PAPER

FACTORS AFFECTING THE SPECTROPHOTOMETRIC QUANTIFICATION OF FLAVONOIDS IN WINE

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ABSTRACT

The quantification of flavonoids in wine and grape skin extract by spectrophotometric evaluation at 280 nm wavelength provides essential information to oenologist concerning wine composition and evolution, and it is commonly applied in wine labs. The measurement of the absorption peak height at 280 nm reported by DI STEFANO and GUIDONI (1989) allows to selectively quantify flavonoids with minor interferences. However, it has proved to be susceptible to SO_2 at low pH or acetone in unpurified grape skin extracts. Moreover, the effect of pH on flavonoids quantification in wine, either containing SO_2 or not, has not been assessed. The effect of SO_2, purification, pH and dilution solvent on spectrophotometric quantification of flavonoids in red wine samples has been evaluated in this work. SO_2 can overrate the flavonoids content in red wine when ethanol and Cl^- ions are contained in acid dilution solvents. A wine sample dilution with a strong acid solvent is mandatory to attain a reliable quantification of flavonoids due to the low anthocyanins absorption at 280 nm in water solution. A minor effect arises from the ethanol content. Eventually, flavonoids can be quantified in SO_2-containing wine diluted with a strong acid solution but a 7% overrating should be expected.

Keywords: polyphenol index, flavonoids, anthocyanins, SO_2; hyperchromic effect, bathochromic effect

1. INTRODUCTION

The amount of polyphenols, especially flavan-3-ols and anthocyanins, affects astringency, bitter taste and green/woody properties of red wine (GIBBINS and CARPENTER, 2013; SOARES *et al*., 2015). Their fast quantification in wine-making and wine ageing is crucial in winery since it makes it possible to carefully address the oenological choices involving the duration and conditions of maceration and ageing. Flavonoids are quantitatively the main phenol fraction in red wine by far; therefore, many analytical methods are based on the quantification of total polyphenols (ALEIXANDRE-TUDO *et al*., 2017). However, many of them are poorly selective or accurate (FOLIN and DENIS, 1912; SINGLETON and ROSSI, 1965) and require quite complex analytical approaches (GARCÍA-GUZMÁN *et al*., 2015) or even expensive analytical instrumentation for quality control laboratories (KENNEDY and JONES, 2001). The spectrophotometric methods are still among the fastest, easy to apply and cheapest for the oenologist; therefore, they are usually applied in the winery laboratories (ALEIXANDRE-TUDO *et al*., 2017). The spectrophotometric analytical approach reported by DI STEFANO and GUIDONI (1989) is widespread and routinely applied in the wineries to achieve a fast and reliable evaluation of flavonoids in grape extract and wine. It is based on the absorption spectrum obtained in the wavelength range 230–700 nm of a diluted sample. The peak height measured at 280 nm (E_{280}) subtracted from the absorbance measured at its valley-to-valley baseline returns the absorbance value mainly due to the flavonoids (E'_{280}). Such an approach allows to avoid the interference due to compounds without an absorption peak at 280 nm like aromatic amino acids, nucleosides and nucleotides (SOMERS and ZIEMELIS, 1985). Wine dilution with strong acid solutions allows to quantify the total anthocyanin content based on the height of the absorbance peak at about 520 nm of the spectra. An easier wine dilution with distilled water is commonly applied to assess the total flavonoids based on the E'_{280}. However, there is a lack of information about the analytical factors affecting the accuracy of this approach, in spite of its widespread use at wine control laboratories. CORONA *et al*. (2015) pointed out the interference exerted by SO_2 (as an undissociated molecular form) in quantifying flavonoids extracted from the grape berry and dissolved in a strong acid solvent like ethanol-hydrochloric acid mixture (EtOH-HCl). A further interference can arise from residual amounts of acetone used as extraction solvent of phenols. Both SO_2 and acetone can be easily removed by solid phase extraction (SPE) packed with a C18 resin (CORONA at al., 2015). It is well-known that sulfites can negatively affect the spectrophotometric quantification of anthocyanins at wine pH and acetaldehyde is needed to effectively remove the SO_2 bound to anthocyanins (USSEGLIO-TOMASSET *et al*., 1982; MAZZA *et al*., 1999). Recently, SO_2 proved capable of forming sulfonated adducts of flavan-3-ols over wine aging (ARAPITSAS *et al*., 2014). The binding involves the C4 position of the flavan ring and only monomeric flavan-3-ols and the terminal flavanol unit of proanthocyanindins are expected to undergo sulfonation in time. Sulfonation of elongation flavanol units has not been reported, possibly owing to steric hindrance issues. The spectrophotometric properties of flavanol-sulfite adducts are unknown, as well as their role in flavonoid quantification. However, their low relative abundance has to be considered, especially in young wine (ARAPITSAS *et al*., 2018). Poor information is available about the role exerted by SO_2 in wine concerning the quantification of flavonoids based on the E'_{280} value, especially when strongly acidic solutions (pH < 1) are used as a dilution solvent to attain the quantification of anthocyanins in the meantime. Moreover, there is a lack of information about how the composition and acidity of the dilution solvent affect the quantification of total flavonoids assessed using the E'_{280} value.

