Food Research International 55 (2014) 150–160

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Oak extract application to grapevines as a plant biostimulant to increase wine polyphenols

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ARTICLE INFO

Article history:
Received 2 July 2013
Accepted 4 November 2013
Available online 9 November 2013

Keywords:
Oak extract
Biostimulant
Polyphenols
Wine
Grapevines

ABSTRACT

Oak extract is a mixture of compounds including phenolics (volatiles and non volatiles), which could act as plant biostimulant if they are able to modulate plant physiological response. It is known that it can modify grape volatile composition after the application over grapevines, impacting on wine aroma, but no studies have been carried out on phenolic composition. So, the aim of this work was to evaluate the phenolic composition of wines elaborated from Monastrell grapevines treated with a commercial oak extract in order to study its biostimulant activity. Several families of polyphenols were studied, including phenolic acids, stilbenes, flavanols, flavonols and anthocyanins, which were analyzed by HPLC-DAD-MS. Results showed oak extract could be considered like an important biostimulant of grape polyphenols, since it affected grape composition, producing less alcoholic and acid wines with higher colour intensity, lower shade and so a more stable colour and higher content of polyphenols such as gallic acid, hydroxycynnamoyltartaric acids, acylated anthocyanins, flavanols and stilbenes.

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1. Introduction

Polyphenols are plant secondary metabolites, studied by many researches as they are implicated in plant resistance, due to their fundamental function in food process and its nutraceutical characteristics (Cheynier, Sarni-Manchado, & Quideau, 2012). In wines, they are responsible of colour, astringency, bitterness, body and aroma (Santos-Buelga, Escribano-Bailón, & Lattanzio, 2010). Among the healthy benefits attributed to wine, when consumed in moderation, it is important to mention the contribution of polyphenols due to their antioxidant properties related to cardio protector effect (Pezzuto, 2008). These facts justify that several approaches have been proposed to increase the phenolic content of wine grapes.

Besides genetic transformation, forbidden in many countries, there are many factors that can induce or increase phenolic content of grapes, including applications of plant products (Parrado et al., 2007) or commercial products whose active components are also found naturally in plants (Tassoni, Durante, & Ferri, 2012). Recently, plant biostimulants have been defined as substances that are able to alter the plant physiological processes providing benefits for growth, development, response to abiotic stress and improve quality. Among the biostimulant categories, it is included as the one formed by a combination of molecules, which have effect on plant physiological response (Du Jardin, 2012).

Oak is commonly used in winemaking to produce wood tanks, especially barrels, where wines are subjected to the aging process (Pontallier, Salagoïty-August, & Ribéreau-Gayon, 1982). Among its most important components are polyphenols and volatile compounds, which are extracted slowly into the wine, and influence their sensory characteristics such as astringency, body, bitterness, colour and aroma. Some wood components are formed or increased during the process of manufacture of the barrel, particularly in the stages where staves are subject to heat treatment (Fernández de Simón, Sanz, Cadahía, Poveda, & Broto, 2006). In that manufacturing process, important residues are generated, which can be added into the wines in the form of chips, pellets and fragments of different sizes giving new alternatives to aging winemaking. Such applications are well regulated by the CEE 1507/2007 regulation. The remaining oak wastes are often burnt but can be used for obtaining oak extracts of different characteristics

Abbreviations: % AEI, anthocyanin extractability index; % SMI, seed maturity index; D3G, delphinidin 3-O-glucoside; C3G, cyanidin 3-O-glucoside; Pt3G, petunidin 3-O-glucoside; Pe3G, peonidin 3-O-glucoside; M3G, malvidin 3-O-glucoside; ΣG, total glucoside antocyanins; Pe3GAc, peonidin 3-O-(6-acetyl)-glucoside; M3GAc, malvidin 3-O-(6-acetyl)-glucoside; ΣGAc, total acetylated antocyanins; C3GCo, cyanidin 3-(6-p-coumaroyl)-glucoside; Pe3GCo, peonidin 3-(6-p-coumaroyl)-glucoside; M3GCo, malvidin 3-(6-p-coumaroyl)-glucoside; ΣGCo, total coumaroyl antocyanins; M3GCa, malvidin 3-(6-t-caffeoyl)-glucoside; ΣA, total antocyanins; VA, vitisin A malvidin 3-O-glucoside; VB, vitisin B malvidin 3-O-glucoside; ΣV, total vitisins. My3Galact, myricetin 3-O-galactoside; My3Glucur + Glucos, myricetin 3-O-glucuronide + myricetin 3-O-glucoside; ΣMyG, total myricetin glicosides; Qu3Galact, quercetin quercetin 3-O-glucoside; Qu3Glucur + Glucos, quercetin 3-O-glucuronide + quercetin 3-O-glucoside; ΣQuG, total quercetin glicosides; La3Glucs/Galact, laricitrin 3-O-glucoside/galactoside; Sy3Glucos, syringetin 3-O-glucoside.

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http://dx.doi.org/10.1016/j.foodres.2013.11.004

which may be an important application for the sustainability of the oak industry.

The most recent works related to the use of aqueous oak extracts showed how their grapevine foliar applications modulated the aroma of the wines produced from these grapes (Martínez-Gil, Garde-Cerdán, Martínez, Alonso, & Salinas, 2011). The main chemical constituents of the aqueous oak extracts are low molecular weight polyphenols, being ellagitannins the most abundant compounds. Recent works (Fernandez & Eichert, 2009) have shown that low molecular weight is needed for absorption of compounds by leaves, either by cuticular pore or by stomata. Due to the high molecular weight of ellagitannins, they could not be absorbed by leaves. The other constituents of the aqueous oak extract are a mixture of a large number of substances which can be assimilated by plants, among which are several compounds derived from shikimic acid pathway, such as the phenylpropanoid eugenol, which is known for its antifungal role and its effect on wine polyphenols (Pardo-García, Martínez-Gil, López-Córcoles, Zalacain, & Salinas, 2013).

It has been reported that grapes can assimilate as glycoside precursors some of the volatile compounds from oak extract foliar applications to grapevines (Martínez-Gil et al., 2013) (Martínez-Gil et al., 2013). Also, wines produced with berries from treated vines had a high content of volatiles present in the applied extract; these volatiles were liberated from their precursors during the winemaking (Martínez-Gil, Garde-Cerdán, Zalacain, Pardo-García, & Salinas, 2012; Martínez-Gil et al., 2011). These treatments impacted on wine aroma at sensory level, giving woody notes as if the wine was aged in oak but, that process did not take place.

As previous works have shown how the aqueous oak extract affects grape and wine aroma, the aim of this work is to expand the knowledge about the oak extracts and their impact on wine polyphenol content when they are applied over grapevines. If the known properties of the oak extracts are expanded with this study, it could be considered as biostimulant with oenological interest.

2. Materials and methods

2.1. Chemicals and standards

The standards employed to identify and quantify phenolic compounds were: (+)-catechin, caffeic acid, *p*-coumaric acid, (−)-epicatechin, ferulic acid, gallic acid, kaempferol, quercetin dihydrate, *trans*-resveratrol, syringic acid and vanillic acid purchase from Sigma-Aldrich (Steinheim, Germany), while malvidin 3-O-glucoside (Mv-3-G), quercetin-3-O-glucoside (Q-3-G) and kaempferol 3-O-glucoside (K-3-G) were obtained from Extrasynthése (Genay, France). HPLC-grade acetonitrile and formic acid were purchased from Panreac (Barcelona, Spain). Water was purified through a Milli-Q system from Millipore (Bedfords, MA, USA). For quantification, different solutions of phenolic standards in ethanol–water (12% v/v, pH = 3.6, 5 g/L tartaric acid) were prepared using concentration ranges for each compound commonly found in red wines.

2.2. Material

2.2.1. Oak extract

The commercial aqueous French oak extract chosen for this study was provided by Protea France S.A.S. (Gensac la Pallue, France) (103 C). This extract was obtained by macerating in water at high temperature French oak chips (*Quercus sessiliflora Salisb*) from natural seasoning toasted at a medium intensity level. The commercial specifications were: pH: 2.8; soluble solids: 1.6°Baumé; total phenols index: 280; % weight: 4.3. This extract is used as food additive in spirits and fruit juices to give its characteristic oak aroma because it is rich in volatile compounds.

2.2.2. Grapevines

Red wine grapes from *Vitis vinifera* variety Monastrell grown during the year 2010 in the experimental field of BSI winery (Jumilla, Southwest of Spain) were used. 8-year-old grapevines were cultivated on a high cordon style trellis system (2.8 × 1.5 m) and the vineyard was equipped with a drip irrigation system to assure the plant water needs. The annual average temperature was 16 °C, with a minimum of −15 °C (January) and a maximum of 40 °C (August).

2.3. Technological methods

2.3.1. Grapevine treatments

Different oak extract treatments were applied to the grapevines during veraison. For all treatments, 0.05% (v/v) of adjuvant Fluvius (BASF, Germany) was added, since this is a superficial wetting agent typically used for treatments with foliar herbicide, constituted by an inert mixture of polymers. The vineyards were treated with different concentrations of the extract. First of all, this extract was diluted with water to four parts. This diluted extract was applied once on the 7 days post-veraison (25%-1 treatment) and also four times, 7, 11, 15, and 18 days post-veraison (25%-4 treatments). Also the undiluted extract was applied once on the 7th day post-veraison (100%-1 treatment) as well. Each treatment was carried out on 16 plants in the same row, leaving other row with untreated plants between the different applications to avoid contamination. 300 mL of each formulation was applied evenly per plant by spraying over leaves. Moreover, an additional row was not treated for control. The treatments were carried out when the environmental temperature was below 20 °C, at approximately 8 a.m. Grapes were harvested on September the 9th at their optimum maturation moment with the °Baumé/titratable acidity ratio around 2.5. Before winemaking some berries were randomly separated in order to measure the oenological parameters.

