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Analytical Methods

Simplification of the DPPH assay for estimating the antioxidant activity of wine and wine by-products

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ABSTRACT

The DPPH· assay is one of the most commonly employed methods for measuring antioxidant activity. Even though this method is considered very simple and efficient, it does present various limitations which make it complicated to perform. The range of linearity between the DPPH inhibition percentage and sample concentration has been studied with a view to simplifying the method for characterising samples of wine origin. It has been concluded that all the samples are linear in a range of inhibition below 40%, which allows the analysis to be simplified. A new parameter more appropriate for the simplification, the EC_{20}, has been proposed to express the assay results. Additionally, the reaction time was analysed with the object of avoiding the need for kinetic studies in the method. The simplifications considered offer a more functional method, without significant errors, which could be used for routine analysis.

1. Introduction

It is widely known that moderate wine consumption is beneficial to health. The benefits of wine are related to its content of polyphenolic compounds and their antioxidant properties (De Beer, Joubert, Gelderblom, and Manley, 2003; Granato, Katayama, and De Castro, 2011; Kanner, Frankel, Granit, German, and Kinsella, 1994; Pinho, Couto, Valentão, Andrade, and Ferreira, 2012; Rockenbach et al., 2011; Santos et al., 2011; Torres et al., 2002). Antioxidant compounds are capable of protecting biological systems against the harmful action of free radicals. Epidemiological evidence indicates that the consumption of food and drinks rich in antioxidants reduces the incidence of various degenerative disorders such as mutagenesis, carcinogenesis, arteriosclerosis, ischaemic heart disease, neurodegenerative diseases and ageing (Bekhit et al., 2011; Briviba, Pan, & Rechkemmer, 2002; De Gaetano, Di Castelnuovo, & Rotondo, 2005; Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012).

Apart from their beneficial effect on health, antioxidants are used in several industries. In the food industry, for example, they are used to prevent the oxidation of lipids and other constituents, for preserving food quality. Nowadays, consumers are increasingly opting for natural compounds against synthetics ones. For this reason, wine by-products, considered a rich source of natural phytochemicals, are particularly suitable for use as ingredient in foods, medicine and cosmetics (Aliakbarian, Fathi, Perego, & Dehghani, 2012; Lafka, Sinanoglou, & Lazos, 2007).

It is therefore, of commercial interest to be able to determine the antioxidant activity of wine, wine derivatives and wine by-products, as well as phytochemicals and food in general (Baoshan et al., 2009; Lutterodt, Slavin, Whent, Turner and Yu, 2011).

Several different methods have been developed for measuring antioxidant activity, but none of them are exempt from problems and limitations (Decker, Warner, Richards, and Shahidi, 2005; Fogliano, Verde, Randazzo, and Ritieni, 1999; Larrauri, Sánchez-Moreno, and Saura-Calixto, 1998; Lee, Kim, Kim, and Jang, 2002; Magalhães, Segundo, Reis, and Lima, 2008; Sánchez-Moreno, Larrauri, and Saura-Calixto, 1998; Šeruga, Novak, and Jakobek, 2011). The DPPH· (2,2-diphenyl-1-picrylhydrazyl) assay is one of the most commonly employed methods because, in general terms, it is simple, efficient and inexpensive. The original method was developed by Blois (1958) and, with the modifications introduced by Brand-Williams, Cuvelier, and Berset (1995), it is widely used as a reference point (Bondet, Brand-Williams, and Berset, 1997; Chen, Bertin, and Froldi, 2013).

DPPH· is a stable free radical which presents a deep purple colour and a strong absorption band in the range of 515–520 nm. In the presence of antioxidant compounds, DPPH· can accept an electron or a hydrogen atom from the antioxidant scavenger molecule,

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to be converted to a more stable DPPH molecule. As the reduced form of DPPH is pale yellow, it is possible to determine the antioxidant activity by studying the change of colour spectrophotometrically. The greater the free radical scavenging capacity of an antioxidant compounds, the more reduction of DPPH and the less purple colour there is in the sample. The results are normally expressed as Efficient Concentration (EC_{50}), which is defined as the amount of sample necessary to decrease the initial DPPH concentration by 50%. The parameter EC_{50} was introduced by Brand-Williams et al. (1995), Molyneux (2004), Kedare and Singh (2011) (otherwise called the IC_{50} value), and it is very useful for comparing results because it is independent of the sample concentration. Many authors use the antiradical power (ARP) parameter, which is defined as the reciprocal of EC_{50}: $ARP = 1/EC_{50}$.

