

Research Note

Evaluation of Small Amounts of Flavonoid Phenols in White Wines by Colorimetric Assays

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For evaluation of flavonoid phenols in the low concentration range in white table wines, four colorimetric assays have been compared: (a) reaction with Folin-Ciocalteu reagent before and after precipitation of flavonoids in HCl-methanal (FC); (b) reaction with vanillin (VAN); (c) reaction with 4-(dimethylamino)-cinnamaldehyde (DAC); and (d) a rapid browning test at 60°C in oxidative medium (POM). There was a highly positive correlation between concentration units obtained by FC, VAN, and DAC, while POM is poorly correlated to concentration units. VAN and, to a lesser extent, FC lead to an overestimation of flavonoid content. DAC displays highest sensitivity and reproducibility. It appears most suitable for appreciation of oxidative aging potential in routine control.

KEY WORDS: flavonoid phenols, oxidative aging, browning, Folin-Ciocalteu reagent, vanillin, 4-(dimethylamino)-cinnamaldehyde

Flavonoid phenols are located in grape solids in variable amounts, depending mainly on cultivar and grape maturity. During vinification, their extraction is highly variable. Maceration with and without stems, mechanical treatment of must by pumping and pressing procedures, the presence of sulfur dioxide prior to alcoholic fermentation, as well as high temperatures are known to enhance extraction.

Since modern analytical means are available for determination of individual phenolic compounds and study of their behavior, it has become known that the flavonoid fraction is primarily responsible for developing bitterness, astringency, and browning during oxidative aging of white wines (2,10,11,12,14,16,18,20). Thus, for white table wine production, rapid processing of must by moderate mechanical influence is recommended.

Oxidation of white grape juices reduces the amount of flavonoid phenols by oxidative polymerization and precipitation. Hyperoxygenation is described as a technique to eliminate them to a great extent. Wines produced in this way are considered more resistant against oxidative aging and the occurrence of astringency (4,6,14,16). The amount of flavonoid phenols in juice is further lowered by adsorption of yeast during fermentation. Fining with phenolic reactive agents such as caseinates and PVPP is often used to remove phenolic compounds when wine appears harsh and bitter. However, these reagents are not specific for flavonoids and reduce the non-flavonoid fraction as well. Furthermore, in comparison to the enzymatically induced precipitation reactions of hyperoxygenation which in many cases lead to practical absence of flavonoids, fining procedures require unusually high amounts of fining compound to obtain similar low levels of residual flavonoids (16).

Flavonoids undergo oxidative polymerization when oxygen is consumed, and their flavor threshold is lowered as polymerization occurs. Thus, they are often not detected by sensorial means in young white wines, but turn out to develop flavor after a certain aging period, specially after bottling (4,11,12).

For European white table wines grown under cool climate conditions, the presence of flavonoids is generally considered undesirable because shelf life and typical cultivar aroma are reduced. Therefore, analytical techniques to control flavonoid content are of increasing interest in order to predict flavor stability.

Experimentation upon hyperoxygenation (14) showed browning behavior of white table wines after alcoholic fermentation to depend on flavonoid content as determined by the HCl-methanal/Folin-Ciocalteu method described by Kramling and Singleton (1). Wines submitted to browning tests in the absence of free sulfur dioxide were stable in color when their flavonoid content was 0 mg/L (as catechin), even in the presence of laccase activity. Under the same conditions, wines containing residual flavonoids displayed browning and oxidative off-flavor, though there was no absolute correlation between browning potential and flavonoid concentration. Oxidation of the non-flavonoid phenol fraction seems to have no sensorial effects when there are no phenols extracted from oak. It could, therefore, be concluded that the flavonoid fraction, as determined by precipitation in HCl-methanal, is responsible for oxidative aging, and that the control of flavonoid content is of extreme importance for quality management (14).

