PHENOLIC CHARACTERIZATION OF AGING WINE LEES: CORRELATION WITH ANTIOXIDANT ACTIVITIES

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Abstract

Aging wine lees are water-wastes produced during the wine aging inside wood barrels that can be considered as alternative sources of bioactive compounds. Phenolic characterization and antioxidant activity (AA) measurements of wines lees solid-liquid extracts have been undertaken on a dry extract (DE) basis. Solvents with different polarities (water, methanol, ethanol, two hydroalcoholic mixtures and acetone) were used. Total phenolic (TPC) and total flavonoid contents (TFC) were determined. The mixture of 75:25(v/v) EtOH:H_2O showed the highest values with 254mg_{GAE}/g_{DE} and 146mg_{CATE}/g_{DE} respectively. HORAC, HOSC and FRAP were used to determine the AA of the extracts being also highest for the mixture of 75:25(v/v) EtOH:H_2O (4,690 $\mu mol_{CAE}/g_{DE}$, 4,527

$\mu mol_{TE}/g_{DE}$ and 2,197 $\mu mol_{TE}/g_{DE}$, respectively). For ORAC method, methanol extract showed the best value with 2,771 $\mu mol_{TE}/g_{DE}$. Correlations between TPC, TFC, phenolic compounds and AA were determined. Most relevant compounds contributing to AA were identified using data from mass spectrometry, being mainly anthocyanins.

Keywords

Aging wine lees, phenolic characterization, antioxidant activity, LC-MS/MS, correlation study, anthocyanins.

1. Introduction

The wine industry is an important sector of the EU economy, with an approximate worldwide production of 280 million hectoliters per year (Dimou et al., 2015). This agricultural activity generates huge amounts of wastes and by-products. In Spain alone, 2-3 million tons of wastes are generated per year (Ruggieri et al., 2009), including grape pomace (62%), lees (14%), stalk (12%) and dewatered sludge (12%). Traditionally, these wastes have been used as a supplement in animal feed with a poor nutrient value, as fermentation nutrient supplement (Dimou et al., 2015) or to recover tartaric acid (Versari, Castellari, Spinabelli, & Galassi, 2001). However, in many cases, they are disposed in landfill contributing to an environmental problem due to their low pH and high content in organic matter (Bustamante et al., 2008). Sometimes they are incinerated, which entails high costs of operation and production of toxic gases potentially dangerous to human health. As an alternative, some environmental friendly technologies have emerged to revalorize and take advantage of these winemaking residues with high contents of natural bioactive compounds (Teixeira et al., 2014).

Wine lees are the least exploited waste from the wine industry. Wine lees are a water-waste residue created during the vinification process of red and white wines and they result from the combination of the yeasts, metabolites and other free phenolic compounds

52 such as released free flavonol aglycones and pyranoanthocyanins (Barcia et al., 2014;
53 Dimou et al., 2015). Depending on the stage of vinification, wine lees can be classified into
54 different groups: first and second fermentation lees (formed during the alcoholic and
55 malolactic fermentations, respectively) and aging wine lees (formed during wine aging in
56 wood barrels). The main factors that may influence the composition of the lees are
57 environmental conditions, the land type, grape variety and the time of aging in the wood
58 barrels (Rankine, Fornachon, Boehm, & Cellier, 1971)

59 Wine lees could be used as rich sources of anthocyanins and other (poly)phenols
60 with a strong potential application in food, cosmetics, and pharmaceutical industries, for
61 their health-promoting effects due to their recognized antioxidant, antimicrobial, anti-
62 inflammatory and cardio protective properties (Barcia et al., 2014; Landeka Jurčević et al.,
63 2017). Furthermore, the exploitation of these dregs would contribute to an environmental
64 equilibrium and lead to extracts of great interest with important bioactive properties that can
65 be used as antioxidant additives. For instance, grape seeds extracts have potential
66 antioxidant properties by inhibiting lipid oxidation and antimicrobial activities against major
67 food borne pathogens (Perumalla & Hettiarachchy, 2011). However, there is a considerable
68 lack of information regarding the polyphenolic composition of extracts derived from wine
69 lees in comparison to other residues, such as grape pomace, seeds and other wine by-
70 products (Teixeira et al., 2014).

71 Different methodologies can be used for determination of antioxidant activity (AA).
72 Among them, the most common assays are Ferric Reducing Antioxidant Power (FRAP) and
73 Oxygen Radical Absorption Capacity (ORAC) assays. They have been already used to
74 measure AA of wine and polyphenolic extracts of winery by-products (Kondrashov, Ševčík,
75 Benáková, Koštířová, & Štípek, 2009). Hydroxyl Radical Averting Capacity (HORAC) and
76 Hydroxyl Radical Scavenging Capacity (HOSC) assays are gaining importance in the
77 measurement of AA of extracts from berries, also rich in anthocyanins (Matias et al., 2016).

78 Furthermore, it is important to correlate and understand which family of polyphenols and/or
79 compounds contribute to the different antioxidant assays, showing specific antioxidant
80 potential for the different radicals (like Fe^{+3}, $OH^{•}$ or $ROO^{•}$) depending on their chemical
81 structure (Kallithraka, Mohdaly, Makris, & Kefalas, 2005).

82 As far as we know, there are only a few studies published studying the antioxidant
83 activities response to extracts of wine lees and their correlations with phenolic composition.
84 Some authors used ORAC assays to measure the antioxidant activity of wine lees extracts
85 prepared using a Soxhlet extraction and a microwave assisted extraction (Pérez-Serradilla &
86 Luque de Castro, 2011) or with ultrasounds (Alonso, Guillén, Barroso, Puertas, & García,
87 2002). $DPPH^{•}$ assay (Wu et al., 2009) and FRAP radical scavenging activity have been also
88 employed to measure the antioxidant ability of wine lees extracts (Landeka et al., 2017) .

89 The work here presented is aimed at contributing to the phenolic characterization of
90 aging wine lees obtained from *Vitis vinifera* grape variety. The total phenolic and flavonoid
91 contents of the extracts prepared were measured as well as the chromatographic peak areas
92 and were correlated with results from antioxidant activity assays to find out which families
93 and specific compounds were contributing to the antioxidant activity. Putative identification
94 of compounds with the major contribution to the antioxidant activity of the extract was
95 carried out.

96 2. Materials

97 2.1 Wine Lees

98 Aging wine lees were provided by the winery *Grupo Matarromera* (41° 38′ 33″ N,
99 4° 17′ 28″ W) after a 12 months aging step of a red wine in American oak barrels. The wine
100 lees were recovered from the bottom of the barrels during the decanting process. The grapes
101 used in the vinification process (*Vitis vinifera*, variety *Tempranillo*) were cultivated in a clay
102 soil in *Valbuena de Duero, Ribera de Duero* Designation of Origin (*Castilla y León*), in
103 2013. The average ambient temperature during this year in the vineyard was 11ºC, the

104 average precipitations were 11 mm and the middling humidity was 32%. Wine lees were
105 centrifuged, (Avanti J-26 XPI with a rotor type *JA-10*) for 90 minutes at 10,000 rpm. The
106 moisture content of the solid phase was 75%. Afterwards, it was freeze-dried for 48 hours
107 (Micro Modulo EDWARDS) and kept isolated from light at ambient conditions. These
108 lyophilized lees were used for further extractions and characterization.

109 **2.2 Reagents**

110 Chemicals used for extractions methodologies were: bidistilled water (Milli-Q®
111 Integral), EtOH absolute grade anhydrous >99.9% was purchased from CARLO ERBA
112 Reagents, methanol absolute 99.99% was from Fisher Scientific (Waltham, MA, USA),
113 acetone with a purity of ≥ 99.5% was from Sigma-Aldrich and citric acid from Sigma-
114 Aldrich (St Quentin Fallavier, France).

115 For phytochemical total phenolic content: sodium carbonate (Na_2CO_3) was from
116 Sigma-Aldrich (St Quentin Fallavier, France), Folin-Ciocalteau reagent was from Panreac
117 (Barcelona, Spain) and gallic acid was from Fluka (Germany).

118 Chemicals used for antioxidant activity assays were: 2`,2`- Azobis (2-
119 amidinopropane) dihydrochloride (AAPH), 6- hydroxy-2,5,7,8-tetramethylchroman-2-
120 carboxylic acid (Trolox), caffeic acid ($C_9H_8O_4$), cobalt fluoride tetrahydrate (CoF_2),
121 hydrogen peroxide (H_2O_2) and picolinic acid ($C_6H_5NO_2$) from Sigma-Aldrich (St Quentin
122 Fallavier, France) and iron chloride ($FeCl_3$) from Riedel-de-Haën (Seelze, Germany).
123 Disodium fluorescein (FS) was from TCI Europe (Antwerp, Belgium). Sodium nitrite
124 ($NaNO_2$ >99%) was purchased from Riedel-de Haen, aluminum chloride ($AlCl_3$ >97) and
125 sodium acetate trihydrate ($C_2H_3NaO_2$ x $3H_2O$ >99%) were acquired from Sigma-Aldrich.

126 HPLC analysis were performed using formic acid 98% PA-ACS,
127 Panreac®(Barcelona, Spain), acetonitrile for HPLC Plus Gradient-ACS+Reag. Ph. Eur.-
128 Reag. USP. Carlo Erba (Val de Reuil, France) and Milli-Q® water (Milli-Q® Integral).

129

130 ## 3 Experimental procedure and analytical methods

131 ### 3.1 Solid-liquid extractions

132 Different solvents were selected to perform the extraction experiments: distilled
133 water, ethanol, acetone, methanol and two mixtures of ethanol:water (50:50 and 75:25 v/v).
134 These extractions were carried out using the same solid:liquid ratio of 1:40 (0.25 g of dry
135 lees in 10 mL of solvent), stirring for 5 min at room temperature followed by 10 min of
136 sonication in a ELMA Transsonic 700/H bath. Afterwards, sample extracts were
137 centrifuged in a Hettich MiKro 220R at 6,000 rpm during 5 min. Supernatants were
138 separated, filtered with PVDF (Polyvinylidene difluoride) filters with a pore size of 0.22 µm
139 and kept at 4 ºC until analysis. In order to express the analytical results in "grams per dry
140 extract" (g_{DE}), sample extracts were evaporated until dryness, using a vacuum centrifuge
141 (Centrivap concentrator, Labconco, Kansas City, MO, USA) with a MD 4C NT vacuum
142 pump (Vacuubrand, Wertheim, Germany).

143

144 ### 3.2 Extracts characterization

145 #### 3.2.1 Total Phenolic Content (TPC)

146 The total polyphenol content was measured by the Folin-Ciocalteu colorimetric method
147 according to the procedure described by *T.Serra et al.* (Serra et al., 2008), which was
148 adapted for the microplate Spectrophotometer (Genesys™ 10UV, ThermoFischer
149 Scientific). The results of TPCs were calculated using a calibration curve for gallic acid
150 (between the range of 50-800 $ppm_{GALLIC\ ACID}$) *(Equation 1)*:

151 $$y = 0.0009x - 0.0133; R^2 = 0.997 \qquad (Eq.1)$$

152 where *'y'* is absorbance at 765 nm and *'x'* concentration of gallic acid in mg/L. TPCs were
153 expressed in mg of gallic acid equivalents (GAE) per gram of dry extract (mg_{GAE}/g_{DE}) ±
154 SD.

155

156 ### 3.2.2 Total Flavonoid Content (TFC)

157 The flavonoid content of the different extracts was also measured as described by *Michalska*
158 *et al.* (Michalska, Cieglińska, & Zieliński, 2007) with a modification, concerning the volume
159 of the reagents used in order to work with a 96 microplate for the microplate
160 Spectrophotometer (Genesys™ 10UV, ThermoFischer Scientific). Absorbance was read at
161 510 nm. The results of TFCs were calculated using a calibration curve for catechin (between
162 the range of 0-1000 $ppm_{CATECHIN}$ *(Equation 2)*:

163 $$y = 2.0421x - 0.0229; R^2 = 0.999 \qquad (Eq.2)$$

164 where *'y'* is absorbance at 510 nm and *'x'* concentration of catechin in mg/L. TFCs were
165 expressed in mg of catechin equivalents (CATE) per gram of dry extract (mg_{CAET}/g_{DE}) ±
166 SD.

167 ### 3.2.3 HPLC-DAD (High Performance Liquid Chromatography)

168 The High Performance Liquid Chromatography (HPLC) system used was a Thermo
169 Finnigan (Surveyor model) equipped with an autosampler, a pump and a photodiode-array
170 detector (PDA). A pre-column (100RP-18, 5µm) and a reversed phase C18 column
171 (LiCrospher® 100 RP-18, 250x4mm; 5µm) in a thermostated oven at 35 ºC were used for
172 separation using a gradient elution, adapted from (Csiktusnádi Kiss et al., 2000), using water
173 acidified with formic acid at 0.5% (v/v) as solvent A and 90% acetonitrile as solvent B. The
174 flow rate was 0.3 mL/min with an injection volume of 20 µL. The linear solvent gradient
175 was as follows: 0 min, 94.4% A; 15 min, 83.3% A; 20 min, 77.8% A maintained for 10 min;
176 55 min, 66.7% A; 80 min, 44.4% A; 120 min, 0% A maintained for 15min; 140 min; 94.4 %
177 A constant for 10 min. The data acquisition systems was the Chromquest version 4.0
178 (ThermoFinnigan—Surveyor, San Jose, CA, USA). Absorption spectra were acquired from
179 210 to 600 nm by a photodiode array detector. Semi-quantitative evaluation of detected

compounds was expressed as the area percentage of each peak respect to the total area of the chromatogram at 280nm and 520 nm, which are the general wavelength for polyphenols and the specific wavelength for anthocyanins, respectively.

3.2.4 HPLC-MS/MS (High Performance Liquid chromatography–mass spectrometry)

The system used was a liquid chromatography Waters Alliance 2695 Separation Module (Waters®, Ireland) consisting on a system of quaternary pumps, degasificator, autosampler and a column furnace. The mass spectrometer (MS/MS) used was a MicroMass Quattromicro® API (Waters®, Ireland). For the data acquisition and processing MassLynx® 4.1 software was employed. Chromatographic separation of compounds was carried out on a LiChrospher® 100 RP-18 (250 x 4.0mm) column in an oven at 35 ºC. Chromatographic separation of compounds was carried out in a reversed-phase LiChrospher® 100 RP-18 5µm LiChroCART® 250-4 column inside a thermostated oven at 35ºC. The mobile phase consisted of formic acid (0.5% v/v in ultrapure water) (eluent A) and acetonitrile (eluent B). The gradient program used was 99:1 A:B for 5 min, from 99:1 A:B to 40:60 A:B in 40 min, from 40:60 A:B to 10:90 A:B in 45 min, held isocratically (90% B) for 10 min, from 10:90 A:B to 99:1 A:B in 10 min, and finally held isocratically (99:1 A:B) for 10 min, at a flowrate of 0.3 mL/min, with an injection volume of 20 µL. Total run time was 120 min. Absorption spectra were acquired from 210 to 600 nm by a photodiode array detector. AC were monitored at 520 nm, flavonols at 360 nm, phenolic acids at 320 nm, and phenolic compounds in general at 280 nm. Mass spectrometry was performed using an electrospray ion source in negative ion mode (ESI-). The ion source temperature was 120°C, the capillary voltage was 2.5 kV, and the source voltage was 30 V. Compounds separated by HPLC were ionized and the mass spectra were recorded in a full scan mode, between *m/z* 100 and 1500. High purity nitrogen was used as drying and

205 nebulizing gas, and ultrahigh purity argon was used as collision gas. Different collision
206 energy values were used in fragmentation experiments.

207

208 **3.3 Evaluation of the Antioxidant activity (AA)**

209 **3.3.1 ORAC (Oxygen Radical Absorbance Capacity)**

210 Oxygen Radical Absorbance Capacity (ORAC) is a method for the evaluation of
211 antioxidative ability of a specific substance based on the fluorescence quenching of
212 fluorescein sodium (FS) salt after exposure to AAPH (2,2-azobis(2-amidino-propane)
213 dihydrochloride), which generates oxygen radicals ($ROO^{\bullet}$) at a constant rate. ORAC assay
214 was carried out by the method described by *Feliciano et al.* (Feliciano et al., 2009) who
215 included some modifications for the FL800 microplate fluorescence reader (Bio-Tek
216 Instruments, Winooski, VT, USA). ORAC values were calculated using a regression
217 equation between the Trolox concentration and the area under the decay of the FS curve
218 (AUC) according to the calibration curve for Trolox (between the range of 5-40
219 $\mu mol/L_{TROLOX}$) *(Equation 3)*:

220 $$y = 0.4328x - 0.7811; R^2 = 0.9931 \qquad (Eq.3)$$

221 where *'y'* is the net AUC and *'x'* concentration of Trolox in µmol/L. The results are given in
222 µmol of Trolox equivalents (TE) per g of dry extract ($\mu mol_{TE}/g_{DE}$) ± SD.

223

224 **3.3.2 HORAC (Hydroxyl Radical Averting Capacity)**

225 Hydroxyl radical averting capacity (HORAC) is an antioxidant method able to measure the
226 capability of a substance to neutralize the hydroxyl radical ($HO^{\bullet}$) generated by Fenton-like
227 reactions employing a Co(II) complex using FS as a probe. HORAC assays were performed
228 by the method developed by *Ou et al.* (Ou et al., 2002) modified for the FL800 microplate
229 reader and tested successfully in more publications (Serra, Duarte, Bronze, & Duarte, 2011).

230 HORAC values were calculated using a regression equation between the caffeic acid
231 concentration and the area under the decay of the FS curve (AUC) according to the
232 calibration curve for caffeic acid (between the range of 0-250 μmol/$L_{CAFEIC\ ACID}$) *(Equation
233 4)*:

234 $$y = 0.0685x - 2.9112; R^2 = 0.983 \qquad (Eq.4)$$

235 where *'y'* is the net AUC and *'x'* concentration of cafeic acid in μmol/L. The results are
236 expressed in μmol of equivalents of caffeic acid (CAE) per g of dry extract ($\mu mol_{CAE}/g_{DE}$) ±
237 SD.

238 **3.3.3 HOSC (Hydroxyl Radical Scavenging Capacity)**

239 Hydroxyl Radical Scavenging Capacity (HOSC) is another method which also uses FS as a
240 probe in order to evaluate the hydroxyl radical scavenging ability of a substance in a classic
241 Fenton reaction with Fe^{+3} and H_2O_2 as a source of hydroxyl radicals. The assay was carried
242 out by the model described by *Moore et al*. (Moore, Yin, & Yu, 2006). HORAC values were
243 calculated using a regression equation between the Trolox concentration and the area under
244 the decay of the FS curve (AUC) according to the calibration curve for Trolox (between the
245 range of 0-30 μmol/L_{TROLOX}) *(Equation 5)*:

246 $$y = 0.7896x - 0.0158; R^2 = 0.997 \qquad (Eq.5)$$

247 where *'y'* is the net AUC and *'x'* concentration of Trolox in μmol/L. The results are given in
248 μmol of Trolox equivalents (TE) per g of dry extract ($\mu mol_{TE}/g_{DE}$) ± SD.

249

250 **3.3.4 FRAP (Ferric Reducing Antioxidant Power)**

251 The FRAP assays has been compared with other antioxidant capacity methods as it is
252 capable to reveal substances that can reduce Fe^{+3} to Fe^{+2}. FRAP assays were carried out by
253 the protocol suggested by *Bolanos de la Torre et al*. (Bolanos de la Torre, Henderson,
254 Nigam, & Owusu-Apenten, 2015). Absorbance was measured at 593 nm in a

255 spectrophotometer (ThermoSpectronic Genesys 10 μV). The FRAP results were calculated
256 according to the calibration curve for Trolox (between the range of 0-600 $\mu mol/L_{TROLOX}$)
257 *(Equation 6)*:

258 $$y = 0.0015x + 0.5585; R^2 = 0.998 \qquad (Eq.6)$$

259 where y is absorbance at 593 nm and *'x'* concentration of Trolox in μmol/L. Results are
260 shown in μmol of Trolox equivalents (TE) g of dry extract ($\mu mol_{TE}/g_{DE}$) ± SD.

261 **3.4 Correlation data treatment**

262 A correlation study using Excel 2013 was performed. Pearson´s regression coefficient
263 '*r*' with P-value was selected. The correlation coefficient '*r*' is employed to assess if two
264 different variable are associated and the p-value is used to quantify the idea of statistical
265 significance of evidence in the context of null hypothesis. A 95% confidence interval for
266 the correlation coefficient was chosen, which means that if the probability is lower than
267 5% ($p<0.05$), the correlation coefficient is statistically significant, according to the t-
268 Student distribution. This correlations was performed between the areas of all detected
269 peaks in the chromatograms at 280 nm with TPC, TFC and the different AA tests for
270 each solvents. Among all peaks, only 11 compounds were selected since they had a '*r*'
271 higher than |0.90|.

272

273 **3.5 Statistical Analysis**

274 All data were expressed as means ± standard deviations (SD). Assays for TPC, TFC
275 and AA measurements were performed, at least, in triplicate. A statistical analysis was done
276 using SigmaStat 3.0® software. These analyses were performed to study if each individual
277 solvent had a statistically significant effect on the measured variables that characterize the
278 extracts (TPC, TFC, ORAC, HORAC, HOSC and FRAP). All values were tested for normal
279 distribution and equal variance. When homogeneous variances were confirmed, data were

280 analyzed by One Way Analysis of Variance (ANOVA) coupled with the post-hoc Holm–
281 Sidak test ($p<0.05$ was accepted as statistically significant in all cases).

282

283 ## 4. Results and discussion

284 For years, the phenolic composition of samples has been determined using
285 spectrophotometric methodologies that are useful for a rapid screening of a large number of
286 samples, and are not particularly expensive. However, they are not able to obtain selective
287 information since results may be influenced by other components present in the samples.
288 Chromatography and mass spectrometry have become important tools for characterization
289 purposes. In the present work wine lees extracts were prepared using different solvents and
290 were analyzed using the methodologies described, in order to characterize their phenolic
291 content.

292 ### 4.1 TPC (Total Phenolic Content) and TFC (Total Flavonoid Content)

293 The solubility of the phenolic compounds into different solvents, which is related
294 with the solvent polarity used (Rocío Teruel, Garrido, Espinosa, & Linares, 2015), plays a
295 major role in the recovery of polyphenols from different sources. Results from the total
296 phenolic content (TPC) for the different extracts prepared in this work are presented in
297 Table 1 and they range from 26±1 mg_{GAE}/g_{DE} to 254±24 mg_{GAE}/g_{DE} (3.6 $mg_{GAE}/g_{DRY\ LEES}$)
298 depending on the solvent used. Water, ethanol and acetone barely extracted the phenolic
299 compounds present in the wine lees, compared with methanol and the mixtures of
300 ethanol:water. Usually mixtures of ethanol:water present better extracting power for these
301 type of compounds and in our case, the mixture corresponding to the ratio 75:25, was the
302 best one with a value of 254±24 mg_{GAE}/g_{DE}. This value was similar to those obtained by
303 *Jia-Jiuan Wu et al.*, who reported a 21% (w/w) recovery of the initial dried wine lees from a
304 Taiwan grape variety with a Soxhlet extraction using 70% (vol.%) aqueous ethanol solution
305 for 6 hours (Wu et al., 2009). In our case, we were able to extract 25% (w/w) of the initial

306 wine lees with a dramatic reduction of time (345 min *vs* 15 min). On the contrary, much
307 higher results were obtained by *Pérez-Serradilla et al*. (Pérez-Serradilla & Luque de Castro,
308 2011). They performed a Soxhlet extraction with a 75:25 EtOH:H_2O (%v/v) from dried
309 Syrah grape variety wine with a solid-liquid ratio of 1/10 lees, during 24 hours, and obtained
310 an extract with 547 mg_{GAE}/g_{DE}. Also *Landeka et al.* (Landeka et al., 2017) described an
311 acidified methanolic wine lees extract from a Bosnia and Herzegovina variety, with a TPC
312 of 23.16 $mg_{GAE}/g_{DRY\ LEES}$. All these extracts were obtained for dry wine lees. The expected
313 recovery using wet wine lees is lower, according to Dimou *et al*. (Dimou et al., 2016). They
314 carried out a simulation of a global valorization process of wet wine lees, from *Merlot*
315 variety grape, and proposed a recovery of antioxidants of only 0.8 % (w/w) by conventional
316 solid liquid extraction with a 70:30 EtOH:H_2O (%vol.), based on lab-scale experiments.

317 The total flavonoid content (TFC) presented a similar behavior as the TPC and
318 values ranged from 16±1 to 146±5 mg_{CATE}/g_{DE}. Higher flavonoid content was obtained with
319 methanol and the mixtures of ethanol:water as shown in Table 1. Acetone, ethanol and water
320 were the solvents with less capacity to extract all the phenolics and flavonoid family.

321 **4.2 Antioxidant activities (AA)**

322 The values obtained for the different antioxidant activities (AA) of the extracts are
323 shown in Table 1. The ethanol:water extracts had higher antioxidant capacities than the rest
324 of the extracts, especially the 75:25 EtOH:H_2O (v/v) mixture. This behavior agrees with the
325 total phenolic concentration: the higher the TPC values, the higher the antioxidant activities
326 (Orak, 2007). However, this tendency was not shown for the ORAC assay where the highest
327 antioxidant activity was found for the methanol extract with 2,771±289 $\mu mol_{TE}/g_{DE}$. These
328 ORAC values were lower compared to 6,100 $\mu mol_{TE}/g_{DE}$ obtained by *Pérez-Serradilla et*
329 *al*. (Pérez-Serradilla & Luque de Castro, 2011) for a wine lees extract prepared from *Syrah*
330 red grapes using a Soxlhet and a 75% ethanol (%, v/v.) aqueous solution with a solid-liquid
331 ratio of 1:10. These differences between extracts may be explained by the different TPC

332 values that was much higher (547 mg_{GAE}/g_{DE}) than ours (254±24 mgGAE/g DE), as
333 previously mentioned). Our ORAC values are also comparable with the one obtained with
334 an extract of grape marc with an ORAC value of 2,644 $\mu mol_{TE}/g_{DE}$ and a TPC of 222
335 mg_{GAE}/g_{DE}. This extract was prepared by traditional solid-liquid extraction of grape marc
336 with a solid-liquid ratio 1:2 (g/mL) at a temperature of 60ºC for a period of 3 hours, using a
337 mixture 50:50 EtOH:H_2O (%vol.) (Moro Gonzaléz, 2009).

338 Concerning FRAP assays, values for aging wine lees extracts ranged from 362± 6 to
339 2,197± 84 $\mu mol_{TE}/g_{DE}$ (44 ± 1 to 583 ± 18 $\mu mol_{TE}/g_{DRY\ LEES}$). These values were similar to
340 those found in the literature for other wine lees waste extracts. For example, *Landeka et al.*
341 (Landeka Jurčević et al., 2017) who obtained a wine lees extract from a Bosnia and
342 Herzegovina winery with a TPC value of FRAP values of 457 $\mu mol_{TE}/g_{DRY\ LEES}$.

343 With the HORAC and HOSC assays, the highest values were obtained for the 75:25
344 EtOH:H_2O (%vol.): 4,690 ± 463 $\mu mol_{CATE}/g_{DE}$ and 4,527 ± 413 $\mu mol_{TE}/g_{DE}$ respectively.
345 These values cannot be compared due to the absence of literature concerning these type of
346 assays for wine lees, wines or grape extracts. However, some works used HORAC assay to
347 measure the capacity against hydrophilic chain-breaking hydroxyl radicals of other red
348 berries. That was the case of *Matias et al.* (Matias et al., 2016) whose cherry extracts
349 presented a higher HORAC value (6874 ± 584 $\mu mol_{CATE}/g_{DE}$) than ours. These differences
350 may result from the different types and concentrations of anthocyanins and phenolic acids
351 found in cherry extracts.

352 Correlations between TPC and TFC values from the six extracts and the AA values
353 are shown in Table 3. A graphic example of these correlations is shown in Figure 1S in
354 Supplementary Material. High positive statistically significant correlations ($r > 0.90$) were
355 found for HORAC, HOSC and FRAP assays. Strong correlations between FRAP and total
356 phenolics and flavonoids have been reported in the literature (Arnous, Makris, & Kefalas,
357 2002; Doshi, Adsule, Banerjee, & Oulkar, 2015). However, ORAC values did not correlate

358 with TPC and TFC ($r \leq 0.665$) since the highest value was achieved for methanolic extract
359 and not for the hydroalcoholic mixture (75:25). This different trend may be explained by the
360 ability of methanol to extract other molecular entities than polyphenols or even by
361 synergetic effects between the main compounds extracted which may potentiate the
362 scavenging of peroxyl free radicals.
363

364 **4.3 Analysis by HPLC –DAD and HPLC-MS/MS**

365 The chromatographic profiles of the extracts were compared using reverse phase
366 chromatography and detection at 280 nm. The employed method has a good repeatability
367 with a variation between 2-5% in peak areas and lower for retention time. Peak area of
368 detected compounds was measured, as well as the total area (TA) of the chromatogram at
369 280 nm (maximum absorption for phenolic compounds) and 520 nm (maximum absorption
370 for anthocyanins) to carry out the correlation with antioxidant activity values. TA values for
371 each extract are shown in Table 1. These values were important to consider, as it might
372 represent the real value of phenolic content since the interferences that occur in the
373 spectrophotometric TPC measurement, were avoided in the chromatographic analysis. The
374 chromatographic profiles from the different extracts analyzed were according to data
375 obtained for TPC and TFC: water, ethanol and acetone extracts chromatograms showed that
376 these solvents were less efficient in the extraction of phenolic compounds from aging wine
377 lees, comparing to MeOH, 50:50 and 75:25 EtOH:H_2O (%vol.) mixtures, being these
378 mixtures diluted twofold. Figure 1 illustrates the chromatographic profiles obtained for
379 aging wine lees extracts with methanol and the two hydro-alcoholic mixtures at 280 nm.
380 Chromatographic profiles for acetone, ethanol and water extracts can be seen in
381 *Supplementary Material*.
382
383

384 #### 4.3.1 Compounds contributing to antioxidant activity

385 Compounds from methanol and hydroalcoholic mixtures were putatively identified
386 by HPLC-MS/MS (Figure 1 and Table 2). All the peaks where present in the three extracts.
387 The main compounds found were anthocyanins, and all the compounds were already
388 reported for red wines (Arnous et al., 2002; Bravo, Silva, Coelho, Boas, & Bronze, 2006;
389 Cantos, Espín, & Tomás-Barberán, 2002) and in wine lees residues (Delgado de la Torre,
390 Priego-Capote, & Luque de Castro, 2015).

391 Even though a large number of peaks were detected in the HPLC chromatograms,
392 only those peaks which showed r values ≥|-0.900| between antioxidant activity and peak
393 areas were considered for discussion, as shown in Table 4.

394 Anthocyanins were the majority of the identified compounds, being malvidin 3-O-
395 glucoside (*7*) and malvidin 3-(6-p-coumaroylglucoside) (*12*) the most concentrated in all
396 extracts as it is shown in Table 2. Most of the compounds were in higher concentration in
397 the hydroalcoholic mixtures, as expected. For example, anthocyanins such as *(5)* petunidin-
398 3-O-glucoside, *(9)* delphinidin 3-(6-p-coumaroylglucoside) and *(10)* petunidin-3-(6-p-
399 coumaroylglucoside) were present in a higher concentration in the 50% vol. ethanol mixture
400 with a percentage of 4.3, 8.0 and 8.5, respectively. Additionally, the 75% vol. ethanol
401 mixture was richer in *(3)* delphinidin-3-O-glucoside (4%), *(6)* malvidin 3-O-glucoside
402 (11.3%) and *(11)* malvidin 3-(6-p-coumaroylglucoside) (15.5%) anthocyanins. In contrast, a
403 different tendency was observed for quercetin-3-glucuronide *(7),* a flavonol that was at
404 higher levels in the MeOH extract (11.7%) than in the 50:50 hydro-alcoholic mixture (5.8%)
405 and similar to the 75:25 hydroalcoholic mixture (10.6%). The other flavonol, *(8)* myrecitin
406 was present in smaller amounts in each extract, being higher for the 75% vol. ethanol
407 mixture. Both flavan-3-ols, *(2)* catechin and *(4)* epicatechin, were found in higher quantities
408 in 50% vol. ethanol extract with 2.7% and 4.4%, respectively.

409 Furthermore, it was possible to establish which compounds or family of compounds
410 contributed to each AA assay and which type of oxygen radicals are affected. Gallic acid *(1)*
411 was the only benzoic acid identified. It presented a statistically significant negative
412 correlation with ORAC and a statistically significant positive correlation with FRAP (-
413 *0.896*, $p<0.050$; *896*, $p<0.05$ respectively*)*. This tendency is explained by the high
414 scavenging power of gallic acid, making it capable of rapidly deactivating a wide variety of
415 radicals via electron transfer (Marino, Galano, & Russo, 2014).

416 Flavan-3-ol presented statistically significant negative correlations with ORAC *(-*
417 *0.840 and -0.947,* $p<0.05$ for catechin *(2)* and epicatechin *(4)*, respectively). Moreover, both
418 showed statistically significant positive correlations with FRAP *(0.940* for *(2)* catechin *and*
419 *0.828,* $p<0.05$ *for* epicatechin *(4)*. However, only catechin *(2)* had a statistically significant
420 positive correlation with HOSC *(0.841,* $p<0.05$*)*. These observations are consistent with
421 published data. It has been strongly substantiated that flavanols, namely catechins and
422 proanthocyanidins, are powerful radical quenchers in various systems (Arnous et al., 2002;
423 Kallithraka, Mohdaly, Makris, & Kefalas, 2005)**.**

424 For the case of flavonols, both identified compounds showed positive correlations
425 but for different assays. Quercetin -3-O-glucuronide *(7)* showed a statistically significant
426 correlation with ORAC *(0.998,* $p<0.05$) and HORAC *(0.815,* $p<0.05$). On the contrary,
427 myricetin *(8)* registered an r value of *0.971 and 0.999 (*$p<0.05$*)* for HOSC and FRAP,
428 respectively.

429 These differences observed between assays are related to the individual molecular
430 structure of each compound. It must be borne in mind that each assay is a measure of the
431 antioxidant activity but using different radicals. Thus, stereoisomerism, functional groups
432 distribution and any other structural parameters such as the oxidation state of the C-ring, the
433 hydroxylation and methylation pattern also are expected to affect the final value (Frankel,
434 Waterhouse, & Teissedre, 1995; Kallithraka et al., 2005). Furthermore, it has also been

435 demonstrated that the substitution of a 3-hydroxyl for a sugar group influences the
436 antioxidant ability of flavonols, decreasing it in a 10-15% (Gardner, McPhail, Crozier, &
437 Duthie, 1999). Thus, the same behavior is expected for the rest of polyphenol families if this
438 substitution takes place.

439 Anthocyanins' contribution seemed to have a completely different effect depending
440 on the method used to measure the AA. For instance, for ORAC and HORAC, the effect was
441 negative while for HOSC and FRAP was positive. However, not all of them were
442 statistically significant. Just petunidin-3-O-glucoside *(5)*, delphinidin 3-(6-p-
443 coumaroylglucoside) *(9)* and petunidin-3-(6-p-coumaroylglucoside) *(10)* displayed
444 statistically significant *r* values for ORAC (*-0.951, -0.912* and *-0.889*, $p<0.05$, respectively*)*.
445 For FRAP, the result was always statistically significant ($r \geq 0.821$, $p<0.05$). Nonetheless,
446 for HOSC, the significance was only ensured for delphinidin-3-O-glucoside *(3)*, malvidin 3-
447 O-glucoside *(6)* and malvidin 3-(6-p-coumaroylglucoside) *(11)* ($r \geq 0.921$, $p<0.05$).

448 Since anthocyanins are the main polyphenols found in wine lees residues,
449 correlations between the peak areas of the identified anthocyanins at 520 nm (maximum
450 absorbance of anthocyanins) and each AA were performed. This wavelength was used to
451 isolate anthocyanins from other possible compounds that can co-elute and can be detected at
452 280 nm. The *'r'* values are listed in Table 5 and, in this case, they showed the same behavior
453 as described in the previous paragraph: negative correlations for ORAC and HORAC,
454 positive correlations for HOSC and FRAP. These individual analyses provided a more
455 accurate pattern regarding significance. All anthocyanins became statistically significant for
456 ORAC ($r \geq |0.824|$, $p<0.05$). Delphinidin 3-(6-p-coumaroylglucoside) *(9),* petunidin-3-(6-p-
457 coumaroylglucoside) *(10)* and malvidin 3-(6-p-coumaroylglucoside) *(11)* were also
458 statistically significant for FRAP ($r \geq |0.888|$, $p<0.05$). Furthermore, malvidin 3-(6-p-
459 coumaroylglucoside) *(11)* had a statistically significant behavior for HOSC *(0.856,* $p<0.05$*)*
460 too. For other anthocyanin/assay, results were not statistically significant.

461 With this new pattern is possible to establish a relation between the significance and
462 individual molecular structure of anthocyanins. Those anthocyanins which have a -3-O-
463 glucoside moiety, negatively contribute to ORAC. Thus, these compounds unsuccessfully
464 scavenge $ROO^{\bullet}$. Also anthocyanins with the 6-p-coumaroyl moiety negatively contribute to
465 ORAC. Nevertheless, they displayed positive statistically significant correlations with
466 FRAP, corroborating they are capable of quenching Fe^{+3} and $HO^{\bullet}$ radicals generated from a
467 Fenton reaction by hydrogen transfer atom (HAT) mechanism with Fe^{+3} (Li et al., 2017).

468 Moreover, it is worth mentioning that correlations, either positive or negative,
469 between anthocyanins and AA were found. Even though most of the researchers concurred
470 that the different antioxidant potential is strongly dependent from total phenolic and flavanol
471 contents, a lot of controversy appears when talking about anthocyanins. Some previous
472 works established there is no relation between ORAC and anthocyanins (Sólyom, Solá,
473 Cocero, & Mato, 2014) or poor correlations (Arnous et al., 2002), but others found strong
474 correlations between AC content and AAs like in our case. As an example, *Moyer et al.*,
475 (Richard A. Moyer, Kim E. Hummer, Chad E. Finn, Balz Frei, & Ronald E. Wrolstad, 2001)
476 whose work reported statistically significant correlations between AC content and ORAC $(r$
477 $\geq |0.460|$, $p<0.005)$ and FRAP $(r \geq |0.440|$, $p<0.005)$.

478

479 ## 5. Conclusions

480 In this work aging wine lees, an underexploited waste stream from the winemaking
481 process, is proposed as an alternative source of phenolic compounds as its extracts could be
482 used as antioxidant additives. An extraction procedure with six solvents with different
483 polarities (water, acetone, methanol, ethanol and two hydro-alcoholic mixtures) was
484 established in order to characterize this raw material in terms of phenolic composition and
485 antioxidant activity, thus providing an important contribution for the valorization of this
486 biomass. It was found that the recovery of phenolic compounds from this raw material is

487 higher (254±24 $mg_{GAE/DE}$) when a mixture of 75:25 (v/v) of EtOH:H_2O is used. Also
488 promising results were obtained for the different antioxidant activities assays. This hydro-
489 alcoholic mixture was also the most advantageous solvent to provide positive antioxidant
490 capacities for HORAC, HOSC and FRAP (4,690 μmol $_{CATE}$/g $_{DE}$, 4,527 μmol $_{TE}$/g $_{DE}$, 2,197
491 μmol $_{TE}$/g $_{DE}$, respectively), meanwhile the methanol extracts showed the highest ORAC
492 value (2,771±289 μmol $_{TE}$/ g $_{DE}$). In addition, a correlation between different antioxidant
493 activities, total phenols and identified compounds was demonstrated. It could be asserted
494 that anthocyanins were the major compounds present in the wine lees extracts. They
495 significantly contribute to ORAC in a negative way. Those of them which presented the 6-p-
496 coumaroyl moiety strongly contribute to FRAP, as well as for gallic acid and both flavan-3-
497 ols detected. Depending on the solvent used different amounts of the individual compounds
498 are extracted which could have higher or lower activity against oxygen radicals ($ROO^{\bullet}$) or
499 ($HO^{\bullet}$) affecting the antioxidant capacity estimation.
500

501 ***Acknowledgements***

502 The authors thank the Marie Curie Industry-Academia Partnerships and Pathways
503 (FP7-PEOPLE-2013-IAPP-612208) actions for funding. This project was carried out in
504 collaboration with the Instituto de Biologia Experimental e Tecnológica iBET (Portugal),
505 Feyecon (The Netherlands) and Bodegas Matarromera (Spain). Soraya Rodríguez Rojo
506 acknowledges Junta de Castilla y Leon and FEDER 2014-2020 for her postdoctoral contract
507 under Project VA040U16. Rut Romero Díez thanks Junta de Castilla y León for her research
508 fellowship. Ana A. Matias thanks FCT for the financial support through the IF Starting Grant –
509 GRAPHYT (IF/00723/2014). We acknowledge also the financial support from Fundação
510 para a Ciência e Tecnologia and Portugal 2020 to the Portuguese Mass Spectrometry
511 Network (LISBOA-01-0145-FEDER-402-022125.

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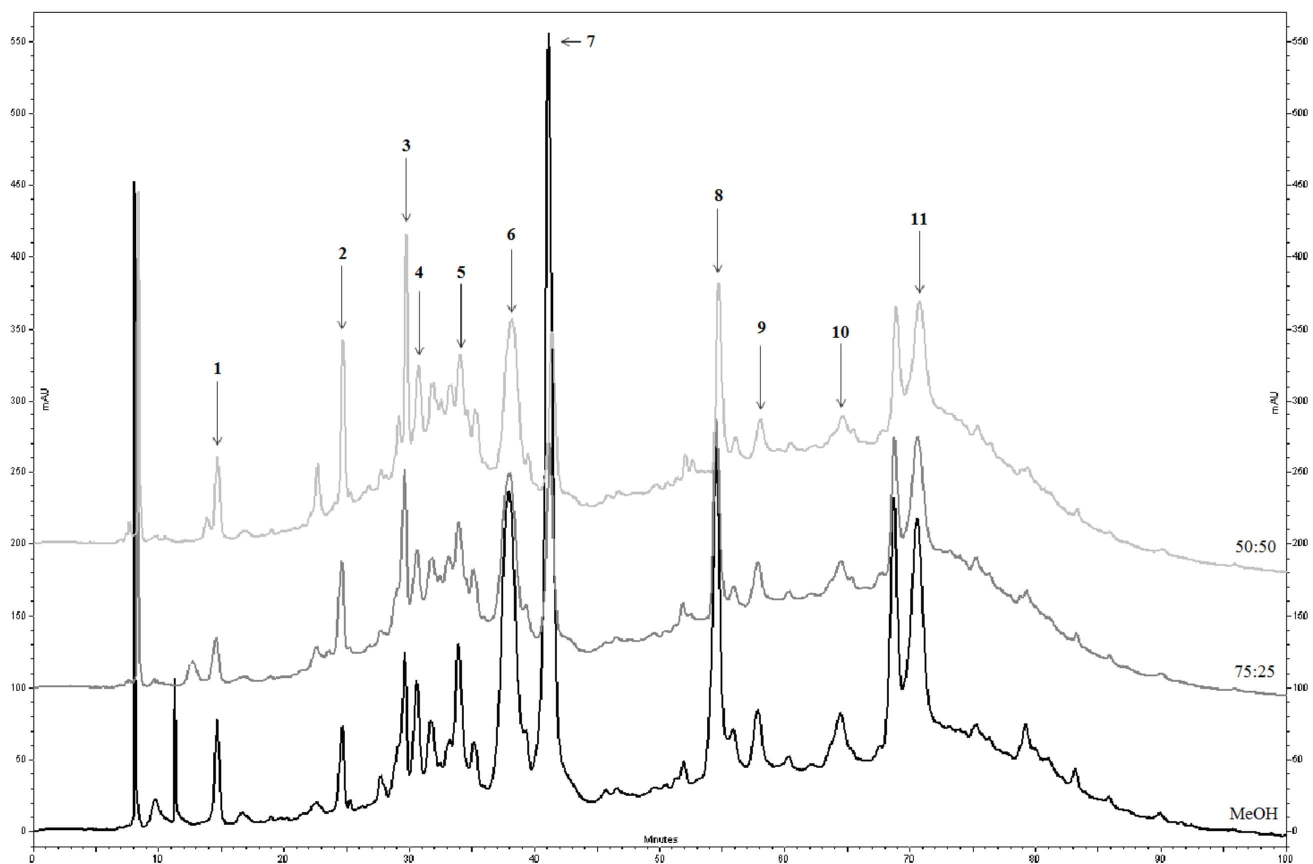
672

FIGURE CAPTIONS

Figure 1: Chromatograms at 280 nm obtained for methanolic and hydro-alcoholic extracts of aging wine lees.

FIGURES

Figure 1



TABLES

Table 1: Total phenolic and flavonoid content and antioxidant activity of aging wine lees extracts obtained with different solvents. The highest values (per g of dry residue) are presented in bold and the lowest values in italics. Values with different lowercase letters in the same column are significantly different ($P<0.05$).

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	TPC mg $_{GAE}$ /g$_{DE}$	**TFC mg $_{CAT}$ /g $_{DE}$**	**ORAC µmol $_{TE}$ /g $_{DE}$**	**HORAC µmol $_{CAT}$ /g $_{DE}$**	**HOSC µmol $_{TE}$ /g $_{DE}$**	**FRAP µmol $_{TE}$ /g $_{DE}$**	**TA 280nm**	**TA 520nm**
H_2O	38 ± 3^{a}	16 ± 1^{a}	471 ± 86^{a}	348 ± 35^{a}	592 ± 39^{a}	461 ± 3^{a}	$8.68 \cdot 10^{7}$	$2.04 \cdot 10^{7}$
EtOH	94 ± 8^{b}	51 ± 18^{b}	1,603 ± 227^{b}	1,245 ± 103^{b}	2,107 ± 134^{b}	1,034 ± 26^{b}	$6.95 \cdot 10^{7}$	$1.90 \cdot 10^{7}$
Acetone	26 ± 1^{a}	30 ± 3^{a}	217 ± 68^{a}	543 ± 59^{a}	281 ± 26^{a}	362 ± 6^{a}	$2.30 \cdot 10^{7}$ *	$2.40 \cdot 10^{6}$ *
MeOH	149 ± 7^{c}	112 ± 12^{c}	**2,771 ± 289^{c}**	3,963 ± 367^{c}	2,732 ± 257^{c}	1,542 ± 38^{c}	$2.72 \cdot 10^{8}$	$1.12 \cdot 10^{8}$
EtOH:H_2O (50:50)	206 ± 28^{d}	145 ± 6^{d}	1,003 ± 90^{d}	2,985 ± 389^{d}	3,912 ± 310^{d}	2,112 ± 65^{d}	**$3.13 \cdot 10^{8}$**	$1.99 \cdot 10^{8}$
EtOH:H_2O (75:25)	**254 ± 24^{e}**	**146 ± 5^{d}**	2,323 ± 289^{e}	**4,690 ± 463^{e}**	**4,527 ± 413^{e}**	**2,197 ± 84^{d}**	$2.75 \cdot 10^{8}$	**$1.77 \cdot 10^{8}$**

*The total areas for the acetone extract was calculated without taking into account the area of the acetone detected in the chromatogram (t_R ~ 15min) (vide Figure 2S from the supplementary material for more information)

Table 2: Putative identification of main compounds in the extracts. Retention time (min), maximum absorbance (nm) (λmáx), MS and MS/MS values (m/z), putative identification, phenolic family and the percentage of each peak area in the different extracts. Numbers in brackets represent the main m/z values.

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Peak nº	Retention time (min)	λmax (nm)	m/z (positive and negative mode)	[M-H] fragments (m/z)	Putative Identification	Phenolic family	Percentage (%) of each peak MeOH	50%v.	75%v.	Reference(s)
1	14.7	270	169 (M^-)	[169], 125	Gallic Acid	Phenolic acid	0.59	1.29	1.16	(Delgado de la Torre et al., 2015; Hernández, Estrella, Carlavilla, Martín-Álvarez, & Moreno-Arribas, 2006), (Bravo et al., 2006)
2	24.7	328	289 (M^-)	[289] 229, 153, 137	Catechin	Flavan-3-ol	1.10	2.67	2.57	(Delgado de la Torre et al., 2015; Hernández et al., 2006), (Cantos et al., 2002)
3	29.7	529	465 (M^+)	[465] 349, 303, 147	Delphinidin-3-O-glucoside	Anthocyanin	1.46	3.62	3.96	(Delgado de la Torre et al., 2015)
4	30.6	283	289 (M^-)	[289] 271, 227, 203, 188	Epicatechin	Flavan-3-ol	1.71	4.43	3.51	(Cantos et al., 2002; Hernández et al., 2006)
5	33.9	529	479 (M^+)	[479] 317	Petunidin-3-O-glucoside	Anthocyanin	2.42	4.27	3.71	(Delgado de la Torre et al., 2015)
6	37.9	527	493 (M^+)	[493] 331	Malvidin 3-*O*-glucoside	Anthocyanin	8.20	9.93	11.35	(Delgado de la Torre et al., 2015), (Cantos et al., 2002)
7	41.4	366	477 (M^-)	[477] 301, 151	Quercetin -3-*O*-glucuronide	Flavonol	11.72	5.75	10.64	(Oszmiański et al., 2015)

8	54.5	368	317 (M^-)	[317] 179, 151	Myricetin	Flavonol	6.25	6.23	7.20	(Delgado de la Torre et al., 2015; Hernández et al., 2006)
9	57.8	529	611 (M^+)	[611] 303	Delphinidin 3-(6-p-coumaroylglucoside)	Anthocyanin	2.94	8.07	6.84	(Hernández et al., 2006; Hokkanen, Mattila, Jaakola, Pirttilä, & Tolonen, 2009)
10	64.4	529	625 (M^+)	[625] 317	Petunidin-3-(6-p-coumaroylglucoside)	Anthocyanin	3.20	8.50	7.55	(Hernández et al., 2006)
11	70.6	530	639 (M^+)	[639] 331	Malvidin 3-(6-p-coumaroylglucoside)	Anthocyanin	7.45	13.29	15.48	(Delgado de la Torre et al., 2015)

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Table 3: Correlation (r values) between TPC, TFC and the antioxidant activity results. Values of $r > |-0.90|$ are in bold. Values with a * are statistically significant ($p < 0.05$).

	TPC	**TFC**
TPC	1.000*	
TFC	**0.970***	1.000*
ORAC	0.665	0.646
HORAC	**0.924***	**0.930***
HOSC	**0.992***	**0.960***
FRAP	**0.990***	**0.983***

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Table 4: Correlation (*r* values) between the peak areas at 280 nm (see Table 2 for identification) and the antioxidant activity results. Values of r >|-0.90| are in bold. Values with a * are statistically significant (p<0.05).

	ORAC	**HORAC**	**HOSC**	**FRAP**
1	-0.896*	-0.414	0.774	0.896*
2	-0.840*	-0.308	0.841*	**0.940***
3	-0.734	-0.139	**0.921***	**0.985***
4	**-0.947***	-0.532	0.682	0.828*
5	**-0.951***	-0.542	0.673	0.821*
6	-0.687	-0.072	**0.946***	**0.994***
7	**0.998***	0.815*	-0.351	-0.551
8	-0.619	0.018	**0.971***	**0.999***
9	**-0.912***	-0.447	0.750	0.879*
10	-0.889*	-0.399	0.784	**0.903***
11	-0.667	-0.046	**0.954***	**0.997***

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682 Table 5: Correlation (*r* values) between the peak areas at 520nm for anthocyanins (see Table

683 2 for identification) and the antioxidant activity results. Values of r >/*-0.90*/ are in bold.

684 Values with a * are statistically significant *(p*<0.05).

	ORAC	HORAC	HOSC	FRAP
3	**-0.989***	-0.673	0.542	0.716
5	**-0.983***	-0.645	0.574	0.742
6	**-0.968***	-0.590	0.629	0.786
9	**-0.903***	-0.428	0.764	0.888*
10	-0.897*	-0.415	0.773	0.895*
11	-0.824*	-0.280	0.856*	**0.950***

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PHENOLIC CHARACTERIZATION OF AGING WINE LEES: CORRELATION WITH ANTIOXIDANT ACTIVITIES

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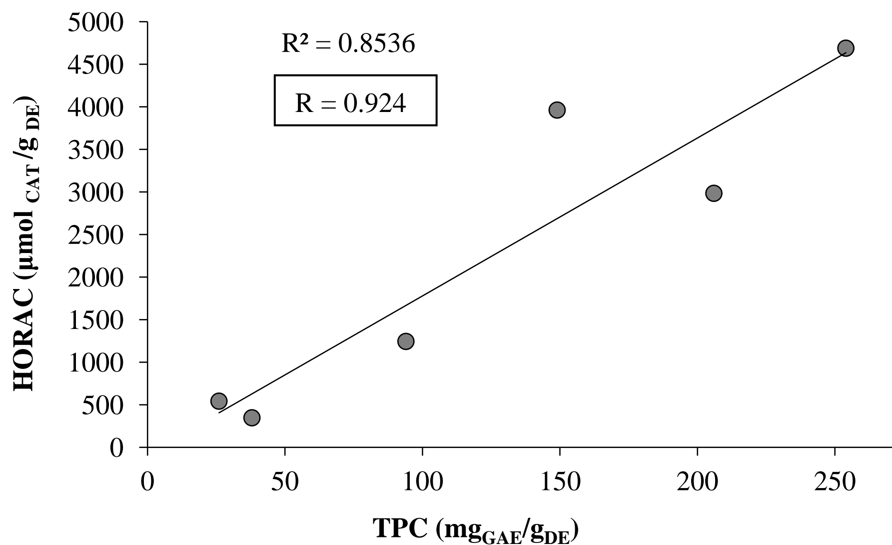
Supplementary Material

Figure 1S: example of correlation values (R^2) between HORAC antioxidant activity and a) TPC and b) TFC.

a)



b)

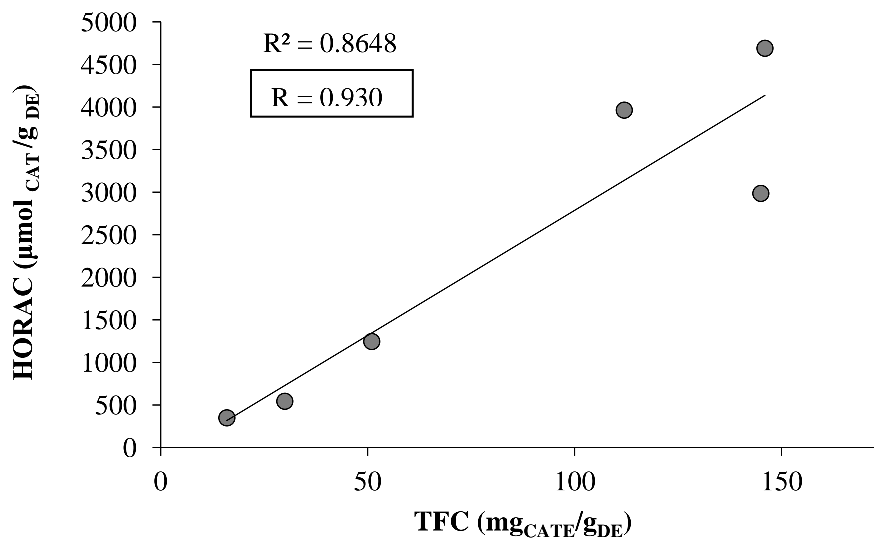


Figure 2S: Chromatograms obtained for acetone, ethanol and water of aging wine lees extracts at a wavelength of 280 nm