2.3.2. Winemaking

Grapes of each treatment were destemed, mixed and processed by the winemaking traditional method. Berries were divided and placed into two 30 L stainless steel tanks (2 wines from each treatment were obtained). 80 mg/L of sulphur dioxide were added to each tank. After that, 20 g/hL of yeast UCLM S377 strain (Springer Oenologie, Maisons-Alfort cedex, France) was inoculated to carry out the alcoholic fermentation, which took place at a temperature of 22 ± 1 °C. After 11 days of maceration–fermentation skins and seeds were removed with a traditional vertical hand-press. Then, each wine was put back to its tank to complete the alcoholic fermentation. Malolactic fermentation was induced using 8 g/hL of a commercial bacterium strain, Lall II-4 (Lallemand, Barcelona, Spain). The malolactic fermentation was carried out in 5 L tanks. Once this step finished, the wines were bottled and stored for six months. Three wine sampling times were performed: after alcoholic fermentation (AF), after malolactic fermentation (MLF) and after six months (6 months). After sensory analysis and measuring the chromatic parameters, wine samples were frozen at −20 °C until analysis.

2.4. Analytical methods

2.4.1. Grape and wines oenological parameters

Grape analysis involved the traditional measurements (°Beaumé, pH and titratable acidity) which were determined according the methodology described in ECC (1990). The classical parameters of wines such as alcoholic degree (°A), pH, titratable acidity (TA) and volatile acidity (VA) were analysed by an equipment based in infrared spectroscopy (FT-IR Multispec of TDI) using the methods (ECC, 1990) as reference. The phenolic potential of grapes (anthocyanin extractability index and seed ripeness index) was calculated according to the method described by Saint-Cricq de Gaulejac, Vivas, and Glories (1998), macerating the grapes for 4 h at two pH values (3.6 and 1.0). The pH 3.6 instead of pH

3.2 was used because it is better suited for Jumilla musts. The anthocyanin content of both solutions as well the total anthocyanin content of wines was assayed by measuring the absorbance of the samples at 520 nm (Puissant & Léon, 1967). The total phenol index (TPI) of grapes and wines was calculated by measuring the optical density at 280 nm of the grape solution at pH 3.6 and directly of the wines, following the method described by Ribéreau-Gayon, Peynaud, Sudraud, and Ribéreau-Gayon (1982). The phenolic potential of grapes was calculated as follows: anthocyanin extractability index (%AEI) = [(ApH1 − ApH 3.6)] × 100 / ApH1; seed maturity index (% SMI) = TPI (pH3.6) − ApH3.6 × 40/1000)] × 100 / TPI (pH 3.6). Wine chromatic parameters were obtained by measuring absorbance at 420, 520, and 620 nm (Glories, 1984): colour intensity (CI) = 420 + 520 + 620 and shade (S) = Abs 420/Abs 520 ratio. Spectrophotometric determinations were made by Lambda 25 UV–vis equipment (Perkin Elmer, Norwalk, Connecticut) with 1 cm (to TPI and anthocyanins analysis) and 0.1 cm (to chromatic parameters, CI) path length glass cells. In all cases, samples were first filtered through a PVDF Durapore filter of 0.45 μm (Millipore, Bedford, Massachusetts). All analyses were made in duplicate.

2.4.2. Determination of phenolic compounds by HPLC

The phenolic characterisation of the oak extract was made in the Spanish Forestry Research Centre (INIA-CIFOR). Oak extract (40 mL) was extracted with diethyl ether and ethyl acetate, and then the aqueous solution remained was freeze-dried. The organic fractions, dried and redissolved in methanol/water (1:1), were used for the HPLC-DAD quantitative determination of low molecular weight phenolic compounds (Cadahía, Muñoz, De Simón, & García-Vallejo, 2001), and an aliquot part of freeze-dried fraction, redissolved in water, was used for HPLC-DAD valuation of ellagitannins (Cadahía, Varea, Muñoz, Fernández De Simón, & García-Vallejo, 2001).

The chromatographic conditions to analyze the wines were adapted from Cozzolino et al. (2004). The samples were filtered through a PVDF Durapore filter of 0.45 μm (Millipore, Bedford, MA, USA) and 20 μL were injected into an Agilent 1200 HPLC chromatograph (Palo Alto, California, USA) equipped with a Diode Array Detector (Agilent G1315D) and a Mass Spectrometer (6130 Quadrupole LC/MS, G1956B SL) multimode electrospray and atmospheric pressure chemical ionisation (MM-ESI/APCI-MS) system, coupled to an Agilent Chem Station (version B.03.01) data-processing station. Separation was performed on a reversed-phase Zorbax-Eclipse XDB-C18 (4.6 mm × 150 mm. 5 μm particle sizes) and a precolumn of the same material at 30 °C. The HPLC grade solvents used were water/formic acid/acetonitrile (97.5:1.5:1 v/v/v) as solvent A and acetonitrile/formic acid/solvent A (78.5:1.5:20 v/v/v) as solvent B. The elution gradient for solvent B was as follows: 0 min, 5%; 2 min, 10%; 7 min, 14.5%; 10 min, 18.5%; 12 min, 20%; 17 min, 20%; 28 min, 30%; 30 min, 30%; 32 min, 50.5%; 38 min, 80%; 40 min, 100%. The system was equilibrated with the starting conditions during 10 min prior to injection of the next sample. The flow rate was 0.5 mL min^{-1}. Phenolic acid, stilbene and flavanol detection was carried out with the DAD detector by comparison with the corresponding UV–vis spectra and retention time of their pure standards in the chromatogram. Standard of piceid-*t*-resveratrol was not available, so it was tentatively identified by spectral parameters (Jeandet, 1997). Acids *t*-caftaric and *t*-coutaric either were not available, so they were identified with the molecular ion and the spectral parameters, which were very close to their analogous caffeic and coumaric acids (Peña-Neira, Hernández, García-Vallejo, Estrella, & Suarez, 2000). Compounds were quantified and identified at different wavelength: (+)-catechin, (−)-epicatechin, gallic and syringic acids at 280 nm; vanillic acid at 256 nm; *t*-caffeic acid and caftaric acid at 324 nm, while *t*-resveratrol, piceid-*t*-resveratrol, *p*-coumaric acid and *t*-*p*-coutaric acid were quantified at 308 nm. Flavonols and anthocyanins, which standard were not available, were identified on the basis of their molecular ion (shown in Table 1), together with the ionisation mode by electrospray ionisation mass spectrometry (MM-ESI-MS) and of bibliography, according to UV–vis spectra and elution order (Castillo-Muñoz, Gómez-Alonso, et al., 2009; Castillo-Muñoz, González, Alonso, Romero, & Gutiérrez, 2009). The parameters employed for MM-ESI-MS were: dry gas, N_2, 10 mL/min; drying temperature, 350 °C; vaporiser temperature, 200 °C; nebuliser, 55 psi; capillary, 2000 V (positive and negative ionisation mode); scan range of 100–700 m/z. Negative mode was used to identify flavonols, while positive mode was used to identify anthocyanins. Quantifications were done using the spectra obtained by DAD; flavonols at 360 nm and anthocyanins at 520 nm. For quantification, calibration curves were obtained with solutions of the available standards in ethanol–water (12% v/v. pH = 3.6. 5 g/L tartaric acid). Piceid-*t*-resveratrol was expressed as *t*-resveratrol equivalents, *t*-caftaric acid as *t*-caffeic acid, *t*-*p*-coutaric acid as *p*-coumaric acid, the flavonol glycosides were quantified as quercetin 3-O-glucoside equivalents, myricetin as quercetin equivalent and the anthocyanins as malvidin 3-O-glucoside equivalents. All analysis were made in duplicate, so n = 4 for each wine.

Table 1
Retention times, ionization mode, molecular weight and molecular ion (m/z) used for identification of hydroxycinnamic ester acids, flavonols and anthocyanins by MM-ESI-MS.

Compound assignation	HPLC. Rt(min)	ESI/MS mode	Molecular weight	Molecular ion (m/z)
Hydroxycinnamic ester acids				
t-caftaric acid	11.34	Negative	312	311
t-coutaric acid	14.65	Negative	296	295
Flavonols				
Myricetin 3-O-galactoside	18.20	Negative	480	479
Myricetin 3-O-glucuronide [a]	18.47	Negative	494	493
Myricetin 3-O-glucoside [a]	18.58	Negative	480	479
Quercetin 3-O-galactoside	23.15	Negative	464	463
Quercetin 3-O-glucuronide[b]	24.05	Negative	478	477
Quercetin 3-O-glucoside[b]	24.11	Negative	464	463
Laricitrin 3-O-glucoside/galactoside[c]	24.57	Negative	494	493
Kaempferol 3-O-glucoside	28.71	Positive	447	447
Syringetin 3-O-glucoside	29.57	Negative	508	507
Myricetin	31.63	Negative	318	317
Quercetine	36.79	Negative	302	301
Kaempferol	38.71	Positive	286	285
Anthocyanins				
Delphinidin 3-O-glucoside	11.79	Positive	464	465
Cyanidin 3-O-glucoside	13.64	Positive	448	449
Petunidin 3-O-glucoside	14.40	Positive	478	479
Peonidin 3-O-glucoside	16.10	Positive	462	463
Malvidin 3-O-glucoside	16.60	Positive	492	493
Petunidin 3-O-(6′-O-acetyl)-glucoside	22.42	Positive	520	521
Peonidin 3-O-(6′-acetyl)-glucoside	26.63	Positive	504	505
Malvidin 3-O-(6′-acetyl)-glucoside	27.20	Positive	533	534
Malvidin 3-(6′-*t*-caffeoyl)-glucoside	29.30	Positive	654	655
Cyanidin 3-(6′-*p*-coumaroyl)-glucoside	29.85	Positive	594	595
Petunidin 3-(6′-*p*-coumaroyl)-glucoside	30.35	Positive	624	625
Malvidin 3-(6′-*p*-coumaroyl)-glucoside	33.05	Positive	638	639
Vitisin A Malvidin 3-O-glucoside	18.02	Positive	560	561
Vitisin B Malvidin 3-O-glucoside	19.53	Positive	516	517

[a] Compounds coeluted.
[b] Compounds coeluted.
[c] It can be glucoside or galactoside of laricitrin.