It has been shown that very small amounts of flavonoid phenols may cause browning (14). On the other hand, the HCl-methanal precipitation method is time-consuming and displays a detection threshold of about 10 mg/L catechin units mainly due to tolerances in the photometric step. Thus, this method of evaluating flavonoids is not very reliable for low concentrations to

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determine a wine's ability to undergo oxidative aging with the sensorial consequences referred to. Since HPLC equipment is not common in quality control laboratories, there is need for a rapid colorimetric assay which meets the following requirements: (1) sensitivity to low flavonoid concentrations; (2) high reproducibility in this concentration range; (3) high specificity; (4) strong correlation to browning potential in absence of SO_2 ; and (5) rapid execution.

The objective of this study was to compare different colorimetric assays in order to determine the most suitable one with special regard to the concentration range of 0 to 50 mg/L catechin.

Materials and Methods

Chemicals: Folin-Ciocalteu phenol reagent (FC), 4-(Dimethylamino)-cinnamaldehyde (DAC), vanillin (VAN), methanol, methanal, 37 % HCl, 97% ethanol, H_2O_2 , and sodium carbonate were reagent grade and purchased from Merck, Darmstadt, Germany. Catechin was purchased from Fluka, Bucks, Switzerland.

Wines: Dry varietal wines of white grape cultivars from Germany (80%), Slovenia, and Portugal were provided by bulk samples submitted to quality control before bottling. Most of them had been made by standard practices, a lesser number by hyperoxygenation of must. No wine had been stored in wooden barrels.

Flavonoids by Folin-Ciocalteu reagent (FC): Flavonoids were precipitated for at least 24 hours following the method outlined by Kramling and Singleton (1) and calculated as the difference between residual non-flavonoids and total phenol content.

The FC procedure was performed by the micro-method described by Singleton and Rossi (19) for both total phenolics and non-flavonoids, except that total phenol evaluation was preceded by titration of interfering total sulfur dioxide by the Ripper method and pH was corrected with NaOH and H_2SO_4 . Dilution and final pH (9.8) were the same in both aliquots (15).

Flavanols by 4-(dimethylamino)-cinnamaldehyde (DAC): DAC has been proposed by Zironi, Buiatti, and Celotti (22) for determination of flavanols in wines. The assay was performed using a chromogen reagent of 1 g DAC dissolved in 250 mL of 37% HCl and 750 mL methanol, following further instructions given by the authors.

Flavanols by vanillin (VAN): The vanillin test was carried out according to Rebelein (8), which is a slight modification of the version described by Pompei and Peri (7), giving very similar results.

POM-test: POM-test is a rapid browning test widely used in German speaking countries, developing browning of polyphenols in oxidative medium. Fifty milliliters of wine are held at 60°C for one hour after addition of 0.2 mL of 3% H_2O_2 (5). Browning produced was estimated at 420 nm.

All concentrations were expressed as mg/L catechin. Calibration curves for DAC, FC, and VAN were

established by catechin dissolved in 10% ethanol.

Absorbances were measured by a Perkin-Elmer (Coleman 571) double-beam spectrophotometer, using a 10-mm path length and water as a blank.

Results and Discussion

Further flavonoid- or flavanol-sensitive colorimetric assays have not been compared, since they all present serious disadvantages with regard to routine control. The butanol-HCl method has poor reproducibility and requires time-consuming handling. Spectral methods (21) lack specificity for the concentration range in concern. The vanillin test carried out in sulfuric acid instead of hydrochloric acid medium is subject to great errors in presence of sugar. There was no correlation between total phenolics and aging potential (11).

Sensitivities of DAC, VAN, and FC procedures have been estimated comparing the concentration-absorption ratios given by the calibration curves at 640, 500, and 720 nm, respectively (Fig. 1). On a catechin concentration base, DAC response is 10 times stronger than VAN response and 27 times stronger than FC response. This means that a possible error of 0.01 AU causes error of 8 mg/L for the FC-method, 3 mg/L for the VAN-method, and 0.3 mg/L for the DAC-method. So far, DAC is to be considered the most sensitive procedure for colorimetric evaluation of low flavonoid concentrations. DAC displays highest photometric resolution, whereas an error of 0.01 AU in the FC-method leads to a deviation not acceptable for the low concentration range.

