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ORIGINAL RESEARCH ARTICLE

Quantification of total polyphenol content in wine lees by conventional optical and photoacoustic spectroscopy

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ABSTRACT

The He-Ne laser (632.8 nm) and photoacoustic spectroscopy (PAS), a variant of the photothermal methods, were combined with the Folin-Ciocalteu colorimetry method to determine total phenolics in wine lees. Utilising this method we found that the total polyphenol content of the nine selected wine lees varied from 1305 to 3907 mg/L. The gallic acid equivalent was determined by means of spectrophotometry using 765 nm as the analytical wavelength. The original Folin-Ciocalteu colorimetry assay was modified for the PAS measurements. Since the PAS does not need dilution, the filtration steps and the Folin-Ciocalteu reagent were used directly on the wine lees samples. Using the original colour reaction process and the modified reaction, the PAS showed linear behaviour between the total polyphenol content and the PA signal; the results of which gave determination coefficients of 0.9946 and 0.9936, while the limit of detection was 232.6 mg/L in both cases.

KEYWORDS: polyphenols, wine lees, Folin-Ciocalteu reaction, photoacoustic spectroscopy

INTRODUCTION

Phenolic compounds play a very important role in winemaking, and the bioactivity of many can positively affect human health (Ferraz da Costa *et al.*, 2020). For example, regarding the "French paradox" (Constant, 1997; Catalgol *et al.,* 2012) - a term used since 1992 - epidemiological data showed that French people had a low incidence of coronary heart diseases, despite the consumption of a diet in high saturated fat. It was established that there is a connection between the daily consumption of red wine (due to its polyphenol content) and coronary heart disease: polyphenols reduce its number and severity. Numerous benefits from these phenolic compounds (antioxidant, anti-cancer, anti-inflammatory effects, anti-mutagenesis, etc.) have been observed; this is very useful information for consumers that can contribute to health and nutrition decisions (Teixeira *et al.,* 2014). Furthermore, grapevine produces phenolic secondary metabolites in large amounts and in extremely diverse chemical forms (Waterhouse *et al.,* 2016a). The quantity and composition of these compounds significantly affect the quality, ageability and sensory properties of wines (Hornedo-Ortega *et al.,* 2020). Flavan-3-ols are present in high concentrations in both the grape berry skin (Prieur *et al.,* 1994) and seeds (Souquet *et al.,* 1996), functioning as important building blocks for polymerised tannins. Grape berry skins are the main source of anthocyanins (Castillo-Muñoz *et al.,* 2009). In *Vitis vinifera*, the most common form of anthocyanins is 3-O-glucoside. The unglycosylated flavonoid ring is called 'anthocyanidin', of which there are five different types in wine grapes: malvidin, petunidin, delphinidin, peonidin and cyanidin. Malvidin-3-glucoside dominates in most cases. Monomeric anthocyanins can also react with other phenolic and non-phenolic compounds found in the wine matrix during winemaking and wine aging processes (co-pigmentation, bisulphite bleaching and formation of wine pigments) (Waterhouse *et al*., 2016b).

The phenolic maturity of the grape berry can be evaluated based on different factors: total or individual concentration of the distinct phenolic molecules, the level of their extractability, or their degree of polymerisation (Ribéreau-Gayon *et al.,* 2006). It is worth mentioning that oak ageing can also add further tannins to the wines (Hornedo-Ortega *et al.,* 2020).

The phenolic composition of wines is affected by the winemaking technology (Singleton and Trousdale, 1983), grape variety, place of cultivation and vintage (De La Presa-Owens *et al.,* 1995; Nagel and Wulf, 1979). Phenolic compounds are transferred from grape to wine, and some of them to the by-products of winemaking. Thus, wine lees also contain significant amounts of phenolic compounds due to the adsorption capacity of the yeast cell wall (Morata *et al.,* 2005). The phenol content of the wine lees also depends on grape variety and winemaking technology (Jara-Palacios, 2019).

