COMPOSITION AND ANALYSIS OF COLLOIDAL MATTER ALONG WINE-MAKING. EXPLOITATION OF ITS ANTIOXIDANT ACTIVITY IN FINAL STABILISATION RESIDUES.

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ABSTRACT:

Colloidal matter formed along vinification has been characterized for two consecutive vintages in order to a better understanding of the final solid residues obtained by freezing stabilisation. The polyphenolic content, metal concentrations and antioxidant activity of these residues have been determined. Vintage 2003 of a *Vitis vinifera* (Tempranillo variety) yielded a concentration of 19.018 $\pm$ 0.226 mg g^{-1} of total anthocyanins (TA) and 13.258 $\pm$ 0.158 mg g^{-1} of total flavonoids (TF) in fresh deposits. It has been found that ca. 50% of initially present Fe is eliminated along wine-making, only 6% of Cu ends-up in final wine and all Mn present at the beginning of the process remains in commercial wine. Antioxidant activity in the residues is found to be related to total polyphenol concentration irrespective of polymerisation degree. An EC_{50} of 0.130 $\pm$ 0.007 g L^{-1} (g of lyophilised dreg per L of buffered solution) was obtained for vintage 2004. Antioxidant capacity of different fractions of wine was not seen to depend upon addition of metals.

KEYWORDS: Antioxidant activity, red wine, solid residues, freezing stabilisation, polyphenols, metals

INTRODUCTION

Free radicals are involved in a large number of illnesses affecting humans such as arthritis, arteriosclerosis, Alzheimer, Parkinson, cancer, etc. [1] Antioxidants (both natural and synthetic) effectively act upon those free radicals, playing a beneficial role on health [2]. There exist a plethora of studies concerning the powerful antioxidant activity of red wine [3]. This antioxidant capacity relies on the large amount of polyphenols it contains that, besides definitely intervening on the organoleptic properties of the beverage [4], are the main responsible for the antioxidant activity of red wines [5-8]. Dependence of health and final quality of a wine on this type of compounds has enhanced the surge of several studies directed to the knowledge of their fate along winemaking [9,10], the relationship between their chemical structure and activity [11-13] and the most appropriate methods to estimate their antioxidant potentiality [14,15].

On the other hand, solid wastes obtained after de-stemming and grape-pressing (skin, pulp, seeds, lees, etc.) that are usually either discarded [16] or used for animal feeding and compost without any previous treatment [17], still contain a certain amount of polyphenols that have not been extracted into the wine. As a consequence, many studies have been done in order to quantify its phenolic content and to ascertain the antioxidant properties of this type of residues and their possible utility [2,16,18-22].

However, this is not the only kind of residue obtained in a wine cellar. After clarification and stabilizing, some other by-products are got. These steps are necessary since just elaborated juices have undesirable suspended particles and substances that render wines turbid [9]. Among all the techniques employed to achieve a limpid wine, freezing is most favoured by wine makers. By this means, tartaric acid crystallizes and

induces precipitation of unstable colloidal substances, together with the carrying over of metal-polyphenol complexes, polysaccharides, proteins and ferric phosphate [9]. Tartaric acid is the only one generally processed sub-product for commercial uses [23]. If we keep in mind that colouring matter is also present, polyphenols are also expected to be available in this residual product. Since it has been formed in a natural way, without adding of reagents, the structure of polyphenols should remain intact.

The aim of this work is to evaluate the content of polyphenols, anthocyanins, flavonoids, selected heavy metals, and the antioxidant activity in solid residues obtainable after freezing stabilisation of wine along its vinification (since must elaboration until fresh wine before bottling or transfer into a barrel). This should provide an insight into stability of colouring material and its antioxidant properties along wine-making period. We will add metals to wine samples to assess whether they exert any influence on the antioxidant power.

The analysis of the different compounds in both the after-freezing solids and in supernatants, and comparison with data obtained for final and commercial wines will facilitate to check if a prolonged cooling time will affect the wine properties as suggested by some authors [9].

MATERIALS AND METHODS

Wine samples

Vitis vinifera (Tempranillo variety) grapes harvested in a supervised experimental vineyard located at La Jeringa in the municipality of Olite (Navarra, Spain) were used to produce the wine studied in this work. After de-stemming and crushing,

71 grape must was allowed to ferment in the presence of 0.25 g L^{-1} yeast (80%
72 *Saccharomyces cerevisae* Na33 and 20% *Saccharomyces bayanus* EC 1118) and 0.08 g
73 L^{-1} potassium metabisulphite.

74 Samples were taken for up to day-73 of fermentation for the first vintage (2003)
75 and for up to day-58 of fermentation for the second vintage (2004). Samples were
76 collected daily for the first two weeks and thereafter sampling was done once every two
77 weeks approximately.

78 Collected samples were frozen at -20ºC for a variable time between 15 and 40
79 days. Before analysis, samples were thawed and centrifuged for 5 min at 4,000 min^{-1} in a
80 Biofuge Stratos (Heraus) apparatus refrigerated at 4ºC to avoid any further fermentation
81 to take place. Supernatants and solid residues were then separated and analyzed
82 independently. Results obtained for supernatants have been reported elsewhere [24].

83 Commercial samples are as bought in a wine store. All of them are Tempranillo
84 variety wines coming from 6 Navarra CBO (N1 to N6) and from 2 CBO close-by location
85 Rioja (R1 and R2) of different vintages (2001:N1; 2002: N2; 2003: N3 and R1; 2004: N4,
86 N5, N6 and R2).