2.4.3. Determination of volatile compounds by GC

The oak extract volatile composition was analyzed according to Marín, Zalacain, De Miguel, Alonso, and Salinas (2005) to wines, by stir bar sorptive extraction and gas chromatography-mass spectrometry (SBSE-GC–MS), but adapting this method to the analysis of such extracts. The polydimethylsiloxane coated stir bar (0.5 × 10 mm, Twister, Gerstel, Mülheim and der Ruhr, Germany) was introduced into 25 mL of sample (oak extract diluted to 25% in water), using γ-hexalactone and 3-methyl-1-pentanol as IS. Thermal desorption was performed on a TD with a PTV injector "Programmed-Temperature Vaporization" CIS-4 Gerstel installed on an Agilent 7890A GC-5975C insert XL MDS

(Agilent Technologies, Palo Alto, CA, USA). The compounds were transferred into the gas chromatograph with a fused silica capillary column (BP21 stationary phase, 50 m length, 0.22 mm i.d., and 0.25 μm film thickness; SGE, Ringwood, Australia). The identification and quantification were carried out in the SCAN mode. The identity of peaks was assigned using the NIST library and confirmed by the retention time of standards. The quantification was based on five-point calibration curves of respective standards (Aldrich, Steinheim, Germany).

2.5. Sensory analysis

The most important descriptors associated to the phenolic content and colour of wines were evaluated: astringency, bitterness, body, colour intensity, as well as blue, red and yellow tones. The wine sensory analysis was made by a panel of eight expert judges between 30 and 55 years old in different sampling times: at the end of the alcoholic and malolactic fermentations and after six months from this. The panelists rated each attribute on a scale from 1 (absence) to 7 (maximum presence).

2.6. Statistical analysis

The statistical treatment of the data was performed using SPSS Version 19.0 statistical package for Windows (SPSS, Chicago, IL). Phenolic compound data were processed using variance analysis (ANOVA). Differences between means were compared using the least significant difference (LSD) test at a 95% probability level. Statgraphics Plus 5.1 (STSC Inc., Rockville, MD, USA) was used to perform the simple regression and principal component analyses.

3. Results and discussion

3.1. Oak extract

Polyphenolic and volatile composition of the oak extract used is show in Table 2. As expected, the oak wood aqueous extract had many phenolic and volatile compounds that occur naturally in oak wood or/and that are intensified during wood toasting process, since it has been obtained from toasted chips. The extract was rich in ellagitanins, the most abundant extractable compounds in oak wood that easily pass to the extract solution due to their high hydrosolubility, showing a concentration (Σ ellagitannins) higher than 900 mg/L. This is because, although the wood toasting produced a considerable decrease of the ellagitannin levels (Cadahía et al., 2001; Chatonnet, Boidron, & Pons, 1989), the extract has been obtained from French oak wood, which has similar concentrations than Spanish oak, but is richer than American oak in these compounds. The macerating conditions of toasted chips applied to obtain the extract, in water at high temperature, possibly have a great influence on the final contents of ellagitannins. The high content of ellagic acid observed (122 ± 3 mg/L), probably derived from the degradation of ellagitanins during wood toasting, as well as the macerating of chips in hot water.

Many lignin derivatives, as phenolic aldehydes and acids, phenyl ketones, and volatile phenols, which are naturally present in oak wood at low concentration, and increase during toasting, have also been found in the extract. The lignin degradation led in the first phase to the formation of hydroxybenzoic acids and especially, hydroxybenzoic aldehydes as syringaldehyde and vanillin, and of hydroxycinnamic aldehydes as sinapaldehyde and coniferaldehyde (Chatonnet et al., 1989; Sarni, Moutounet, Puech, & Rabier, 1990), resulting in high concentrations in the extract. Volatile phenols such as guaicol, eugenol and 6-methoxyeugenol were also present in the extract although in lower concentration, coming from aldehydes and acid thermolysis in a second phase of the wood toasting. Among them, guaiacol was the most abundant compound (0.21 ± 0.01 mg/L), as it was in other kind of aqueous oak extracts (Martínez-Gil et al., 2011, 2012).

Table 2
Concentrations of polyphenolic and volatile compounds in aqueous oak extract.

Volatile compounds (by GC-MS)	
Trans-β-methyl-γ-octalactone	0.247 ± 0.014
Cis-β-methyl-γ-octalactone	0.231 ± 0.009
Furfural	2.869 ± 0.5
5-hidroxymethylfurfural	0.74 ± 0.03
Eugenol	0.011 ± 0.001
6-methoxyeugenol	0.18 ± 0.02
Guaiacol	0.21 ± 0.01
Benzaldehyde	0.034 ± 0.005
Acetovanillone	2.16 ± 0.06
Low molecular weight polyphenols (by HPLC-DAD[14])	
Vanillin	14.1 ± 0.3
Syringaldehyde	48 ± 5
Coniferaldehyde	29 ± 2
Sinapaldehyde	94 ± 4
Vanillic acid	12.5 ± 0.9
Syringic acid	32 ± 2
Gallic acid	55.4 ± 1.1
Ellagic acid	122 ± 3
Ellagitannins (by HPLC-DAD[15])	
Castalagin	146 ± 12
Vescalagin	161 ± 12
Roburin E	188 ± 2
Grandinin + roburin D	279 ± 6
Roburin A + B	110 ± 2
Roburin C	68 ± 3
Σ *Ellagitannins*	952 ± 20

Volatile compounds, low molecular weight polyphenols and ellagitanins are given in mg/L.

Referring to carbohydrate derivatives, that show negligible concentrations in fresh oak wood and very important increases in toasted wood, only the products of Maillard reactions such as furfural and 5-hydroximethylfurfural have been detected in the extract, being furfural the most abundant volatile compound (2.9 ± 0.5 mg/L). When wood is heated, pentoses, the main constituent of hemicelluloses, produce furfural, while from the hexose units of cellulose 5-hydroxymethyl and 5-methylfurfural are formed (Cutzach, Chatonnet, Henry, & Dubourdieu, 1999). Although cellulose is more abundant in wood than hemicellulose (Sarni et al., 1990), its higher resistance to degradation could explain the differences in the concentrations of these compounds.

The most characteristic compounds of oak wood with woody and coconut aroma, *cis* and *trans* β-methyl-γ-lactone, were present in the extract at significant concentrations, showing *cis*/*trans* ratio of 0.93, which confirms that the extract is from French oak (Garde-Cerdán et al., 2010; Waterhouse & Towey, 1994). These lactones together with vanillin are the most odorant compounds present in the oak extract (the vanillin olfactory threshold in wines is 60 μg/L according to Culleré, Escudero, Cacho, & Ferreira, 2004; the olfactory threshold in wine of *cis* oak lactone is 67 μg/L and of *trans* oak lactone 790 μg/L, according to Boidron, Chatonnet, & Pons, 1988) so, they are the main responsible of the characteristic aroma of these extracts.

3.2. Grape and wine oenological parameters

The parameters associated to technological maturity and the phenolic potential of the grapes at harvest are shown in Table 3. As Baumé values show, control grapes have greater sugar content than grapes from treated grapevines, being the lowest values to 100%-1 treatment. Control also presented higher pH value and lower acidity than grapes from treated vines. As a consequence, °Be/TA ratio was significantly lower in grapes from extract treatments. Therefore, even though the same quantity of extract arrived to the plant in both treatments (100%-1 and 25%-4), the results show that the timing of oak extract distribution (one application or distributed in four applications) affected sugar and acids synthesis.

Table 3
Grape oenological parameters associated to the technological maturity and phenolic potential of grapes at the harvest day.