Nowadays, an extremely wide range of analytical techniques are used to identify phenolic components in grape juice and wines. Among these, there are relatively simple chemical methods and more complex instrumental techniques, which are expensive and time consuming.

A very common method in wine research for determining phenolic content is ultraviolet-visible (UV/Vis) spectrophotometry. This technique is used to measure both colour and other phenolic compounds in grapes and wine. In the UV spectrophotometry, 280 nm is the analytical wavelength for the evaluation of total phenolics (Aleixandre-Tudo and du Toit,, 2018). However, Waterhouse (2002) has noted that not all phenolic substances are detectable at 280 nm.

The light absorption maximum for anthocyanins is 520 nm. There are, however, disadvantages to this method compared to liquid chromatography; for example, the lack of specificity: several non-phenolic compounds in wine absorb light at these specific wavelengths too, thus the probability of overestimating the real concentration of phenolics is high (de Villiers *et al.,* 2012).

Our study focused on wine lees, a winemaking by-product that has been studied for a long time, since it is rich in phenolic compounds, of which mainly flavonols and anthocyanins (Jurčević *et al.,* 2017; Zhijing *et al.,* 2018; Romero-Díez *et al.,* 2018; Jara-Palacios, 2019). Therefore, there is great interest in the reuse of wine lees in the wine industry itself, as well as in other food or non-food industries (Matos *et al.,* 2019; Troilo *et al.,* 2021). Wine lees was proposed as a dietary supplement for humans and animals in 2000 (Gómez *et al.,* 2004). At present, the extraction of bioactive residues from wine lees is being intensely investigated using different methods (Chakka and Babu, 2022; Ciliberti *et al.,* 2022).

Opportunities for measuring phenolic content in winemaking have been thoroughly discussed by Harbertson and Spayd (2006). Of the described techniques, the Folin-Ciocalteu assay is one of the most widely used and easiest ways to determine total wine phenols. The Glories Gelatin index can also be used to quantify both total tannins and protein reactive tannins, and the use of methyl cellulose is the most recent technique for precipitating tannins (Sarneckis *et al.,* 2006). All three of these methods require spectrophotometric measurements in order to calculate the results. Fourier-transform mid-infrared (FT-MIR) instrumentation has also been utilised for decades in routine wine analysis (including total phenolics) (Patz *et al.*, 2004; Kupina and Shrikhande, 2003).

Besides UV/Vis, a wide range of different spectroscopic techniques are used in wine research, such as infrared/visible spectrophotometry, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS). High-performance liquid chromatography (HPLC) is used to precisely determine different groups of phenolics with different chemical properties (Dóka *et al.,* 2011; Ficzek *et al.*, 2011; Odgerel *et al.,* 2022). In wine research, HPLC is nowadays very commonly applied (Merkytė *et al.*, 2020).

A more in-depth summary is written by de Villiers *et al.* (2012) about the chemical (including phenolics) analysis of wines.

Merkytė *et al.* (2020) also summarise numerous analytical methods for the precise determination of the distinct molecules that account for total phenolics.

As well as the aforementioned analytical methods, a relatively new method, the optothermal window technique, can also be used after the Folin-Ciocalteu reaction to determine the total polyphenol content of wines (Dóka and Bicanic, 2002).

In this study, photoacoustic spectroscopy (PAS) combined with the Folin-Ciocalteu reaction was applied to determine the total polyphenol content of different types of wine lees. The colour reaction was modified, the time of the analysis was significantly reduced and the obtained results measured on wine lees samples were compared to the original reaction in the same samples.

MATERIALS AND METHODS

1. Growing conditions

The experimental vineyard (Kőlyuktető) is located in the Eger wine region of Hungary (47°86′N, 20°38′E, 173 m above sea level). It belongs to Eszterházy Károly Catholic University. The climatic conditions of the region are humid continental with an average annual temperature of 10.5 °C and average annual precipitation of 577 mm. The soil is brown forest soil on rhyolte tuff bedrock. The grapes were grown under a middle-high cordon, the spacing was 3x1 m, and the crop load was set to 8 bud/plant. The varieties were grown on 'Teleki 5C' rootstock (*Vitis berlandieri* x *V. riparia*, breeder S. Teleki, 1924, Hungary). The orientation of the rows was north-south.

Four red varieties were grown, Syrah, Kadarka, Cabernet franc I and Blauburger, and one white, Leányka. The white cuvée comprised a mixture of wines prepared from berries of numerous white varieties from a gene bank belonging to the university. The red cuvée was made by blending the lees of the following officially-permitted varieties grown on the university's vineyard: Medoc noir, Syrah, Kékfrankos (syn. Blaufränkisch), Blauburger, Merlot, Cabernet franc and Cabernet-Sauvignon. Additional samples (Cabernet franc II and Cabernet-Sauvignon) were also collected from another professional winery in the Eger wine region with the same growing conditions as described above.

TABLE 1. The main parameters of the wine making process.

Variety	Harvest date	Nutrition before fermentation (g/hL[a], g/100kg[b])	Nutrition during fermentation (g/hL)	Yeast (g/hL[a], g/100kg[b])	Skin contact (d)	Racking
			Red			
Syrah	22nd October	Ecobiol PDC Arom, 28[a]	Activit O Ecobiol, 28 Rouge Volutan, 35	IOC Befruit, 28[a]	23	8th December
Kadarka	18th October	Aromcolor, 4[b] Oxyless U, 28.5[b] Ecobiol PDC Arom, 28[a]	Ecobiol PDC Arom, 28 EcobiolSafe, 28 Activit O, 28	IOC Befruit, 28[a]	29	3rd December
Cabernet franc I	20th October	Oxyless U, 28.5[b] Aromcolor, 4[b] Ecobiol PDC Arom, 28[a]	Ecobiol PDC, 28 Ecobiol Safe, 28 Activit O, 28	IOC Befruit, 28[a]	29	15th December
Blauburger	08th October	Aromcolor, 4[b] Ecobiol PDC Arom, 28[a]	Ecobiol PDC, 28 Ecobiol Rouge, 35 Volutan, 28 IOC Sentinel, 25 Essentiel, 15	Gaia, 20[b] IOC Befruit, 28[a]	3	15th December
Cabernet-Sauvignon	11th October	Uvavital 20[a]	Uvavital, 10-10-10-10	Uvaferm BDX, 20[a]	40	15th December
Cabernet franc II	9th October	Uvavital 20[a]	Uvavital, 10-10-10-10	Uvaferm BDX, 20[a]	23	5th December
Red cuvée	-	Oxyless U, 15[a] IOC Fullprotect, 20[a]	Ecobiol PDC, 20 Activit, 30 Actibiol, 30	Blastosel Lambda/ Blastosel Grand Cru/ La Claire Extreme, 20[a]	-	8th December
			White			
Leányka	24th September	Ecobiol PDC Arom, 20[a]	Glutarom Extra, 30 Ecobiol ICE, 30 Bouquet B45, 10 Activit, 40	Boreal, 20[a]	-	23rd November
White cuvée	-	Oxyless U, 28.5[b] Cleaspeed, 1[a]	Ecobiol PDC Arom, 20 Glutarom extra, 30 Ecobiol ICE, 30 Bouquet B45, 10 Activit, 40	EM2, 20[a]	-	8th December

2. Wine making process

During the wine making process the destemmed and crushed berries were directly pumped into stainless steel tanks and treated with 5 g/hL potassium metabisulphite. Before inoculation and during the fermentation, nutrients were added to the must or the destemmed grape depending on the grape variety. Fermentation was carried out with *Saccharomyces cerevisiae* under controlled conditions with 3-40 days of skin contact in the case of red varieties. The temperature inside the tanks was maintained between 24-25 °C during the fermentation process. In the case of the red varieties, mash caps were punched down twice a day. At the end of malolactic fermentation, the free SO_2 level of the new wines was adjusted to 30 mg/L. Generally, the wines were racked 1 month after pressing and stored at 15 °C (see detailed parameters of the wine making process, Table 1). After racking, the wine lees were stored at -18 °C until the measurements were taken.