87 **Total metal quantification**

88 **Sample digestion and preparation**

89 A fraction of the residues was dried till constant weight in an oven at 100ºC. Dried
90 residues with weights ranging from 0.1 to 0.7 g were treated with 6.00 mL of sub-boiling
91 HNO_3 under the same experimental conditions employed before [10] in an Ethos Plus
92 microwave labstation (Milestone) with computer–controlled easywave software. Once

digested, samples were made up to 10.00 mL with ultra-pure water (Waserlab G.R. Type I-reagent grade-water system, Millipore).

Atomic Absorption Spectroscopic (AAS) measurements

Metals were quantified by AAS by using an acetylene-air flame in a Perkin-Elmer Atomic Absorption Spectrometer A Analyst 800. Experimental conditions for each metal are summarized as follows:

Experimental conditions	Metal			
	Zn	Fe	Cu	Mn
λ (nm):	213.9	248.3	324.8	279.5
Slit width:	0.7	0.2	0.7	0.2
Lamp current (mA):	15	30	15	20
Calibration interval:	0 – 0.3 mg L^{-1}	0 – 1.2 mg L^{-1}	0 – 0.6 mg L^{-1}	0 – 1.8 mg L^{-1}
Calibration line	$y = 0.307x + 1.86 \cdot 10^{-3}$	$y = 0.058x - 1.2 \cdot 10^{-4}$	$y = 0.0764x + 1.43 \cdot 10^{-4}$	$y = 0.067x + 5.1 \cdot 10^{-4}$
Correlation coefficient:	0.9999	0.9998	0.9999	0.9999
n:	4	4	4	4
Detection limit*:	0.002 mg L^{-1}	0.024 mg L^{-1}	0.007 mg L^{-1}	0.022 mg L^{-1}
Quantification limit*:	0.006 mg L^{-1}	0.082 mg L^{-1}	0.025 mg L^{-1}	0.075 mg L^{-1}

*Detection and Quantification limits have been calculated according to MacDougall et al. [25].

Quantification of Polyphenols, anthocyanins, flavonoids and antioxidant activity

Sample conditioning: Lyophilisation and extraction of colouring matter from solid residues

A portion of solid residue obtained after centrifugation was lyophilized in a Selecta Lyophilize BT3-SL. Approximately, 0.1 g of lyophilized was weighted and treated with 5.00 mL of a pH 4 acetate/acetic acid buffer (Suprapur, Merck) containing 8% ethanol and 50% methanol and left to lay still for 24 hours. Afterwards, the sediment was isolated from the liquid and it was subjected to another set of two subsequent extraction steps with two

aliquots of 10.00 mL of buffer. Finally, a fourth extraction with a 10.00 mL buffer was done by allowing the solid to keep in contact with the buffered solution for a longer period of 72 h. At the end, the solid was notably absent of colour. Extracts obtained in the successive mentioned steps were gathered and taken to a final volume of 50.00 mL with buffer. This very same dilution ratio and buffer composition was used henceforth since it had been proved adequate to preserve the natural equilibriums of wine and minimizes chromatic alteration of wine [26].

Total polyphenol (TP) quantification

A modification of the *Prussian blue* method [27] has been used. Onto variable volumes (0.10 to 0.80 mL) of the extracts (*vide supra*), 0.15 mL of 0.1 M $FeCl_3$ (Panreac P.R.S.) plus 0.15 mL of 0.08 M $K_3Fe(CN)_6$ (Panreac, P.A.) were added and made up to 10.00 mL. After exactly 15 min, absorbance is measured at 720 nm vs. a reagent blank using disposable Plastibrand®(Brand Gmbh, Wertheim, Germany) cuvettes of 1 cm length in a UV-VIS Spectrophotometer (model 1203, Shimadzu). Since a time-dependent kinetics was observed, time was scrupulously offset and spectroscopic measurement process was repeated in full 4 times for each of the triplicate aliquots, so that in total we have 12 absorbance data for each sample. Method was previously validated according to the Asociación Española de Farmacéuticos de la Industria (Spanish Association of Industry Pharmacist, AEFI) standard [28] for the studied samples.

Resulting equation as average of 3 separate calibration graphs obtained for 5 gallic acid standards is:

$y = 0.060 + 0.282\ x$; $R^2 = 0{,}998$;

Detection limit (DL) : 0.120 mg L^{-1}; Quantification limit (QL) : 0.401 mg L^{-1}

where x is gallic acid concentration expressed in mg L^{-1} and y is the absorbance at 720 nm. Detection and Quantification limits have been calculated according to the classical procedures [25].

Total anthocyanin (TA) quantification

Anthocyanin quantification is based on their absorbance at 520 nm in the alcoholic pH 4 buffer diluted extracts filtered through a 0.45 µm Low Protein Binding Durapore (PVDF; Millex®-HV, Millipore, Ireland) filters . Measurements were made by triplicate for each of the three aliquots per sample.

Validation was done according to the same standards of AEFI (*28*); three independent calibration graphs obtained for seven standards of malvidin-3-glucoside yielded the following results:

$y = - 0.0044 + 3.273 x$; $R^2 = 0.999$; DL : 0.006 mg mL^{-1} ; QL : 0.021 mg mL^{-1}

where y is the absorbance at 520 nm and x is the malvidin-3-glucoside concentration in g L^{-1}.

Individual anthocyanin (IA) measurements

Four anthocyanins were measured, namely petunidin-3-glucoside (Pt-3-gluc), cyanidin-3-glucoside (Cy-3-gluc), malvidin-3-glucoside (Mv-3-gluc) and malvidin-3-glucoside acylated with *p*-cumaric acid (Mv-3-*p*-cm-gluc), following the experimental conditions published elsewhere [10].