In this work the effects of SO_2, ethanol concentration, acid and pH on the absorbance values E_{280} and E'_{280} assessed in diluted red wine samples were assessed to monitor their role on the quantification of wine flavonoids.

2. MATERIALS AND METHODS

2.1. Chemicals

Methanol, ethanol, sulphuric acid, hydrochloric acid, tartaric acid, ethanol, sodium hydroxide, citric acid monohydrate, potassium phosphate monobasic, sodium phosphate dibasic, hydrogen peroxide solution (30% w/w in water), ethyl acetate, polyvinylpolypyrrolidone (PVPP) and bromocresol green methyl red indicator were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium metabisulphite and phosphoric acid were purchased from J.T. Baker (Deventer, Holland). Seeds and white and red grape tannins were provided by Bono and Ditta s.p.a. Italian Grape Juice from Campobello di Mazara (Trapani, Italy).

2.2. Wine Samples

Sixty different commercial red wine samples produced in the years 2013–2015 were collected at the market and submitted for the evaluation of total anthocyanin and total flavonoid contents. Moreover, the spectrophotometric response obtained following different dilution conditions of eight samples of red wine obtained from *Nero d'Avola* grape (vintages 2013–2015), *Nerello Mascarese* grape (vintage 2015), *Cabernet Sauvignon* grape (vintage 2014 and 2015) and *Merlot* (vintage 2014) was assessed. All measurements were performed in triplicate.

2.3. Purification of flavonoids

Half millilitre of wine sample was diluted with 5 mL H_2SO_4 5 mM and loaded into a 400 mg C18 SPE cartridge (Sep-Pak, Waters, Milan, Italy) previously conditioned with 2 mL methanol and then 3 mL H_2SO_4 5 mM. The polar compounds were eluted with 3 mL H_2SO_4 5 mM to drying and discarded, then the phenols were collected into a 25 mL volumetric flask by eluting with 3 mL methanol and brought to volume with one of the following solvents: H_2O, 0.5 M H_2SO_4, ethanol:H_2O:12 M HCl 70:30:1 (v/v/v) (EtOH-HCl). The same solutions were also used for diluting 0.5 mL of the wine samples to 25 mL in volumetric flasks. Triplicate preparations were carried out.

2.4. Determination of total flavonoids

Flavonoids were purified by treatment with SPE procedure. The UV-visible absorption spectra in the range 230–700 nm wavelength of either unpurified or purified flavonoids were recorded, and the absorption values at 280 nm (E_{280}) were measured. Triplicate preparations were carried out. The E'_{280} value was also measured according to DI STEFANO and GUIDONI (1989) and modified by Corona *et al*. (2015). The total flavonoid content was calculated according to DI STEFANO and GUIDONI (1989) and CORONA *et al*. (2010) as follows:

$$\text{Total flavonoids (as mg/L (+)-catechin equivalent)}: 82.4 \times E'_{280} \times 50.$$

2.5. Absorbance parameters of white grape skin extract

Buffered solutions at pH 1.1, 3.0, 5.0 and 7.0 were prepared according to KÜSTER *et al*. (1979) and used for dissolving 30 mg/L grape skin extract. Their UV-visible absorption spectra in the range 230-400 nm wavelength were recorded and the values of λ_{max}, E_{280} and E'_{280} were measured. Triplicate preparations were carried out.