Even though this method is considered very simple and efficient, it does present various limitations which make it complicated to perform. Several experimental studies have shown that the relationship between the antioxidant concentration and the DPPH radical scavenging activity is non-linear (Locatelli et al., 2009; Villaño, Fernández-Pachón, Troncoso, & García-Parrilla, 2005), so measurement of the EC_{50} may be quite problematic. This lack of linearity means that it is necessary to study the behaviour of every sample and to obtain a standard curve for each one. Several aliquots containing different sample concentrations must be tested and the results must be transferred onto a graph showing the percentage of residual or inhibited DPPH against the concentration of sample divided by the amount of initial DPPH. The EC_{50} is then obtained from this curve by interpolation. This gives rise to two inconveniences: first, it is possible, especially when testing complex samples, that these curves do not present good fits, so the results could be subject to significant errors. Secondly, when the number of samples to be studied is large, considerable time and work is required because every sample involves the analysis of at least five or six aliquots (normally in duplicate or triplicate). Thus the number of determinations can be enormous, putting into question the viability of the procedure.

Furthermore, several authors have demonstrated that it is important to measure incubation at the steady state instead of at a fixed time, in order not to underestimate the results (Mishra, Ojha, & Chaudhury, 2012). The reaction time to reach the plateau will depend on the nature and the concentration of the antioxidants; however, the relation between reaction time and concentration is not linear (Huang, Boxin, & Prior, 2005). This means that the kinetic behaviour of every aliquot analysed must be studied, which requires considerable time. Despite this method being technically simple, and only requiring a UV–Vis spectrophotometer, if it is going to be used routinely it is almost essential to use an automated spectrophotometer which can measure multi-cell kinetics in parallel.

Finally, it is important to mention that this method has been applied using many different protocols, in which there are differences in respect of the solvents, the initial $DPPH^{\cdot}$ concentration, the sample volume, incubation time and presentation of results; consequently, it is known that the results obtained according to different protocols are not comparable. Several authors have called for the method to be properly standardised (Dawidowicz, Wianowska, & Olszowy, 2012; Sánchez-Moreno, 2002).

In the present work, we have focused on the fact reported by several authors that for only a limited range of concentration a reliable linear relationship does exist between antioxidant concentration and percentage of inhibition (Buenger et al., 2006; Locatelli et al., 2009; Villaño et al., 2005). The aims of this study are to determine the range of linear behaviour for wine and wine by-product samples; then to study the advantages of working within this range, to be able to reduce some of the problems cited above; and to propose a simplified procedure for estimating the antioxidant activity of a wine product, which could be used for routine analysis.

2. Materials and methods

2.1. Chemicals

Gallic acid, caffeic acid, (+) catechin (all with purity >98%) and 1,1-diphenyl-2-picrylhydrazil (DPPH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethanol was provided by Panreac (Barcelona, Spain). Ultrapure water used was from a Milli-Q system.

2.2. Samples

Three standard polyphenolic compounds (gallic acid, caffeic acid and (+) catechin), five different wines, one vinegar and six extracts of wine by-products were analysed.

2.2.1. Polyphenolic compounds

The standard phenolic compounds analysed were chosen because they usually occur in grape and wine; gallic acid, caffeic acid, and (+) catechin were selected as examples of hydroxybenzoic acid, hydroxycinnamic acid and flavonol, respectively. Different concentrations of the samples were prepared in water.

2.2.2. Wine and vinegar

The initial differentiation made is between white wine and red wine. Because, from a chemical standpoint, all red wines have a similar composition, and all white wines are also similar to each other, various wines each made by different wine-making process were selected, with a view to studying the method in typical wines but, at the same time, in the most different wines of each main type. We selected three white wines: a young wine and two Sherry wines (manzanilla); two red wines: one young and one aged, and one Sherry vinegar too. The samples of all six products were obtained from the Cadiz area, Spain. The samples were diluted to different concentrations with water.