150 **Total flavonoid (TF) quantification**

151 Total flavonoid quantification was accomplished by following an adaptation of the
152 method described in the German Pharmacopoeia DAB10 [29]. Aliquots of 2.00 mL of the
153 extracts present in the 50.00 mL alcohol-containing buffer solution are mixed with 2.00
154 mL of 0.08 M $AlCl_3$ (Probus) and made up to the mark with water to 5.00 mL. After 30
155 min, absorbance vs. a blank is measured at 425 nm. Procedure was repeated twice for
156 every triplicate.

157 In this case, three separate calibration plots were done from 9 quercetin standards
158 giving rise to the final equation:

159 $y = 0.0104\ x - 0.0065$; $R^2 = 0.996$; DL : 2.54 mg L^{-1} ; QL : 8.46 mg L^{-1}

160 where x is stands for the quercetin concentration expressed in mg L^{-1} and y is the
161 absorbance at 425 nm.

162 ***In vitro* antioxidant activity**

163 Antioxidant activity was measured in vintage 2004 along its wine-making process
164 (both in supernatants and deposits) and in commercial wines of same variety and
165 geographical origin. Given the cumbersome experimentation involved in the determination
166 of antioxidant activity, and given the similar profiles obtained for the rest of parameters
167 studied for two vintages, it was decided to analyze the antioxidant potential in just one
168 selected vintage, namely 2004.

169 Antioxidant activities were evaluated by means of a modification of the DPPH (1,1-
170 diphenyl-2-picrylhydrazyl) method as proposed by Brand-Williams *et al.* [30] A pH 4
171 acetate buffer was used to dilute samples as well as the reagent instead of methanol,

172 thus avoiding any modification of polyphenols due to pH variation with respect to that of
173 wine [26]. Spectrophotometric measurements were done by means of a Universal
174 Microplate Spectrophotometer (Power wave^{TM}xs BIOTEK) in conjunction with microplates
175 incorporating 96 wells with 300 μL of capacity each.

176 A calibration graph was constructed for DPPH measurements at 517 nm, that
177 helped us to check that its initial concentration in buffer was 0.035 mM throughout all
178 experiments. Data adjusted to the straight line $y = 0.1932 + 179\ x$; $R^2 = 0.998$, where y
179 represents the absorbance at that wavelength and x stands for the DPPH concentration
180 expressed in g L^{-1}

181 For every single sample to be analyzed, three sets of ten consecutive dilution steps
182 were carried out. Each dilution was done with the pH 4 acetate buffer and rendered a
183 concentration reduced by half. Thus, samples ranged from most concentrated (original
184 sample) to most diluted (1,024-fold dilution).

185 Three rows of the microplate were filled with 150 μL of every diluted sample plus
186 150 μL of a freshly prepared 0.035 mM DPPH standard. This procedure was done by
187 triplicate in a decreasing order of sample concentration.

188 In another row, 150 μL of each assayed diluted sample were placed onto which
189 150 μL of buffer were added. The absorbance of each of these wells was used as the
190 blank (A_{bl}) value to be subtracted. Finally, other 20 wells were filled with 150 μL of the
191 freshly prepared DPPH together with 150 μL of the buffer solution. The average
192 absorbance value of these solutions was taken as the reference for the totally oxidized
193 reagent ($A_{DPPHref}$).

194 Once the microplate was thus prepared, it was introduced in the
195 spectrophotometer, it was shaken for 10 s, and absorbencies were measured after three
196 different waiting times: 0, 30 and 60 min.
197 Percentage of reduced DPPH was readily calculated by applying the following
198 expression:

199 $\%\,\text{reduction} = \frac{A_{DPPH_{ref}} - A_{dil}}{A_{DPPH_{ref}}} \cdot 100$ in which A_{dil} is the measured absorbance for the reagent in

200 contact with diluted sample once the blank absorbance of the sample itself (A_{bl}) has been
201 subtracted.
202 In order to ensure the reliability of results, three microplates were processed for
203 every triplicate sample, so that nine antioxidant values were obtained for every dilution of
204 each sample.
205 By plotting the %reduction obtained values vs. sample concentration, the efficient
206 concentration (EC_{50}), that is to say the sample concentration able to reduce 50% of the
207 DPPH, is easily calculated. We have expressed this parameter as mL of supernatant per
208 L of solution; mL of commercial wine per L of solution; mL of turbid wine per L of solution
209 necessary for the residue to quench 50% of the initial DPPH activity. Needless to say that
210 the less the EC_{50} the larger the antioxidant activity of analyzed sample.
211 Validation of the method used was done by applying the proposed protocol to the
212 determination of antioxidant activity of two well known standards: butyl-hydroxy-anysol
213 (BHA) and ascorbic acid (vitamin C) [30]. This has also served as a way to get
214 comparative reference values for our antioxidant activity measurements.
215 Potential influence of both Fe and Cu on the antioxidant role of a wine was
216 examined by checking the EC_{50} of selected samples both commercial and under

vinification. Commercial studied wines were those designed N4, N5, N6 and R1; supernatants of vintage 2004 wine were studied at days 1, 5, 7, 32 and 58 of vinification. First dilution of samples was done with a buffer containing certain known amount of metal. Subsequent dilutions were carried out by adding metal-free buffer. Certipure (Merck) 1,000 ppm standards of both Fe and Cu were used. Dual experiments were performed for every sample at two levels of concentration of added metals. Metal concentrations after the first sample dilution with metal-containing buffer were 0.1248 mg L^{-1} (essay 1) and 6.000 mg L^{-1} (essay 2) for Fe, and 0.025 mg L^{-1} (essay 1) and 1.5 mg L^{-1} (essay 2) for Cu.