2.6. Purification of anthocyanins from red grape skin extract

Phenols from red grape skin extract were obtained from the skin of 50 berries by using a tartaric buffer (5 g tartaric acid, 22 mL NaOH 1 N, 2 g $Na_2S_2O_5$, 125 mL ethanol 95–96%, brought to 1 L with H_2O). Anthocyanins were obtained from the extract as follows. Three millilitres of H_2SO_4 0.5 M and 6 g of PVPP were added to 60 mL of skin extract. The mixture was stirred for 2 min, then centrifuged at 2000 *g* × 10 min and the PVPP was recovered and then rinsed with 20 mL of H_2SO_4. The mixture was centrifuged as above and the PVPP was recovered. The anthocyanins absorbed on the PVPP were dissolved by dispersing the PVPP into 15 mL EtOH-HCl solution and centrifuging at 2000 *g* × 10 min. The addition of EtOH-HCl solution and the centrifugation were carried out four times again, and all the five supernatants were collected and blended in a 100 mL evaporation flask. The ethanol contained in the anthocyanins solution was removed by vacuum-drying and the water solution was transferred in a 100 mL extraction funnel. The residual flavan-3-ols were removed by a triplicate extraction with 10 mL ethyl acetate each. The purified anthocyanin extract was transferred in a 100 mL evaporation flask and the residual ethyl acetate was removed by vacuum drying. Finally, the dried anthocyanins were dissolved with 50 mM H_2SO_4 10 mL and recovered.

2.7. Absorbance parameters of anthocyanins

One millilitre of either red skin extract or purified anthocyanins solution was diluted to 25 mL with buffer solutions at pH 1.1, 3.0, 5.0 and 7.0 prepared according to KÜSTER *et al*. (1979) or with 0.1 M HCl solutions containing 10, 20, 40 or 80% ethanol. Their UV-visible absorption spectra in the range 230–700 nm wavelength was recorded, and the values of maximum absorption wavelengths in the range 275-282 nm (λ_{maxUV}) and in the range 510-550 nm (λ_{maxVIS}) were measured, as well as their absorption values (E_{280}, E'_{280}, E_{520}).

2.8. Determination of SO_2 in wine samples

The SO_2 content in wine was carried out according to the Functional EEC in 2376 (1990) standard procedures. Triplicate determinations were carried out.

2.9 Statistical analysis

Analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test to calculate significant differences between treatments were carried out. All tests were performed at a significance level of $p < 0.05$ using the statistical program SPSS (ver. 13, IBM, Armonk, NY, USA).

3. RESULTS AND DISCUSSION

A fast quantification of total flavonoids and anthocyanins in wine can be achieved by measuring the spectrophotometric values E'_{280} and E_{520} of the sample diluted with an acid solution. However, such an approach can overrate the flavonoid content owing to the presence of gallic acid. SO_2 has an absorption peak close to 280 nm (276 nm) and diluting wine in strong acid solutions might increase the E'_{280} value, thus inducing a major overrating of the flavanol concentration (CORONA *et al*., 2015). Wine dilution with ethanol-HCl can further increase the absorbance of SO_2 owing to the bathochromic and hyperchromic effects induced by ethanol and Cl^-, respectively. SO_2 can be removed from the wine sample by SPE packed with a C18 resin (CORONA *et al*., 2015). To assess the effect of sample purification on the spectrophotometric quantification of flavonoids, the analytical responses of 61 SPE-treated and untreated red wine samples, both of them diluted in an ethanol-HCl solution, were compared (Fig. 1).