DAC, VAN, FC, and POM-test were carried out on

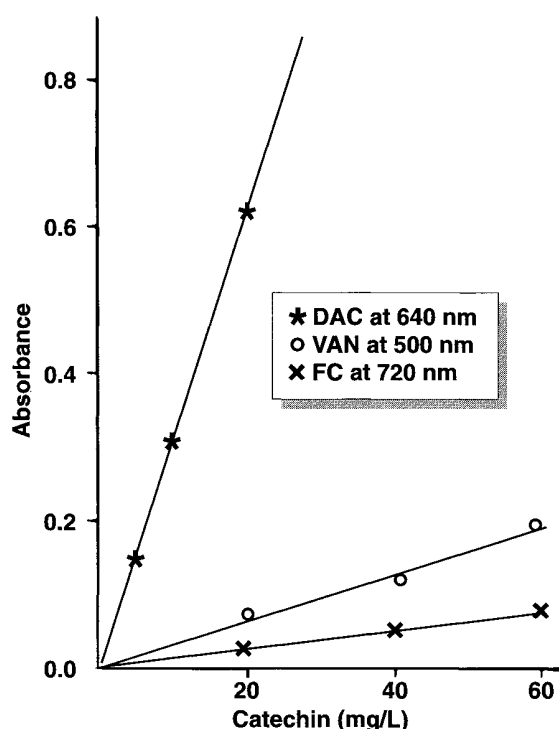


Fig. 1. Calibration curves of methods for flavonoids.

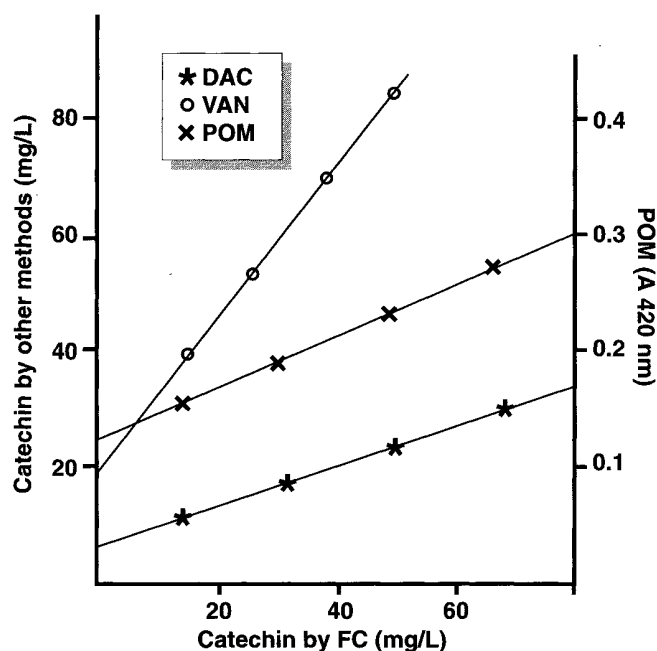


Fig. 2. Correlation between FC and other methods for flavonoid estimation.

36 wines and results submitted to simple regression analysis in order to establish the relationship between the methods. Figure 2 reports the correlation between FC and other methods applied. Average of methods for all wines are 13.6 mg/L for FC, 36.7 mg/L for VAN, 9.9 mg/L for DAC, and 0.174 AU for POM. In contrast, with the hydro-alcoholic solutions used for the calibration curves, DAC response in wine is lower than VAN and FC response.

Coefficients of variation have been established in white wines for DAC, VAN, and FC as 1.2%, 2.2%, and 24%, respectively ($n = 20$).

As can be seen in Table 1, highest correlation ($r = 0.96$) was obtained between DAC and VAN. Both methods are based on reactions of an aromatic aldehyde with 6- and 8-positions of the A-ring of flavanols. High correlation is obtained as well between FC and VAN and between FC and DAC.

Different analytical approaches to the same parameter lead to different results. Furthermore, DAC and VAN are sensitive to flavanols, whereas FC determines the somewhat larger flavonoid fraction. On the other

Table 1. Coefficients of correlation and regression equations between methods for flavonoid determination applied to 36 white wines.

Methods	n	Coefficient of correlation	Regression equation
DAC:VAN	36	0.96	$DAC = 0.87 + 0.25 \text{ VAN}$
DAC:FC	36	0.91	$DAC = 5.22 + 0.343 \text{ FC}$
FC:VAN	36	0.86	$VAN = 19.4 + 1.27 \text{ FC}$
FC:POM	36	0.76	$FC = -52.6 + 434.5 \text{ POM}$
DAC:POM	36	0.59	$DAC = -7.45 + 116.6 \text{ POM}$

hand, since FC is also the most commonly used method for total phenolics determination, flavanols obtained by FC are directly comparable to total phenolics when conditions of color development are standardized in both cases. However, poor sensitivity, reproducibility, and accuracy of FC for flavonoid determination make it inappropriate to match low flavanol or flavonoid concentrations encountered in most white wines. Comparing the coefficients of correlation listed in Table 1 and taking into consideration the coefficients of variation of each method, it appears that flavanol determination by DAC and, to a lesser extent, VAN could substitute for FC. Using the respective regression equations of Table 1, results can be transformed into values obtainable by the other methods.

Results obtained by VAN have been reported to decrease with increasing degree of polymerization of flavanols in red wines (9,13), and DAC may be expected to show similar behavior since both reagents react in the same way at the 6- and 8-positions. However, in white wines, flavanol concentration is too low to give results affected by flavanol polymerization. There is no influence of sugar, sulfur dioxide and iron on VAN and DAC (7,8,13,22). Thus, results can be compared before and after fermentation without corrections.

VAN leads to an overestimation of flavanol content in the way that in wines rich in phenolics, VAN-assayed flavanols are higher than total phenolics as determined by FC. It seems that reaction is not absolutely according to theory. This systematic error can be reduced by dilution and is due to interferences within the flavonoid fraction (13). Actually, VAN and DAC do not respond after removal of flavanols by precipitation in HCl-methanol.

FC gives higher results than DAC, too. It has been reported that FC response is not proportional to dilution (3), but since dilution was the same (1:100) in both aliquots for total and non-flavonoid phenols, slight overestimation of flavanols assayed by this method is not well understood and may be due to removal of FC-interfering substances by the precipitation step. More recently, it has been proposed (17) to overcome interference of sulfur dioxide distilling it from the wine with the aid of phosphoric acid and methanol. The dilution obtained in this way is considered to not affect fractionation in flavonoid phenols followed by the FC-procedure.

POM-test displays poor correlation with concentration units (Table 1). Accelerated browning tests at 50°C have been reported to be poorly related to long term storage tests at 20°C (18), and highly oxidative medium can be expected to be less comparable to real aging conditions.

A separate type of analysis was carried out only on young white wines produced by hyperoxygenation of must (data not reported). In these cases, DAC and VAN gave results as low as 1 to 5 mg/L, respectively, while there was no substantial browning developed by POM-Test. DAC and POM both give no response at a zero flavonoid level.

Conclusions

Flavonoid phenols are considered primarily responsible for oxidative aging of white wines. For their quantitative appreciation in the low concentration range, DAC displays high photometric revelation, good reproducibility, and simple execution in routine control. VAN, and to a lesser extent, FC lead to overestimation of flavonoid content. POM as a rapid browning test at 60°C in oxidative medium is poorly correlated with concentration units as determined by DAC, FC, and VAN since POM test conditions are not comparable to real aging conditions. However, DAC and POM both give no response at a zero flavonoid level as it might be encountered in wines made from hyperoxygenated musts. DAC responds to low flavonoid concentrations not more detected by FC. Control of hyperoxygenation or fining procedures as technical means to lower aging potential by flavonoid removal is rapidly run by DAC. Like VAN and in contrast to FC, DAC is not affected by interferences of sugar, sulfur dioxide, and iron. Thus, results before and after alcoholic fermentation can be compared without further corrections. DAC-derived results may be expected to correlate better with white wines' shelf-life than FC-data since the latter have poor reproducibility and precision. Further studies involving long term storage tests are required.

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