RESULTS AND DISCUSSION

Deposits composition after freezing-thawing and centrifuging

Wines under vinification, once thawed, presented a high turbidity. After centrifuging solutions remained limpid whereas gelatinous, sludge-like residues of intense purple colour and nacre aspect were produced. Average percentage humidity calculated by either drying at 100ºC or by lyophilisation (freeze-drying) method was found to be 64.6 $\pm$ 2.5 %. Table 1 reflects the amount of matter in suspension found for both 2003 and 2004 vintages wines along vinification that ended-up in the solid residue after freezing, thawing and centrifuging. It is readily seen that suspension matter is most abundant in the first few days of vinification but, after day six or so, it progressively decreases till the end of the vinification process.

Total polyphenols, anthocyanins and flavonoids

Figure 1 shows the evolution of the concentration (mmol per L of turbid wine) of total polyphenols (TP), total anthocyanins (TA) and total flavonoids (TF) along vinification for both vintages. As it can be seen evolution profiles are very much the same for both instances, in which maximum values are reached between day-5 and day-6, with a progressive decreasing tendency henceforth.

The concentration profiles for all the assayed compounds are such that there exists a relative accumulation of soluble and measurable substances in the suspension matter that later on shall become part of the residues. As vinification proceeds, the obtained residue contain but little of these analytes, implying that they have remained soluble in the clear juices. As a matter of fact, results published for the supernatants of these very same samples [24], show that polyphenols, anthocyanins and flavonoids are fully extracted from grapes into the wine after ca. the same lapse of time (10-11 days) reaching a more or less constant concentration from then onwards. Moreover, TP, TA and TF concentrations measured in the last days of vinification was found to be very close to values corresponding to commercial wines of the same geographical origin and variety, what induces to suspect that freezing treatment did not much affect the composition of the antioxidant components of wine.

It is worth to note that values plotted in Figure 1 belong to antioxidants present in suspended matter, that are only a minor portion with respect to the total content in the wine. This can be more clearly seen in Table 2 and Table 3, which present percentages of these components in suspension with respect to the whole wine.

Data demonstrate that, at the beginning of vinification, up to a 20% TP, 30% AT and 32% TF extracted from grapes remain mainly in suspension and, consequently, will never be a

261 part of the final wines, since eventually they flocculate and sediment in the bottom of the
262 deposit.

263 Further measurements were carried out in order to calculate how much of these
264 antioxidant compounds could be recovered from residues obtained after a freezing
265 stabilisation process when done at the very final stage of vinification. Results, expressed
266 as mg per g of fresh residue obtained for last day of vinification, are as follow: for vintage
267 2003, 19.018 $\pm$ 0.226 mg g^{-1} of TA and 13.258 $\pm$ 0.158 mg g^{-1} of TF; for vintage 2004:
268 11.600 $\pm$ 0.129 mg g^{-1} of TA and 6.716 $\pm$ 0.174 mg g^{-1} of TF. Taking into account, on one
269 side, the volume of the barrel employed, for example, in vintage 2003 (2.200 L) and, on
270 the other side, the amount of residue obtained per L of wine (5.11 g), a total weight of
271 11.24 Kg and 7.12 Kg of sludge would be available for processing. Consequently, 213.76
272 $\pm$ 2.54 g of TA and 149.01 $\pm$ 1.78 g of TF would be achievable for vintage 2003.

273 **Individual anthocyanins quantification**

274 Chromatograms recorded for all samples containing solid residues showed no
275 analytical signal for any of the individual anthocyanins assayed. From these experimental
276 evidence it is readily concluded that anthocyanins are either condensed or polymerized.
277 In the literature it is reported that a purple colour is related to anthocyanin-tannin
278 complexes [31] that could very well be the case.

279 **Metals quantification**

280 Figure 2 shows the concentrations found for the four target metals analyzed, Fe,
281 Cu, Mn and Zn, for the residues obtained along vinification for the two vintages. Larger
282 amounts of metals can be found in the solid fraction of the wine at the beginning of

283 fermentation, but shortly afterwards a marked decrease follows, concentrations eventually
284 plummeting at the end of wine-making.

285 These experimental results, together with described pathways for the fate of Fe
286 and Cu [32], make it plausible that only a minor part of Fe is carried away into the
287 sediments while most of it remains soluble in solution. A diverse pattern is ascribable to
288 Cu, which appears to mainly deposit at the bottom of the container, thus vanishing from
289 either supernatants or suspension matter. In order to verify this hypothesis, total
290 concentrations (in clear supernatant plus dregs originated from suspension matter, that is
291 to say in *turbid wine*) of both metals were determined for the first and last day in the
292 fermentation tank, as detailed in Table 4.

293 Thus, according to these experimental data, it can be seen that between 37.3%
294 and 40.5% of total Fe present at the beginning of the vinification process ends up at the
295 bottom of the tank, whereas a much larger proportion of Cu vanishes from the wine (87.4
296 to 90.3%). Besides, up to a 22% of Fe and up to a 48.7% of Cu present in turbid wine are
297 associated with matter in suspension that will eventually be eliminated after clarification
298 and stabilisation processes.

299 Therefore, a young wine has approximately a 50% of total initial Fe and around 6%
300 of total initial Cu. This numerical facts resemble the real risk situation of a ferric *casse*
301 occurring in a bottled wine, while cupric *casse* is very much precluded since it has already
302 taken place along vinification.

303 As for Zn and Mn, results show that their presence in the sludge, as it also
304 happened for supernatants [24], is more or less constant except for variations found for
305 the very first days. Table 5 collects comparative data for the total concentration found in

306 both supernatants and solid residues for these two metals between first and last day in
307 fermentation process.

308 It is remarkable that total Mn concentration scarcely experience any variation along
309 the whole wine-making process, as well as the fact that its percentage associated to
310 suspension matter is very small (see Table 6), so that it may be concluded that all initially
311 available Mn remains in the clarified wine and very little, if any, losses take place along
312 vinification. This statement is in good agreement with experimental results found for
313 commercial wines which show very similar content of Mn to those wine samples
314 belonging to last days of vinification, when both of them come from the same variety of
315 grape and very close geographical area [24]. This could also be an explanation for the
316 Mn being chosen as a geographical tracer [33].