3. Determination of polyphenol content

The polyphenol content was determined in the presence of Folin-Ciocalteu's reagent (Sigma-Aldrich Co.) at 765 nm using spectrophotometry (Hitachi U2800A, Japan) in three repetitions, on the basis of a calibration curve made from gallic acid, according to Singleton and Rossi (1965). Figure 1 shows the calibration curve prepared from gallic acid in the range of 0 and 1000 mg/L in 100 mg/L stages. The calibration curve shows linear behaviour at 765 nm with a determination coefficient of $R^2 = 0.9917$. This curve was also used for the determination of total polyphenol content in the optical spectroscopy measurements.

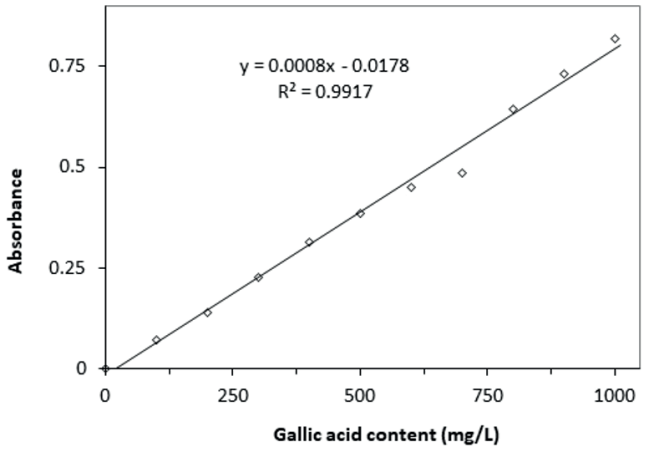

FIGURE 1. The obtained calibration curve, prepared from gallic acid in the 0 and 1000 mg/L concentration range. The absorbance was measured by optical spectrophotometry at 765 nm.

4. Modified sample preparation

0.5 mL of wine lees was poured into a 50 mL Falcon-tube, and 2.5 mL of Folin-Ciocalteu's reagent was added. After 5 min, a 7.5 mL 0.7M Na_2CO_3 (CAS: [497-19-8]) solution was added. The mixture was not filled to 50 mL with distilled water (as the original method requires). The mixture was kept in the dark at room temperature for two hours and was measured with photoacoustic spectroscopy.

As a result of the modification, the samples - after the reaction - were more suitable for photoacoustic measurement. Without the dilution and filtration processes the samples were expected to have a higher concentration of polyphenols. In the case of PAS, the samples did not have to be transparent, as described in the following paragraph. Higher absorption results in a higher PA signal.

5. Photoacoustic spectroscopy

In the photoacoustic spectroscopy the sample to be investigated is irradiated by a modulated beam of radiation. The fraction of the energy absorbed by the sample is converted to heat, a result of which is that the temperature of the sample oscillates periodically at a frequency identical to that of the modulated radiation itself. Generated thermal waves eventually reach the sample's surface and cause periodic heating and cooling of the contacting layer of the surrounding gas. Finally, the expansions and contractions of the gas give rise to acoustic waves; these are detected as a voltage (termed photoacoustic [PA] signal) using a microphone. The optical and thermal parameters of the sample and the contacting gas all play a decisive role in the generation of the PA signal. The home-made PA spectrometer (Figure 2) used in this study is composed of a 5 mW He-Ne laser (Melles Griot). The laser beam was mechanically chopped (26 Hz), and by using a plane mirror it was directed into the PA cell. Radiation entered the PA cell through a quartz window with a 12.7 mm in diameter. A 3 mm long capillary (inner diameter 300 µm) connected the miniature electret microphone (Sennheiser KE 4-211-2) with the part of the cell that accommodates the sample. The microphone sensitivity was 10 mV/Pa at 1000 Hz. The PA signal was processed by a dual phase lock-in amplifier (Stanford SR530) with a 3 s time constant coupled to a computer.

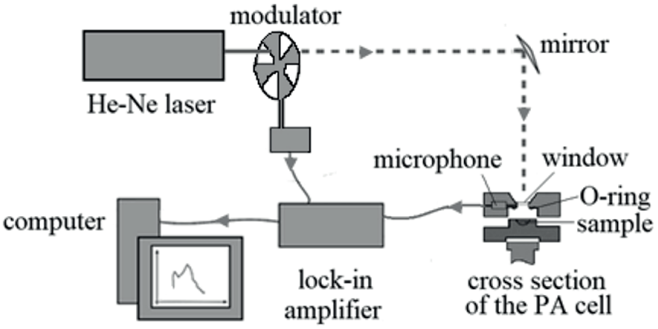

FIGURE 2. The home-made PA setup.

The wine lees samples were measured after the colour reaction. With each loading, 256 successive readings of the lock-in amplifier were taken and the average value and standard deviation were computed. The wine lees were then removed and the PA cell was cleaned using a kitchen towel. A fresh quantity of the same wine lees was then loaded into the PA cell and the whole procedure was repeated. The loadings were repeated three times and the average of the measured values was considered as the outcome for the analysis.

RESULTS

1. Optical spectroscopy

The concentration (determined by optical spectroscopy at 765 nm) and the corresponding standard deviation (expressed as gallic acid equivalents in mg/L) of the total polyphenols in nine wine lees samples are shown in Table 2. The highest polyphenol contents were mostly found in red wine lees (Syrah, Cabernet franc I, Blauburger). However, there are some red wine samples (Kadarka, Red cuvée, Cabernet franc II and Cabernet-Sauvignon) that contained similar amounts of polyphenols as the white wines, and in the case of two samples (Cabernet franc II and Cabernet-Sauvignon), the polyphenol content was even lower than in the white wine samples.

TABLE 2. The total polyphenol content and standard deviations of wine lees measured by spectrophotometry.

Variety	Total polyphenol mg/L
Syrah	3906.67 ± 85.1
Kadarka	1785.83 ± 70.7
Cabernet franc I	2452.50 ± 57.5
Blauburger	2398.33 ± 88.7
Red cuvée	1609.58 ± 56.4
Cabernet franc II	1347.08 ± 35.5
Cabernet Sauvignon	1305.42 ± 75.3
Leányka	1823.33 ± 61.7
White cuvée	1548.33 ± 47.3

2. PA spectroscopy

First, the PA measurements on six prepared samples (four red and two white wine lees samples) - namely Syrah, Kadarka, Cabernet franc I, Blauburger, Leányka and White cuvée - were analysed applying the Folin-Ciocalteu method. Figure 2 shows the amplitude of the PA signal at 632.8 nm plotted against the TPC (determined by SP) in the wine lees samples. In all the PA experiments, the laser power, experimental geometry and modulation frequency (26 Hz) were the same. The relationship between the amplitude of the PA signal and TPC indicates linear proportionality. The correlation between the PA response and TPC was linear ($R^2 = 0.9946$), as shown in Figure 3. The data shown in Figure 3 are the averages of four to six consecutive single measurements. Each single measurement represents 256 successive readings of the lock-in signal. The standard deviation in such "single load" type of measurements ranged between 1.5 and 7 % of the measured value, with an average of 3.6 %.

In the second step, all nine wine lees samples (see Table 2) were prepared using the modified Folin-Ciocalteu method and then analysed. Figure 4 represents the PA signal versus TPC (determined by SP) in the wine lees samples. The conditions were the same as in the first step. Once again, the relationship between the amplitude of the PA signal and TPC indicates linear proportionality. The correlation between the PA response and TPC is linear ($R^2 = 0.9935$), as shown in Figure 4.

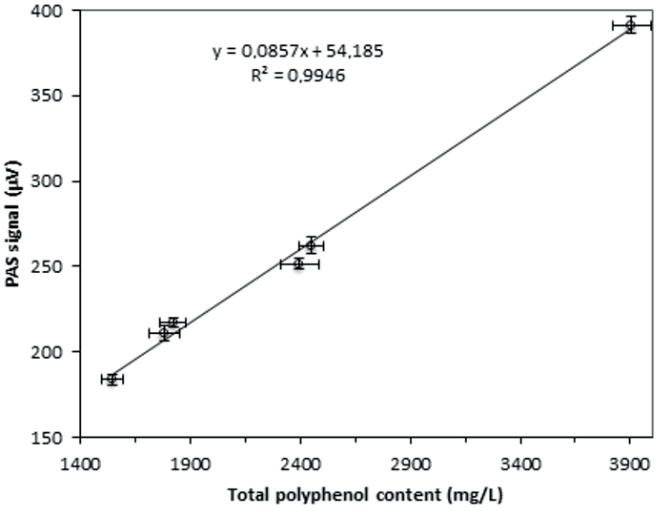

FIGURE 3. Measured PA signal versus total polyphenol content according to the Folin-Ciocalteu method.

The data shown in Figure 4 are the averages of three to five consecutive single measurements. Each single measurement represents 256 successive readings of the lock-in signal. The standard deviation in such "single load" type of measurements ranged between 0.6 and 5.9 % of the measured value, with an average of 2 %. Table 3 contains statistical analysis and regression data for both series of the prepared wine lees samples.

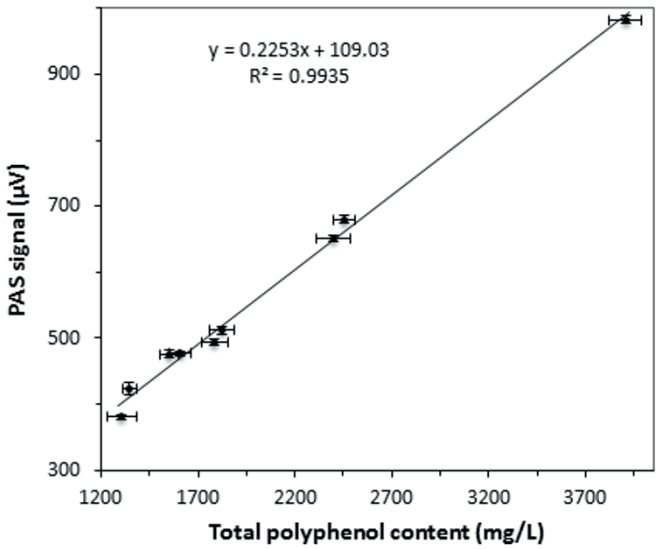

FIGURE 4. Measured PA signal versus total polyphenol content according to the modified Folin-Ciocalteu method.

DISCUSSION AND CONCLUSION

Wine lees are wine making by-products that have a high content of antioxidant molecules consisting of mainly phenolic compounds (Mazauric and Salmon, 2005; Mazauric and Salmon, 2006). The most abundant phenolic compounds in wine lees are flavonols and anthocyanins (primarily in red grapes). In several previous studies, the polyphenol

TABLE 3. Regression data for total polyphenol content in the wine lees samples[a].

Technique	Best-goodness of linearity fit							Precision	
	Conc.range (mg/L)/ N/n/n′	*R/RSS/*$s_{y/x}$	Slope		Intercept		LOD estimate (mg/L)	Instrument precision (%)	Measurement repeatability (%)
			Mean (μV /mg/L)	*RSD* (%)	Mean (μV)	*RSD* (%)			
PAS (632.8 nm)[b]	1548.3-3906.7/ 6/3-2/6-4	0.997/146.1/6.0	0.086	0.023	54.19	0.089	232.6	1.5-7 (256 readings)	0.3-1.3 (*n* = 6-4)
PAS (632.8 nm)[c]	1305.4-3906.7/ 9/1-3/3-5	0.997/1765.2/15.9	0.225	0.030	109.03	0.136	232.6	0.6-5.9 (256 readings)	0.5-1.2 (*n* = 3-5)

LOD (limit of detection) = 3.3 $s_{y/x}$/slope, N – number of concentration levels, n – number of prepared samples from each wine lees, n′ - number of independent measurements (loadings) at each concentration, PAS – photoacoustic spectroscopy, RSD – relative standard deviation, R – coefficient of correlation, RSS – residual sum of the squares, $s_{y/x}$ – residual standard deviation of the regression line.

[a] Concentrations are reported for the liquid phase measured by SP at 765 nm (3 independent analyses).

[b] PA measurements according to Folin-Ciocalteu method.

[c] PA measurements on the modified Folin-Ciocalteu samples.

content of wine lees was evaluated in GAE/g dry matter. However, a wide range of values were obtained depending on grape variety, oenological technology employed, extraction process and solvent used: Pérez-Serradilla and Luque de Castro (2011) reported 547 mg GAE/g, Romero-Díez *et al.* (2018) 26-254 mg GAE/g dry extract (DE) total phenol content, and Tao *et al.* (2014) measured 44–59 mg GAE/g DE; while other studies found lower values: 30.86 mg GAE/g DE (Reis *et al.*, 2016) and 23.16 mg GAE/g DE (Jurčević *et al.*, 2017).

We expressed our results as mg/L, because we wanted to avoid the time-consuming determination of dry matter content. However, in relevant literature we could not find results for wine lees expressed as mg/L. Therefore, our results cannot be compared to the data in the literature. There are studies in which the TPC is determined in mg/L, but only for wine: in Welschriesling, Lužar *et al.* (2016) obtained 444 mg/L TPC, while in Sauvignon blanc, they obtained 451 mg/L. de Lima *et al.* (2011) analysed TPC in red wines obtaining values of between 1582 and 2896 mg/L. When comparing our values with the aforementioned data, we can conclude that in terms of magnitude we obtained sound results. In conventional Folin-Ciocalteu's colorimetry, detection is usually performed at 765 nm, at which the absorbance is maximal. A laser emitting at 765 nm is not currently available in our laboratory; therefore, 632.8 nm radiation of He-Ne was used instead for excitation in the PAS experiment. At 632.8 nm, the absorbance is approximately 90 % of that at 765 nm, because the polyphenols have a very wide absorption band around the maximum (Dóka and Bicanic, 2002).

In both the standard and modified Folin-Ciocalteu reactions, the PA signal is linearly proportional to the results of the spectrophotometry. This suggests that the PAS is also suitable for measuring polyphenols in wine lees and has some advantages regarding the sample preparation: the dilution and filtration processes are not necessary, nor is the determination of dry matter content. The extent of interference in the PA measurement of phenolic content is the same as that in optical spectrophotometry.

The precision of the instrument was under 6 and 7 %, while the repeatability of the measurements was 1.2 and 1.3 % respectively. The LOD values were the same; i.e., 232.6 mg/L at the analytical wavelength. Additionally, the intermediate dilution and filtration steps and hyphenated Folin-Ciocalteu PA colorimetry are not needed for the determination of total polyphenolic content in wine lees. Furthermore, our analytical procedure offers several additional attractive features, such as relatively low cost, speed, simple sample loading, and an easy cleaning procedure.

To summarise, the results of our study show a high correlation between the two different methods, with similar accuracy; therefore, this simple analytical procedure can be used, and can replace the Singleton and Rossi (1966) method, to determine the polyphenol content of wine lees. This procedure can also be used in the identification of further uses for wine lees as a by-product of the wine industry, such as in the pharmaceutical, food (e.g., ice-cream, jam and yoghurt) and cosmetic industries.

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