrated to occur with monomeric anthocyanins; therefore, copigmentation in older red wines is negligible. Young red wines are typically bright red with some purplish hues, while older red wines tend to have a more brick-red appearance because of the different spectral characteristics of free anthocyanin and polymeric pigments (Somers 1971).

Red wine coloration can be monitored with a spectrophotometer and can be broken down into measurements that determine color specification and component analysis. A tri-stimulus measurement provides numerical values

more directly related to the color perceived by the human eye than pigment concentration. Color intensity and tint can be measured independently or can be derived from data collected during the tri-stimulus analysis. The color components that contribute to red wine color can also be measured by determining the amount of copigmentation, anthocyanins, and polymeric pigments.

White wine color. The principal compounds responsible for coloration of white wines are the catechins and hydroxycinnamates, which are initially colorless but can be oxidized either enzymatically or chemically to form yellow and brown products. In white and red musts, browning can occur through oxidation of phenolics by polyphenol oxidase in the presence of oxygen (Singleton et al. 1985). As white wine ages, catechins and hydroxycinnamates become oxidized and polymerize with other, nonoxidized phenolics to make brown polymers that eventually precipitate (Cheynier et al. 1990). In model wine systems, catechin will react with glyoxylic acid to form a yellow xanthylium compound (Es-Safi et al. 2000).

Spectral Properties of Phenolics

Wine is a heterogeneous mixture of phenolics. Each compound has its own specific spectral properties, but within each class the compounds are similar enough that generalized characteristics can differentiate them. There is one spectral property common to all phenolics, which is an aromatic ring that allows absorption in the ultraviolet region. Flavonoid compounds such as catechins and tannins (condensed and hydrolyzable) have a characteristic peak at 280 nm, while the flavonols such as quercetin and kaempferol absorb at 365 nm. Of the nonflavonoid compounds, hydroxycinnamic acids and stilbenes absorb maximally between 300 and 330 nm. Although the phenolic classes each have unique chemical signatures, there is enough overlap that it is difficult to determine the individual components in a mixture such as wine.

Anthocyanins are an exception because they have high molar extinction coefficients at wine pH and their absorption maxima are dependent upon pH. The absorption maxima of anthocyanins at wine pH are also at a wavelength (520 nm) sufficiently distant from other classes not to overlap. The structurally undefined polymeric pigments and anthocyanin-derived pigment classes (pinotins, portosins, and vitisins) each have absorption maxima close to that of the pigment from which they were derived. The polymeric pigments are generally regarded to absorb at 520 nm (Somers 1971), while portosins, pinotins, and vitisins absorb maximally at 580, 520, and 510 nm, respectively (de Freitas and Mateus 2004, Schwarz and Winterhalter 2004, Bakker and Timberlake 1997, Fulcrand et al. 1998).

Red Wine Color Characteristics

Tri-stimulus. Tri-stimulus is the most widely used system for color specification and was defined by the Commission Internationale de l'Eclairage (CIE). Within this system, any color can be matched or defined by combining three primary colors represented by mathematical constructs known as tri-stimulus values. A spectrophotometer is used to measure the transmittance or absorbance of a colored sample at intervals across the visible spectrum. Collected data are then multiplied by three color-matching functions and summed to obtain the X, Y, and Z tri-stimulus values. Once the values are determined, they are normalized and the Z value is ignored. The X and Y coordinates are mapped onto a two-dimensional color spectrum graph, and the sample's color can be defined.

Natural products are known to be spectrally smooth within the visible light region, and therefore data collected across this spectrum can be reduced using principal component analysis (PCA). Several attempts have been made to determine the wavelengths that optimize different types of wine (Glories 1984, Ayala et al. 1999, Perez-Caballero et al. 2003) so that a wine's color can be more easily determined using characteristic wavelengths, instead of measuring absorbance across the entire visible spectrum. The current International Organization of Vine and Wine (OIV) method uses four characteristic wavelengths at 625, 550, 495, and 445 nm (OIV 1990). The most recent methodology uses four different wavelengths (450, 520, 570, and 630 nm) and CIELAB space (Perez-Caballero et al. 2003). It converts the tri-stimulus values into L*, a*, and b* and then plots them in CIELAB. The authors report an error of only 2.5 CIE units, where the minimum color difference between two wine samples discernable by human subjects is 2.7 CIE units.