2.2.3. Wine by-products

The by-products used were obtained from a commercial wine-making facility. Pomace and seeds from white grape (var. *palomino*); pomace (without stems), stems and seeds from red grape (var. *tempranillo*), and red wine lees were selected. Each by-product was dried in a climate chamber at 40 °C, in darkness. Samples were then ground and sieved to obtain homogeneous particles of size between 100 and 300 μm. Powdered samples (1 g) were extracted with 25 ml $EtOH/H_2O$ (1:1) in an ultrasound bath (P-Selecta, Spain) and filtered in vacuum. The samples were diluted in water to obtain different concentrations.

2.3. Methods

DPPH scavenging activity was determined according to the method reported by Brand-Williams et al. (1995) with some modifications. For each sample, ten concentrations were tested in order to obtain their calibration curves. As the method works equally well with methanol or ethanol (Cheng, Moore, & Yu, 2006), ethanol was chosen because of its lower toxicity. It is usual that some wines and vinegars present the phenomenon of precipitation when the alcoholic proportion is increased. This could happen when the sample is mixed with the alcoholic medium of the DPPH. Despite that, no precipitation occurs if the samples are diluted first with water. None of the aliquots used in this study showed precipitation problems.

200 μl of sample or EtOH (blank) were added to 3.3 ml of a 50 μM solution of DPPH in ethanol prepared daily (0.069 ppm of initial DPPH). The initial amount of DPPH was chosen to obtain initial and final absorbance values within the range of accuracy of spectrophotometry (Sharma & Bhat, 2009), and the sample volume selected is appropriate for obtaining good results even for wines with low activity. The exact initial concentration of DPPH solution in EtOH was calculated spectrophotometrically from a calibration curve determined by linear regression:

$$y = 0.0286[\mathrm{DPPH}] + 0.004; \quad r^2 = 0.9999$$

where: [DPPH] is the concentration of DPPH in ppm. This parameter is expressed in ppm because the final result is given as: mg of sample/mg of initial DPPH.

The absorbance was read at 515 nm, using a spectrophotometer model Cary 50 Bio (Varian, Australia), every ten minutes until the reaction reached a plateau. The temperature of the sample chamber was controlled at 20 °C using a thermo-regulating system (Frigiterm, P-Selecta, Spain). For each sample concentration tested, the reaction kinetic was plotted and the inhibition percentage of DPPH at the steady state was determined using the following equation:

$$I\% = (\text{Abs blank-Abs sample})/\text{Abs blank} \times 100$$

These percentages were transferred onto a graph against the concentration of sample divided by the amount of initial DPPH.

2.4. Statistical analysis

All measurements were carried out in triplicate and the results are presented as mean ± standard deviation (SD). Linearity was studied by quantification of the correlation coefficients. Comparison of mean values between methods was performed using the *t* and *F* statistics. The repeatability of the method was studied from one way ANOVA analysis. Statistical significance was declared at $p < 0.05$. Results were processed using the software Statgraphic Plus 5.1.

3. Results and discussion

3.1. Linearity study

Studying the inhibition percentage of DPPH (*I*%) against sample concentration, we have found differences in free radical scavenging behaviour for each sample. After applying several common regression models to the samples, it was found that some samples do not present a good fit with these models, as can be seen in the example in Table 1.

The existence of a linear relationship between antioxidant concentration and percentage of inhibition has been tested for all the samples studied; however, the linearity range varies considerably depending on the type of sample. The standard polyphenolic compounds studied are the samples with widest range of linearity: they are linear below 70% of DPPH inhibition. The wine by-product extracts show differences in linearity ranging from less than 40% inhibition for red grape stem, to less than 80% inhibition for red wine lees. The samples with the least linearity are the wines and the vinegar tested. For the white wines and the aged red wine the linearity barely reached 40% inhibition. The young red wine is linear below 60% and the Sherry vinegar below 50% of DPPH inhibition (Fig. 1).

Table 1
Aged red wine. Example of a sample which presents poor fits. All the fits applied are presented.

Regression model	Equation	R^2
Linear	$y = 0.2497x + 32.535$	0.6759
Logarithmic	$y = 25.81\ \ln(x) - 45.607$	0.9539
Exponential	$y = 30.661e^{0.0048x}$	0.5329
Polynomial	$y = -0.0021x^2 + 0.9029x + 12.727$	0.9648
Power	$y = 5.7176x^{0.5438}$	0.9022

This means that, when testing some of the samples, such as white wine or aged red wine, to obtain the EC_{50}, this is found to be in a part of the curve which is not very well defined, and consequently the results will be associated with large errors. Based on the results, it has been found that all the samples analysed show, at least, a common range of linear relationship between sample concentrations and DPPH inhibition percentage at less than 40% of DPPH inhibition. Given that it is very advantageous to work with linear fits and that all the samples studied are linear below 40% of DPPH inhibition, it would be most convenient to work with dilutions which produce DPPH inhibition below 40% with the object of ensuring linearity.

However, if DPPH inhibitions below 40% are considered in order to be able to work in the linear range, the results cannot be expressed as EC_{50}. By analogy with the EC_{50}, we are proposing that the antioxidant activity should be expressed as EC_{20} (i.e. the amount of sample necessary to decrease the initial DPPH concentration by 20%). A DPPH inhibition of 20% falls exactly in the middle of the linear zone ensured.

Working in the linear range offers significant advantages: first, there is the assurance that all the samples will have simple and good fits, and the results will present lower errors; and second, it is possible to reduce the number of dilutions necessary to obtain the calibration curve without significant errors.

3.2. Reaction time study

Previously, reference has been made to the importance of measuring at the steady state so that the results obtained are not underestimations (Mishra et al., 2012). Nevertheless, this form of measuring requires excessively long times, and the possibility of testing a lot of samples at the same time is lost. Also mentioned before is the need to use specific equipment, an automatic spectrophotometer, for multicell kinetics, when this method is used frequently. If an efficient but practical and functional method is wanted, the need for kinetic studies must be avoided. This requires selecting a reaction time which allows us to measure at a fixed time, without significant errors for any sample. Analysing the reaction time data, it is seen, first, that the reaction times depend on the sample concentration: the lower the sample concentration, the shorter the reaction times. It has been found that, particularly in complex samples like wines, it is more difficult to achieve the plateau as the concentration of the aliquot is much higher, as can be seen in Fig. 2. This means that the dilutions used to obtain the EC_{20}, with lower concentrations than many of the aliquots necessary to analyse in the original method (Brand-Williams et al., 1995), achieve stability better and in less time. It is also seen that the time required to reach the plateau is very different depending on the sample tested. The reaction times for extracts of wine by-products take from 170 to 240 min depending on the aliquot. The wine and vinegar samples present reaction times of 300 min for the majority of aliquots. The standard polyphenols are the samples with the fastest kinetics, presenting reaction times of between 30 and 240 min. Checking all the reaction times for the aliquots analysed which produce DPPH inhibitions below 40%, and considering the minimum waiting time with the minimum error in the result, we have concluded that a reaction time of 240 min is a suitable choice. All the aliquots analysed for the standard polyphenols and wine by-products complete the reaction in a maximum time of 240 min; however, wines and vinegars need more than

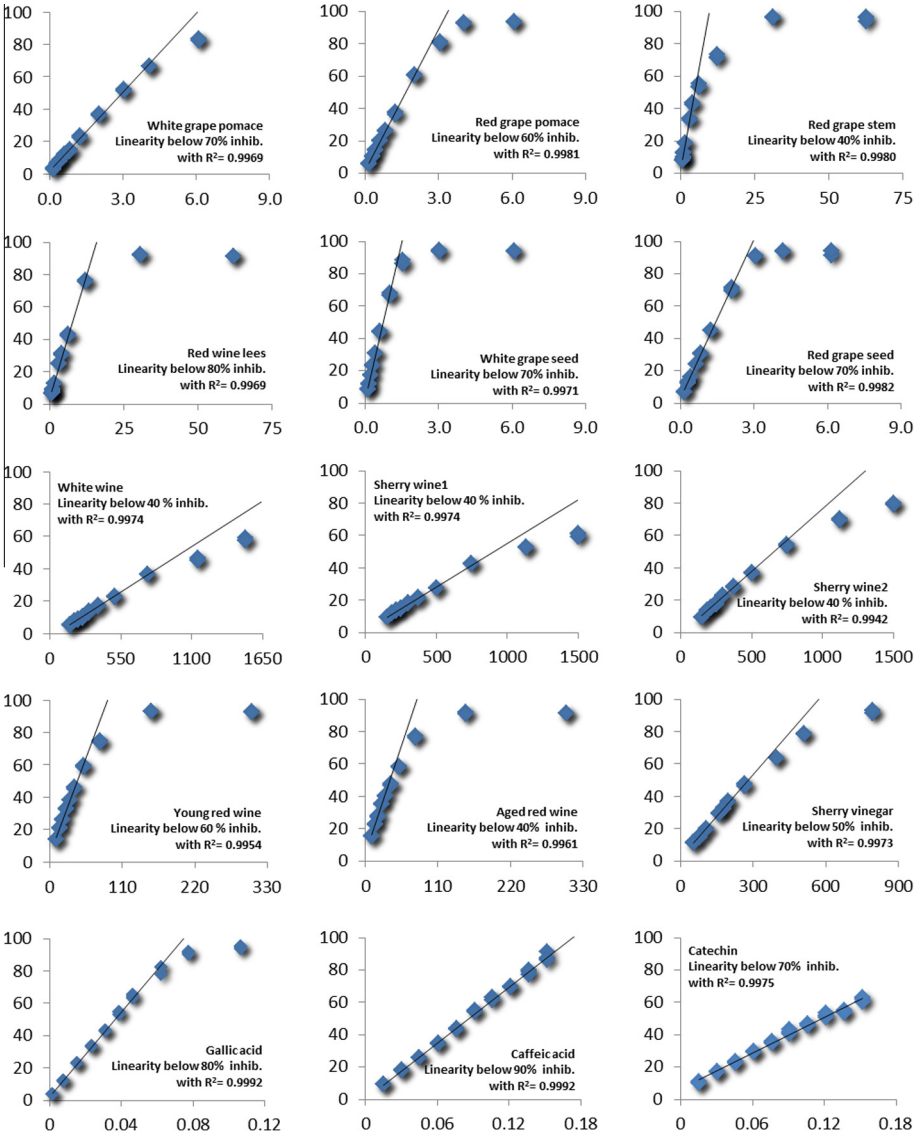

Fig. 1. Representation of the antioxidant activity for the samples studied. The antiradical curves have been plotted as the percentage of DPPH inhibition on the ordinate against the milligrams of sample divided by the initial milligrams of DPPH on the abscissa.

240 min to reach the plateau. Comparing the results obtained measuring at steady state with the results obtained measuring at 240 min, it is seen that wines and vinegar present similar relative errors below 5%, except for the white wine for which the relative error is 5.69% (Table 4). Consequently, when measuring at a fixed time of 240 min, errors do not appear, with the exception of wine and vinegar for which a relatively low error must be accepted in return for achieving a much more practical and useful method.

3.3. Simplification of the method

Although the normal practice would be to make the calibration with at least 4 or 5 dilutions, the possibility has been studied of obtaining the EC_{20} from a linear fit made with only three points (in duplicate) in the range of 40% inhibition, with the object of simplifying the procedure as far as possible. From the analysis of the results, it has been demonstrated that it is possible to obtain the EC_{20} without significant errors, on condition that at least one point is below 20% and another is between 20% and 40% of DPPH inhibition. In other words, it is necessary for the EC_{20} to be among the points studied to minimise the error. If no information about the antioxidant activity of the sample is available beforehand, it would be necessary to perform an initial assay, measuring a single aliquot, to be able to determine easily the ideal concentrations to obtain inhibitions one below 20% and another between 20% and 40%. Table 2 shows the EC_{20} obtained in the linear range with several points in triplicate, and those obtained with only three points in duplicate, under the condition mentioned before. From the statistical analysis no significant differences have been found between the two results at $p < 0.05$.

Additionally, and as a further step in the simplification, the possibility has been studied of obtaining the EC_{20} by measuring just one aliquot. Theoretically, if one dilution with a concentration x_1 is analysed, and an inhibition y_1 is obtained, and we want to know the EC_{20} for an inhibition of $y_2 = 20$:

$$EC_{20} = \frac{y_2 \times X_2}{y_2} + \frac{b}{m}\left(\frac{y_2 - y_1}{y_1}\right)$$

where: b is the y-intercept, m the slope of line, and $\frac{b}{m}\left(\frac{y_2-y_1}{y_1}\right)$ is the factor which is not considered when we work with just one aliquot and the calibration curve is unknown. Developing this expression to obtain an error of less than ±0.05 EC_{20}, and considering that the b

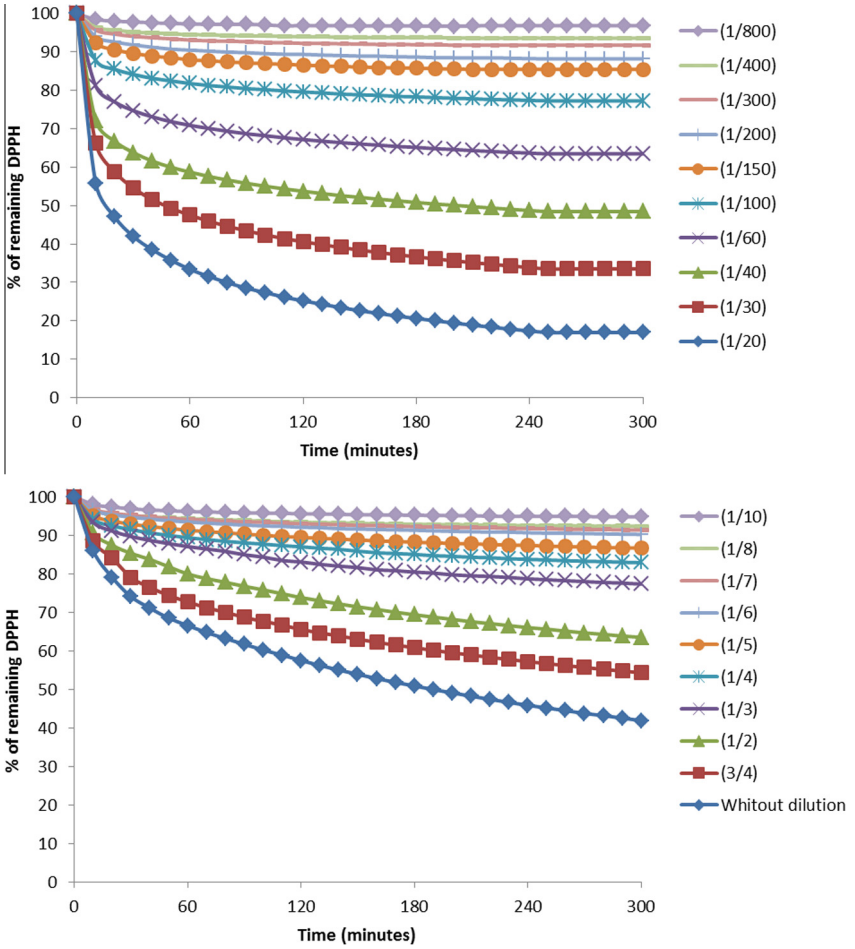

Fig. 2. Examples of different kinetic behaviour. The percentage of remaining DPPH is represented on the ordinate against time on the abscissa. Samples: (a) white grape pomace, (b) white wine.

Table 2
Differences between the results obtained at the steady state and at a fixed time of 240 min.

Samples	EC20 at steady state	EC20 at 240 min	Relative error
White grape pomace	25.37 ± 0.84	25.37 ± 0.84	0.00
Red grape pomace	13.96 ± 0.38	13.96 ± 0.38	0.00
Red grape stem	40.44 ± 1.45	40.44 ± 1.45	0.00
Red wine lees	59.00 ± 2.60	59.00 ± 2.60	0.00
White grape seed	5.98 ± 0.23	5.98 ± 0.23	0.00
Red grape seed	11.84 ± 0.38	11.84 ± 0.38	0.00
White wine	438.49 ± 10.23	463.42 ± 10.93	5.69
Sherry wine1	344.26 ± 10.10	357.94 ± 12.65	3.97
Sherry wine2	274.16 ± 9.21	283.86 ± 9.47	3.54
Young red wine	14.27 ± 0.56	14.68 ± 0.52	2.90
Aged red wine	13.36 ± 0.51	13.99 ± 0.64	4.73
Sherry vinegar	103.33 ± 3.05	103.33 ± 3.66	4.47
Gallic acid	$0.013 \pm 3.2 \times 10^{-4}$	$0.013 \pm 3.2 \times 10^{-4}$	0.00
Caffeic acid	$0.034 \pm 6.9 \times 10^{-4}$	$0.034 \pm 6.9 \times 10^{-4}$	0.00
Catechin	$0.038 \pm 12.0 \times 10^{-4}$	$0.038 \pm 12.0 \times 10^{-4}$	0.00

value is low and not very different for all the samples studied, if we estimate a b value as the maximum value obtained in our group of real samples, it is found that: $17 \leqslant y_1 \leqslant 25$. So, theoretically, if we analyse an aliquot with an inhibition close enough to the EC_{20}, specifically with an inhibition of between 17 and 25%, we can obtain the EC_{20} with an error of less than 5%. All the samples were analysed from a single aliquot in triplicate, following the aforementioned premise, and it was verified experimentally that it is fulfilled in all cases, as shown in Table 3. To obtain inhibitions in the range considered, it was only necessary to make a preliminary measurement of the samples to determine the appropriate concentration.

Therefore, it is possible to reduce the number of aliquots necessary to obtain the calibration curve, from five or six of the original method to three or even to one, working in the linear zone, with DPPH inhibition below 40%, and following the premises mentioned, results without significant errors can be obtained.

3.4. Repeatability study

In order to know the repeatability of the simplified method, the EC_{20} was determined for a real sample, a young red wine, following the considerations mentioned to obtain the EC_{20} from the

Table 3
Comparison of the EC_{20} achieved with several points in triplicate, and with three points in duplicate, in the linear range. The last column shows the relative error of the results.

Samples	Points	Linear regression	R^2	EC20 ± DS	Points	Linear regression	R^2	EC20 ± DS	Error
White grape pomace	7	$y = 0.7564x + 0.8120$	0.9969	25.37 ± 0.84	3	$y = 0.7695x - 0.3063$	0.9946	25.59 ± 1.16	0.88
Red grape pomace	6	$y = 1.3037x + 1.7959$	0.9981	13.96 ± 0.38	3	$y = 1.2606x + 2.6035$	0.9993	13.80 ± 0.27	1.17
Red grape stem	6	$y = 0.4420x + 2.1277$	0.9980	40.44 ± 1.45	3	$y = 0.4415x + 2.3491$	0.9963	39.98 ± 1.62	1.12
Red wine lees	7	$y = 0.2948x + 2.6048$	0.9969	59.00 ± 2.60	3	$y = 0.3350x + 1.1321$	0.9983	56.33 ± 1.11	4.53
White grape seed	6	$y = 3.0455x + 1.7820$	0.9971	5.98 ± 0.23	3	$y = 3.0163x + 2.5100$	0.9850	5.80 ± 0.32	3.07
Red grape seed	6	$y = 1.5304x + 1.8796$	0.9982	11.84 ± 0.38	3	$y = 1.5208x + 1.9869$	0.9920	11.84 ± 0.49	0.03
White wine	8	$y = 0.0510x - 2.3616$	0.9974	438.49 ± 10.23	3	$y = 0.0513x - 2.5942$	0.9975	440.76 ± 10.74	0.52
Sherry wine1	8	$y = 0.0542x + 1.3308$	0.9974	344.26 ± 10.10	3	$y = 0.0506x + 2.2065$	0.9939	351.75 ± 10.60	2.18
Sherry wine2	7	$y = 0.0779x - 1.3526$	0.9942	274.16 ± 9.21	3	$y = 0.0782x - 0.8246$	0.9987	266.08 ± 3.65	2.95
Young red wine	5	$y = 1.1669x + 3.3446$	0.9954	14.27 ± 0.56	3	$y = 1.3502x + 0.6145$	0.9976	14.38 ± 0.24	0.58
Aged red wine	5	$y = 1.2263x + 3.6138$	0.9961	13.36 ± 0.51	3	$y = 1.3659x + 1.6682$	0.9931	13.42 ± 0.40	0.44
Sherry vinegar	6	$y = 0.1753x + 1.8829$	0.9973	103.33 ± 3.05	3	$y = 0.1795x + 0.8967$	0.9974	106.42 ± 2.25	2.99
Gallic acid	5	$y = 1324.8x + 2.2477$	0.9992	$0.013 \pm 3.2 \times 10^{-4}$	3	$y = 1291.8x + 3.1084$	0.9994	$0.013 \pm 2.5 \times 10^{-4}$	2.42
Caffeic acid	5	$y = 561.7x + 0.7572$	0.9992	$0.034 \pm 6.9 \times 10^{-4}$	3	$y = 537.5x + 1.7310$	0.9974	$0.034 \pm 8.9 \times 10^{-4}$	0.78
Catechin	5	$y = 398.4x + 4.8943$	0.9975	$0.038 \pm 12.0 \times 10^{-4}$	3	$y = 403.8x + 4.6880$	0.9970	$0.038 \pm 9.3 \times 10^{-4}$	0.01

Table 4
EC_{20} obtained from the measurement of one aliquot in triplicate, in the linear range, following the premise mentioned. The last column shows the relative error of the results obtained, compared with those obtained with the measurement of several points in triplicate.

Samples	Points	EC20 ± DS	Error
White grape pomace	1	24.97 ± 0.60	1.59
Red grape pomace	1	13.74 ± 0.26	1.59
Red grape stem	1	42.04 ± 0.85	3.97
Red wine lees	1	56.46 ± 1.18	4.30
White grape seed	1	6.14 ± 0.04	2.70
Red grape seed	1	11.64 ± 0.05	1.67
White wine	1	444.63 ± 4.56	1.40
Sherry wine1	1	347.39 ± 5.23	0.91
Sherry wine2	1	278.707 ± 9.35	1.66
Young red wine	1	14.25 ± 0.17	0.16
Aged red wine	1	13.30 ± 0.20	0.46
Sherry vinegar	1	106.54 ± 1.61	3.11
Gallic acid	1	$0.013 \pm 1.4 \times 10^{-4}$	0.27
Caffeic acid	1	$0.034 \pm 5.2 \times 10^{-4}$	1.88
Catechin	1	$0.040 \pm 7.5 \times 10^{-4}$	4.40

measurement of just one aliquot. The samples were kept in darkness and the measurement was made after 240 min. This process was carried out 9 times within 1 day and it was repeated on 3 different days. From the ANOVA analysis non-significant errors were verified among all the results obtained. The intra-day RSD was 0.93% and the inter-day RSD was 0.31%. These low relative standard deviations indicate very good repeatability of the method.

4. Conclusions

Working with DPPH inhibition percentages below 40% ensures a linear relationship between DPPH inhibition and sample concentration for wine and wine by-products. In all cases this makes it possible to have good fits and to simplify the DPPH assay procedure with the analysis of considerably fewer aliquots. A new and more appropriate parameter, EC_{20}, has been proposed to express the results. This parameter would replace the current EC_{50}, which is, in many cases, in a non-linear zone and not well defined because it is difficult to determine accurately. Additionally this parameter would allow results to be obtained for many samples with limited reactivity, which do not reach an inhibition of 50%. The EC_{20} can be obtained, in the linear zone, from the analysis of just three aliquots, on the condition that the EC_{20} is among the points analysed, in order to reduce errors. It is even possible to obtain the EC_{20} from the analysis of just one aliquot if the inhibition of this aliquot is close enough to 20%, specifically between 17% and 25% of DPPH inhibition. On the basis of this condition the errors found are non-significant. Working in the range below 40% of inhibition, the aliquots reach the plateau earlier and more clearly. The samples can be measured at the fixed time of 240 min without significant errors, obviating the need for kinetic studies. Although at first sight, having to wait 240 min to obtain results may not seem a simplification, avoiding the need for kinetic studies means that many samples can be measured at the same time and that the measurements can be made in a simple spectrophotometer (with no need for scanning kinetic equipment).

It must be borne in mind that, from a chemical view point, all the samples of wine origin have a similar matrix. Considering the group of white wines, it can be seen that within this group the calibration lines are quite similar and the EC_{20} values are within the same order of magnitude. Observing the red wines, again very similar fits and results are obtained. This means that, each group contained wines that were very different because of differences in the wine-making process, and even so the antioxidant behaviour has been similar; it would therefore be possible to infer the results and to use the procedure for other different samples of wine origin. Given all these considerations, the simplified procedure proposed in this study constitutes a more practical and functional method that gives good results and promises to be very useful for the routine analysis of samples of wine origin.

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