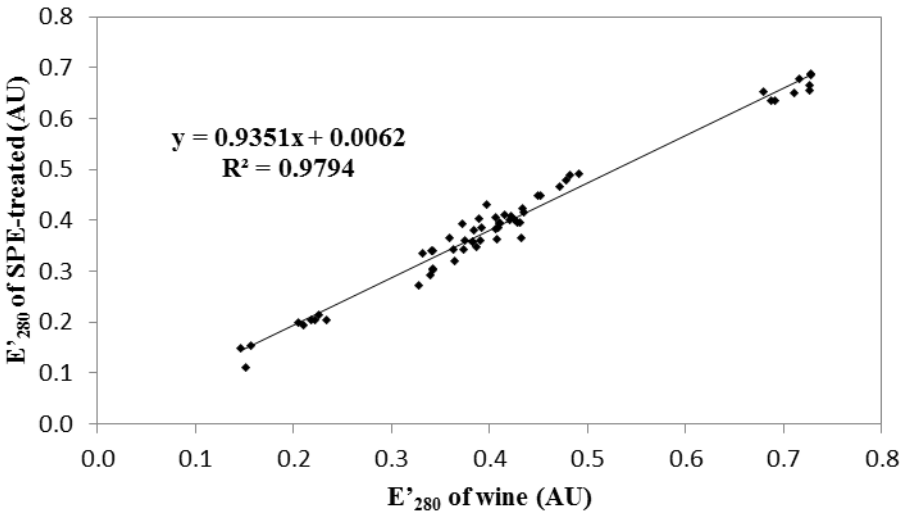

Figure 1. Comparison of E'_{280} values obtained for wine samples and their corresponding SPE-treated wine (n=3). All the samples were diluted with EtOH-HCl solution.

A good correlation (r^2 = 0.979) was obtained; however, the slope of the regression line shows the E'_{280} values of wine were 6-7% higher than the corresponding SPE-treated wine. Such an overrating was expected as SPE purification removes the polar phenols unretained on the SPE resin, namely gallic acid, tyrosine and tyrosol (DI STEFANO and GUIDONI, 1989). However, the role of SO_2 is hard to assess in unknown samples, even though it was proved in the previous work of CORONA *et al*. (2015). Therefore, a known addition of SO_2 in real wine samples is expected to increase the E'_{280} value, but such an interference is hard to quantify owing to the occurrence of different pH values as well as quality and content of SO_2-binding compounds (ethanal, anthocyanins, pyruvate or other carbonyl compounds). To better focus the interference of SO_2 on the quantification of flavonoids, the absorption spectra obtained from red wine samples containing different concentrations of SO_2 either submitted or not to SPE purification of flavonoids and diluted with acid solutions with different pH values were compared (Table 1). Following the purification step, the E_{280} values of the acid-diluted samples decreased by up to -20% in accordance with the work of SOMERS and ZIMELIS (1985) (Table 1).

Table 1. Value of λ_{max}, E_{280} (as AU) E'_{280} (as AU), flavonoids (as mg/L (+)-catechin equivalent) and SO_2 (mg/L) in wine and SPE-treated wine samples diluted with different solutions.

