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Comparison of Three Extraction Methods Used To Evaluate Phenolic Ripening in Red Grapes

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Because there is not a single method for carrying out phenolic ripening analysis, it is very difficult to compare the results obtained by different researchers. In this study, the three most widely used extraction methods of polyphenols (Glories, AWRI, and ITV) have been analytically compared by evaluating two of the most important parameters for the wine industry: total polyphenols and total anthocyanins. Samples from different grape varieties (Tempranillo, Garnacha, Cariñena, Syrah, Merlot, and Cabernet Sauvignon), from three different vintages (2006, 2007, and 2008), and at different ripening states (from the beginning of ripeness until harvest) were analyzed to obtain a wide range of representative phenolic contents. To avoid external interferences on the comparisons, the same grape puree was used to make the maceration assays using the different solvents according to each extraction method. Although every extraction method exhibits a different extraction efficiency, the correlation between the results obtained with each one was very good both for total anthocyanins and for total polyphenols. Thus, after having determined a parameter value of the phenolic ripeness using a specific method, the relationship found can be used to predict the parameter value of the phenolic ripeness provided by the other two methods.

KEYWORDS: Phenolic ripening; anthocyanins; polyphenolic compounds; red grape; extraction methods; correlations

INTRODUCTION

The vintage date has been traditionally decided by the technological maturity of grapes. This term is used to define the state in which grapes achieve an adequate amount of sugars and acids, because they would give to wine its characteristic alcohol−acidity balance after alcoholic fermentation. Nowadays, it is known that it is also necessary to get an optimal phenolic ripeness, because phenolic compounds play an important role in the color and mouthfeel properties (*1*, *2*). Among the different parameters that enologists use when evaluating the phenolic ripeness of grapes, two of the most important are the total anthocyanins and the total polyphenols.

To determine the phenolic maturity, winemakers need a methodology as fast and simple as possible to extract the phenolic compounds from grape berries and to analytically measure their concentration. Because the extraction is usually a critical step, several methods have been proposed in the literature, although some of them are unsuitable for cellar quality control because they are tedious, slow, and not repeatable enough. For this reason, instead of the time-consuming methods in which grape solid parts (skins and seeds) and pulp are analyzed separately (*3*−*6*), it is preferable to use methods in which polyphenols are extracted from the whole grape puree.

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Within these methods, the most widely used are the most traditional method, developed by Glories and co-workers (*7*), the one implemented by the Australian Wine Research Institute (AWRI) (*8*), and, finally, the ITV Standard (*9*), a method implemented by the Institute Technique de la Vigne et du Vin in France. All of them measure spectrophotometrically the contents of total anthocyanins and polyphenols. However, the time required and the solvent media used (pH value and ethanol contents) on the maceration process are different for each method and, therefore, the extraction efficiency in each case will be also different.

Because all of the proposed methods are equally valid from an enological point of view, there is a lack of consensus in the choice of one of them. This fact makes difficult the comparison between the results found in the bibliography and obtained by different researchers if they do not use the same method.

The aim of this study was to find correlations between the results obtained with the three extraction methods abovementioned. With these correlations we would have a very useful tool that, from the values obtained in the analysis of a sample with a specific method, would provide the values that the same sample would provide if it was analyzed with the other methods.

MATERIALS AND METHODS

Samples. Fresh grape berries of six red cultivars (Tempranillo, Merlot, Syrah, Garnacha, Cariñena, and Cabernet sauvignon) were obtained from the experimental vineyard belonging to the Faculty of Enology

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(Rovira i Virgili University) in Constantí (Tarragona, Spain) during 2006, 2007, and 2008 vintages.

The sampling was carried out by taking into account the variability of the positions of the fruit on the cluster, of the cluster on the vine, and of the vine in the vineyard as well as from variations in sun exposure to asses a representative sample of the vineyard. Each sample consisted of 300 berries randomly collected. Special care was taken to obtain a good distribution between berries from the inside and the outside of the cluster: one was taken from the top, one from the bottom, and one from the middle of the cluster. Moreover, the sampling also considered the complete biological cycle; thus, it was done one day a week from the beginning of ripeness until harvest.