Wine Color Composition Measurements

The color of red wine is mainly due to anthocyanins, which exist in different forms depending on the surrounding pH. The flavylium form is red and has an absorption maximum at 520 nm. This form is easily decolorized by the addition of bisulfite (HSO_3^-), a form of antimicrobial agent commonly added to wine and referred to as SO_2. When wine color is measured in a spectrophotometer, the measurement does not just represent anthocyanins. Copigmentation and other anthocyanin-derived pigments collectively known as "polymeric pigments" contribute to color as well. Recent findings have demonstrated that anthocyanins can react with other wine components to form colored and noncolored products that are both polymeric and nonpolymeric in nature and are not all completely resistant to bisulfite bleaching. Thus, the original definition of polymeric pigments is somewhat of a misnomer, as it does not include all possible anthocyanin-derived pigments and does includes compounds that are not polymeric in nature. Although the label "polymeric pigments" is problematic, the practice of measuring bisulfite-stable color continues to be very useful. While nonpolymeric compounds resistant to bisulfite bleaching have been discovered, their abundance in wine and significance has yet to be established. Furthermore, there are no current

methodologies available to measure them in a winery setting. Thus, for the purposes of this review, the term "polymeric pigments" despite its flaws is used and the methods developed to measure bisulfite stable color are examined.

Copigmentation. Currently, there is only one method (Levengood and Boulton 2004) for measuring red wine color that includes a copigmentation measurement, despite other authors making reference to copigmentation as early as 1977 (Somers and Evans 1977). In this method, each sample is adjusted to pH 3.6 with base (NaOH) or acid (HCl) to eliminate color differences due to pH. Copigmentation is measured by comparing diluted and undiluted wine samples. Because of the high molar extinction coefficients of anthocyanins, it is necessary to place undiluted wines into a cuvette with a 1- or 2-mm path-length cuvette to obtain an accurate measurement. A dilution of 20- to 100-fold reduces the concentration of anthocyanins for most wines to below the minimum concentration necessary for copigmentation and also liberates anthocyanins that were previously colorless because of the binding of bisulfite. A small amount of acetaldehyde is added to the undiluted sample. Acetaldehyde reacts readily with bisulfite and liberates bisulfite bound anthocyanins, thus compensating for the second effect of dilution and increasing parity. These measurements are calculated as follows:

Copigmentation color (AU) [C] = (A_{520}Undiluted – A_{520}Diluted)
Anthocyanin color (AU) [A] = (A_{520}Diluted – $A_{520}SO_2$)
Polymeric pigment color (AU) [P] = ($A_{520}SO_2$)

Anthocyanins are calculated by comparing the diluted sample to an undiluted sample that has been swamped with bisulfite. The sample that has been decolorized with bisulfite is a measure of the polymeric pigments. All of these measurements are reported in absorption units and not converted using an extinction coefficient or standard.

In the Somers and Evans (1977) method, anthocyanins are measured by comparing a wine sample adjusted to pH <1 to a wine sample that has been decolorized with bisulfite. At low pH the anthocyanins are in the colored flavylium form, and color due to polymeric pigments is almost completely unaltered. According to the method, all measurements can be carried out in a 1-, 2-, or 5-mm path-length cuvette and all absorbance measurements are converted to a 10-mm path-length equivalent. The absorbance of a sample at its natural pH less the absorbance due to polymeric pigments provides an estimate of the amount of anthocyanins in the colored form at the unique pH of the wine. Polymeric pigments are estimated by dividing the absorbance of the bisulfite-decolorized sample by the absorbance of the acidified sample. These measurements are calculated as follows:

Total anthocyanins (mg/L) = 20 (A_{520}HCl – 5/3 x $A_{520}SO_2$)
Ionized anthocyanins (mg/L) = 20 (A_{520} – $A_{520}SO_2$)
Polymeric pigments = $A_{520}SO_2$/A_{520}HCl