317 A diverse picture is got for the fate of Zn which total concentration experiences
318 decreases of 32% and 60% from the first to last day in the fermentation container for the
319 two consecutive vintages, respectively. These losses are due, in part, to consumption by
320 yeast [34]; but no doubt, since its concentration remains reasonably constant in the clear
321 supernatants [10,24] and shows a decrease in the suspension matter (see Table 6), they
322 are ascribable as well to dragging of Zn to the bottom by polymerized polyphenols that
323 have the ability to complex this metal [35,36].

324 **Measurement of antioxidant activity in solid residues, supernatants and**
325 **commercial wines**

326 Preliminary experiments were carried out to optimize the time offset of
327 spectrophotometric measurements at the wavelength of the DPPH when in contact with
328 antioxidants. The three types of samples -that is to say extracts from the residues formed

329 in the freezing stage, clear supernatants obtained after centrifuging the solid residue, and
330 commercial wines- were subjected to *in vitro* measurements of EC_{50} at 0 min, 30 min and
331 60 min after mixing with the reagent. It was observed that a decrease in EC_{50} ranging
332 from 33% to 50% could take place after 30 min of sample and DPPH mixing, whereas
333 EC_{50} remained relatively constant for higher times up to 60 min. From these results we
334 chose a time offset of 30 min as optimum to perform measurements that guaranteed a
335 good repeatability.

336 **Antioxidant activity in supernatants**

337 Vintage 2004 was selected as adequate to perform antioxidant measurements
338 along vinification process. Measured EC_{50} values can be seen in Table 7, which show a
339 decreasing tendency for EC_{50} (in other words, an increasing tendency of the antioxidant
340 capacity) as vinification proceeds.

341 The antioxidant activity increases rapidly for the first few days and stabilises from
342 day-10 onwards, resembling the evolution pattern of polyphenols for the same wine
343 samples [24], as expected, since those compounds are the main responsible for the
344 antiradical activity of wines. A Pearson correlation matrix was constructed from all
345 parameters available for those vinification samples and correlation factors are collected in
346 Table 8.

347 Values are quite high indicating an extremely nice correlation of all phenolic
348 compounds with the antioxidant power of assayed wine. Besides, amongst all monomeric
349 anthocyanins, cyanidin-3-glucoside and malvidin-3-glucoside provide highest correlation
350 factors. This finding is in accordance with reports indicating that a catechol group in ring B
351 make flavonoids exhibit better antioxidant properties [12,13,37,38]. In that sense, it should

352 be expected that cyanidin-3-glucoside would play a dominant antioxidant role, but since
353 malvidin-3-glucoside is most abundant in wine its contribution to antioxidant power is
354 higher as reflected in the correlation factors.

355 The good correlation between chromatic parameters such as IC, L* and a* and
356 EC_{50} comes as no surprise for -as it has been proved before- all these parameters are
357 highly correlated with polyphenols, and especially anthocyanins [24].

358 A simple and straightforward way of estimating EC_{50} from chromatic parameters L* and a*
359 is proposed, as evidenced by linear regression analysis of data, which were found to
360 adjust to the following expression:

361 $EC_{50} = 1.147\ L^* + 0.592\ a^* - 90.584;\ R^2: 0.910$

362 On the other hand, reasonably good correlations were found for EC_{50} with Fe and
363 Cu, especially with the latter. Capacity of inhibiting Fenton reaction [13,39,40] by
364 complexing potential catalysers in the formation of free radicals, such as Fe^{2+} and Cu^{2+}, is
365 known to be part of the antioxidant properties of flavonoids. This would justify the good
366 statistical correlations found. However, this information is not sufficient as to deduce if an
367 increase or decrease of the metals concentration would modify the antioxidant beneficial
368 properties of a given wine. This has prompted us to check whether adding of either Fe or
369 Cu to both clear supernatants and commercial wine samples would reveal an alteration in
370 EC_{50} values. Experiments carried out at the pH 4 buffered conditions showed no
371 significant variations for EC_{50} with respect to the original metal-free situations, so that we
372 may conclude that antioxidant capacity of wine is not altered, neither improved neither
373 worsened, by adding either Fe or Cu. This conclusion seems to be in good terms with
374 other author's assertions [41] that Fe-flavonoids complexes do not interfere in the *in vitro*
375 antioxidant potential of phenolic compounds.

Antioxidant activity of commercial wines; comparison with vitamin C and BHA

Commercial wines antioxidant measurement is not something to fuss about, but we were tempted to see whether their EC_{50} values would differ from those calculated for wines of the same variety and geographical origin that have been experimentally monitored in the EVENA oenological institute. Table 9 summarises Ec_{50} as calculated for the eight commercial wines, which values are very close, or even less, than those reported above (Table 7) for wines in the last day of vinification. From these results one can obviously conclude that antioxidant properties of wine is not deteriorated by freezing stabilisation treatment.

When methodology was confronted by using either BHA or vitamin C, EC_{50} values obtained for a 30 min period employing the same DPPH as before, were 3.385 ± 0.245 mg L^{-1} and 2.936 ± 0.299 mg L^{-1}, respectively. These results indicate that antioxidant capacity of commercial wines fall within the same order of magnitude of both standards, and even -in some instances- higher than those of vitamin C. This behaviour was also found for wines in their last days of vinification.

Antioxidant activity in solid residues

Remnant antioxidant potential in polyphenols associated to suspension matter, that later becomes sludge-like residue, was also quantified and, for comparison reasons, results in Table 10 are expressed as mL of turbid wine (without eliminating the suspension matter) per L of buffered solution necessary for the residue to quench 50% of the free radical.