Treatment	°Be	pH	TA	°Be/TA	ApH1	ApH3.6	%AEI	%SMI
Control	13.00 ± 0.07 c	3.68 ± 0.04 b	4.50 ± 0.37 a	2.88 ± 0.08 b	442 ± 4 b	263 ± 3 a	41 ± 1 b	49 ± 2 a
25%-1	12.85 ± 0.07 b	3.64 ± 0.01 ab	4.96 ± 0.04 c	2.59 ± 0.01 a	456 ± 6 c	267 ± 4 a	42 ± 1 b	56 ± 1 b
25%-4	12.70 ± 0.01 b	3.64 ± 0.01 ab	4.94 ± 0.02 c	2.57 ± 0.01 a	431 ± 4 a	286 ± 7 b	42 ± 1 b	57 ± 1 b
100%-1	12.55 ± 0.07 a	3.63 ± 0.00 a	4.89 ± 0.01 b	2.57 ± 0.01 a	451 ± 5 c	264 ± 3 a	39 ± 1 a	54 ± 2 b

Control: untreated grapevines; 25%-1: grapevines treated with oak extract diluted to a quarter applied one time; 25%-4: grapevines treated with oak extract diluted to a quarter applied four times; 100%: grapevines treated with oak extract without dilution. °Be: Baumé degrees; TA: total acidity expressed as g/L of tartaric acid; A: total anthocyanins (mg/L); % AEI: anthocyanin extractability index; % SMI: seed maturity index. Different letters in the same column indicate significant differences according to the LSD test ($p < 0.05$). Different letters in the same column indicate significant differences according to the LSD test ($\alpha < 0.05$). The values are shown with their standard deviations ($n = 3$).

Looking at the phenolic potential of grapes at harvest, it can be observed that the content of total anthocyanins at pH 1 was around 40% higher than at pH 3.6 (approximately must pH). When anthocyanin values at pH 1 and 3.6 are close, their extractability is higher and the %AEI (also known as phenolic maturity index) is lower. The application of oak extract at 25% in one time (25%-1) or four times (25%-4), did not affect the anthocyanin extractability respect to control; however, 100%-1 treatment decreased the value, so anthocyanins are probably more easily extractable during winemaking. All oak extract treatments significantly increased the %SMI, representing the percentage of seed tannins which contribute to their total content in wine, so it is to be expected that they diffuse more easily to wine.

Oenological parameters of wines at the end of alcoholic fermentation, after malolactic fermentation and six month later are shown in Table 4. All parameters have values into the suitable range for high quality wines. It has been considered the parameters related to their technological quality (alcoholic degree, pH, titratable acidity, volatile acidity) as well as the global phenolic quality (total phenol index, total anthocyanins) and colour (colour intensity, and shade) were studied. Regarding to technological parameters, as it was expected, the same differences detected in grapes were observed among treated and control wines; control had higher alcoholic degree, pH and total acidity than wines from treated vines. Wine from 100%-1 grapevine treatment had the lowest values of alcoholic degree and pH. The highest volatile acidity was obtained in control wines, although it was below 0.6 g/L, which means there were no microbiological problems. After malolactic fermentation it was observed a lightly decrease of alcoholic degree and total acidity, while pH and volatile acidity increased. The main differences observed among control wines and the ones from treated grapevines were also maintained at this sampling time and after six months, since the control wines had higher alcohol content and pH that wines from the treatments.

Six months after malolactic fermentation, a decrease of alcoholic degree, pH and total acidity values together with an increase of volatile acidity values were observed in all wines respect to the end of malolactic fermentation.

Both basic technological parameters, alcoholic degree and total acidity, were affected by oak extract treatments. All treatments produced a significant decrease of them respect to control, although in a different degree, depending on the treatment carried out, highlighting 100%-1 treatment because produced the greatest decrease while 25%-4 one produced the lowest after alcoholic fermentation. These results are in agreement with the ones reported in grapes.

In terms of wine global polyphenol content and colour (Table 4), it was observed that TPI of wines from oak extract treated grapevines was significantly higher than control in all the sampling times, which was in agreement with %SMI of grapes phenolic potential, being the highest content in 25%-1 wine after alcoholic fermentation. Anthocyanins are the main compounds responsible for the colour of red grapes and the wines produced from them; the concentrations found were the habitual in Monastrell wines with long macerations. Total content of anthocyanins (A) was also higher in wines from treated grapevines with oak extract than control one after alcoholic fermentation, although the differences decreased through the next sampling times, when 100%-1 treated wine had the highest content of anthocyanins, which is in agreement with the estimation of grapes phenolic potential. In order to evaluate wine colour, the parameters used were colour intensity (CI) and shade (S) which give information about the colour quality and its stability. Both parameters should be interpreted together, so the wine colour quality is better when higher is CI and S is lower. All

Table 4
Oenological parameters of wines at the different sampling times.

Wines	°A	pH	TA	VA	TPI	A	CI	S
At the end of the alcoholic fermentation								
Control	13.32 ± 0.48 c	3.80 ± 0.03 c	5.60 ± 0.11 d	0.46 ± 0.05 b	59 ± 3 a	374 ± 10 a	10.86 ± 0.49 a	0.70 ± 0.01 b
25%-1	12.60 ± 0.20 b	3.73 ± 0.03 b	4.98 ± 0.01 b	0.16 ± 0.01 a	76 ± 5 c	417 ± 15 b	12.66 ± 0.02 c	0.65 ± 0.01 a
25%-4	12.72 ± 0.11 b	3.74 ± 0.01 b	5.10 ± 0.06 c	0.14 ± 0.01 a	69 ± 2 b	440 ± 8 b	11.49 ± 0.11 b	0.65 ± 0.02 a
100%-1	12.30 ± 0.02 a	3.72 ± 0.02 a	4.67 ± 0.01 a	0.16 ± 0.01 a	66 ± 3 b	454 ± 5 c	11.70 ± 0.03 b	0.63 ± 0.01 a
At the end of the malolactic fermentation								
Control	13.21 ± 0.25 c	3.96 ± 0.01 c	4.81 ± 0.06 c	0.56 ± 0.15 a	59 ± 2 a	366 ± 8 b	9.07 ± 0.19 a	0.87 ± 0.01 b
25%-1	12.54 ± 0.08 b	3.91 ± 0.01 b	4.81 ± 0.06 c	0.42 ± 0.03 a	70 ± 3 b	346 ± 10 a	9.90 ± 0.12 c	0.79 ± 0.01 a
25%-4	12.60 ± 0.11 b	3.88 ± 0.01 a	4.75 ± 0.01 b	0.34 ± 0.04 a	68 ± 2 b	386 ± 5 c	9.74 ± 0.06 c	0.79 ± 0.01 a
100%-1	12.03 ± 0.02 a	3.86 ± 0.02 a	4.67 ± 0.01 a	0.42 ± 0.01 a	65 ± 2 b	396 ± 4 d	9.29 ± 0.02 b	0.80 ± 0.01 a
After 6 months of the malolactic fermentation								
Control	13.02 ± 0.15 c	3.96 ± 0.02 c	4.66 ± 0.08 b	0.59 ± 0.01 a	49 ± 2 a	262 ± 10 a	8.57 ± 0.12 a	0.87 ± 0.01 b
25%-1	12.34 ± 0.07 b	3.90 ± 0.01 b	4.49 ± 0.09 b	0.48 ± 0.04 a	58 ± 3 b	255 ± 4 a	9.73 ± 0.02 b	0.82 ± 0.01 a
25%-4	12.42 ± 0.08 b	3.84 ± 0.01 a	4.44 ± 0.08 a	0.51 ± 0.01 a	58 ± 1 b	268 ± 6 a	9.79 ± 0.03 b	0.84 ± 0.01 a
100%-1	11.97 ± 0.11 a	3.84 ± 0.01 a	4.40 ± 0.14 a	0.44 ± 0.05 a	58 ± 2 b	291 ± 5 b	8.67 ± 0.05 a	0.83 ± 0.01 a

Control: untreated grapevines; 25%-1: grapevines treated with oak extract diluted to a quarter applied one time; 25%-4: grapevines treated with oak extract diluted to a quarter applied four times; 100%: grapevines treated with oak extract without dilution. °A: alcohol degree as %v/v; TA: total acidity expressed as g/L of tartaric acid; VA: volatile acidity expressed as g/L of acetic acid; TPI: total phenol index; A: total anthocyanins expressed as mg/L; CI: colour intensity; S: shade. Different letters in the same column indicate significant differences according to the LSD test ($\alpha < 0.05$). The values are shown with their standard deviations ($n = 4$).

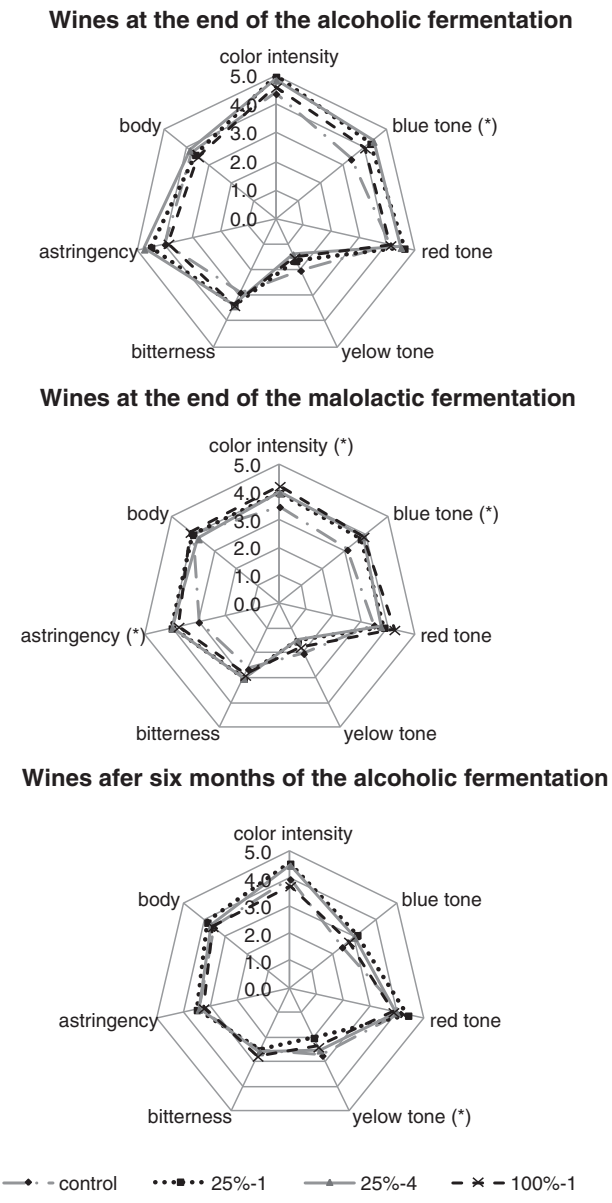


Fig. 1. Sensory analysis of wines. (*) Significant differences respect to the control wine according to the LSD test ($\alpha < 0.05$).

wines from treatments had higher CI and lower S than control wine, which is related to their greater TPI value, as other authors reported (Gómez-Cordovés & González-SanJosé, 1995), being that 25%-1 wine had the highest CI/S ratio at all sampling times. So, it is possible to think that this treatment produced the wine with the highest colour quality and stability.

Fig. 1 shows the sensory analysis of wines at the three sampling times. At the end of the alcoholic fermentation significant differences respect to control were only observed in the blue tone score, which was higher in wines from treatments than in control. At the end of the malolactic fermentation, scores of all the descriptors were lower than in the previous sampling, especially for wine control, which had the lowest values of astringency, colour intensity and blue tone. However, significant differences of the evaluated descriptors were not appreciated among wines from the different treatments. After six months yellow tone score for wine from 25%-1 treatment was significant lower than the control. Therefore, sensory analysis results suggest that wines from grapevines treated with oak extract had more stable and better colour than the control, especially the one from 25%-1 treatment, which agrees with results from the CI and S parameters.

3.3. Detailed polyphenolic composition

Tables 5 and 6 show the results of the 33 phenolic compounds analyzed in wines, grouped as non-flavonoid (phenolic acids and stilbenes) and flavonoid (anthocyanins, flavanols and flavonols) families. Only the most important compounds were measured, taking into account different aspects like their abundance in wines, contribution in reactions related to colour stabilisation and importance to human health. Phenolic acids of wines (Table 5) are mainly hydroxybenzoic acids (C6–C1 structure) and hydroxycinnamic acids (C6–C3 structure). Hydroxycinnamic acids exist in free forms or like esters of tartaric acid; caffeic and coumaric acids, as well their tartaric esters namely caftaric (caffeoyltartrate) and coutaric (coumaroyltartrate) acids were determined. Hydroxycinnamoyltartaric acids (HCTs) are the most abundant group of non-flavonoids in grapes and wines prevailing in wines before malolactic fermentation (MLF), while the free forms, caffeic and *p*-coumaric acids, are most abundant in wines that have undergone MLF and after aging because they are originate from hydrolysis of HCTs (Cabrita et al., 2008; Ferrandino, Carra, Rolle, Schneider, & Schubert, 2012; Gómez-Gallego, Gómez García-Carpintero, Sánchez-Palomo, González-Viñas, & Hermosín-Gutiérrez, 2013; Hernández, Estrella, Carlavilla, Martín-Álvarez, & Moreno-Arribas, 2006). Oak extract treatments increased the total content of hydroxicinnamic acids and HCTs in wines. They were more abundant in wines from treated grapevines than in control, especially, after malolactic fermentation. However, after malolactic fermentation an important decrement was observed for *t*-caftaric and *t*-coutaric acids in 25%-1 wine together with an increase of *t*-caffeic and *p*-coumaric acids, being the greatest HCTs content in wines from 25%-1 to 25%-4. It is known that caftaric and *p*-coutaric are the major substrates of polyphenol oxidase, to *o*-quinones, and the intensity of browning depends on their concentration; so, it is described that during wine aging HCT contents decrease with a parallel increase in oxidative browning (which is observed by a CI decrease and a shade increase chromatic parameters) (Ferrandino et al., 2012). The present results support the discussed previously about the higher colour quality observed in wines from treated grapevines with oak extract. The decrease of HCTs in wine 25%-1, until it reaches similar values as the control wine after malolactic fermentation, can also be related to the decreased of CI in 25%-1 wine, which means that wines from treatments with more quantity of oak extract (25%-4 and 100%-1) are more resistant against oxidation. On the other hand, total hydroxibenzoic acids increased gradually with aging, being the gallic acid the most abundant compound followed by syringic acid, while vanillic acid remained constant. These results are in agreement with other authors (Cabrita et al., 2008). However, the content of gallic and syringic acids of wines from treatments was significantly greater than in control, while vanillic content was lower. These variations can be due to the fact that oak extracts are rich in syringic and gallic acids, while vanillic acid is in lower concentrations. Gallic acid is one of the most potent antioxidant found in wines (Teissedre, 2012).

The studied stilbenes were *t*-resveratrol and its glucoside piceid-*t*-resveratrol or *t*-resveratrol 3-O-β-D-glucopyranoside (Table 5). *T*-resveratrol is one of the major stilbene phytoalexins produced by various families of plants, but grapes and related products are considered the most important dietary sources of these substances. These compounds have antioxidant properties and beneficial effects on human health related to protection against cardiovascular diseases (Teissedre, 2012), and also the chemopreventive activity of *t*-resveratrol had been studied (Pezzuto, 2008). In our study the standard of *t*-resveratrol aglycone allowed us to confirm its identity in the samples, but the piceid compound was tentatively identified attending to the bibliography and the spectra obtained at 308 nm (Jeandet, 1997). Assuming that *t*-resveratrol and piceid-*t*-resveratrol isomers are more abundant in wines than the *c*-resveratrol, the peak obtained was assigned to the isomer piceid-*t*-resveratrol. The treatments carried out produced an

Table 5
Non-flavonoid composition (mg/L) of wines at the different sampling times (n = 4).

	At the end of the alcoholic fermentation				At the end of the malolactic fermentation				After 6 months of the malolactic fermentation			
Phenolic acids	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1
t-caffeic	0.74 ± 0.02 a	0.97 ± 0.01 c	0.99 ± 0.01 c	0.83 ± 0.01 b	1.34 ± 0.04 a	5.70 ± 0.02 c	1.27 ± 0.03 a	1.60 ± 0.08 b	1.44 ± 0.03 ab	4.74 ± 0.03 c	1.20 ± 0.04 a	1.68 ± 0.03 b
p-coumaric	0.68 ± 0.01 a	0.93 ± 0.05 d	0.80 ± 0.02 c	0.74 ± 0.02 b	1.38 ± 0.03 a	3.73 ± 0.05 d	1.60 ± 0.02 b	2.02 ± 0.06 c	2.00 ± 0.02 a	3.64 ± 0.06 c	1.97 ± 0.01 a	2.79 ± 0.02 b
ΣHidroxycinnamic acids	*1.42*	*1.90*	*1.79*	*1.57*	*2.72*	*9.43*	*2.86*	*3.62*	*3.44*	*8.68*	*3.17*	*4.47*
t-caftaric	17.9 ± 0.1 a	27.4 ± 0.2 c	26.8 ± 0.3 b	26.6 ± 0.2 b	16.5 ± 0.2 b	14.3 ± 0.1 a	24.9 ± 0.3 c	25.0 ± 0.1 c	16.6 ± 0.1 b	14.0 ± 0.2 a	25.3 ± 0.1c	25.1 ± 0.2 c
t-coutaric	5.34 ± 0.1 a	9.9 ± 0.3 b	10.96 ± 0.1 c	11.08 ± 0.1 c	5.4 ± 0.1 a	5.5 ± 0.1 a	10.4 ± 0.2 b	10.3 ± 0.3 b	5.5 ± 0.1 a	5.4 ± 0.2 a	10.7 ± 0.1 b	10.6 ± 0.1 b
ΣHydroxicinnamic ester	*23.24*	*37.75*	*37.67*	*23.18*	*19.72*	*35.28*	*35.27*	*21.91*	*19.46*	*35.95*	*35.78*	*22.16*
gallic	22.3 ± 0.4 a	35.9 ± 0.6 c	27.9 ± 0.9 b	26.1 ± 0.9 b	27.6 ± 0.5 a	46.3 ± 0.7 c	35.9 ± 0.6 b	34.9 ± 0.5 b	35.7 ± 0.2 b	23.20 ± 0.3 a	47.5 ± 0.8 c	45.8 ± 0.9 c
vanillic	2.98 ± 0.05 d	2.51 ± 0.02 c	2.22 ± 0.03 b	2.08 ± 0.02 a	2.81 ± 0.03 d	2.05 ± 0.04 b	2.18 ± 0.02 c	1.79 ± 0.02 a	3.14 ± 0.01 d	2.37 ± 0.02 b	2.66 ± 0.06 c	2.04 ± 0.05 a
syringic	7.2 ± 0.2 b	8.7 ± 0.1 c	8.6 ± 0.1 c	6.7 ± 0.2 a	7.4 ± 0.1 a	9.0 ± 0.1 c	8.5 ± 0.1 b	7.3 ± 0.2 a	7.8 ± 0.1 a	9.0 ± 0.1 b	8.8 ± 0.1 b	7.9 ± 0.1 a
ΣHydroxibenzoic acids	*32.52*	*47.13*	*38.69*	*34.90*	*37.87*	*57.33*	*46.59*	*43.95*	*46.80*	*36.95*	*58.96*	*55.73*
Σ ACIDS	*57.14*	*86.78*	*78.14*	*59.66*	*60.31*	*102.04*	*84.72*	*69.48*	*69.70*	*81.58*	*97.90*	*82.36*
Stilbenes	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1
p-t-resveratrol	0.82 ± 0.01 a	1.51 ± 0.01 c	1.67 ± 0.02 d	1.40 ± 0.02 b	0.84 ± 0.01 a	1.34 ± 0.03 c	1.50 ± 0.04 d	1.16 ± 0.01 b	0.82 ± 0.01 b	0.72 ± 0.02 a	1.46 ± 0.01 d	1.09 ± 0.01 c
t-resveratrol	0.150 ± 0.002	0.365 ± 0.013	0.513 ± 0.015	0.545 ± 0.009	0.229 ± 0.004	0.270 ± 0.008	0.457 ± 0.015	0.183 ± 0.009	0.341 ± 0.010	1.071 ± 0.008	0.568 ± 0.010	0.400 ± 0.013
	a	b	c	d	b	c	d	a	a	d	c	b
ΣStilbenes	*0.971*	*1.876*	*2.182*	*1.943*	*1.073*	*1.611*	*1.960*	*1.348*	*1.161*	*1.793*	*2.033*	*1.494*

Control: untreated grapevines; 25%-1: grapevines treated with oak extract diluted to a quarter applied one time; 25%-4: grapevines treated with oak extract diluted to a quarter applied four times; 100%: grapevines treated with oak extract without dilution. p-*t*-resveratrol: piceid-*p*-resveratrol. Different letters in the same row indicate significant differences among treatments according to the LSD test ($\alpha < 0.05$). The concentrations are shown with their standard deviations (n = 4).

important increase of both *t*-resveratrol and piceid content, being more important in the case of piceid, which varied respect to control, from 25 to 125%, depending on treatment and sampling time. Table 6 shows the flavonoid composition of wines: anthocyanins, flavanols and flavonols and their evolution in wines at three sampling times are shown. The total content of 11 individual monomeric anthocyanins (as quantified by HPLC) in wines does not necessarily correlate with wine absorbance at 520 nm (absorbance at which total anthocyanins are spectrophotometrically measured) (Table 4), since reactions with other phenolic compounds and the formation of derived pigments that also absorb at 520 nm may explain the observed differences. However, a similar behaviour was observed in both measures by treatment effects. Non-acylated anthocyanins (82–85%) are predominating over the acylated ones (15–19%). Among acylated anthocyanins, coumarylated anthocyanins were found in higher percentages than acetylated (13–18% and 5–6%, respectively) and to a lesser extent the caffeoyl ones, being the malvidin derivatives the most abundant, in both non-acylated and acylated forms. Ratio of *p*-coumarylated to acetylated anthocyanins has been proposed as a distinctive analytical parameter to differentiate wines made with different grape cultivars (Holbach, Marx, & Ackermann, 1997). The presence of acylated anthocyanins is very important for the colour of wine, as they may participate in intramolecular copigmentation reactions, stabilizing and increasing the colour (Gómez-Cordovés & González-SanJosé, 1995). It is observed that wines from 100%-1 treatment had acylated anthocyanins significantly higher than control one at the three sampling times. Moreover, the evolution of these forms is slower than non-acylated, independently from the type of wine. The oak extract effect on acylated anthocyanins percentage and the ratio coumarylated/acetylated forms were quantified (Fig. 2). Both values were higher in wines from treated vinegrapes than in control one, especially in wines from 100%-1 treatment, although they decreased in all wines through the time.

Vitisin A and B belong to a group of minor pigments, called piranoanthocyanins, that have been detected in red wine but not in fresh grapes (Asenstorfer, Markides, Iland, & Jones, 2003), and they contribute to the colour with orange-yellow tones (Bakker & Timberlake, 1997). These anthocyanin-derived pigments are formed by reaction between malvidin 3-glucoside with pyruvic acid and acetaldehyde respectively, being present in relatively high amounts during fermentation (Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998). Both of them were present in similar concentrations in wines after alcoholic fermentation, although they decreased after MLF, mainly vitisin B. This decrease can be attributed to the negative effect of MLF. It has not been observed a clear effect by oak extract treatments.

The main monomer flavanols present in grapes are (+)-catechin and (−)-epicatechin, whose composition and evolution in the studied wines is shown in Table 6. Significant differences can be observed among wines from treatments and the control, showing the highest content in wines from 100%-1 treatment and the lowest in wine from 25%-1 treatment. There were not important variations in their concentrations with time, which remained constant in all wines except in wine from 100% treatment after six months of the MF, where their total content increased due to (−)-epicatechin. These compounds form complexes polymers and can combine with some anthocyanins to give more stable pigments and as consequence more stable colour in wines. These results are in agreement with the higher colour quality of the wines resulted from oak extract treatments previously discussed. Besides, it was demonstrated that (+)-catechin and (−)-epicatechin inhibited the oxidation of LDL 20 times greater that vitamin E, which is considered as a reference antioxidant (Teissedre, 2012). Wines from oak extract treatments had higher average content than control (the increases ranged from 16 to 70% at the end of the alcoholic fermentation, from 40 to 89% at the end of the malolactic fermentation, and from 7 to 81% after six months from the malolactic fermentation). It seems there is a relation between oak extract treatment and the increment of flavanols in wines (25%-1 < 25%-4 < 100%-1). These results suggest

Table 6
Flavonoid composition (mg/L) of wines at the different sampling times (n = 4).

	At the end of the alcoholic fermentation				At the end of the malolactic fermentation				After 6 months of the malolactic fermentation			
Anthocyanins	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1
D3G	11.2 ± 0.1 b	10.2 ± 0.1 a	17.4 ± 0.2 d	14.7 ± 0.6 c	10.4 ± 0.3 a	10.6 ± 0.1 a	12.1 ± 0.1 b	14.4 ± 0.1 c	7.2 ± 0.1 b	6.8 ± 0.1 a	7.1 ± 0.2 b	11.3 ± 0.3 c
C3G	0.87 ± 0.10 b	0.91 ± 0.01 b	1.09 ± 0.04 c	0.65 ± 0.06 a	0.66 ± 0.05 a	0.87 ± 0.05 c	0.95 ± 0.06 d	0.77 ± 0.02 b	0.63 ± 0.03 a	0.63 ± 0.05 a	0.82 ± 0.01 b	0.82 ± 0.04 b
Pt3G	26.8 ± 0.3 b	22.4 ± 0.3 a	37.2 ± 0.3 d	31.3 ± 0.4 c	23.3 ± 0.1 b	20.9 ± 0.3 a	23.8 ± 0.2 b	29.0 ± 0.1 c	16.1 ± 0.08 b	11.8 ± 0.07 a	13.3 ± 0.04 c	21.4 ± 0.04 d
Pe3G	6.2 ± 0.2 b	5.3 ± 0.1 a	8.9 ± 0.2 c	6.0 ± 0.2 b	6.1 ± 0.2 b	5.2 ± 0.1 a	6.3 ± 0.1 b	6.1 ± 0.2 b	4.14 ± 0.1 c	3.0 ± 0.1 a	3.6 ± 0.1 b	4.4 ± 0.12 c
M3G	148 ± 9 a	142 ± 6 a	189 ± 12 b	179 ± 10 b	141 ± 2 c	114 ± 1 a	127 ± 4 b	155 ± 4 d	92 ± 2 c	62 ± 3 a	72 ± 1 b	111 ± 2 d
ΣG	192.68	180.68	253.28	232.13	181.78	151.69	170.65	205.08	120.21	83.84	96.73	149.04
Pe3GAc	1.02 ± 0.02 b	0.71 ± 0.02 a	1.15 ± 0.07 c	1.33 ± 0.09 d	0.99 ± 0.08 c	0.76 ± 0.04 a	0.85 ± 0.02 b	0.97 ± 0.05 c	0.69 ± 0.05 b	0.35 ± 0.01 a	0.35 ± 0.03 a	0.79 ± 0.05 b
M3GAc	12.2 ± 0.1 b	10.80 ± 0.1 a	16.9 ± 0.1 d	15.6 ± 0.3 c	12.3 ± 0.1 c	10.8 ± 0.1 a	11.7 ± 0.4 b	15.3 ± 0.3 d	8.0 ± 0.6 c	5.7 ± 0.2 a	6.2 ± 0.8 b	10.8 ± 0.1 d
ΣGAc	13.25	11.50	18.03	16.93	13.30	11.57	12.51	16.24	8.66	6.07	6.56	11.57
C3GCo	1.33 ± 0.04 a	1.87 ± 0.03 d	1.58 ± 0.09 b	1.71 ± 0.02 c	1.18 ± 0.04 b	1.04 ± 0.09 a	1.10 ± 0.04 ab	1.32 ± 0.6 c	0.67 ± 0.02 c	0.37 ± 0.01 a	0.46 ± 0.01 b	0.85 ± 0.02 d
Pe3GCo	3.76 ± 0.07 b	3.21 ± 0.05 a	5.87 ± 0.02 c	5.95 ± 0.13 c	3.20 ± 0.07 b	2.67 ± 0.03 a	3.20 ± 0.09 b	4.79 ± 0.02 c	1.73 ± 0.07 b	1.21 ± 0.08 a	1.38 ± 0.09 a	2.88 ± 0.02 c
M3GCo	17.5 ± 0.5 a	17.1 ± 0.3 a	29.3 ± 0.5 b	28.3 ± 0.6 b	15.8 ± 0.1 b	14.4 ± 0.1 a	17.2 ± 0.1 c	24.1 ± 0.2 d	8.8 ± 0.1 c	7.1 ± 0.1 a	7.9 ± 0.2 b	15.1 ± 0.5 d
ΣGCo	22.61	22.22	36.76	35.95	20.18	18.11	21.51	30.19	11.14	8.67	9.80	18.79
M3GCa	1.39 ± 0.04 c	0.76 ± 0.01 a	0.97 ± 0.03 b	0.79 ± 0.02 a	1.18 ± 0.04 c	0.64 ± 0.03 a	0.65 ± 0.02 a	0.70 ± 0.03 b	0.62 ± 0.01 c	0.26 ± 0.01 a	0.27 ± 0.01 a	0.46 ± 0.02 b
ΣAcylated	37.25	34.48	55.76	53.68	34.66	30.32	34.67	47.12	20.42	15.00	16.63	30.82
ΣA	*229.93*	*215.16*	*309.04*	*285.81*	*216.45*	*182.01*	*205.33*	*252.20*	*140.63*	*98.85*	*113.36*	*179.86*
Vitisins	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1
VA	3.00 ± 0.04 c	2.78 ± 0.06 b	2.45 ± 0.02 a	3.40 ± 0.01 d	2.26 ± 0.04 d	1.77 ± 0.03 b	2.01 ± 0.07 c	1.57 ± 0.01 a	2.31 ± 0.01 c	1.61 ± 0.04 a	1.84 ± 0.03 b	1.69 ± 0.05 a
VB	2.99 ± 0.10 c	3.94 ± 0.06 d	2.50 ± 0.14 a	2.83 ± 0.06 b	0.53 ± 0.03 b	0.89 ± 0.05 d	0.72 ± 0.03 c	0.45 ± 0.01 a	0.54 ± 0.05 b	0.40 ± 0.03 a	0.99 ± 0.03 c	0.44 ± 0.03 a
ΣV	5.99	6.73	4.96	6.22	2.79	2.67	2.73	2.03	2.85	2.01	2.83	2.12
Flavanols	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1
(+)-catechin	29.3 ± 1.0 a	39.1 ± 0.6 b	43.9 ± 1.3 c	50.0 ± 0.5 d	27.9 ± 0.8 a	39.0 ± 0.5 b	41.0 ± 0.6 c	53.5 ± 0.3 d	27.3 ± 1.0 a	34.4 ± 0.4 b	35.3 ± 0.6 b	49.3 ± 0.4 c
(−)-epicatechin	12.4 ± 0.5 a	16.6 ± 0.2 b	18.6 ± 0.4 c	21.0 ± 0.4 d	12.2 ± 0.2 a	16.9 ± 0.1 b	16.5 ± 0.3 b	22.2 ± 0.9 c	17.2 ± 0.8 b	13.4 ± 0.7 a	22.3 ± 0.2 c	31.2 ± 0.1 d
ΣFlavanols	*41.7*	*55.7*	*62.5*	*71.0*	*40.1*	*55.9*	*57.5*	*75.7*	*44.5*	*47.8*	*57.6*	80.5
Flavonols	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1	Control	25%-1	25%-4	100%-1
Qu3Galact	4.69 ± 0.01 c	4.24 ± 0.02 b	4.03 ± 0.01 a	4.68 ± 0.05 c	4.20 ± 0.04 b	3.88 ± 0.06 a	3.82 ± 0.03 a	4.33 ± 0.02 b	3.43 ± 0.05 b	3.18 ± 0.01 a	3.20 ± 0.02 a	3.38 ± 0.01 b
Qu3Glucur + Glucos	22.0 ± 0.5 c	20.6 ± 0.7 b	19.3 ± 0.2 a	22.1 ± 0.5 c	18.4 ± 0.2 b	16.1 ± 0.6 a	16.7 ± 0.7 a	18.7 ± 0.4 b	15.6 ± 0.4 b	14.4 ± 0.6 a	15.8 ± 0.3 b	16.1 ± 0.2 b
ΣQuG	26.74	24.81	23.33	26.78	22.63	20.00	20.48	23.03	19.06	17.63	19.05	19.51
My3Galact	4.60 ± 0.01 b	4.57 ± 0.02 b	4.40 ± 0.03 a	4.50 ± 0.12 ab	4.16 ± 0.01 b	4.00 ± 0.02 a	4.09 ± 0.10 ab	4.16 ± 0.03 b	3.81 ± 0.02 a	3.83 ± 0.06 a	3.90 ± 0.02 b	3.90 ± 0.01 b
My3Glucur + Glucos	24.5 ± 0.1 b	25.5 ± 0.1 c	24.3 ± 0.2 b	21.2 ± 0.2 a	18.2 ± 0.1 c	15.0 ± 0.1 b	18.0 ± 0.2 c	14.0 ± 0.4 a	9.8 ± 0.1 a	10.1 ± 0.1 b	13.2 ± 0.1 c	10.4 ± 0.2 b
ΣMyG	29.12	30.10	28.73	25.74	22.39	19.03	22.06	18.15	13.67	13.94	17.08	14.28
La3Glucs/Galact	4.71 ± 0.02 b	4.80 ± 0.05 d	4.48 ± 0.03 a	4.65 ± 0.04 b	4.50 ± 0.01 b	4.35 ± 0.04 a	4.41 ± 0.02 a	4.38 ± 0.01 a	3.98 ± 0.03 b	3.87 ± 0.03 a	4.19 ± 0.02 c	3.85 ± 0.02 a
Sy3Glucos	3.94 ± 0.01 b	3.98 ± 0.02 b	3.81 ± 0.07 a	3.96 ± 0.04 b	3.69 ± 0.05 a	3.61 ± 0.05 a	3.61 ± 0.02 a	3.74 ± 0.02 b	3.52 ± 0.04 a	3.54 ± 0.03 a	3.56 ± 0.01 a	3.65 ± 0.01 b
ΣGlycosides	64.51	63.70	60.35	61.13	53.22	46.99	50.55	49.30	40.24	38.98	43.88	41.29
myricetin	8.5 ± 0.1 a	8.8 ± 0.3 b	9.0 ± 0.1 c	8.5 ± 0.1 a	11.9 ± 0.1 a	13.0 ± 0.2 b	11.6 ± 0.3 a	12.5 ± 0.3 b	15.3 ± 0.1 a	17.4 ± 0.1 b	15.2 ± 0.1 a	20.23 ± 0.2 c
quercetine	14.5 ± 0.1 a	17.4 ± 0.3 c	17.5 ± 0.1 c	16.8 ± 0.4 b	17.0 ± 0.3 a	19.2 ± 0.1 b	16.3 ± 0.5 a	19.0 ± 0.2 b	18.1 ± 0.3 b	20.6 ± 1.4 c	17.2 ± 0.1 a	23.0 ± 0.2 d
kaempferol	0.33 ± 0.004 a	1.11 ± 0.01 b	1.23 ± 0.02 c	1.35 ± 0.02 d	0.49 ± 0.01 a	1.11 ± 0.01 b	1.15 ± 0.03 b	1.43 ± 0.01 c	0.61 ± 0.04 a	1.34 ± 0.01 c	1.21 ± 0.02 b	1.67 ± 0.01 d
ΣAglycones	23.34	27.32	27.73	26.68	29.39	33.26	29.05	32.91	34.02	39.34	33.60	44.90
ΣFlavonols	*87.85*	*91.02*	*88.08*	*87.81*	*82.61*	*80.25*	*79.60*	*82.21*	*74.26*	*78.32*	*77.49*	*86.19*

Control: untreated grapevines; 25%-1: grapevines treated with oak extract diluted to a quarter applied one time; 25%-4: grapevines treated with oak extract diluted to a quarter applied four times; 100%: grapevines treated with oak extract without dilution. *Abbreviations*: D3G: delphinidin 3-O-glucoside; C3G: cyanidin 3-O-glucoside; Pt3G: petunidin 3-O-glucoside; Pe3G: peonidin 3-O-glucoside; M3G: malvidin 3-O-glucoside; ΣG: total glucoside antocyanins; Pe3GAc: peonidin 3-O-(6-acetyl)-glucoside; M3GAc: malvidin 3-O-(6-acetyl)-glucoside; ΣGAc: total acetylated antocyanins; C3GCo: cyanidin 3-(6-p-coumaroyl)-glucoside; Pe3GCo: peonidin 3-(6-p-coumaroyl)-glucoside; M3GCo: malvidin 3-(6-p-coumaroyl)-glucoside; ΣGCo: total coumaroyl antocyanins; M3GCa: malvidin 3-(6-t-caffeoyl)-glucoside; ΣA: total antocyanins; VA: vitisin A malvidin 3-O-glucoside; VB: vitisin B malvidin 3-O-glucoside; ΣV: total vitisins. My3Galact: myricetin 3-O-galactoside; My3Glucur + Glucos: myricetin 3-O-glucuronide + myricetin 3-O-glucoside; ΣMyG: total myricetin glicosides; Qu3Galact: quercetin quercetin 3-O-glucoside; Qu3Glucur + Glucos: quercetin 3-O-glucuronide + quercetin 3-O-glucoside; ΣQuG: total quercetin glicosides; La3Glucs/Galact: laricitrin 3-O glucoside/galactoside; Sy3Glucos: syringetin 3-O-glucoside. Different letters in the same row indicate significant differences among treatments according to the LSD test ($\alpha < 0.05$). The concentrations are shown with their standard deviations (n = 4).

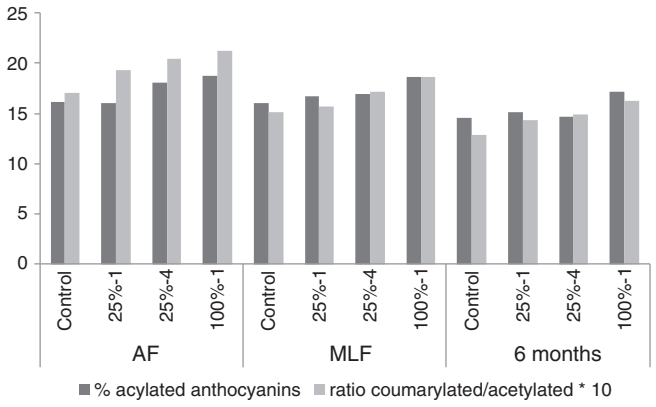
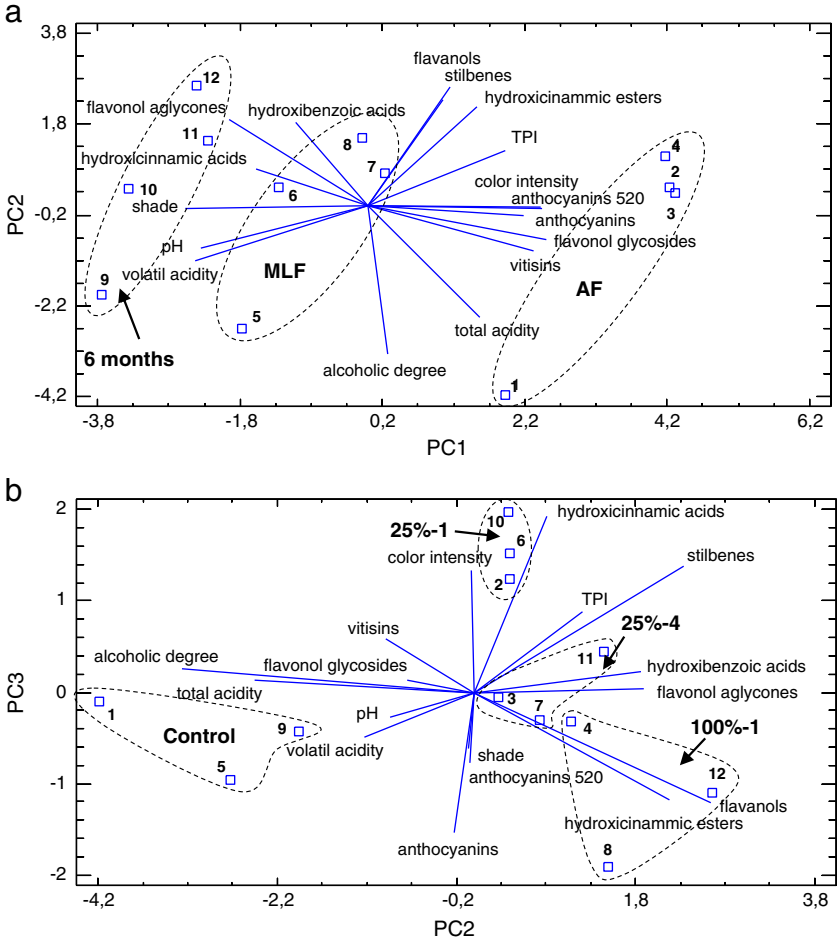


Fig. 2. Percentage of acylated anthocyanins and ratio between coumarylated and acetylated anthocyanins in wines at the end of the alcoholic fermentation (AF), malolactic fermentation (MLF) and after six months of this (6 months).

that wines from oak extract treatments could be important positive contributors to human health.

Flavonols are largely localised in the grape skins, where they are found as flavonol glycosides. Free flavonol aglycones can be produced from these flavonol glycosides, as a result of hydrolysis of the glycosidic bound by enzymes or acid conditions in the wine (Hermosín-Gutiérrez, Castillo-Muñoz, Gómez-Alonso, & García-Romero, 2011). They are yellow pigments that contribute directly to the colour of white wines, but in red wines flavonols are masked by anthocyanins, the red pigments. Flavonols are involved in the phenomenon of copigmentation in red wines, which can cause an enhancement of the extraction of anthocyanins during winemaking, being reflected in a more intense red colour. In addition, some flavonols such as quercetin and myricetin, have antioxidant activity but lower than flavanols (Teissedre, 2012). In Table 6, it can be observed that Monastrell wines had high proportion of quercetin-type flavonols, being glucuronide derivative the most abundant the, while the quercetin glucoside was in very low concentration (data not shown), as it has been reported in a previous work (Hermosín-Gutiérrez et al., 2011). This fact can be justified by the lability of quercetin-3-O-glucoside that is hydrolyzed to a large extent (Castillo-Muñoz, González, et al., 2009).). There was also high proportion of myricetin 3-O-glycosides, having similar profile than quercetin type about the type and proportion of glycosides. Laricitrin and syringetin flavonols were detected in low quantity in wines. On the other hand, although kaempferol-3-glucoside could be guaranteed by comparison with authentic standard, peak was not found in the samples and no other flavonol 3-O-glycoside of kaempferol was identified in the studied wines. Moreover, kaempferol aglycone was found in very low proportion in comparison with the other flavonols (1–4% of the total flavonols). The applied treatments did not induce a clear effect on flavonol content. A decrease of derivative glycosides was observed a long



1: Control after AF; **2**: 25%-1 after AF; **3**: 25%-4 after AF; **4**: 100%-1 after AF; **5**: Control after MLF; **6**: 25%-1 after MLF; **7**: 25%-4 after MLF; **8**: 100%-1 after MLF; **9**: Control after 6 months; **10**: 25%-1 after 6 months; **11**: 25%-4 after 6 months; **12**: 100%-1 after 6 months.

Fig. 3. Projection of wines, polyphenol families and oenological parameters in the spaces defined by the three principal components.

time, due to their hydrolysis and the correspondent aglycones releasing. After six months of MLF similar proportions of glycosides and aglycones were found.

Finally a principal component analysis was carried out with the total concentration of the phenolic compounds determined by HPLC-MS grouped into their corresponding chemical families, together with the enological parameters of the different wines studied at the different sampling times. Fig. 3(a) shows that samples were separated along the first principal component (PC1), which explained 51.2% of the variance, while PC2 explained 22.7%. It can be observed that flavonol glycosides, total vitisins, anthocyanins, and the spectrophotometric measures of anthocyanins and colour intensity had a high weight on PC1, and they were negatively correlated with shade, pH, volatile acidity and hydroxycinnamic acids. On the other hand, flavanols, stilbenes, hydroxibenzoic acids and alcoholic were associated to PC2. Wine samples after 6 months were distributed at negative values of PC1, while samples after alcoholic fermentation were distributed at positive values of PC2, showing cases with a higher content of anthocyanins and glycosidic flavonols. The distribution along the PC1 was in accordance to the evolution of wines. Taking into account wines from the same sampling time (AF, MLF and after 6 months), it can be observed that wines from treatments were located on the right side of control wine, meaning that these wines had higher content of anthocyanins and glycosidic flavonols than the control. The same behaviour took place in the three sampling times studied, which means that treatments affected the wines increasing such compounds. Wines from treatments and control were separated on PC2, as well being the highest difference between control and 100%-1 wine in all cases. If PC1 was removed from the plot (which was correlated with the evolution of wines through the time) and PC2 is represented versus PC3 (7.6%) (Fig. 3(b)), samples were grouped according to the treatment carried out. Colour intensity, hydroxicinnamic acids, together with stilbenes and TPI had high and positive weight on PC3, while anthocyanins had the highest negative weight on this component. Wines from treatments were distributed on the right of PC2, showing higher content of stilbenes, flavanols and hydroxicinnamic acids than control wine. These compounds are antioxidant, which means that the high stability of these wines against their evolution is due to them.

In conclusion, it has been observed that oak extract treatments to grapevines, affected to the grape parameters used in wineries to evaluate their oenological potential. Such parameters are related with the technological maturity of grapes (sugar and acids) and with the phenolic potential, especially the one related to seeds maturity. These quality differences result on less alcoholic and acid wines, higher polyphenolic content (fact that favour the aging capacity of wines) and better colour, whose evolution through the time is more stable. The detailed monomeric polyphenol content of wines allowed us to know why treated wines with oak extract improved in such an important way respect to control. Thus, it has been shown that wines have higher content of compounds directly related to colour quality and stability, such as HCTs, acylated anthocyanins, flavanols (with a strong antioxidant effect) and stilbenes, whose cardioprotective and chemopreventive benefit to human health had been demonstrated on the literature. Moreover, it has also been shown that phenolic acids present in the oak extract increased significantly in wines, highlighting gallic acid due to its antioxidant action, which suggest that it could be accumulated directly in grapes and after pass to wine. The other analyzed compounds did not come directly from the extract, so their presence in wine could only be as result of a stimulating action on the secondary metabolism of grape by oak extracts. Consequently, oak extract applications as shown in this paper, could be considered as a biostimulant of phenolic compounds in grapes implicated in wine quality and its benefit on human health.

Acknowledgments

Many thanks for the financial support given by the Ministerio de Ciencia e Innovación of Spain to the Project AGL2009-08950 and for the FPI scholarship of A.I.P.-G (BES-2010-038613). Also we are gratefully to the Junta de Comunidades de Castilla-La Mancha (Spain) of A.M.M-G scholarship (EXP 422/09) and to PROTEA (France) by supporting the commercial oak extract.

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