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Analytical Methods

Selective methods for polyphenols and sulphur dioxide determination in wines

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ABSTRACT

A critical review to the methods recommended by international bodies and widely used in the winery industry and research studies was performed. A Laccase biosensor was applied to the selective determination of polyphenols in wines. The biosensor response was characterised and it responds mainly to o-diphenols which are the principal polyphenols responsible for the stability and sensory qualities of wines. The spectrophotometric method to determine free and total sulphur dioxide recommended for beers was applied directly to wines. A sampling of 14 red and white wines was performed and they were analysed for biosensor polyphenol index (I_{BP}) and sulphur dioxide concentration (SO_2). The antioxidant capacity by the $ABTS^{\cdot+}$ spectrophotometric method was also determined. A correlation study was performed to elucidate the influence of the polyphenols and SO_2 on the wines stability. High correlations were found between I_{BP} and antioxidant capacity and low correlation between SO_2 and antioxidant capacity. To evaluate the benefits of wine drinking a new parameter (I_{BP}/SO_2) is proposed.

1. Introduction

Wine is a complex mixture of several hundred compounds present at different concentrations. Many of them are found at very low concentrations, even though they play an important role in wine evolution and quality. Usually, the parameters to be analysed are simplified and are focused on the sample evolution, sensory characteristics or to fulfil food laws. Among the parameters more often analysed are polyphenols index (I_{BP}) and sulphur dioxide (SO_2).

Many analytical methods recognised by the international community as official methods of analysis are useful for monitoring wine composition, but in many cases obsolete and show lack of selectivity. However, there are methods described in the scientific literature to provide better analytical results, therefore is time to change, to break the inertia and to move to more advanced methodologies.

Polyphenols compounds are a complex group of substances of plant origin. The basic features of all polyphenolics are the presence of one or more hydroxylated benzene rings. The two main groups of polyphenolics are phenolic acids and flavonoids (Blasco, Rogerio, Gonzalez, & Escarpa, 2005). Their concentration in wines depends on grape variety, geographical origin, soil type, harvest and winemaking technique. These compounds are responsible of the sensory properties of the wines. Due to its antioxidants properties they have been associated with reduced risk of cancer, heart disease and diabetes (Sies, 2010).

The individual identification of all polyphenols is not possible and furthermore, it would be a difficult task because of their chemical complexity. Some authors determine individually chemical compounds (Russo, Andreu-Navarro, Aguilar-Caballos, Fernández-Romero, & Gómez-Hens, 2008) but in such a complex samples could be more useful to determine groups of similar compounds. Very often these results can be more valuable to assess the quality of wines. Therefore, it is important to underline the interest of "polyphenols indexes" (Gamella, Campuzano, Reviejo, & Pingarron, 2006). Traditionally, the term "total phenolics contents" refers to the results obtained by spectrophotometric methods, specially the Folin–Ciocalteu method. The reagent used in this method is an oxidant mixture that reacts with reducing substances without selectivity to form chromophores that can be detected spectrophotometrically. In the case of the wines, yields an overestimation of the total polyphenolic content, as it is known, the main interferents are: sulphur dioxide, reducing sugars and ascorbic acid. In spite of that, many authors to validate a method to determine polyphenolic content in food samples compare with the ones obtained with the Folin–Ciocalteu method. In all cases, as expected, the results with

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this method are higher than when a more selective method for polyphenols is used. Therefore, the Folin–Ciocalteu method is a measure of the reducing capacity rather than a determination of polyphenols (Martinez-Periñan et al., 2011). In the cases, when the relationship polyphenols versus other reducing agents is high, the positive error is lower than when this relationship is low.

In recent times, different electrochemical methods have been proposed for the characterisation of polyphenols in wine on the basis that practically all polyphenolic molecules present in wine are electrochemically active (Sanchez-Arribas, Martinez-Fernandez, & Chicharro, 2012). Cyclic voltammetry was the first electrochemical method used for characterisation of polyphenols and determination of total polyphenols content in wines (Marijan, Novak, & Jakobek, 2009). Flow injection analysis with electrochemical detection for determination of total polyphenols in wines (Blasco et al., 2005; Gamella et al., 2006) and differential pulse voltammetry (Marijan et al., 2009) has also been used. These methods also can present interferences from other compounds with similar redox potentials. Biosensors have been proposed as efficient analytical tools for the detection of polyphenol compounds, exhibiting advantages mainly a high selectivity. An amperometric Laccase biosensor with the enzyme immobilised onto a glassy carbon electrode has been described for wines (Gamella et al., 2006) and also an amperometric determination by Laccase immobilised onto silver nanoparticles/zinc oxide nanoparticles modified gold electrode (Chawla, Rawal, Kumar, & Pundir, 2012). In this work, a Laccase–Sonogel-Carbon biosensor (Lac/SNGC) previously developed by us (ElKaoutit et al., 2008) and applied to beers has been used to determine polyphenols in wines. A study of the biosensor response to the different polyphenols present in wines was performed.

The sulphur dioxide is found naturally in wines at low concentrations, besides is added to prevent oxidation and bacteria growth and to control enzymatic reactions during elaboration and storage. Without this additive is not possible to guarantee the wine quality, nevertheless, an excess can spoil wine since it produces discolouration, gives a spicy odour and change the flavour. Sulphur dioxide has been the subject of legislation as food additive since the discovery that it causes asthmatic attack, allergic reactions to hypersensitive people and dermatological problems (Vally, Misso, & Madan, 2009). In the USA and EU, a directive requires food manufactures to label if it is present at concentration higher than 10 mg L^{-1} in prepacked foods (Spricigo et al., 2009), since it is potentially toxic.

The sulphur dioxide can be present in wine under two forms: free (HSO_3^- or SO_2) or bound to carbonyl and unsaturated compounds and to phenols (Barbe, De Revel, Joyeux, Lonvaud, & Bertrand, 2000) existing in a reversible equilibrium between the two forms. The free sulphur dioxide has antiseptics and reductant properties and its levels should be adjusted before packing.

As above mentioned, it is necessary to develop a precise, sensitive and selective method for sulphur dioxide determination. Most of the used methods are based on sulphur dioxide oxidation to sulphuric acid. The International Organisation of Vine and Wine method is based on the sulphur dioxide separation by a nitrogen or air stream followed by an oxidation with hydrogen peroxide and the sulphuric acid formed is titrated with sodium hydroxide. The differentiation between free and bound sulphur dioxide is performed by temperature (10 °C for free and 100 °C for bound sulphur dioxide). The quantitative separation is not assured and depends on the sulphur dioxide wine content. Its accuracy, precision and reproducibility are not adequate.

The Ripper method (Ripper, 1892) is the most widely used in wineries; it is based on the sulphur dioxide titration with iodine and starch as indicator. It presents two drawbacks: difficulties to detect colour changes, especially in red wines and positive errors due to the interference of polyphenols, ascorbic acid and other reductants.

It is possible to find more advanced methodologies based on instrumental techniques: electroanalytical techniques (Baldo, Salvatore, & Mazzocchim, 1994; Kawamura et al., 1994), piezoelectric sensors (Palenzuela, Simonet, Rios, & Valcarcel, 2005), atomic spectroscopic techniques (Cmelik, Machát, Niedobová, Otruba, & Kanický, 2005; Huang, Becker-Ross, Florek, Heitmann, & Okruss, 2005) and chromatographic techniques (Lim et al., 2014). In the case of beers, sulphur dioxide is determined by UV–Vis molecular absorption spectrophotometry after the reaction of sulphur dioxide with organic reagents which provides a high selectivity; the most commonly used is p-rosaniline-formaldehyde (method recommended by the American Society of Brewing). This method has been applied to wines but separating previously the sulphur dioxide by pervaporation and followed by flow injection analysis (Mataix & Luque de Castro, 1998).

In this work, a method is proposed to determine directly sulphur dioxide in wines based on the reaction with p-rosaniline-formaldehyde with a high selectivity and that allows the differentiation between free and bound sulphur dioxide.

These two selective methods for polyphenols and sulphur dioxide have been applied to 14 samples of wine (reds and whites). The antioxidant capacity by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method has also been determined. The results agree with the ones found in bibliography. With the help of statistic tools, the influence of polyphenols and sulphur dioxide on stability and wine quality was studied. High correlations between antioxidant capacity and polyphenol index have been found, it indicates the potential role of the Lac/SNGC biosensor for determining polyphenols responsible of the antioxidant capacity in red wines.

2. Experimental

2.1. Reagents

2.1.1. Electrochemical transducer preparation and biosensor fabrication

Acetic acid glacial 100% (Merck, Darmstadt, Germany); sodium acetate (Merck, Darmstadt, Germany); methyltrimethoxysilane (MTMOS) (Merck, Hohenbrunn, Germany); nafion-perfluorinated ion-exchange resin 5% (w/v) in a mixture of lower aliphatic alcohols and water was obtained from Aldrich (Steinheim, Germany); glutaraldehyde 25 wt.% solution water (Aldrich, Steinheim, Germany); gallic acid monohydrated (Sigma–Aldrich, Steinheim, Germany); Laccase from trametes versicolor (Fluka, Germany); graphite powder, natural, high purity 200 mesh (Alfa Aesar, Karlsruhe, Germany).

All reagents were of analytical grade and used as received without further purification. Nanopure water was obtained by passing twice-distilled water through a Milli-Q system (18 MΩ cm, Millipore, Bedford, MA). Glass capillary tubes, i.d. 1.15 mm, were used as the bodies of the composite electrodes.

2.1.2. Polyphenol assays

Gallic acid 98% (Sigma–Aldrich, Steinheim, Germany); quercetin (Sigma–Aldrich, Steinheim, Germany); rutin hydrate 94% (Sigma–Aldrich, Steinheim, Germany); tannic acid 90% (Fluka, Buchs SG, Switzerland); ferulic acid 98% (Fluka, Buchs SG, Switzerland); (+) catechin 96% (Sigma–Aldrich, Steinheim, Germany); (−)epicatechin 90% (Sigma–Aldrich, Steinheim, Germany); tyrosol 95% (Sigma–Aldrich, Steinheim, Germany); caffeic acid 95% (Fluka, Buchs SG, Switzerland); vanillic acid (Fluka, Buchs SG, Switzerland); syringic acid (Kodac Rochester, NY); p-coumaric acid 95% (Sigma–Aldrich,

Steinheim, Germany); 4-methyl catechol (Sigma–Aldrich, Steinheim, Germany); sodium hydroxide (Panreac, Barcelona, Spain); tartaric acid (Sigma–Aldrich, Steinheim, Germany); D-glucose monohydrate 99.5% (Fluka, Buchs SG, Switzerland); ethanol absolute (Merck, Darmstadt, Germany).

2.1.3. Sulphur dioxide determination

Formaldehyde 37–38% W/W (Panreac, Barcelona, Spain); mercury(II) chloride; p-rosaniline chloride (Merck, Darmstadt, Germany); hydrochloric acid 35% (Panreac, Barcelona, Spain); sodium chloride (Scharlau, Mas dÉulisa, Spain); sodium hydroxide (Panreac, Barcelona, Spain); sulphuric acid 96% (Merck, Darmstadt, Germany); sodium hydrogen sulphite solution 40% w/v (Panreac, Barcelona, Spain); starch from potato (Panreac, Barcelona, Spain), iodine (Panreac, Barcelona, Spain); potassium iodine (Panreac, Barcelona, Spain).

2.1.4. Antioxidant capacity assay

2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) (Sigma, Steinheim, Germany); potassium peroxydisulphate (Panreac, Barcelona, Spain).

2.2. Instrumentation

Spectrophotometric measurements were carried out with UV/Vis Spectrophotometer Jasco V-550 (Japan). Chronoamperometric measurements were performed with an Autolab PGSTAT20 (Ecochemie, Utrecht, The Netherlands) connected to a personal computer and 663 Metrohm VA stand module, using the GPES software for data acquisition and elaboration. A 600-W model, 20 kHz high power ultrasonic generator, SONICATOR 3000 from Misonix (Misonix Inc., Farmingdale, NY) equipped with a 13 mm titanium tip was used to synthesise the Sonogel-Carbon material.

2.3. Sampling

Wine samples were obtained from a local store. For previous studies "Viña Albali" and "Prada" red wines and "Antonio Barbadillo" white wine were used. For wine analysis and correlation study, nine red wines and five white wines were used. Red wines: Red 1: *Gibalbin*, Red 2: *Cariñena joven*, Red 3: *Cariñena Crianza*, Red 4: *Cariñena Reserva*, Red 5: *Dominio de la Fuente Joven*, Red 6: *Dominio de la Fuente Crianza*, Red 7: *Dominio de la Fuente Reserva*, Red 8: *Organic Piedra Luenga* and Red 9: *Organic Matalagrana*. White wines: White 1: *Verdejol*, White 2: *El Coto*, White 3: *Estero*, White 4: *Señorío de Ojailen* and White 5: *Organic Piedra Luenga*. They were selected taking into account their different geographical origin, grape variety, type of soil and different cultivation techniques. Inside the group of the red wines two series of the same trademark with different aged time in oak cask and bottle were selected. Each group includes a young (not aged) wine, an aged wine "*crianza*" (at least 24 months in oak cask) and an aged wine "*reserva*" (at least 36 months in oak cast).

2.4. Biosensor preparation for polyphenols determination

Electrochemical Sonogel-Carbon transducer was prepared as described previously (Cordero-Rando, Hidalgo-Hidalgo de Cisneros, Blanco, & Naranjo-Rodriguez, 2002). Before biological modification, the electrodes were electrochemically pre-treated by dipping them in 0.05 mol L^{-1} sulphuric acid and polarised by voltage cycling from −0.5 to +1.5 V for 5 cycles with a scan rate of 0.05 V s^{-1}; electrodes with similar current backgrounds were selected, washed carefully with Milli-Q water and left to dry at room temperature.

The Sonogel-Carbon biosensors based on Laccase were fabricated as follows (ElKaoutit et al., 2008): an adequate quantity of enzyme in order to obtain an acceptable numbers of units of enzyme per electrode was dissolved in 60 μL of acetate buffer, 0.2 mol L^{-1} pH 5. Afterwards, 2.5 μL of glutaraldehyde was added and the mixture immersed in ultrasonic bath for 3 min, and then modified by adding 7 μL of Nafion. From the resulting solution, adequate quantities were deposited on the top of the Sonogel-Carbon electrodes with a μ-syringe and allowed to dry under room conditions. Hence, the resulting biosensors have 23 units of Laccase, 0.9% of glutaraldehyde and 0.5% of Nafion per electrode. Before using, the enzymes electrodes were dipped in stirred buffer solution for 15 min, to eliminate the excess of enzymes adsorbed, rinsed with the same buffered solution and stored immersed in the buffer at 4 °C when they were not in use.

2.5. Polyphenol index determination

Analyses of wine samples were performed by the standard addition method. Fifty microliter of red wine diluted 1:5 with Milli-Q water or 100 μL of white wine without dilution, were added to a electrochemical cell that contains 25 mL of a buffer solution acetic acid/sodium acetate at pH = 4.5. The electrochemical measurements were carried out by chronoamperometry using Ag/AgCl/KCl (3 M) as reference electrode, a Pt electrode as auxiliary electrode and a Laccase–Sonogel-Carbon as working electrode. The possible mechanism of the biosensor for polyphenol index determination involves the oxidation of polyphenols to quinones and subsequent electrochemical reduction of the quinones on the working electrode. A constant potential of −150 mV and a stirring rate of 50 rpm was applied. An intensity decrease was observed at room temperature and the signal was recorded when it was stabilised. After that, 50 μL of gallic acid 200 mg L^{-1} were added. The intensity decrease was observed and the signal was recorded when it was stabilised; this last step was repeated three times.

2.6. Sulphur dioxide determination

2.6.1. Standard preparation

One millilitre of wine was transferred into each of a series of eight 100 mL volumetric flasks. The amount of $NaHSO_3$ water solution corresponding to obtain the standards set between 0 and 160 μg SO_2 was added to these flasks and made to volume with Milli-Q water. Twenty-five millilitres aliquots of each of these wine solutions were transferred to separate 50 mL volumetric flasks. Five millilitres of p-rosaniline and 5 mL of formaldehyde solution were added to each flask. The flask were made to volume with Milli-Q water and held in 25 °C water bath for 30 min. The colour developed was read with the spectrophotometer at 560 nm.

2.6.2. Sample preparation

In a 100 mL volumetric flask were pipetted 2 mL mercury stabilising solution (prepared dissolving 27.2 g $HgCl_2$, 11.7 NaCl and diluting to 1 L with Milli-Q water) and 5 mL of 0.05 M sulphuric acid solution were pipetted. One millilitre of wine was taken and transferred to this volumetric flask. Fifteen millilitres of 0.1 M sodium hydroxide were added. The solution was mixed by swirling and held for 15 s. Ten millilitres of 0.05 M sulphuric acid solution were added. The flask was made to volume with Milli-Q water and mixed thoroughly. A twenty-five millilitres aliquot of mixed solution was pipetted into 50 mL volumetric flask. Five millilitres of colour reagent were added to solution and swirled to mix. Five millilitres of formaldehyde solution were added and made to volume with Milli-Q water. The solution was mixed thoroughly and

held in 25 °C bath for 55 min in the case of red wines and 35 min for white wines. The absorbance was measured at 560 nm.

2.6.3. Blank preparation

In a 100 mL volumetric flask, one millilitre of wine was pipetted. Half millilitre of starch indicator was added. After that, concentrate iodine was added dropwise until the colour change was observed. The flask was made to volume with Milli-Q water. A twenty-five millilitres aliquot was pipetted into 50 mL volumetric flask. Five millilitre of colour reagent and 5 mL of formaldehyde were added and made to volume with Milli-Q water. The solution was mixed and held 55 min for red wines and 35 min for white wines. The absorbance was measured at 560 nm.

2.7. ABTS radical preparation for antioxidant capacity determination (Re et al., 1999)

ABTS was dissolved in water to a 7 mM concentration, ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting with 2.45 mM potassium peroxydisulphate and allowing the mixture to stand in the dark at room temperature for 16–20 h before use. Half millilitre was taken and diluted to 25 mL with Milli-Q water. Fifteen microlitres of the Milli-Q water were added to 2.5 mL of $ABTS^{\cdot+}$ and the absorbance was measured at 734 nm. After that, 15 μL of wine were added to 2.5 mL of $ABTS^{\cdot+}$ and the absorbance was measured at 734 nm. The decrease in absorbance was recorded.

3. Results and discussions

3.1. Polyphenol index for wines and characterisation of the response of the Laccase–Sonogel-Carbon biosensor

The authors have developed several amperometric sensors based on phenoloxidase-modified-Sonogel-Carbon materials for the determination of polyphenols in beer. The best results were obtained with the Lac/SNGC biosensor (ElKaoutit et al., 2008). The possibility of interferences concerning sulphur dioxide and ascorbic acid has been discarded due to some experiments carried out previously (Di Fusco, Tortolini, Deriu, & Mazzei, 2010; Martinez-Periñan et al., 2011).

Previous tests were performed with the Laccase electrode in red wine samples by means of chronoamperometry. No response was obtained with the same experimental conditions used for beers. A decrease in the magnetic stirring speed allowed us obtaining a signal. After that, it was observed that when biosensor is newly manufactured, the signal intensity was not reproducible for different aliquots of the same wine. This problem was solved conditioning the electrode performing measurement tests at −150 mV until the signal is reproducible before starting the analysis.

Several samples of red "Viña Albali" and white "Antonio Barbadillo" wines were analysed as described in Section 2. Aliquots of 50 μL of 200 mg L^{-1} gallic acid were added to a sample of red wine diluted and the chronoamperogram was registered (Fig. 1). The calibration graph by standard addition method was obtained (y (A) = $2.0{\cdot}10^{-11}$ × (A L mg^{-1}) + $1.1{\cdot}10^{-8}$ (A), R^2 = 0.997). By this way the polyphenols index was 833.6 mg L^{-1} expressed as gallic acid with a coefficient of variation of 3.2%. The method was also applied to white wines with good results.

The figures of merit calculated for 200 mg L^{-1} gallic acid solution were: sensitivity $3.65{\cdot}10^{-7}$ A L mg^{-1}; limit of detection 0.011 mg L^{-1} and reproducibility 2.6%. The reproducibility was determined using three Lac/SNGC biosensors with the same method applied to the samples by triplicate with different operators and using different equipment. The biosensor was stable for one week.

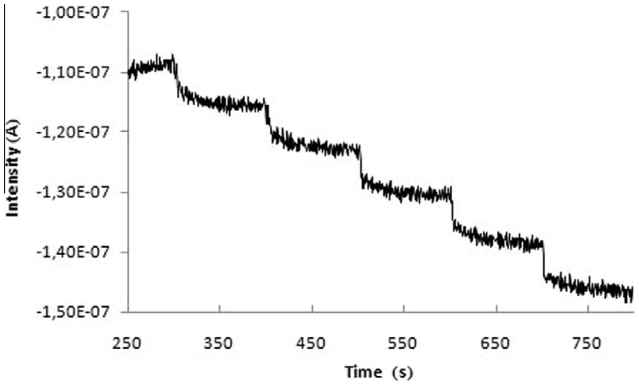

Fig. 1. Chronoamperogram for the polyphenol index determination in "Viña Albali" red wine with Lac/SNGC: 0.05 mol L^{-1} acetate buffer pH = 4.5, applied potential −150 mV. The first intensity decrease corresponds to the sample wine addition and the following intensity correspond to the additions 50 μL gallic acid 200 mg L^{-1}.

In order to characterise the response of the Lac/SNGC biosensor to polyphenols in wines, some experimental work has been carried out. As it is known, the "Laccase Trametes Versicolor" enzyme responds mainly to o-diphenols (Jolivalt et al., 1999), but its selectivity could be modified when it is immobilised on a biosensor.

For the selection of polyphenols to be tested, it was considered the polyphenols reported to be found in wines. An o-diphenol of each family and polyphenols found at relevant concentrations were chosen. Besides other polyphenols which were supposed not respond were also tested. 4-Methyl catechol was included as representative of the o-diphenols although it is not present in wine (Elias & Waterhouse, 2010). The polyphenols selected were: gallic acid; quercetin; rutin; tannic acid; ferulic acid; (+)catechin; (−)epicatechin; tyrosol; caffeic acid; vanillic acid; syringic acid; p-coumaric acid; 4-methyl catechol. They were added to model samples prepared with the main components of the wines: ethanol (12%), tartaric acid (2.5 g L^{-1}) and glucose (0.9 g L^{-1}). Sodium hydroxide was added to adjust the pH to 3.3.

First of all, it was tested that this matrix sample did not give any response. Two groups of experiments were carried out: the first assay with individual polyphenols at 100 mg L^{-1} concentrations and the second assay with polyphenols at typical concentrations found in wines (Flanzy, 2003).

3.1.1. Individual polyphenols

The biosensor responds with good sensitivity to most of the o-diphenols: gallic acid, caffeic acid, (+)catechin, (−)epicatechin and 4-methyl catechol. Other polyphenols that are not o-diphenols give also a signal, they are ferulic acid and vanillic acid (from the oak cask) it can be explained by its similar structure to o-diphenols. The sensitivities for these polyphenols are shown in Table 1. Other polyphenols do not give signal (quercetin, rutin, tannic acid, tyrosol, syringic acid and p-coumaric acid) mainly due to its big volume and to steric hindrance.

Table 1
Sensitivity of individual polyphenols.

Polyphenol	Sensitivity (A L mg^{-1})
Gallic acid	$4.7{\cdot}10^{-11}$
Caffeic acid	$1.0{\cdot}10^{-9}$
(+)Catechin	$1.6{\cdot}10^{-10}$
(−)Epicatechin	$8.2{\cdot}10^{-11}$
4-Methyl catechol	$1.2{\cdot}10^{-9}$
Ferulic acid	$1.9{\cdot}10^{-10}$
Vanillic acid	$1.5{\cdot}10^{-11}$

3.1.2. Collective polyphenols

In order to study interactions among the polyphenols which could modify the signal, two different tests with polyphenols added to model samples were carried out. The first sample was prepared only with the polyphenols which give a signal in the individual experiments, described above, at the concentrations found in wines. These polyphenols for red wine samples and their concentrations were: gallic acid (95 mg L^{-1}); ferulic acid (0.1 mg L^{-1}); (+)catechin (190 mg L^{-1}); (−)epicatechin (80 mg L^{-1}); caffeic acid (10 mg L^{-1}); vanillic acid (4 mg L^{-1}). This sample was analysed by triplicate as described in Section 2. The polyphenol index expressed as gallic acid was 338.3 mg L^{-1} and the coefficient of variation was 1.2%. Another model sample was prepared adding the polyphenols mentioned before and besides other polyphenols studied which gave no signal in the individual polyphenol study. These polyphenols were: gallic acid (95 mg L^{-1}); quercetin (10 mg L^{-1}); rutin (15 mg L^{-1}); ferulic acid (0.1 mg L^{-1}); (+)catechin (190 mg L^{-1}); (−)epicatechin (80 mg L^{-1}); tyrosol (25 mg L^{-1}); caffeic acid (10 mg L^{-1}); vanillic acid (4 mg L^{-1}); syringic acid (8 mg L^{-1}); p-coumaric acid (6 mg L^{-1}). The result obtained by triplicate for the polyphenol index expressed as gallic acid was 609.4 mg L^{-1} with a coefficient of variation of 1.6%. This increase in the signal compared with the signal obtained in the experimental described above can be due to synergic effect among the polyphenols or to the sum of small contribution to the signal from several not individually detectable polyphenols.

3.2. Spectrophotometric method for sulphur dioxide determination

The sulphur dioxide determination by UV–Vis molecular absorption spectrophotometry with p-rosaniline is based on the modified Schiff reaction to form a coloured compound. The colour intensity of the compound is directly proportional to the sulphur dioxide concentration of the sample. It presents an absorption band with a maximum around 548 nm which permits its spectrophotometric determination with a high selectivity.

This procedure is based on the standard addition method which uses aliquots of the sample with increasing sulphur dioxide additions, since other chemical compounds from the matrix may influence the absorbance values and therefore the slope of the calibration curve. An advantage of this method is the possibility to differentiate free sulphur dioxide from total sulphur dioxide.

The preparation of a blank, a wine sample without sulphur dioxide, is necessary since there are other components in the wine matrix able to absorb in the same zone. These absorbance values will be subtracted from the absorbance values corresponding to the sulphur dioxide/formaldehyde/p-rosaniline complex. To determine the total sulphur dioxide content, sodium hydroxide is added previously to the reagents responsible for the colour, with the aim to release the sulphur dioxide bound to carbonyl compounds, unsaturated compounds and amines. The free sulphur dioxide is determined using the first standard that does not contain additional sulphur dioxide.

This method is recommended for determining sulphur dioxide in beers. As it is known, the wine matrix is more complex than beer matrix; therefore its application to wines requires a study of the adequate experimental conditions for its direct determination.

In order to apply this sulphur dioxide determination method to wines, some modifications of the method were carried out. First of all, the adequate sample volume to prepare sample and standards was evaluated. It was estimated that one millilitre of sample would be adequate, so that it fits in the linear range of the calibration curve. After adding the reagents, as described in Section 2, a spectrophotometric scan was carried out. The spectrum showed a maximum at λ = 560 nm, a light shift from the 550 nm obtained from beers is observed. The absorbance maximum was read at 560 nm after 25 min as described for beers in order to allow the formation of the coloured complex. Nevertheless, this absorbance signal was not stable, since it was increasing with time. Therefore, a stability study was carried out. Every 5 min the signal was read, finding that it was slowly increasing. Only after 55 min for red wines and 35 min for white wines, the absorbance signal was stabilised. It can be explained by the more concentrated and complex matrix, in the case of red wines, that influences the kinetic of the sulphur dioxide/formaldehyde/p-rosaniline reaction.

Several samples of red and white wines were prepared as described in Section 2. After that, the absorbances were measured and the results for the red wine "Viña Albali" are shown in Fig. 2 where two calibration curves are shown. The one with lower origin ordinate is obtained after subtracting the blank value and it is used to calculate free and total sulphur dioxide in the wines. As can be observed, the slope is adequate to differentiate the sulphur dioxide concentration of the wines; therefore, the method shows a good sensitivity and linearity. The sample was analysed in triplicate. The result was 65.5 mg L^{-1} of total sulphur dioxide with a coefficient of variation of 3.2% and 20.5 mg L^{-1} of free SO_2. The mean value is inside the range of the results published in the bibliography for red wines and is below the maximum values allowed by the regulation.

Next, the method was applied to the white wine "*Antonio Barbadillo*". Standards and sample were prepared as described in Section 2. The calibration curve results are $y = 0.291x + 0.186$ and $R^2 = 0.999$. The mean value for the triplicates was 75.9 mg L^{-1} of total sulphur dioxide and 1.53% for the coefficient of variation. The result for free sulphur dioxide was 24.7 mg L^{-1}. Also in this case of white wines, the results are in accordance with the published in the bibliography. Therefore, the methodology is adequate for white wines.

Obviously, the results obtained with this method are lower than those obtained when the Ripper method is used (Mataix & Luque de Castro, 1998), since it shows positive interferences mainly due to ascorbic acid and other reductants. It is quite clear the necessity of the elaboration of a reference material with certified values in sulphur dioxide concentration. This method is clearly adequate to assess the value in reference materials.

3.3. Antioxidant capacity using ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid))

A number of assays are available for the measurements of the total antioxidant activity which is the cumulative capacity to scavenge free radicals in food. The methodology more adequate to

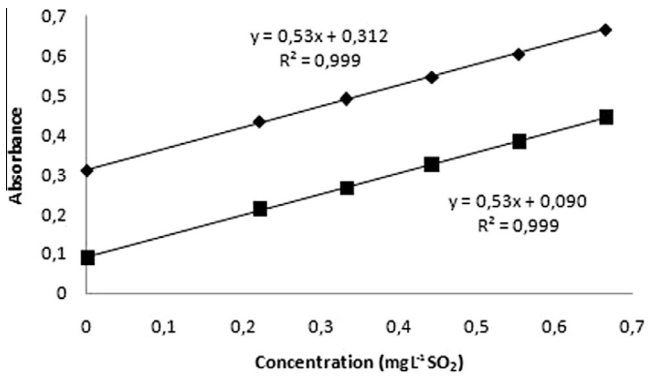

Fig. 2. Calibration curves for the SO_2 determination in "Viña Albali" red wine. (♦) Obtained by increasing SO_2 additions. (■) After subtracting the blank value.

detect OH radicals is the electron spin resonance, but it is an expensive instrumental technique and requires experience. Some methodologies have been proposed based on simulations adding exogenous free radicals to samples by measuring the capacity of sample to capture these free radicals. These exogenous free radicals show absorbance by UV–Vis molecular absorption spectrophotometry and the decrease experimented by adding the sample is measured. They have been used for wines for several authors (Cimino, Sulfaro, Trombetta, Saija, & Tomaino, 2007; Fernández-Pachón, Villaño, García-Parrilla, & Troncoso, 2004; Gomez-Gallego, Gómez, Sánchez-Palomo, González Viñas, & Hermosín-Gutiérrez, 2012; Paixão, Perestrelo, Marques, & Câmara, 2007). The most widely used is the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). It has been presented as an excellent tool for determining the antioxidant capacity of plant material and beverages (Arnao, Cano, Hernandez-Ruiz, Garcia-Canovas, & Acosta, 1996; Cano, Hernandez-Ruiz, Garcia-Canovas, Acosta, & Arnao, 1998; Cano-Lario, Acosta-Echevarria, & Bañon-Arnao, 1998).

There are two ways to obtain the ABTS radical: chemical oxidation with $K_2S_2O_8$ and enzymatic oxidation with H_2O_2 and peroxidase. This organic compound shows a spectrum which permits to measure at λ_{max} = 414 nm and λ_{max} = 734 nm. We have obtained the free radical using $K_2S_2O_8$ due to the fact that the radical stability is higher (Martinez-Periñan et al., 2011). This reagent is used at a lower concentration (as necessary to oxidise the ABTS), in order to avoid an excess that would oxidise the wine samples. Therefore, after oxidation some ABTS (λ_{max} = 312 nm) has remained in the medium. The tail of this maximum is slightly overlapped with the ABTS radical maximum (λ_{max} = 414 nm) as can be observed in Fig. 3. For this reason is better to read the decrease in absorbance at 734 nm to avoid this interference.

Several types of wines have been tested and in all cases, important absorbance differences are observed (ΔAbs). These differences vary depending on the wine nature. The sulphur dioxide and polyphenolics content can be responsible for this ΔAbs. In the case of beer it was attributed to sulphur dioxide concentration rather than to polyphenols (Hernández-Artiga & Bellido-Milla, 2009).

These ΔAbs are increasing with time, it can be due to the different kinetic of the compounds implied in the reaction with the ABTS radical. After 20 min the ΔAbs is stabilised and these values are recorded at 734 nm.

Some authors transform this data into gallic acid equivalents, but as the aim of this work is to study the sulphur dioxide and polyphenols concentration influence on this property, this transformation is not needed (Martinez-Periñan et al., 2011; Oñate-Jaén, Bellido-Milla, & Hernández-Artiga, 2006).

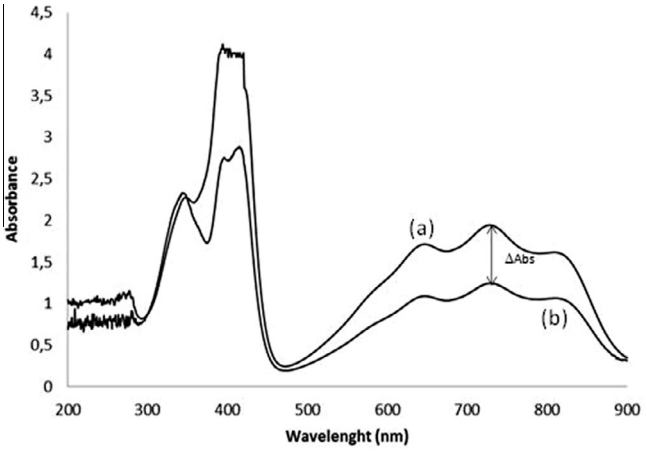

Fig. 3. Absorbance spectrum of ABTS radical. (a) With addition of 15 μL of Milli-Q water; (b) with addition of 15 μL of "Prada" red wine.

Table 2
Analysis results for red and white wines.

Wines	Polyphenol index (I_{BP}) as gallic acid (mg L^{-1})	Antioxidant capacity (ΔAbs)	Total SO_2 (mg L^{-1})	Free SO_2 (mg L^{-1})	I_{BP}/ SO_2 total
Red 1	945	1.529	32.8	6.9	28.8
Red 2	834	1.290	48.7	10.3	17.1
Red 3	1059	1.777	81.8	9.6	12.9
Red 4	1139	1.943	80.6	19.9	14.1
Red 5	1006	1.665	30.5	24.4	32.9
Red 6	1238	1.894	74.6	16.2	16.6
Red 7	1392	1.961	51.5	14.6	27.0
Red 8	726	1.590	61.6	13.7	11.8
Red 9	1225	2.084	67.6	3.9	18.1
White 1	129	0.222	128.4	28.8	1.0
White 2	124	0.201	89.1	27.5	1.4
White 3	172	0.086	49.9	4.5	3.4
White 4	152	0.196	49.7	5.1	3.1
White 5	99	0.064	78.3	7.7	1.3

3.4. Wine analysis

After considering the appropriate methods to determine a polyphenol index, sulphur dioxide and the antioxidant capacity with selectivity and precision, a group of red and white wines were analysed. The data obtained, by this way, can lead to conclusions nearer to the reality, than in the case of using methods with poor figures of merits (with interferences, instability and/or high coefficients of variations). The methods recommended and accepted by international bodies can be useful for the industry but are not always adequate for research studies.

A group of 14 wines samples available to consumers were purchased, in order to analyse the parameters mentioned and study its correlations. The results are shown in Table 2.

The data for sulphur dioxide, polyphenols and ABTS assay were obtained in triplicate and the variation coefficients are below 5%. The total sulphur dioxide concentrations are always higher than free sulphur dioxide, as expected. In all cases the total sulphur dioxide concentration is below the values permitted by the regulation. All the bottles were labelled "contain sulphites" which regulations oblige must be shown when the concentration is higher than 10 mg L^{-1} sulphur dioxide (Spricigo et al., 2009). In all cases the concentration is higher than this value. On the other hand, the maximum contents permitted for red wines are 150 and 200 mg L^{-1} for white wines. In the case of organic wines these values are lower, for red wines 100 and 150 mg L^{-1} for white wines (European Commission, 2012). In all cases, the results obtained are below these maximum values.

The mean values of total sulphur dioxide obtained for the red wines group is lower than the obtained for white wines. The biosensor polyphenol index (I_{BP}) is always higher for red wines than for white wines, as expected. The antioxidant capacity is also higher for red wines than for white wines.

The two groups of three wines of the same trade mark, but with increasing ageing in oak casks, show an increasing in the polyphenol index and in the antioxidant capacity. It can be due to the polyphenols extracted or modified from the oak cask.

A correlation study among the parameters shown in Table 2 has been performed with the aim to obtain more information enclosed into the data than for a simple inspection. A p-value = 0.05 was established as critical.

The correlation between antioxidant capacity (ΔAbs) and polyphenol index (I_{BP}) for all the analysed wines is 0.98; other

authors have arrived to similar conclusions Cimino et al., 2007; Fernández-Pachón et al., 2004). Another author (Gomez-Gallego et al., 2012) highlighted a lack of such a relationship for red wine (the total phenolic content are estimated by measuring absorbance at 280 nm) and suggested that the antioxidant capacity of wines is more related to the kind of polyphenolics compounds present in the wine than to their global content. It suggests that complex, not yet well understood, interactions between the different classes of phenolic compounds could be responsible for actual antioxidant capacity shown by wines.

In our opinion, the high positive correlation obtained by us for all the wines analysed are also due to the method used to determine polyphenols and the antioxidant capacity. It can be interpreted also that the electrochemical biosensor used in this work is able to detect the polyphenols of red wines group with higher antioxidant capacity.

Other correlations between the parameters analysed shown in Table 2 were tested but without success. The antioxidant capacity is not well correlated with the total sulphur dioxide in the case of all the wines analysed. Only in the case of the white wines the correlation with free sulphur dioxide shows an acceptable value (0.68). The correlation between free sulphur dioxide and total sulphur dioxide was not good at all the wines. But in the case of white wines this correlation is 0.88. These results for white wines can be due to its lower content in polyphenols and higher total sulphur dioxide concentration.

No correlation has been found between polyphenols index and free or total sulphur dioxide.

In relation with the consumer health, the polyphenols can be considered as beneficial and the sulphur dioxide can involve some health risk. Therefore, the relationship of both (I_{BP}/SO_2 total) could be an interesting parameter to be considered. As can be observed in Table 2, the red wines appear grouped with the higher values and the white wines with the lower values. It can be concluded that consumption of wines with moderation is healthier in the case of red wines due to its higher polyphenol content and lower SO_2 concentration.

4. Conclusions

The method used to determine polyphenols and sulphur dioxide in wines recommended by international bodies and used in research studies so far are not selective, in spite of the fact, that analytical methods which provide better analytical results are described in the scientific literature. This can be extended to food analysis in general and to other fields where chemical compounds are analysed.

A Laccase–Sonogel-Carbon biosensor designed by the authors and applied previously to beers, has proven to be an important tool to determine the polyphenol content responsible for the antioxidant capacity and sensory quality of red wines. It is also able to detect ageing in oak cask. A spectrophotometric selective method recommended for beers based on the reaction of sulphur dioxide with p-rosaniline to determine directly free and bound sulphur dioxide has been modified for wines.

The high correlation obtained between antioxidant capacity and polyphenol index show the important role of the polyphenol detected on the stability and quality of wines.

Since the recommended methods present positive interferences and cannot be used to compare the results with the new proposed methods, for this reason reference materials for wines certified in sulphur dioxide and polyphenols should be developed.

The ratio between the polyphenol index and total sulphur dioxide concentration described in this work is proposed as a useful tool for researchers studying the effect of food quality in the health.

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