Wine and vintage year		Wine			SPE-treated wine			SO_2 level in wine	
		H_2O	5×10^{-1} M H_2SO_4	Ethanol-HCl	H_2O	5×10^{-1} M H_2SO_4	Ethanol-HCl	Total	Free
Nero d'Avola 13	λ_{max}	276.0±0.00^{a}	277.5±0.71^{b}	278.5±0.71^{b}	278.0±0.00^{A}	279.0±0.00^{A}	280.0±0.00^{A}	59.5±1.2	17.3±0.3
	E_{280}	0.428±0.01^{a}	0.466±0.02^{b}	0.484±0.00^{b}	0.378±0.00^{A}	0.400±0.00^{B}	0.390±0.00^{AB}		
	E'_{280}	0.129±0.00^{a}	0.168±0.00^{b}	0.175±0.00^{b}	0.126±0.00^{A}	0.162±0.00^{B}	0.163±0.00^{B}		
	Total Flavonoids	1068±11^{a}	1384±87^{b}	1445±9^{b}	1037±12^{A}	1337±22^{B}	1345±11^{B}		
Nero d'Avola 13	λ_{max}	277.0±0.00^{a}	277.0±0.00^{a}	279.0±0.00^{a}	278.5±0.71^{A}	279.0±0.00^{A}	280.0±0.00^{A}	61.1±1.1	17.9±0.1
	E_{280}	0.450±0.00^{a}	0.504±0.01^{b}	0.543±0.00^{c}	0.384±0.07^{A}	0.446±0.01^{B}	0.435±0.00^{B}		
	E'_{280}	0.148±0.00^{a}	0.186±0.01^{b}	0.201±0.01^{b}	0.125±0.00^{A}	0.184±0.01^{B}	0.187±0.00^{B}		
	Total Flavonoids	1219±29^{a}	1533±33^{b}	1660±10^{c}	1034±19^{A}	1514±11^{B}	1542±10^{B}		
Nero d'Avola 14	λ_{max}	276.0±0.00^{a}	276.5±0.71^{b}	278.0±0.00^{b}	278.0±0.00^{A}	278.0±0.00^{A}	280.0±0.00^{A}	1.3±0.0	0.6±0.0
	E_{280}	0.384±0.01^{a}	0.403±0.00^{ab}	0.419±0.01^{b}	0.342±0.00^{A}	0.358±0.00^{B}	0.345±0.00^{A}		
	E'_{280}	0.114±0.00^{a}	0.131±0.00^{ab}	0.140±0.00^{b}	0.106±0.00^{A}	0.136±0.00^{B}	0.130±0.00^{B}		
	Total Flavonoids	938±22^{a}	1076±43^{b}	1154±43^{b}	873±4^{A}	1122±22^{C}	1071±11^{B}		
Nero d'Avola14	λ_{max}	276.0±0.00^{a}	276.5±0.71^{ab}	278.0±0.00^{b}	278.0±0.00^{A}	278.5±0.71^{A}	280.0±0.00^{A}	1.3±0.0	0.6±0.0
	E_{280}	0.415±0.00^{a}	0.431±0.00^{ab}	0.456±0.02^{b}	0.368±0.00^{A}	0.395±0.01^{A}	0.397±0.00^{A}		
	E'_{280}	0.122±0.00^{a}	0.139±0.01^{ab}	0.160±0.01^{b}	0.120±0.00^{A}	0.146±0.01^{B}	0.154±0.01^{B}		
	Total Flavonoids	1007±33^{a}	1145±20^{ab}	1314±120^{b}	992±11^{A}	1203±20^{B}	1273±9^{C}		
Nero d'Avola 15	λ_{max}	276.0±0.00^{a}	277.0±0.00^{a}	279.0±0.00^{b}	278.0±0.00^{A}	279.0±0.00^{A}	280.0±0.00^{A}	24.3±1.2	8.7±0.5
	E_{280}	0.415±0.00^{a}	0.432±0.01^{a}	0.447±0.01^{a}	0.379±0.00^{A}	0.385±0.00^{B}	0.389±0.02^{B}		
	E'_{280}	0.122±0.01^{a}	0.146±0.01^{ab}	0.157±0.01^{b}	0.124±0.00^{A}	0.151±0.00^{B}	0.155±0.01^{B}		
	Total Flavonoids	1005±11^{a}	1204±20^{b}	1291±43^{b}	1018±4^{A}	1245±21^{B}	1276±22^{B}		
Nero d'Avola 15	λ_{max}	278.0±0.00^{a}	278.5±0.71^{a}	279.0±0.00^{a}	279.0±0.00^{A}	279.0±0.00^{A}	280.0±0.00^{A}	34.3±4.2	11.3±2.4
	E_{280}	0.357±0.00^{a}	0.379±0.00^{a}	0.381±0.01^{a}	0.323±0.00^{A}	0.319±0.00^{A}	0.319±0.00^{A}		
	E'_{280}	0.110±0.00^{a}	0.112±0.00^{a}	0.113±0.01^{a}	0.105±0.00^{A}	0.107±0.00^{A}	0.108±0.00^{A}		
	Total Flavonoids	910±4^{a}	923±15^{a}	930±11^{a}	869±11^{A}	884±11^{A}	892±20^{A}		

Nerello Mascalese 15	λ_{max}	276.0±0.00^{a}	277.0±0.00^{a}	278.0±0.00^{a}	278.0±0.00^{A}	278.0±0.00^{A}	279.5±0.71^{A}	35.7±4.2	13.0±2.9
	E_{280}	0.344±0.01^{a}	0.346±0.00^{a}	0.360±0.01^{a}	0.285±0.01^{A}	0.292±0.00^{A}	0.286±0.00^{A}		
	E'_{280}	0.125±0.00^{a}	0.130±0.00^{a}	0.144±0.00^{b}	0.119±0.01^{A}	0.118±0.00^{A}	0.126±0.00^{A}		
	Total Flavonoids	1030±22^{a}	1069±10^{a}	1184±22^{b}	982±20^{A}	970±21^{A}	1038±11^{B}		
Cabernet Sauvignon 15	λ_{max}	276.0±0.00^{a}	277.0±0.00^{a}	279.0±0.00^{b}	278.0±0.00^{A}	277.0±0.00^{A}	279.0±0.00^{A}	86.8±7.2	27.9±4.1
	E_{280}	0.565±0.00^{a}	0.591±0.01^{b}	0.626±0.00^{c}	0.514±0.01^{A}	0.585±0.00^{C}	0.542±0.01^{B}		
	E'_{280}	0.185±0.00^{a}	0.217±0.01^{b}	0.236±0.01^{c}	0.176±0.01^{A}	0.212±0.00^{B}	0.215±0.01^{B}		
	Total Flavonoids	1524±28^{a}	1786±30^{b}	1948±15^{c}	1453±15^{A}	1736±48^{B}	1772±6^{B}		
Nero d'Avola 14	λ_{max}	276.0±0.00^{a}	277.5±0.71^{b}	278.5±0.71^{b}	n.d.	n.d.	n.d.	62.1±1.3	22.5±0.75
	E_{280}	0.416±0.00^{a}	0.473±0.01^{b}	0.491±0.01^{b}					
	E'_{280}	0.127±0.00^{a}	0.171±0.01^{b}	0.174±0.01^{b}					
	Total Flavonoids	1045±15^{b}	1407±23^{b}	1430±17^{b}					
Nero d'Avola 15	λ_{max}	276.0±0.00^{a}	276.5±0.71^{ab}	278.0±0.00^{b}	n.d.	n.d.	n.d.	27.2±1.5	10.8±0.05
	E_{280}	0.398±0.00^{a}	0.432±0.00^{b}	0.479±0.00^{b}					
	E'_{280}	0.107±0.00^{a}	0.137±0.00^{b}	0.141±0.01^{b}					
	Total Flavonoids	884±8^{a}	1130±18^{b}	1161±12^{b}					
Cabernet Sauvignon 14	λ_{max}	276.0±0.00^{a}	277.0±0.00^{a}	278.0±0.00^{a}	n.d.	n.d.	n.d.	33.4±1.7	15.3±0.61
	E_{280}	0.369±0.00^{a}	0.402±0.00^{b}	0.429±0.00^{b}					
	E'_{280}	0.124±0.00^{a}	0.152±0.00^{b}	0.160±0.00^{b}					
	Total Flavonoids	1022±10^{a}	1253±12^{b}	1322±31^{b}					
Merlot 14	λ_{max}	276.0±0.00^{a}	277.0±0.00^{a}	279.0±0.00^{b}	n.d.	n.d.	n.d.	89.7±9.1	30.4±0.60
	E_{280}	0.413±0.01^{a}	0.454±0.01^{b}	0.492±0.01^{b}					
	E'_{280}	0.132±0.01^{a}	0.166±0.01^{b}	0.172±0.01^{b}					
	Total Flavonoids	1084±38^{a}	1368±19^{b}	1414±10^{b}					

n = 3 samples; mean value ± standard deviation. n.d.: not determined.
Different letters in the same row indicate significant differences between wine samples or SPE-treated wine samples (Tukey's HSD test, $p < 0.05$).

It is mainly due to the loss of purines, pyrimidines, nucleosides, amino acids and aromatic alcohols occurred following the purification procedure. Therefore, the E_{280} value confirms to be an unsuitable index of the phenol content in wine. The quantification of flavonoids based on the E'_{280} value shows the major role of the dilution solvent on the measured values. The E'_{280} values obtained by water dilution of the wine samples were always significantly lower than the sample diluted with acid solutions. The E'_{280} value increases as the pH decreases, thus suggesting that the SO_2 plays a role. Moreover, the hyperchromic effect detectable only in the wine samples diluted with ethanol-HCl solution further supports such a conclusion. However, a comparable or even higher increase of the E'_{280} value can be detected after the removal of SO_2 by SPE, even in the samples containing negligible SO_2 level (Nero d'Avola 14). Neither the bathochromic nor the hyperchromic effects detected in the wine samples, Nero d'Avola 14, ought to be observed when ethanol-HCl instead of a H_2SO_4 solution is used as dilution solvents. Moreover, no increase of the E'_{280} value should be detected in the SPE-treated samples following dilution with H_2SO_4 if SO_2 had a major role. Nonetheless, the calculated flavonoids content strongly increases as the pH of the dilution solvent decreases, even in the SPE-treated wine samples (Table 1). All these data highlight the minor contribution of SO_2 to the E'_{280} value and quantification of flavonoids in wine, while pH and solvent composition strongly affect the analytical response. Since the variation of E'_{280} values also occurs with the SPE-treated samples where the hydrophobic compounds eluted with methanol from the C18 resin are contained, phenols are likely involved in this behaviour. However, flavan-3-ols, either monomer or polymer, are not expected to be affected by pH values lower than 7, as their pK_a exceeds 9. Therefore, pH variations in the range 0-7 should attain negligible dissociation effects whatever the alcohol content of the adopted diluting solvent (DANILEWICZ, 2003; FRIEDMAN and JÜRGENS, 2000). As expected trials carried out at pH values spanning from 1 to 7 with different ethanol content did not show any significative effect on the E'_{280} values recorded for grape phenols extracted from white skin (Table 2) and comparable results were obtained when phenols extracted from grape seeds were evaluated (data not shown). Therefore, flavan-3-ols and proanthocyanidins, as well as other colourless skin or seed phenols (phenolic acids, flavonols, hydroxystilbenes), can hardly be responsible for the E'_{280} variations induced by pH and solvent differences. Consequently, the role of anthocyanins was investigated. The absorption spectra of anthocyanins are affected by the pH. Moreover, the E_{280} value of their flavilium ion is higher than its neutral form occurring in wine at pH values lower than 6 (MARÇO *et al*., 2011). The E'_{280} value obtained assessing anthocyanins from red grape skin extract treated by SPE packed with PVPP significantly decreases as the pH increases. The E'_{280} values increase more than 15% by wine acidification (pH 3–4) down to pH 1 (Table 1). Such a change is lower than it occurs when the absorption peak at 520–540 nm is considered (see $E_{lmaxVis}$ in table 3); nonetheless, it can strongly affect the spectrophotometric evaluation of flavonoid by the E'_{280}, especially when wines containing a high amount of anthocyanins are considered (Table 3). As the role of ethanol on the absorbance of anthocyanins in diluted wine is well-known (LEE *et al*., 2005), the increase in E_{520} values following the increased ethanol concentration was expected. Ethanol does not affect the E'_{280} value and only an ethanol level as high as 80% (v/v) shows a minor role. Same results were obtained when HCl was replaced with H_2SO_4 (data not shown). If grape skin extract is concerned, increasing E'_{280} values are clearly visible when the ethanol content increases due to the hyperchromic effect arising from the presence of SO_2 in the skin extract (Table 3).

Table 2. Effect of pH and dilution solvent on the analytical response parameters of phenols extracted from white grape skin.

	λ_{max}	E_{280}	E'_{280}
pH			
1.1	283±0.6^{a}	1.193±0.00^{a}	0.279±0.00^{a}
3.0	284±0.6^{a}	1.197±0.01^{a}	0.280±0.00^{a}
5.0	283±0.6^{a}	1.195±0.00^{a}	0.284±0.00^{a}
7.0	283±0.6^{a}	1.195±0.00^{a}	0.284±0.00^{a}
Ethanol %, 0.1 M HCl			
0%	283±0.6^{a}	1.145±0.00^{a}	0.277±0.00^{ab}
10%	283±0.6^{a}	1.146±0.00^{a}	0.279±0.00^{b}
20%	282±0.0^{a}	1.143±0.01^{a}	0.275±0.00^{ab}
40%	282±0.6^{a}	1.143±0.00^{a}	0.274±0.00^{ab}
80%	282±0.0^{a}	1.149±0.00^{a}	0.270±0.00^{a}

n = 3 samples; mean value ± standard deviation.
Different letters in the same column indicate significant difference between dilution solvents (Tukey's HSD test, $p < 0.05$).

Table 3. Effect of pH, dilution solvent and purification on some spectrophotometric parameters of grape skin anthocyanins.

	λ_{maxUV}	E_{280}	E'_{280}	λ_{maxVis}	$E_{\lambda maxVis}$
pH			**purified anthocyanin**		
1.1	277.0±0.0^{b}	0.466±0.01^{c}	0.221±0.02^{c}	519±0.0^{a}	0.618±0.01^{d}
3.0	277.0±0.0^{b}	0.414±0.00^{b}	0.186±0.00^{bc}	519±0.0^{a}	0.464±0.00^{c}
5.0	276.5±0.7^{b}	0.371±0.00^{a}	0.155±0.00^{b}	519±0.0^{a}	0.192±0.00^{b}
7.0	275.0±0.0^{a}	0.351±0.01^{a}	0.099±0.00^{a}	551±0.0^{b}	0.088±0.00^{a}
Ethanol %, 0.1 M HCl			**purified anthocyanin**		
0%	277.0±0.0^{a}	0.436±0.00^{a}	0.241±0.00^{b}	519±0.0^{a}	0.609±0.00^{a}
20%	278.0±0.0^{a}	0.437±0.00^{a}	0.243±0.00^{b}	528±0.0^{b}	0.650±0.00^{b}
40%	279.0±0.0^{a}	0.435±0.00^{a}	0.246±0.00^{b}	536±0.0^{c}	0.684±0.00^{c}
80%	280.0±0.0^{a}	0.431±0.00^{a}	0.229±0.00^{a}	544±0.0^{d}	0.730±0.00^{d}
Ethanol %, 0.1 M HCl			**red grape skin extract**		
0%	278.0±0.0^{a}	0.854±0.04^{a}	0.315±0.00^{a}	520±0.0^{a}	0.305±0.01^{a}
20%	278.0±0.0^{a}	0.882±0.01^{a}	0.331±0.01^{a}	527±0.0^{b}	0.336±0.00^{ab}
40%	279.0±0.0^{a}	0.892±0.02^{a}	0.334±0.01^{a}	535±0.0^{c}	0.358±0.01^{bc}
80%	280.0±0.0^{a}	0.916±0.03^{a}	0.341±0.01^{a}	542±0.0^{d}	0.381±0.01^{c}

n = 3 samples; mean value ± standard deviation.
Different letters in the same column indicate significant difference between treatments (Tukey's HSD test, $p < 0.05$).

4. CONCLUSIONS

Our data highlight that the dilution of wine with water to assess the total flavonoid content by the E'_{280} value prevents from the spectrophotometric interference of SO_2 in the analytical response unless it occurs at concentration values exceeding the permitted

amounts in wine. However, under such conditions the interference exerted by the polar non-flavonoid phenols and acid equilibrium of anthocyanins induces a biased quantification. On the other hand, wine dilution with EtOH-HCl can induce an overrated quantification due to both the interference of SO_2 in low pH solutions and the hyperchromic effect exerted by Cl^-. Such interferences can be avoided by carrying out the SPE purification of wine and then diluting the sample with an acid solution (pH < 1).

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