397 There is a marked decrease of EC_{50} values when passing from day-1 to day-2. A
398 gradual decrease follows reaching a minimum (a maximum in antioxidant activity) ca.
399 day-6 of vinification. Afterwards, a steeping increase of EC_{50}values is observed. As a
400 whole, the antioxidant pattern is a mirror image of the behaviour showed by phenolic
401 compounds in these very same samples as corroborated by reached Pearson correlation
402 coefficients: TP vs EC_{50} = -0.767; TA vs EC_{50} = -0.746 and TF vs EC_{50} = -0.763.
403 On the other hand, these EC_{50} values are much smaller than correspondent values
404 calculated in their clear supernatants (that end-up in the bottle of wine).
405 In principle, this drop could be due to the lesser concentration of polyphenols
406 present in suspension matter as well as to the fact that these polyphenols are mainly
407 polymerized. However, when ratios of polyphenols -rather than absolute values- are
408 compared for both supernatants and suspension matter, it is found that ratio decrease of
409 phenolic compounds is similar to drop in antioxidant activity (that is to say, increment of
410 EC_{50} values). This finding would imply that, in these measurement conditions, variation of
411 antioxidant potential from one sample to another is related to the ratio of polyphenols
412 present in the sample rather than to their degree of polymerization.
413 Polymerization reactions in the pro-cyanidins is known to proceed through bonds
414 C4-C8 or C4-C6, and reactions between anthocyanins and tannins occur through C4-C4
415 or C4-C8 positions [42]. This means that, in majority of situations, catechol groups
416 present in aromatic ring B remain unaffected, what redounds in an improved antioxidant
417 activity. This would explain why concentration of total polyphenols present, independently
418 of the polymerization degree, would be the responsible for antioxidant power of the
419 sample.

420 Last, but not least, it was our interest to find out precisely how much of antioxidant
421 power is associated to sludge-like residue obtained in the very last day of vinification just
422 before bottling or transfer into an oak barrel. EC_{50} was measured for those residues once
423 extracted and lyophilised, obtaining an $EC_{50\ 30\ min}$ of 0.130 $\pm$ 0.007 g L^{-1} (g of lyophilised
424 per L of buffered solution). This value for antioxidant activity associated to the solid
425 residues points to the possible industrial interest in the exploitation of these residues
426 produced at the end of the chain in wine-making cellars.

CONCLUSIONS

- Freezing stabilisation step does not alter antioxidant properties of a wine
- Up to a 20%TP, 30%TA and 32% TF are associated to suspension matter that will never end-up in the final wine.
- All anthocyanins present in the suspension matter are polymerised within the colloidal colouring matter.
- Practically all Mn initially present in grape must is transferred to the final wine.
- Antioxidant activity of a wine under vinification may be readily estimated from simple measurement of two chromatic parameters, L* and a*.
- Polyphenol polymerisation does not account for variation of antioxidant capacity from one wine to other, but rather it is due to their ratio in the sample.
- Around 50% of Fe and 6% of Cu initially present in the fermentation reactor, turns out to be in the final young wine.
- Solid residues obtained after freezing stabilisation are rich in polyphenols and have good antioxidant properties, making them attractive to exploit.

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Captions for Figures & Tables

Figure 1: Total polyphenols (TP), anthocyanins (TA) and flavonoids (TF) found in suspension along vinification for vintage 2003 (**a**) and vintage 2004 (**b**).

Figure 2: Metallic content (mg per L of turbid wine) in the solid residues obtained along vinification for vintage 2003 (a, b) and vintage 2004 (c, d).

Table 1: Solid residue obtained per litre of turbid wine.

Table 2: Percentage of TP and TA in suspension matter with respect to total concentration found in whole wine for vintage 2003 (mean±SD).

Table 3: Percentage of TP, TA and TF in suspension matter with respect to total concentration found in whole wine for vintage 2004 (mean ± SD).

Table 4: Total concentrations of Fe and Cu for the initial and final stages of wine-making in fermentation container.

Table 5: Total concentrations of Mn and Zn for the initial and final stages of wine-making in fermentation container.

Table 6: Percentage of Mn and Zn in suspension matter with respect to total concentration found in whole wine for vintages 2003 and 2004 (mean ± SD).

Table 7: EC_{50} values obtained for supernatants expressed as mL of supernatant per L of buffer solution necessary to quench 50% of DPPH.

Table 8: Pearson's correlation coefficients for parameters measured in vintage 2004 wine's supernatants.. all results are significant at the 0.01 level (2-tailed).

Table 9: EC_{50} values obtained for commercial wines expressed as mL of wine per L of buffer solution necessary to quench 50% of DPPH.

Table 10: EC_{50} values obtained along vinification. Results are expressed as mL of turbid wine necessary for the residue to quench 50% of the initial DPPH activity.

Table 1: solid residue obtained per liter of turbid wine.