The main difference between the Levengood and Boulton (2004) and the Somers and Evans (1977) method is the inclusion of the copigmentation assessment. Most commercial laboratory wine assessment is done within the first two years of a wine. Since the phenomenon is found primarily in young wines and can account for up to 40% of the observable differences, its measurement is important. The drawback of the Somers and Evans method arises from the often-lengthy pH adjustment required for each sample. However, this step is required to standardize the analysis because free and copigmented anthocyanins display different pH responses. The other practical constraint that hinders analysis is the 45-min incubation period required for the undiluted wine to react with acetaldehyde. When the longest incubation periods for the two methods are compared, the 3-hr incubation required for the Somers and Evans method is far more tedious. The major drawback of the Levengood and Boulton method is that it does not measure copigmentation or anthocyanins observed at a wine's native pH or provide a measure of total anthocyanins present in the sample. However, it is the only comprehensive method available for measuring all main wine color components and thus is very useful. The Somers and Evans method does measure total and ionized anthocyanins in a sample, but does not provide a measure of copigmentation and is more tedious to practice.

Color intensity and hue. Wine color intensity has been traditionally represented by the sum of A_{420}, A_{520}, and A_{620} (Glories 1984) or the sum of A_{420} and A_{520} (Sudraud 1958). The tone or hue of wine is calculated as the ratio of A_{420} and A_{520} (Glories 1984, Sudraud 1958). Color intensity typically measures how much color a wine has, while changes in hue are used to monitor wine aging because of the shift from red to brick red that occurs during aging. These measurements are useful in the context of monitoring a single wine as it ages, but their utility for comparing multiple wines is limited because the color of wine is pH-dependent and wine pH is variable. The measurements are also unable to provide information about how different phenolic components will change during wine processing and aging, making it somewhat difficult to interpret the results. However, the simplicity and lower cost of the methods make them an attractive alternative to measuring the different color components. Results using Sudraud's methodology were summarized by Somers, in which a survey of Cabernet Sauvignon and Shiraz wines showed that the range of color intensity varies between the varieties; however, both populations had identical mean values (Somers 1998). The color intensity of the wines varied over 8-fold. The variability in hue of over 400 Cabernet Sauvignon and Shiraz was similar and found to vary only 2.4-fold.

Total Phenolics Measurements

There are several methods available to measure total phenolics in red and white wines with a spectrophotometer. Two popular methods are the measurement of the absorbance in the ultraviolet (UV) region at 280 nm (A_{280}) and

the use of the Folin-Ciocalteu reagent. Several new methods have also been introduced for measuring total phenolics. Each method has benefits and drawbacks.

Absorbance at 280 nm (A_{280}). The simplest method for measuring phenolics is based on absorption of UV light by compounds with aromatic rings. Measurement requires a quartz or polycarbonate cuvette because silicate and plastic cuvettes will absorb in the UV spectrum, causing significant interference. The utility of this method is its simplicity. Ultraviolet absorbance is not influenced by pH, and Beer–Lambert's law for concentration and light path length are followed (Somers 1998), making data simple and easy to collect.

One drawback is interference caused by other nonphenolic compounds in wines that contain aromatic rings. Such compounds include nucleotides, proteins, peptides containing aromatic amino acid residues, and free aromatic amino acids. Research on many wines showed that interference in wines and musts was statistically constant (Somers 1998). After back calculation to account for initial dilution, the researchers found an average nonphenolic background of 4 absorbance units and that value can be subtracted to yield a more precise measure (Somers 1998). A drawback of A_{280} is that it is a general measurement and provides no information about subclasses of phenolics. Such information can only be obtained with chromatography or the use of chemical reagents and fractionation.

Folin-Ciocalteu. The Folin-Ciocalteu method is probably the most-studied chemical reagent used for measuring total phenolics in red and white wine and has been reviewed in several papers (Singleton et al. 1999, Singleton 1974). The reagent is a mixture of phosphomolybdic and phosphotungstic acids. It is difficult to formulate correctly but can be purchased from laboratory suppliers. The Folin-Ciocalteu reagent (FCR) is the end product of research into improving the original Folin-Denis reagent for stability and sensitivity (Singleton and Rossi 1965). The Folin-Ciocalteu reaction has its basis in oxidation/reduction chemistry. First, wine is added to the FCR and the phenolate ions are mixed with oxidizing agents, which change from yellow to blue once reduced. The mixture of oxidizing agents and wine is then added to an alkaline solution. Under alkaline conditions, phenolics ionize completely to their phenolate form and can be readily oxidized by the FCR. The oxidized phenolate changes to the quinoid structure, while the oxidizing agents gain an electron, going from a 6+ to a 5+ oxidation state. The color change is monitored with a spectrophotometer and converted into a concentration using a standard.

Because the FCR is unstable under alkaline conditions, the wine must be first mixed with the FCR and then the base. The reaction takes about two hours for completion at room temperature. The temperature can be raised to speed up the reaction; however, there is a slight loss in sensitivity (Singleton and Rossi 1965). A sample blank without FCR is necessary because of significant background interference (Singleton et al. 1999).

The FCR is a mixture of different oxidizing agents that reacts with compounds that donate electrons easily. It reacts with mono- and dihydroxylated phenolics; however, methoxyl substitution blocks reactivity (Singleton et al. 1999). Phenolics exhibit different molar color yields depending on the number of phenolic groups (Singleton et al. 1999, Singleton 1974). One of the greatest strengths of the FCR is that the chromophore produced is independent of the source of electrons. Different substrates have no influence on the spectral property of the chromophore, which simplifies standardization.

Originally, tannic acid was used as a standard for phenolics measurements. Preparations of tannic acid vary, so gallic acid was chosen as a standard instead. Values are expressed in gallic acid equivalents (GAE). Gallic acid is relatively inexpensive, stable when dry, and readily soluble in aqueous solutions. The analysis is reproducible, with a standard error of 1 to 5% (Singleton and Rossi 1965).

The highly reactive nature of FCR is both a benefit and drawback. Many other compounds in wine can donate electrons and cause interference. Fructose, bisulfite, tyrosine, tryptophan, cysteine, proteins, guanine, xanthine, and ascorbic acid interfere. Of these, the most significant are fructose and bisulfite. "Swamping" the sample with acetaldehyde will bind free bisulfite and reduce interference by 50% (Singleton et al. 1999). A correction factor can be subtracted from the total value to compensate for bisulfite (Ough and Amerine 1988), as follows:

$$\text{Total } SO_2 \text{ (mg/L)} \times 0.122 = \text{GAE correction}$$

Fructose, a reducing sugar, also interferes with the FCR measurement. Dividing the phenolic concentration by a correction factor based on wine sugar concentration can compensate for errors caused by sugar. A table containing correction factors is available (Ough and Amerine 1988).

Other methodologies. While there are other published methodologies for measuring phenolics, most have been neglected in favor of modern liquid chromatographic methods. Nevertheless, these methods remain valid and readily accessible to winery laboratories equipped with a few basic pieces of equipment and a spectrophotometer. Singleton (1999) provides an extensive review of different techniques that can be used in conjunction with FCR. Under highly acidic conditions, formaldehyde can be used to cross-link and precipitate flavonoid compounds. The precipitation reaction requires a 24-hr incubation and centrifugation. The nonflavonoid phenolic content can be determined by subtracting the flavonoid component from the total phenolics. However, formaldehyde will not precipitate anthocyanins or flavonols unless there are sufficient flavonoids available to cross-link them (Singleton et al. 1999). The greatest drawback of this technique is that it uses at least 40 mg of formaldehyde per sample. Formaldehyde is a known health hazard and requires the use of a specialized fume hood not normally found in wineries.

It has been demonstrated that nonflavonoid content of wine does not vary appreciably with wine processing and

can be assumed constant. Since barrel-derived phenolics are nonflavonoid hydrolyzable tannins, the phenolics extracted from oak can be estimated (Singleton et al. 1971).

Hydrolyzable tannin can also be determined by using cinchonine sulfate (Peri and Pompei 1971). Combining cinchonine sulfate with formaldehyde allows measurement of tannin, hydrolyzable tannin, flavonoids, and nonflavonoid phenolics (Peri and Pompei 1971). The technique used thin-layer chromatography (TLC), which is also used to monitor malolactic fermentation, thus the technology may be available in some wineries (Harris 2001). Sodium chloride with ethyl acetate has also been used to precipitate tannins, but it is more variable than cinchonine sulfate (Margheri et al. 1976, Margheri and Tonon 1977, Margheri and Versini 1979). This method also uses TLC, but may eventually be adapted for use with a spectrophotometer.

Iron chloride. Iron chloride has recently been reintroduced as a reagent for measuring phenolics, in a protein precipitation assay for tannin (Harbertson et al. 2002, 2004). Methods using iron as a reagent had been abandoned because it did not react with monohydroxylated phenolics and because vicinal dihydroxylated and trihydroxylated phenolics gave different colored products under some conditions (Singleton et al. 1999). Use of ferric chloride to measure total phenolics in conjunction with protein precipitation could allow greater standardization of phenolics assessment in red wine. Caffeic acid, caftaric acid, catechins, quercetin, and gallic acid all react with ferric chloride. However, anthocyanins do not react, but all phenolics containing vicinal dihydroxyls do react. The FCR and ferric chloride methods were highly correlated ($r^2 = 0.92$, $n = 26$), indicating that ferric chloride is a suitable reagent for all phenolics in wine except anthocyanins and monohydroxylated phenolics (Harbertson et al. 2004). The strength of the system is that ferric chloride can be used to measure both tannin (discussed later) and total phenolics with the same standardized units. It can also be combined with measurement of polymeric pigments, anthocyanins, and copigmentation to provide a convenient assessment of the functional classes of phenolics in grapes and wines. There is no interference from bisulfite or reducing sugars (Harbertson et al., unpublished data, 2003), but because the iron chloride reagent does not measure monohydroxylated phenolics or anthocyanins, the assay is best termed a measure of iron-reactive phenolics rather than total phenolics. The vicinal dihydroxylated phenolics that are measured are the most readily oxidizable phenolics and are therefore of great importance. The iron-reactive phenolics measurement is not as sensitive as the FCR but is relatively nontoxic and less problematic for waste removal and lab safety.

Enzymatic method. An enzymatic method for measuring total phenolics based on the Trinder reaction (Trinder 1969) was recently developed for wine (Stevanto et al. 2004). The new analysis uses an enzyme to measure the phenolic content of wine by monitoring the production of a colored quinone-imine product. Colorless 4-aminophenazone is added to a sample containing a phenolic compound and hydrogen peroxide in the presence of horseradish peroxidase to produce water and a colored quinone-imine product. Hydrogen peroxide oxidizes the enzyme, which in turn reacts with the phenolics to make phenoxyl radicals. The phenoxyl radicals covalently bond to the colorless 4-aminophenazone (4-AMP) to form a colored quinone-imine product. The reaction is linear and is performed as an end-point analysis in a spectrophotometer. It requires only a 5-min reaction time and is linear over a broad range. Catechin was linear from 10 to 200 μM while wine samples were linear from 10 to 250 μL samples. The importance of linearity in laboratory procedures is discussed in (Butzke and Ebeler 1999).

Although the author stipulates that horseradish peroxidase (HRP) shows little selectivity for its phenolic substrates (Stevanto et al. 2004), it has not been demonstrated whether the most abundant phenolics present in grapes are reactive with either the HRP or 4-AMP. Caftaric acid, the most abundant nonflavonoid present in grapes, was not tested. Tannins and anthocyanins, the most abundant flavonoids in red wine, also were not tested. Catechin, gallic acid, kaempferol, quercetin, ferulic acid, resveratrol, and catechol all reacted with HRP and displayed a product with a similar visible spectrum, but with slightly different molar absorbances ranging from 4500 to 5900 M^{-1}.

The enzymatic reaction correlated well with the Folin-Ciocalteu reaction. In three sets of data examining 23 red wine samples, the correlation coefficient ranged from 0.85 to 0.95. The enzyme system did not correlate as well with white wines ($n = 16$), with correlation coefficients ranging from 0.70 to 0.81. The standard error of the analysis was less than 2%. Compounds that interfered with the FCR, such as ascorbic acid, citric acid, and sulfite, did not interfere with the enzymatic assay. The enzymatic system is not as sensitive (about 30 to 40% less sensitive with red and white wines). However, when the two methods were tested with tea samples, both methods correlated well and gave approximately the same value. It is not clear why there is a discrepancy between tea samples and wine samples; however, tea lacks condensed tannin and anthocyanin, which are the two main phenolic classes in red wine and neither has yet been tested with HRP or 4-AMP.

Protein Precipitation Methods for Measuring Tannins

Tannin is the common name given to several classes of phenolic compounds. Tannins are further divided into two subcategories: condensed proanthocyanidins and hydrolyzable. Both can interact with protein, forming soluble and insoluble complexes (Makkar 1989). The tannin-protein complexes readily precipitate under the right conditions of pH, ionic strength, solvent strength, and temperature (de Freitas and Mateus 2001). The ability of tannin to bind protein is dependent on the molecular weight of the tannin and the number of sites on the tannin capable of interact-

ing with the protein (Hagerman et al. 1998). Most protein precipitation analyses are designed to maximize precipitation, such as in the Hagerman–Butler assay in which the buffer pH is near the isoelectric point of the protein (Hagerman and Butler 1978).

The efficiency and mechanism of protein precipitation is different for the different classes of tannin. This was demonstrated using purified condensed and hydrolyzable tannin with bovine serum albumin (BSA) (Hagerman et al. 1998). Purified condensed tannins precipitated more protein on a molar basis than purified hydrolyzable tannins. Condensed tannins precipitate protein independently of temperature and the presence of organic solvent, whereas protein precipitation by hydrolyzable tannins increases with temperature and decreases when alcohol is present. Precipitation of protein by the two classes of tannin is believed to be due to different mechanisms. It is thought that hydrolyzable tannins form a hydrophobic coating on the surface of the protein, while condensed tannins form hydrogen-bonded cross-links between protein molecules (Hagerman et al. 1998). The practical sensory implications of this have yet to be explored.

The insoluble tannin-protein complexes are of particular interest to researchers and assays for them have been developed (Bate-Smith 1973, Hagerman and Butler 1978, Glories 1984, Makkar et al. 1988). Many protein precipitation assays for tannin use a spectrophotometer (Makkar 1989); however, measurement of tannin in wine by protein precipitation is a relatively new technique. Several published methods have been developed (Glories 1984, Adams and Harbertson 1999, Harbertson et al. 2002, Llaudy 2004) and a brief description follows.

One of the first protein precipitation assays used with red wines is the Glories method, which takes advantage of the breakdown of proanthocyanidins to colored anthocyanidin, delphinidin, or cyanidin products (Glories 1984). It was initially used by Swain and Hillis (1959) and Bate-Smith (1954b) and modified for wine by Ribéreau-Gayon and Stonestreet (1966). The sample is boiled in acidic ethanol for 30 min, and then the absorbance is taken at 550 nm. Gelatin is then added to another sample of wine to form a tannin-protein precipitate. After three days the tannin protein precipitate is centrifuged, and the supernatant is boiled in an acidic ethanol solution for 30 min and compared with the nonprecipitated sample. The use of both measurements allows for the quantification of both total and protein-reactive tannins. The difference is known as the gelatin index, because the precipitated tannin is expressed as a percentage of the total tannin in the solution. The value can be expressed as a concentration, but this is not widely practiced:

Gelatin index =
(Total tannin – Protein precipitated tannin/Total tannin) x 100

One of the difficulties for the Glories Gelatin Index method is that it relies upon a long precipitation step, making the assay tedious. A protein precipitation assay was recently developed that uses purified chicken egg protein, ovalbumin (Llaudy et al. 2004). The Llaudy method is similar to the Glories Gelatin Index because it takes an initial measurement, and after precipitation and centrifugation it measures the supernatant. The Llaudy method uses A_{280} to measure total phenolics, and then assumes that the phenolics left in the supernatant after precipitation with protein are nontannin phenolics. After the initial A_{280} measurement, 12 vol of serially diluted ovalbumin (0 to 4 mg/mL) is added to fixed amounts of either wine or tannic acid (used as a standard). After a 10-min incubation, the samples are centrifuged and the supernatant is measured at A_{280}. The log of the absorbance is plotted against the concentration of tannic acid added for the standard curve. An experiment was performed (Llaudy et al. 2004) that compared wines rated for astringency by sensory methods and the amount of tannin as measured by the Llaudy method and the Glories Gelatin Index. Ten panelists rated wines for astringency with a 100-point scale anchored with three standard wines scored 30, 50, and 85. Both methodologies correlated well with astringency scores from the same wines. The Gelatin Index and Llaudy method had linear regression coefficients of 0.71 and 0.77, respectively. However, the absolute tannin concentrations given by the two methods did not correlate well and the ovalbumin methodology values were much lower (Llaudy et al. 2004), suggesting that the ovalbumin method is less sensitive than the gelatin method, but quicker and not as tedious. One drawback of the ovalbumin method is that it is somewhat cumbersome, because it requires 12 samples of each wine to be run with various concentrations of protein. That is necessary because the analysis utilizes A_{280} to monitor phenolics, and it is known that protein interferes. Thus, the amount of protein found in the supernatant must be minimized to optimize reproducibility, and the absolute amount of protein precipitation must be found for each sample at the expense of efficiency.

Another widely used tannin-protein precipitation methodology was first developed in 1978 by Hagerman and Butler and was later adapted for use with grapes and wine (Harbertson et al. 2002). The tannin protein-precipitate is formed after incubating for 15 min and the pellet is washed and resuspended in an alkaline detergent. After measuring background absorbance at 510 nm, the tannin is measured directly by adding ferric chloride and remeasuring the absorbance at 510 nm.

This method can be combined with a measurement of total phenolics using iron chloride reagent discussed earlier. It can also be combined with bisulfite bleaching to separate polymeric pigments by size into small and large fractions (SPP and LPP, respectively), adding further utility to the method (Harbertson et al. 2003). Research on grapes and wine (Harbertson et al. 2003, Adams et al. 2004) demonstrated that there were small amounts of SPP in the fruit and preferential formation of LPP during wine aging, which agrees with other results (Somers 1971).

A recent study (Boselli et al. 2004) compared wines evaluated by sensory methods for bitterness and astringency to the same wines measured for tannin, SPP, LPP (Harbertson et al. 2002, 2003), anthocyanins, copigmentation (Levengood and Boulton 2004), monomeric phenolics by HPLC (Donovan et al. 1998), and sugars, organic acids, ethanol, and glycerol by HPLC. Partial least squares (PLS) analysis was used to predict sensory results based on the chemical data. Astringency was positively correlated with tannins, LPP, gallic acid, and an unidentified catechin derivative and was negatively correlated with catechin, organic acid content, and measurements indicative of color (except LPP). It is has been hypothesized that tannin-anthocyanin adducts may have different protein-binding properties than tannin (Singleton 1992). These results associate polymeric pigments with astringency; however, the authors do not discuss any differences between polymeric pigments (LPP) and tannin. Further discussion of recent findings with regard to the reduction of astringency by anthocyanins and polymeric pigments isolated from wine are discussed in (Cheynier et al. 2006).

Indirect methodologies for measuring tannins. Methodologies that do not measure tannin directly but instead measure either the protein precipitated in the tannin-protein complex or an enzymatic activity (like HRP) are not widely practiced. Although many methods have been developed, only a few have been used in wine (Bate-Smith 1973, Asquith and Butler 1985, Makkar et al. 1989, Marks et al. 1987). A method adapted for use with wine was developed recently that is based on the ability of tannin to precipitate both alkaline phosphatase and BSA from a mixture of the two proteins (Adams and Harbertson 1999). Alkaline phosphatase is a unique enzyme in that it is not inhibited by tannin (Adams and Harbertson 1999, Ittah 1991, Goldstein and Swain 1965). The tannin-protein precipitate is pelleted by centrifugation and washed to remove residual unprecipitated alkaline phosphatase. The tannin-protein complex is then dissolved in an alkaline buffer, and the alkaline phosphatase activity in the dissociated precipitate is determined by the addition of para-nitrophenylphosphate (pNPP), a substrate converted by the enzyme to a yellow product that absorbs at 405 nm. The alkaline phosphatase activity in the dissolved pellet is proportional to the tannin used to form the precipitate. The enzyme activity is linear; thus, the assay can be conducted as an end-point assay or the absorbance at 405 nm can be monitored with a spectrophotometer for one minute.

Advantages and disadvantages of methods. Methods that use protein precipitation for measuring tannins are dependent on the conditions of analysis. If protein is available in extreme excess, then it can remove all phenolics rather than selectively removing polymers. None of the methods available uses protein in such excess, so this problem is generally not encountered. However, it does point out that the distinction between large and small polymeric species depends upon the tannin composition of the sample and the experimental conditions of the analysis (such as protein, pH, dilution range). Protein precipitation methods should undergo validation by comparison with more selective analytical methods such as those available with HPLC and gel permeation chromatography (GPC) coupled with mass spectroscopy (MS) to confirm which fractions of tannins are being removed. An example is the work of DeBeer and colleagues (DeBeer et al. 2004), who compared a normal-phased HPLC technique for measuring phenolics to various colorimetric analyses including one protein precipitation tannin assay (Harbertson et al. 2002). Results showed that tannins measured by protein precipitation correlated strongly to the high molecular weight polymers and total polymer content measured by NP-HPLC ($r > 0.9$, $p < 0.001$), but not the low molecular weight polymers.

The advantage in using protein precipitation methods for measuring tannins is that generally there is no interference from compounds found in significant quantities in wine (such as sugar, acid, bisulfite, or ethanol). The most significant advantage of protein precipitation is that it measures the biological activity of tannin (Makkar 1989). Human salivary proteins interact with tannin to form insoluble complexes, which forms the mechanistic basis of the tactile sensation known as astringency (Lu and Bennick 1998, Sarni-Manchado et al. 1998, Noble 1998). Despite the disadvantages of tannin analysis using protein precipitation, the methods show good correlation with sensory data for astringency. Eventually, quantification of phenolic parameters including color, copigmentation, anthocyanins, tannins, and total phenolics will be more commonplace in wineries and may provide better information for winemakers as they make production decisions and quality assessments.

Conclusions

A spectrophotometer can be used to measure wine phenolics easily and quickly in a winery setting. Wine color can be described using the tri-stimulus method and phenolic concentration by measures of copigmentation, anthocyanin, and polymeric pigment concentration. The tri-stimulus measurement of wine color is a useful tool, and the methodology developed by Perez-Caballero et al. (2003) shows much promise. Measuring color composition is a difficult task. The method developed by Levengood and Boulton (2004) for measuring copigmentation, anthocyanin, and polymeric pigment is highly recommended because of its comprehensive approach to describing wine color composition.

Measuring the phenolic content of wine has been a controversial subject among researchers. The ideal analytical system would describe the total phenolic content of a wine and break it down into the corresponding subclasses. That would require standardized units and reproducibility. To achieve this with a spectrophotometer and no chemical reagents is impossible because of the spectral overlap of

each of the phenolic classes. Some researchers consider that chemical reagents are too problematic and that attempts to use colorimetric methods have "retarded progress in this aspect of oenology" (Somers 1998). The FCR can be combined with other techniques to evaluate each phenolic component of wine successfully. Given the emphasis on using less hazardous and carcinogenic reagents in laboratories, formaldehyde and FCR may no longer be appropriate. Methods involving iron chloride may offer effective and less hazardous alternatives. Current research on iron chloride has been limited to total iron reactive phenolics and tannin, so its usefulness should be explored further. Gelatin and ovalbumin assays are useful for measuring tannins, but cannot measure total phenolics with the same standardized units, although ovalbumin may prove useful since it uses A_{280}. The assay with 4-AMP and HRP may become valuable when more is known about its reactivity with the most abundant wine and grape phenolics. Eventually 4-AMP and HRP may be combined with protein precipitation to give a measure of tannin. The use of A_{280} is still the most rapid and simple measurement of total phenolics, but it provides limited information because it is difficult to use with fractionating techniques.

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