Instrumentation. The grapes were homogenized using a high-speed homogenizer Ultra-Turrax T-18 (IKA, Wilmington, NC) equipped with an S18N-19G rotating shaft (dispersing tool). Sample centrifugation was carried out by a Hettich Universal 32 R centrifuge (Tuttlingen, Germany). The absorbance of the extracts was determined using a Thermo Spectronic ultraviolet−visible spectrophotometer model Helios γ (Thermo Electron Corp., Cambridge, U.K.).

Reagents and Standards. The standard of malvidin-3-glucoside (purity $\geq$ 90%) was supplied by Fluka (Madrid, Spain) and gallic acid monohydrate (99.5%) by Scharlab (Barcelona, Spain). The other chemicals used for the study were ethanol, hydrochloric acid (HCl), and L-(+)-tartaric acid (H_2T), all of them of analytical reagent grade and supplied by Scharlab.

Maceration Conditions. Every set of 300 berries of each sample was destemmed and homogenized (including seeds) at room temperature to obtain a smooth paste using an Ultra-Turrax high-speed at 24000 rpm. This homogenate was distributed into eight different flasks to carry out the maceration in duplicate according to the three methods considered, and which were slightly improved (see below).

Glories Method. This method provides two different extracts because it determines two parameters: total polyphenols and easily extractable polyphenols. Therefore, two different acidic solutions are used: one at pH 1 (by using 0.1 M HCl) and another at pH 3.2 (by using 0.034 M H_2T). Two replicates of 25 g of paste were macerated in 25 mL of different pH solutions in flasks of 250 mL, under agitation, during 4 h and at room temperature. Then, the samples were centrifuged at 8000 rpm during 10 min. The supernatant obtained was decanted, and the volume was brought to 50 mL with extracting solution to guarantee a final constant volume.

AWRI Method. Two grams of paste was put into the centrifuge plastic tube with 20 mL of a 50% v/v ethanol−water solution. The tube was shaken on a rotating wheel at 30 rpm for 1 h at room temperature. Finally, it was centrifuged and the supernatant brought to 25 mL with ethanol−water 50% v/v to ensure a final constant volume.

ITV Standard Method. Fifty grams of paste was macerated in a hydroalcoholic acid solution (85 mL of HCl 0.1% v/v + 15 mL of ethanol 96%) in a flask of 250 mL by 1 h under manual agitation every 15 min. After this time, the sample was centrifuged and the supernatant was brought to 200 mL with the extracting solution to achieve a final constant volume.

Analytical Measurements. The contents of total polyphenols and total anthocyanins were the parameters used to follow the phenolic ripening. They were determined by spectrophotometric analysis of the extracts (diluted at the specified pH conditions in each case) at 280 and 520 nm, respectively.

Total Polyphenols Analysis. The contents of total polyphenols was determined by measuring at 280 nm the absorbance of diluted extracts obtained in each case of study. For the ITV and Glories methods, the extract was diluted 50 times on deionized water (*10*). For the AWRI method, the extract was diluted 25 times on 1 M HCl (*11*).

Total Anthocyanins Analysis. Total anthocyanins content was determined by measuring the absorbance at 520 nm of the diluted extract. For the ITV and Glories methods, the extract was diluted 25 times on HCl 1% (w/w) (*10*). For the AWRI method, the extract was diluted 25 times on 1 M HCl (*11*).

Calibration Lines. The external standard method was used to quantify the total polyphenols as milligrams of gallic acid per kilogram of grape and anthocyanins as milligrams of malvidin-3-glucoside per kilogram of grape. Calibration lines were evaluated by the determination coefficient (R^2) and the online linearity (LOL) (*12*). All of the parameters of the calibration lines have been calculated with ULC 2.0 (Univariate Linear Calibration) computer software (*13*).

When dealing with polyphenols, we prepared a stock solution of gallic acid of 5000 mg L^{-1} in ethanol−water (50% v/v) for the AWRI method, an other two stock solutions at pH 1.0 (with HCl) and pH 3.2 (with H_2T) for the Glories method, and another one in a hydroalcoholic acid solution (85 mL of HCl 0.1% v/v + 15 mL of ethanol 96%) for the ITV method. In all cases, we prepared six calibration solutions (concentration ranges between 2 and 20 mg L^{-1}) by diluting the stock solutions with suitable amounts of the required solvent according to each method. We built the calibration lines by plotting the absorbance values at 280 nm of each calibration solution against their corresponding concentrations.

With regard to anthocyanins, a single stock solution of malvidin-3-glucoside of 2000 mg L^{-1} was prepared in HCl 1% (w/w) to be used for the three methods. As with polyphenols, we prepared six calibration solutions (concentration range from 5 to 15 mg L^{-1}) by diluting the stock solutions with suitable amounts of the required solvent according to each method. In this case, we built the calibration lines by plotting the absorbance values at 520 nm of each calibration solution against concentrations.

For the Glories method, only a calibration line was built for anthocyanins because, in this determination, there is a dilution 1:25 of the extract in HCl, which means that, when measured spectrophotometrically, the medium is practically the same regardless of the pH at which maceration takes place.

Comparative Study of the Three Methods. To carry out this study, the results obtained with one of the three methods were plotted versus the results obtained with each of the other two methods. In this way, we obtained six graphs for each of the two parameters studied (total polyphenols and anthocyanins). Because these representations showed a linear trend, the regression and the performance parameters were calculated and evaluated again by using ULC 2.0.

RESULTS AND DISCUSSION

Extraction Method Precision. As is well known, the recovery of an analyte depends on the extraction method used. Therefore, because the different cellars are using different extraction procedures to determine the phenolic content of grapes, the results obtained will be also different. To compare these results, which is the objective of this study, evaluation of the precision of the extraction methods used is essential since the viability of this comparison depends on the value of this parameter.

To estimate the repeatability of the three methods considered in this study, suitable amounts of the same grape variety harvested on the same date were crushed to obtain a single grape homogenate. This homogenate was divided into 24 different portions to get 24 identical samples. In that way, we could take 6 identical replicates to be macerated under the conditions specified for each of the three methods studied. Once the different extracts were obtained, the absorbances at 280 and 520 nm were measured to determine the total polyphenols and total anthocyanins values, respectively. The results obtained showed relative standard deviation values of 3.9 and 4.6 for AWRI, 2.2 and 0.7 for ITV, 1.4 and 1.6 for Glories (whatever the pH used), respectively. A statistical comparison showed that all of these values were comparable for all of the methods evaluated. Therefore, considering the similar and high precisions in all cases, comparison between the results obtained by using the three different methods could be carried out.

Calibration Lines. We consider it to be of interest to study the linearity of the models, determining both the determination coefficient (R^2) and the LOL values, because they indicate the dispersion degree of the data around the calibration line, helping to evaluate the linearity of the model.

The calibration lines obtained for total polyphenols by using gallic acid as standard showed very good determination coefficient

values ($R^2 \geq 0.999$) and excellent linearity was confirmed by LOL > 99.5% regardless of the method evaluated.

For anthocyanins, the calibration lines obtained from the solutions of malvidin-3-glucoside also presented very good coefficient values ($R^2 \geq 0.999$) and good linearity over the concentration ranges evaluated, because LOL values are >98.5% in all cases.

Table 1. Concentration Ranges of Total Polyphenols (Milligrams of Gallic Acid per Kilogram of Grapes) and Total Anthocyanins (Milligrams of Malvidin-3-glucoside per Kilogram of Grapes) Determined by Using the Extraction Methods Evaluated

method	no. of samples	range (mg kg^{-1})	
		total polyphenols	total anthocyanins
ITV	120	1042−2097	350−1310
AWRI	66	1323−2869	255−1307
Glories pH 1.0	58	719−1587	265−1227
Glories pH 3.2	58	500−1082	151−597

Comparative Study of the Methods. After the calibration lines for each of the three different methods had been obtained, the total polyphenols and total anthocyanins were quantified in many different samples. The samples analyzed included a wide range of varieties and ripening state (see Samples, above) to consider the most possible variability in the study. **Table 1** summarizes the concentration ranges of the parameters determined by the three methods. The concentration values for both parameters are the usually expected in red grapes, including both grape variety and ripening state. As can be noted, whereas the AWRI method provides the more effective extraction for total polyphenols, the ITV method provides the more effective one for total anthocyanins. The different extraction efficiencies obtained by each method could be explained by two factors: the extraction capacity of the solvent used and the total time employed in the extraction. Thus, an increase in the ethanol content facilitates the seed polyphenols extraction (*14*). However, although an acidic medium does not provide a relevant extraction over the seeds as it does ethanol media, when this content increases, a higher degradation of vacuolar membrane and therefore a higher extraction from the

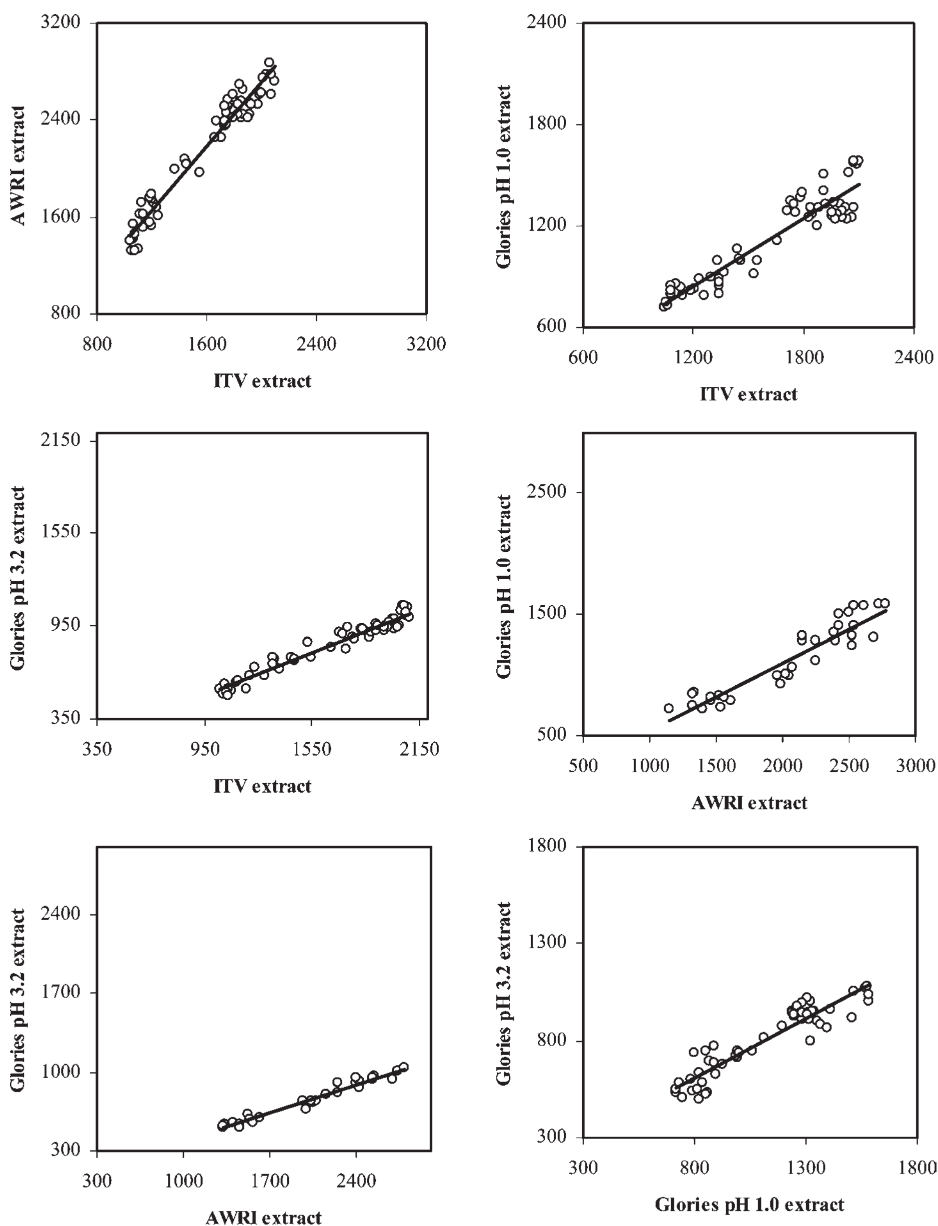

Figure 1. Graphical representation of the linear regression between total polyphenols quantified in all extracts (in mg of gallic acid per kg of grape).

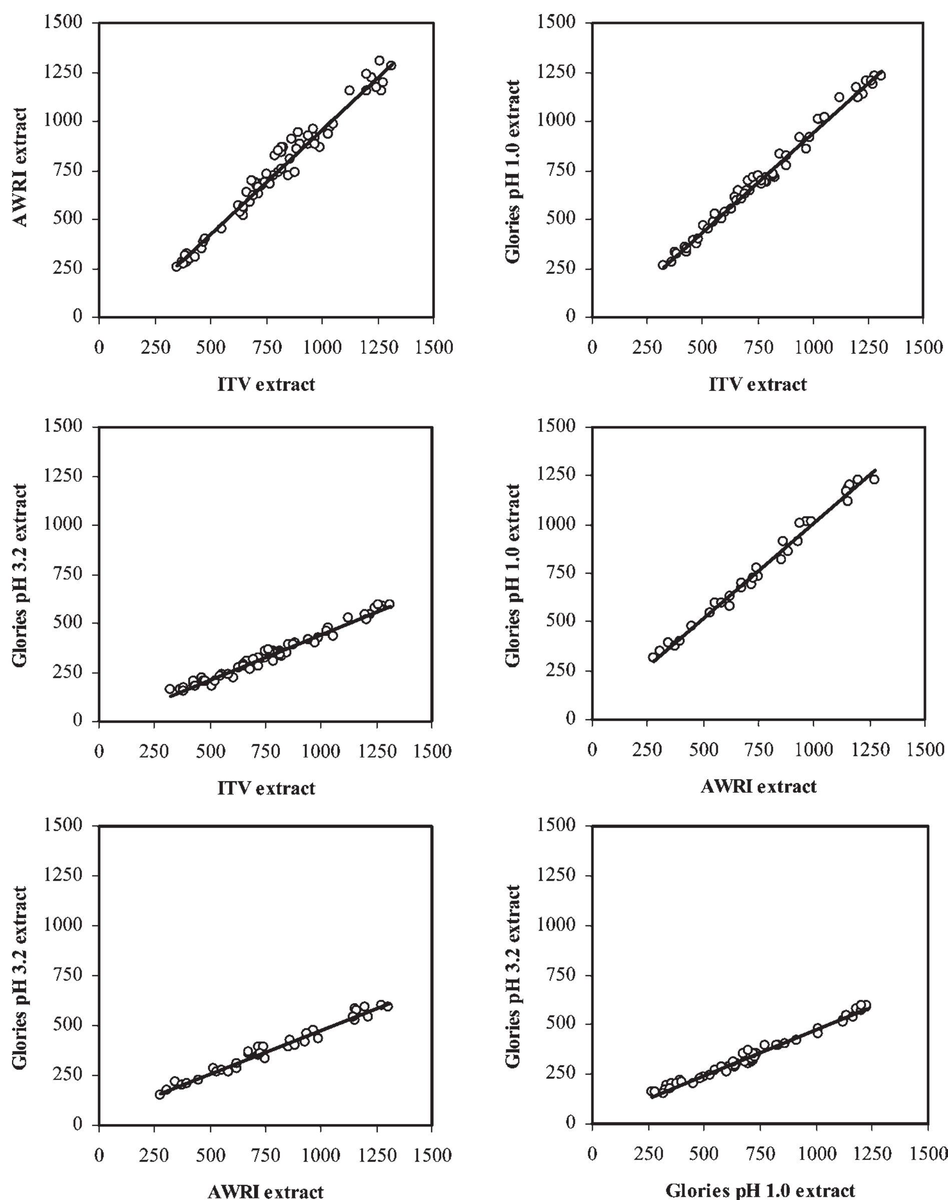

Figure 2. Graphical representation of the linear regression between total anthocyanins quantified in all extracts (in mg of malvidin-3-glucoside per kg of grape).

skins will be achieved. All of these effects are most favored the longer the maceration time.

To proceed with the comparative study, we calculated and evaluated the regression parameters of the different lines obtained when the results obtained were plotted with the methods studied (two by two). **Figures 1** and **2** show graphically these regressions.

For total polyphenols, there is a very good correlation and linearity in all cases, as shown by the values of R^2 and LOL (**Table 2**). Therefore, this mathematical expression can be used for predicting the value of the studied variable by using a different method from that used to obtain the extract analyzed.

On the other hand, it can be noted that the extent of extraction clearly differs between methods, because the slopes obtained when correlating the results from the pair-to-pair comparison are different from 1. Thus, for example, a value of 0.56 in the slope of the regression line obtained when the Glories method is compared with the AWRI method means that the first method extracts only about 56% of what is extracted by using the second one. Therefore, from the different slope values we can conclude that, whereas the AWRI method gives the highest extraction yield, the Glories pH 3.2 method provides a lower extraction efficiency value. This behavior coincides with the fact that whereas the AWRI method provides a nearly complete extraction, the method of Glories at pH 3.2 extracts only the polyphenols called "easily extractable polyphenols".

Table 2. Regression Parameters on the Comparative Study of the Three Methods for Total Polyphenols

methods compared [method 1 (x) vs method 2 (y)]	slope ± CI[a]	intercept ± CI[a]	R^2	LOL (%)
AWRI−Glories pH 1.0	0.56 ± 0.08	−17 ± 165	0.870	93.16
AWRI−Glories pH 3.2	0.36 ± 0.03	25 ± 57	0.964	96.30
ITV−Glories pH 1.0	0.67 ± 0.07	36 ± 113	0.874	94.93
ITV−Glories pH 3.2	0.47 ± 0.03	53 ± 47	0.953	96.97
ITV−AWRI	1.32 ± 0.07	66 ± 121	0.953	97.17
Glories pH 1.0−Glories pH 3.2	0.61 ± 0.06	115 ± 76	0.869	94.66

[a] CI, confidence intervals with a significance level (α) = 0.05.

The correlation parameters for the results of anthocyanins obtained by the three methods were even better than those obtained for total polyphenols (**Table 3**).

Table 3. Regression Parameters on the Comparative Study of the Three Methods for Total Anthocyanins

methods compared [method 1 (x) vs method 2 (y)]	slope ± CI[a]	intercept ± CI[a]	R^2	LOL (%)
AWRI−Glories pH 1.0	0.98 ± 0.04	27 ± 34	0.988	97.90
AWRI−Glories pH 3.2	0.43 ± 0.03	42 ± 22	0.972	97.01
ITV−Glories pH 1.0	1.01 ± 0.03	−72 ± 24	0.989	98.58
ITV−Glories pH 3.2	0.46 ± 0.02	−24 ± 16	0.977	97.94
ITV−AWRI	1.08 ± 0.05	−118 ± 39	0.971	97.84
Glories pH 1.0−Glories pH 3.2	0.46 ± 0.02	16 ± 13	0.982	98.13

[a] CI, confidence lintervals with a significance level (α) = 0.05.

Table 4. Mean Values of EA% and SM% Indices Calculated from the Extracts Obtained with the Glories Method and the Values Predicted from Regression Lines

		EA%				SM%			
	no. of samples	calcd	predicted	recovery (%)	bias	calcd	predicted	recovery (%)	bias
ITV	58	52.0	52.3	100.6	0.36	44.2	44.0	99.5	0.18
AWRI	35	51.8	51.6	99.6	0.13	48.5	48.2	99.4	0.24

With regard to the extraction efficiency of the total anthocyanins obtained with the different methods, we can observe two different behaviors. On the one hand, the slopes ITV−AWRI, ITV−Glories pH 1.0, and AWRI−Glories pH 1.0 are very close to 1, so the extraction efficiencies of anthocyanins obtained by the three methods are comparable. This behavior is due to the fact that the three extraction methods carry out an almost total extraction of anthocyanins. On the other hand, the comparison of the Glories pH 3.2 method with the other ones gives slope values of the linear regression close to 0.5, so in this case the extraction efficiency of this method is only half that achieved by using the other three methods.

Measurement of Phenolic Ripening Indeices. To demonstrate the application feasibility of the correlations found in this study to the enological field, we applied the correlation models developed to predict two indices widely used in enology: the cellular extractability index or extractable anthocyanins index (EA) and the phenolic maturity index or seed maturity index (SM).

The EA% index represents the percentage of anthocyanins that have not been extracted during winemaking and, usually, decreases during ripening. The SM% index represents the fraction of polyphenols provided by seeds, commonly known as astringent tannins, and its value also decreases with ripening. Both indices are determined in an empirical way and allow us to know the quality phenolic ripening and the state of grapes and, therefore, to set the harvest date.

These phenolic potential parameters (in mg L^{-1}) were calculated as

$$\mathrm{EA}(\%) = \left[\frac{(C_{\mathrm{pH1}} - C_{\mathrm{pH3.2}})}{C_{\mathrm{pH\,1.0}}}\right] \times 100 \quad (1)$$

$$\mathrm{SM}(\%) = \left[\frac{(C_{280(\mathrm{pH3.2})} \times \mathrm{DF}) - \left(C_{\mathrm{pH3.2}} \times \frac{40}{1000}\right)}{(C_{280(\mathrm{pH3.2})} \times \mathrm{DF})}\right] \times 100 \quad (2)$$

where $C_{\mathrm{pH\,1.0}}$ = total content of anthocyanins (mg L^{-1} malvidin-3-glucoside), $C_{\mathrm{pH3.2}}$ = extractable anthocyanins (mg L^{-1} malvidin-3-glucoside), and $C_{280\,(\mathrm{pH3.2})} \times \mathrm{DF}$ = total polyphenols easily extracted at pH 3.2 measured at 280 nm, previously diluted in deionized water. This parameter is widely called the total polyphenols index. $C_{\mathrm{pH3.2}} \times (40/100)$ = skin polyphenols estimated by extractable anthocyanins (*15*), and $(C_{280(\mathrm{pH3.2})} \times \mathrm{DF}) - [C_{\mathrm{pH3.2}} \times (40/100)]$ = seed polyphenols.

Because these indices are from the measure of the extracts obtained with the time-consuming Glories method, the challenge was to predict these indices from the extracts obtained with the ITV and AWRI methods, which are much faster.

Thus, several samples of different varieties and at different ripening stages were analyzed under the Glories method conditions and the EA% and SM% were calculated. In that way we had a wide range of values for the indices to be predicted from AWRI and ITV and, therefore, we could check the prediction ability in a wide range of concentrations.

Table 4 shows the mean values of both indices calculated from the extracts obtained according to the Glories method and also the predicted values from the extracts obtained with the AWRI and ITV methods. To assess the goodness of the predictions we defined the recovery parameter as the ratio predicted value/calculated value and expressed it as a percentage. As can be seen, in all cases the recovery was very satisfactory, close to 100%. Because the values shown corresponded to the mean of several samples, it was interesting to examine also the value of bias, which should be as close as possible to 0. The statistics showed that for both methods and also for both parameters, the bias values are comparable to 0 for a significance level of 0.05. However, given that the parameter providing more direct information about the results obtained is the prediction error, we also evaluated it from the ratio bias/calculated value. When the values were predicted from the AWRI method, the prediction error for EA% was $<0.3\%$ and $<0.5\%$ for SM%. For the prediction from the ITV method, the results were also very satisfactory because the prediction error for EA% was $<0.7\%$ and $<0.3\%$ for SM%.

Therefore, although the recoveries of the polyphenolic compounds obtained with each of three methods are not comparable, we can conclude that the regression lines obtained for total polyphenols and total anthocyanins analyzed by different methods are suitable tools to compare information about phenolic ripening obtained in laboratories that use different methodologies.

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