	Vintage 2003	Vintage 2004
Vinification time (day)	**g L^{-1}**	**g L^{-1}**
1	25.55	10.33
2	34.15	25.96
3	40.53	28.18
4	19.94	17.74
5	22.74	18.40
6	16.35	36.15
7	13.96	15.51
8	13.68	15.29
9	11.76	9.41
10	7.37	5.60
11	9.18	4.39
16	15.47	
18		5.30
25	6.15	4.06
31	6.86	
32		4.85
38	5.39	
45	5.28	
51	5.17	2.46
58		2.85
59	4.99	
67	4.11	
73	5.11	

Table 2: Percentage of TP and TA in suspension matter with respect to total concentration found in whole wine for vintage 2003 (mean±SD)

Vinification day	% TP suspended	% TA suspended
1	11.394 ± 0.453	13.634 ± 1.068
2	8.908 ± 0.230	17.252 ± 0.485
3	8.146 ± 0.259	20.310 ± 1.264
4	6.526 ± 0.215	17.763 ± 0.107
5	7.664 ± 0.118	20.165 ± 0.249
6	5.464 ± 0.198	16.637 ± 0.393
7	4.296 ± 0.112	11.831 ± 0.083
8	4.956 ± 0.360	18.366 ± 0.180
9	4.153 ± 0.060	15.145 ± 0.214
10	2.514 ± 0.065	8.209 ± 0.221
11	5.106 ± 0.184	12.934 ± 0.145
16	7.748 ± 0.190	15.498 ± 0.109
25	3.151 ± 0.139	10.442 ± 0.012
31	2.446 ± 0.073	9.072 ± 0.063
38	2.080 ± 0.039	7.981 ± 0.079
45	2.951 ± 0.093	10.175 ± 0.162
51	2.437 ± 0.064	8.371 ± 0.011
59	2.485 ± 0.157	7.715 ± 0.087
67	1.921 ± 0.086	6.222 ± 0.082
73	2.555 ± 0.144	8.075 ± 0.096

Table 3: Percentage of TP, TA and TF in suspension matter with respect to total concentration found in whole wine for vintage 2004 (mean ± SD)

Vinification day	% TP suspended	% TA suspended	% FT suspended
1	3.520 ± 0.798	12.849 ± 1.640	12.828 ± 0.771
2	14.584 ± 0.527	26.238 ± 1.581	35.856 ± 2.062
3	18.183 ± 0.462	20.571 ± 0.612	21.772 ± 0.464
4	13.543 ± 0.132	18.844 ± 0.520	19.858 ± 0.325
5	11.350 ± 0.395	18.749 ± 0.653	21.289 ± 0.896
6	21.881 ± 0.602	30.821 ± 1.122	32.161 ± 0.427
7	14.133 ± 0.283	17.417 ± 0.497	19.801 ± 0.109
8	13.690 ± 0.777	15.164 ± 0.119	20.619 ± 0.194
9	13.135 ± 0.458	15.210 ± 0.974	25.889 ± 1.215
10	7.940 ± 0.463	11.182 ± 0.159	16.530 ± 0.169
11	4.961 ± 0.206	8.154 ± 0.208	12.994 ± 0.094
18	4.433 ± 0.280	5.672 ± 0.143	7.370 ± 0.163
25	2.566 ± 0.234	4.882 ± 0.092	6.338 ± 0.200
32	3.523 ± 0.115	6.472 ± 0.015	8.389 ± 0.073
51	1.587 ± 0.069	2.799 ± 0.046	3.551 ± 0.225
58	1.842 ± 0.048	3.531 ± 0.039	4.703 ± 0.122

Table 4: Total concentrations of Fe and Cu for the initial and final stages of wine-making in fermentation container

	Vintage 2003		Vintage 2004	
	First day	Last day	First day	Last day
Fe (mg L^{-1})	3.422 ± 0.033	2.036 ± 0.049	1.162 ± 0.025	0.728 ± 0.031
Cu (mg L^{-1})	1.423 ± 0.021	0.179 ± 0.007	1.361 ± 0.122	0.132 ± 0.009

Table 5

Table 5: Total concentrations of Mn and Zn for the initial and final stages of wine-making in fermentation container

	Vintage 2003		Vintage 2004	
	First day	Last day	First day	Last day
Mn (mg L^{-1})	1.312 ± 0.056	1.071 ± 0.031	0.772 ± 0.064	0,794 ± 0.035
Zn (mg L^{-1})	0.526 ± 0.010	0,353 ± 0.013	0.507 ± 0.007	0,201 ± 0.005

Table 6: Percentage of Mn and Zn in suspension matter with respect to total concentration found in whole wine for vintages 2003 and 2004 (mean ± SD)

	Vintage 2003		Vintage 2004	
Vinification time	**Mn%**	**Zn %**	**Mn%**	**Zn %**
1	5.777 ± 0.402	6.927 ± 1.006	3.563 ± 0.463	4.429 ± 0.136
2	10.728 ± 0.439	20.601 ± 1.016	23.268 ± 1.068	14.568 ± 0.710
3	25.890 ± 0.643	21.643 ± 0.696	17.502 ± 4.518	17.899 ± 0.560
4	9.557 ± 0.280	8.850 ± 0.484	12.534 ± 0.705	14.663 ± 0.380
5	9.131 ± 0.396	11.555 ± 1.215	11.046 ± 0.626	17.185 ± 0.874
6	6.121 ± 0.215	8.083 ± 0.708	27.068 ± 1.354	23.398 ± 0.344
7	9.169 ± 0.305	12.189 ± 0.826	9.455 ± 0.436	8.893 ± 0.390
8	4.317 ± 0.062	5.699 ± 0.130	10.431 ± 0.530	12.922 ± 0.391
9	4.038 ± 0.100	5.649 ± 0.388	6.101 ± 0.398	8.458 ± 0.398
10	1.498 ± 0.526	5.503 ± 1.289	3.549 ± 0.259	2.943 ± 0.247
11	2.753 ± 0.091	3.983 ± 0.142	2.683 ± 0.188	3.825 ± 0.109
16	7.062 ± 0.100	5.796 ± 0.322		
18			3.059 ± 0.193	2.134 ± 0.177
25	1.509 ± 0.209	2.914 ± 0.513	1.767 ± 0.124	1.008 ± 0.116
31	2.044 ± 0.162	5.180 ± 0.518		
32			1.789 ± 0.109	1.807 ± 0.160
38	1.375 ± 0.082	3.826 ± 0.281		
45	0.994 ± 0.107	3.038 ± 0.320		
51	1.264 ± 0.027	2.190 ± 0.091	0.760 ± 0.083	0.866 ± 0.095
58			0.694 ± 0.099	1.423 ± 0.046
59	1.212 ± 0.056	3.009 ± 0.082		
67	0.972 ± 0.026	1.918 ± 0.026		
73	0.987 ± 0.107	1.810 ± 0.245		

Table 7

Table 7: EC_{50} values obtained for supernatants expressed as mL of supernatant per L of buffer solution necessary to quench 50% of DPPH.

Vinification day	EC_{50} 30 min (mL L^{-1})
1	17.998 ± 1.798
2	11.111 ± 1.468
3	5.929 ± 0.552
4	5.306 ± 0.639
5	3.557 ± 0.367
6	3.828 ± 0.453
7	2.718 ± 0.467
8	2.796 ± 0.327
9	2.598 ± 0.339
10	2.670 ± 0.541
11	2.135 ± 0.226
18	2.102 ± 0.198
25	2.282 ± 0.126
32	1.626 ± 0.194
51	2.053 ± 0.301
58	2.061 ± 0.427

Table 8: Pearson's correlation coefficients for parameters measured in vintage 2004 wine's supernatants.. all results are significant at the 0.01 level (2-tailed)

	TP (g L^{-1})	TA (g L^{-1})	TF (g L^{-1})	Pt-3-gluc (mg L^{-1})	Cy-3-gluc (mg L^{-1})	Mv-3-gluc (mg L^{-1})	Mv-3-*p*-cm-gluc (mg L^{-1})	IC	L*	a*	Fe (mg L^{-1})	Cu (mg L^{-1})
EC_{50} (mL L^{-1})	-0,928	-0,944	-0,911	-0,882	-0,900	-0,944	-0,809	-0.775	0.843	-0.650	-0,659	0,969

TP: total polyphenols; TA: total anthocyanins; TF: total flavonoids; Pt-3-gluc: petunidin-3-glucoside; Cy-3-gluc: cyanidin-3-glucoside; Mv-3-gluc: malvidin-3-glucoside, Mv-3-*p*-cm-gluc: malvidin-3-*p*-cumaroil-glucoside.

Table 9

Table 9: EC_{50} values obtained for commercial wines expressed as mL of wine per L of buffer solution necessary to quench 50% of DPPH.

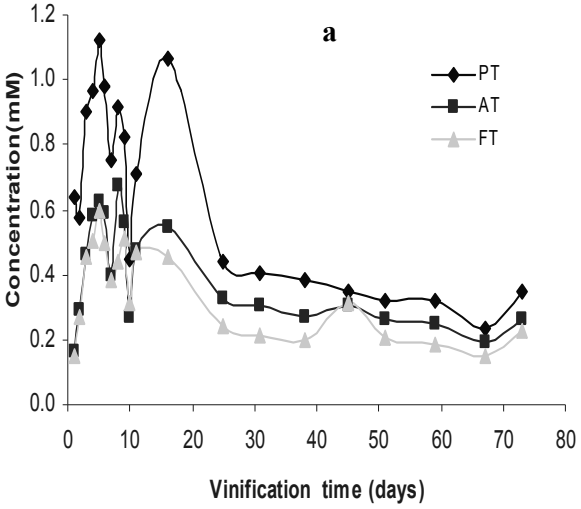
Commercial wine	EC_{50} 30 min (mL L^{-1})
N1	1.588 ± 0.130
N2	2.912 ± 0.291
N3	1.360 ± 0.084
N4	1.958 ± 0.153
N5	3.394 ± 0.520
N6	3.456 ± 0.226
R1	3.444 ± 0.332
R2	2.588 ± 0.224

Table 10

Table 10: EC_{50} values obtained along vinification. Results are expressed as mL of turbid wine necessary for the residue to quench 50% of the initial DPPH activity

Vinification day	EC_{50} 30 min (mL L^{-1})
1	80.001 ± 4.833
2	15.218 ± 1.407
3	12.851 ± 0.498
4	12.691 ± 0.749
5	12.472 ± 0.945
6	6.247 ± 0.219
7	13.514 ± 0.946
8	10.491 ± 1.391
9	11.226 ± 0.456
10	17.317 ± 2.367
18	23.528 ± 1.315
25	37.197 ± 3.897
32	28.880 ± 0.967
51	44.143 ± 3.343
58	33.512 ± 2.232

Figure 1: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PT
AT
FT
Concentration (mM)
Vinification time (days)
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0.4
0.6
0.8
1.0
1.2
0
10
20
30
40
50
60
70
80

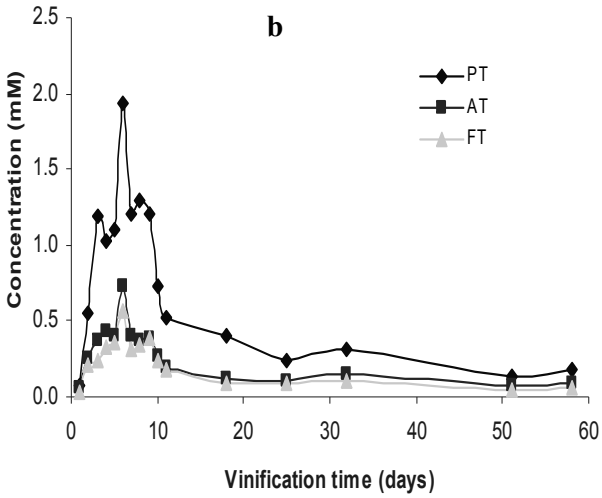
b
PT
AT
FT
Concentration (mM)
Vinification time (days)
0.0
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40
50
60

Figure 2

Figure 2: