Application of LC-MS and Tristimulus Colorimetry to asses the ageing aptitude of Syrah wine in the Condado de Huelva D.O. (Spain), a typical warm climate region

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Pigment composition and colour quality of aged red wines

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Abstract

The study of the evolutions of different wine pigment families, copigmentation/polymerisation processes and colour characteristics during the first year of ageing in oak barrel has allowed the assessment of the ageing aptitude of Syrah wines from "Condado de Huelva D.O.", a warm climate region. A total of 32 anthocyanic pigments were identified, including 14 major compounds from grape and 18 minor derivatives formed during the vinification. The anthocyanin profile changed toward more chemical complexity, being vitisin-like pyranoanthocyanins the predominant minor pigments during the first months of ageing. As wine became older, a progressive increase on the content of 4-vinylcatechin, 4-vinylphenol and 4-vinylcatechol compounds took place. Results showed that copigmentation occurred during the whole process of ageing inducing visual perceptible colour effects. Simultaneously to the copigmentation decrease, the degree of polymerisation increased during ageing, being maximum at 9 months old wines (77%). The colour of wines evolved progressively in a positive way from 3 to 9 months of ageing, becoming darker and with more vivid colour. However, from 9 to 12 month of ageing, the chemical structure of wines was negatively affected resulting in lighter, with more red-orange hues and less vivid colours. The inclusion of the chemical and colorimetric information on the PCA model allows us to reach very good discriminations among the Syrah wines with different wood contact period.

Keywords: ageing process; copigmentation; detailed pigment composition; quality colour; oak wood.

1. Introduction

The climatic conditions of a given geographical area is one of the most important factors influencing the overall wine style of a region since determine the types and the quality of grapes that can be grown [1].

In warm climate regions, red grapes have not been traditionally cultivated because the high night temperatures and the severe light exposures during the ripening affects negatively the anthocyanic biosynthesis and its accumulation in the fruit skin, giving rise to poor coloured grapes [2]. As a consequence, red wines elaborated from them usually show important problems with colour quality and stability during winemaking maturation, especially when they are subjected to ageing processes.

However, due to the market demand, the interest for red wine vinifications in warm regions is becoming more and more increasing in the last decades. Particularly, in the ‘‘Condado de Huelva’’ Designation of Origin, a restricted wine-producing zone in south-western Spain with the typical climatological conditions of warm climate, new red grape varieties are being introduced in order to diversify the wine choice of the DO and to expand their potential market [3]. This D.O. includes approximately 6000 hectares of neutral or slightly alkaline soil, having a typical Mediterranean climate with a clear Atlantic influence: gentle winters and springs and long and warm summers (average temperature 18ºC, minimum over 10ºC in winter and over 40 ºC in summer), relative humidity ranging between 60% and 80%, and mean rainfall around 700 mm year $^{-1}$.

Vitis vinifera cv. Syrah grape is the main and more extensively grown in the zone. It was selected because has been described as an excellent and robust

74 grape, well-adapted to warm climate, easy to cultivate and little vulnerable to
75 diseases.

76 In fact, the elaboration of young Syrah wines with better phenolic
77 composition and higher colour intensity in the “Condado de Huelva D.O.” has
78 been already possible thanks to important investments in new technologies
79 such as the application of low maceration temperatures prior to fermentation,
80 which resulted in a more effective extraction of the anthocyanin pigments and
81 improved the colour stabilization during the earlier stages of wine maturation [4,
82 5]. Notwithstanding, as was previously stated, to produce high quality wines
83 from Syrah grapes besides to preserve the levels of phenols in the winemaking,
84 perform a long fermentation and watch carefully the temperature, summiting
85 them to ageing processes is also recommended since they are especially fit for
86 ageing in oak barrels [6]. Taking into account the absence of previous studies
87 about the ageing potential of this variety during longer ageing processes in this
88 region, further investigations are still necessary.

89 Nowadays, is well-known that the expression of the colour in red wines
90 depends not only from the anthocyanin concentration originally extracted from
91 grapes but also on physicochemical phenomena of copigmentation that led to
92 the formation of new derived pigment which stabilize wine colour [7].

93 Recently, different groups of anthocyanin-derived pigments with
94 particular colour characteristics have been identified in red wines, such as
95 pyranoanthocyanins, direct flavanol–anthocyanin condensation products and
96 acetaldehyde-mediated flavanol–anthocyanin condensation products [8].
97 Although some of these pigments have only been detected in very small
98 quantities in wines, they present unique spectroscopic features that may

somehow contribute together to the overall colour of aged red wines by means of additive, synergistic, or suppressive effects [9]. A common colorimetric characteristic of all these derived pigments is the greater resistance to degradation reactions that could further enhance their sensorial importance.

Different mechanisms have been proposed and confirmed the formation of this compounds [10,11] being some of them favoured by the presence of oxygen [12]. For this reason, although wine ageing in oak barrels implies a high cost outlay for the wineries, it is commonly used because the positive effects storage tends to improve the quality of wines. Low oxidative processes, playing an important role in reactions as well as increasing colour stability, spontaneous clarification and wine complexity [13].

Therefore, the main objective of this paper is to study more in depth the detailed anthocyanic composition of the aged Syrah wines elaborated in the typical "Condado de Huelva D.O.", a warm climate region, as well as to assess their role in the wine colour quality according to the ageing time. For these purposes, not only the occurrence of the main anthocyanins was studied, but also those of the anthocyanin-derived pigments in order to establish the contribution of each pigment family at each moment of wine ageing. As the identification of minor pigments has proven difficult, especially because their levels are much lower than those of original anthocyanins, high performance liquid chromatography–diode array detection coupled to mass spectrometry (HPLC–DAD–MS) has been used in pigment identification.

Regarding colour assessment, Tristimulus Colorimetry, based on objective and direct spectral measurements, represent a useful methodology for colour

specifications in red wines by means of the quantitative and qualitative colorimetric variables, which also provide high precision and accuracy to follow the integral colorimetric effect induced by copigmentation processes [14]. Moreover, applying principal component analysis, the usefulness of the pigment composition and colorimetric variables in the differentiation of the wines as a function of the ageing time was also assessed.

2. Materials and methods

2.1. Wine samples and ageing process

A red wine made from grapes *Vitis vinifera* var. Syrah (2007 vintage) cultivated in "Condados de Huelva" Designation of Origin, in Spain, was used for the experiment. The wine was elaborated by "prefermentative cold-maceration", a vinification technique designed to obtain high concentrations of phenolic compounds, particularly anthocyanin pigments. It consisted of two stages: a first stage of 11 days of pre-fermentative cold maceration (between 5-8ºC), followed by 15 days of traditional maceration (between 20-25ºC). The cold-maceration process was carried out by using an industrial refrigeration system (HITSA-TOPAIR, mod RAE-101, Madrid, Spain) for the recirculation of refrigerant liquid through cooling water jackets to keep low temperatures. At the end of the stabilization process in stainless steel tanks during 5 months, the wine was distributed into three new American oak barrels (225 L, medium toast), where it was maintained for a 12 months. Samples were analyzed at 3, 6, 9, and 12 months of ageing, so a total of 12 wines (200 mL) were submitted to study.

2.2. HPLC–DAD–MS analysis

The HPLC used was an ELITE LaChrom fitted with a L-2130 quaternary pump, a L-2200 autosampler and a L-2455 DAD. The stationary phase was a MERK RP – C18 column 250 mm x 4 mm i.d. (5 µm). The injection volume was 20 µL and the mobile phase was composed by two solvents: solvent A, a 10% (v/v) aqueous formic acid solution, solvent B, formic acid: acetonitrile: water (1:3:6) (v/v). The elution conditions were: flow rate set at 1 mL min^{-1}, starting with a linear gradient from 20% to 66% of B in 50 min, a second linear gradient from 66% to 100% of B for 10 min followed by an isocratic elution of 100% of B for another 10 min. The column was washed with 100% of acetonitrile for a 7 min period proceeded by a re-equilibration step of 15 min of isocratic 20% of B. The UV–vis absorbance spectra were recorded from 250 to 700 nm and the peaks recorded at 280 and 520 nm.

MS analyses were performed using a Finnigan LCQ MS detector (Thermoquest) equipped with an API source, using an electrospray ionization (ESI) interface. The HPLC system was connected to the probe of the mass spectrometer via the UV cell outlet. Both the sheath and the auxiliary gas was nitrogen. The sheath gas flow was 1.2 $Lmin^{-1}$ and the auxiliary gas flow 6 $Lmin^{-1}$. The source voltage and the capillary voltage were 4.50 kV and 26 V, respectively, and the capillary temperature 195 ºC. Spectra were recorded in positive ion mode between m/z 150 and 2000. The mass spectrometer was programmed to do a series of consecutive scans: a full mass and a MS2 scan of the most abundant ion in the full mass. The normalized energy of collision was 45%.

The quantification of the anthocyanins compounds was made by comparing the areas and the retention times with the malvidin 3-glucoside standard, and anthocyanin concentration was expressed as mgL^{-1}. On the basis of their chemical structure, the overall pigment profile was divided into four major pigment families: a) Monomeric anthocyanins, grouped as Anthocyanidin-3-*O*-gluc (GL), acetylated anthocyanins (AC) and *p*-coumaroyl anthocyanins (COUM), b) pyranoanthocyanins (PY), c) acetaldehyde-mediated flavanol–anthocyanin products (ACD), and d) direct flavanol–anthocyanin condensation products (DC). The proportion of the different pigment families estimated by summing the content of each member found and the total pigment content estimated as the sum of the all compounds identified (SUM_TA) were also calculated.

2.3. Copigmented and polymerized anthocyanins determination

The contribution of copigmented anthocyanins (%CA) and polymeric pigments (%PP) to the total wine colour at pH 3.6 were determined following the method proposed by Boulton [15]. Wine samples were firstly adjusted to pH 3.6. The colorimetric effect of the copigmentation phenomenon was also evaluated by Tristimulus Colorimetry as reported by Gordillo et al. [14].

Colorimetric measurements

The whole visible spectrum of the wines (380–770 nm) was recorded at ($\Delta\lambda$ = 2 nm) constant intervals with a Hewlett-Packard UV-vis HP8452 spectrophotometer (Palo Alto, CA), using 2 mm pathlength glass cells and distilled water as a reference. The CIELAB parameters (L*, a*, b*, C^*_{ab} and h_{ab}) were determined by using the original software CromaLab® [16], following the Commission Internationale de L'Eclariage recommendations: the 10° Standard

198 Observer and the Standard Illuminant D65 [17]. Colour differences (ΔE^*_{ab}) were
199 calculated as the Euclidean distance between two points in the three-
200 dimensional space defined by L*, a* and b*: $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$.

201 *2.4. Statistical analysis*

202 Significant differences among wines and for each variable were
203 assessed by analysis of variance (ANOVA) using the Statistica® v 8.0 software
204 [18].

205

206 **3. Results and discussion**

207 *3.1. Detailed pigment composition in aged Syrah wines*

208 In the Syrah wines studied 32 anthocyanic pigments were identified from
209 suitable information concerning their UV-vis or mass spectral characteristics by
210 comparing them with those reported by relevant literature references [9,19,20]
211 (Table 1). Figure 1 shows the HPLC chromatograms of the representative wine
212 sample recorded at 520 nm, corresponding to 3 and 12 months of ageing.

213 In agreement with previous reports about other young and aged red
214 wines [13,20,21], the major peaks in the HPLC chromatograms in all samples
215 corresponded to monoglucoside anthocyanins, particularly of delphinidin (Dp 3-
216 glc), petunidin (Pt 3-glc), peonidin (Pn 3-glc) and malvidin (Mv 3-glc) (peaks 2,
217 3, 5 and 6). Their identities were easily identified by UV-vis and MS analysis,
218 providing a very high response factor. On contrast, cyanidin 3-glucoside was
219 not detected in any of the aged wine samples revealing the lower stability of this
220 anthocyanin against oxidative reactions due to its *o*-diphenolic chemical
221 structure [22].

In the same way, their corresponding acetic (peaks 8, 13, 17, and 18) and coumaric (peaks 19, 21, 22, 28 and 29) esters were present in several samples but in minor proportions. The particular identity of all acyl derivatives was elucidated from their molecular ions, which showed other peaks [M - 204]+ and [M - 308]+, representing the aglycone after the loss of an acetylglucoside or coumaroylglucoside group. Specially, the peaks of Pn 3-glc derivatives (peaks 17 and 28) are overlapped by those of Mv 3-gl derivatives, being the MS^3 analysis very useful for the discrimination of their identities since malvidin had the characteristic elimination mass of 16 u. whereas peonidin derivatives of 15 u.

The occurrence of esters of the caffeoyl acid with all monoglucoside anthocyanins were identified by Alcalde-Eón [9] in Tempranillo wines. However, in the Syrah wines studied, only Mv 3-caffeoylglucoside was detected, particularly in three month ageing samples (peak 4). It was assigned according to its mass (molecular ion at *m/z* 655, MS^2 fragment at *m/z* 331 and MS^3 at *m/z* 315) and HPLC retention characteristics, eluting immediately before Pt 3-glc.

Besides the main anthocyanin compounds referred, other 18 minor anthocyanin-derived pigments were identified. Monagas et al. [23] stated that the occurrence of anthocyanin-derived pigments in different red wines was related to the concentration of the corresponding anthocyanin precursors. In the aged wines studied, they were basically derived from Mv 3-glc and Pn 3-glc, which were the most abundant anthocyanidin monoglucosides previously described in young Syrah wines elaborated by cold maceration in warm climate [4,5].

As far as pyranoanthocyanins are concerned, 11 different compounds belonging to diverse sub-families were identified. A first sub-familiy, the A-type vitisins, corresponded to peaks 9, 11 and 20. Peak 9 was identified as vitisin A through its molecular ion (*m/z* 561) and by a characteristic fragment (*m/z* 399), which eluted soon after Mv 3-gl. Pyruvic acid derivatives of Mv 3-acetyl-glucoside (peak 11) and Mv 3-coumaroyl-glucoside (peak 20) were also detected at *m/z* values of 603 and 707, respectively. A fragment at *m/z* 399 was present for each of these compounds, resulting from the loss of acyl- and coumaroyl-glucoside groups. Additionally, the new ring in the structure of all these three compounds causes a typical hypsochromic shift of λ_{max} around 510 nm in the visible absorption maximum of the cromophore, which was confirmed in the DAD-vis analysis.

Vitisin B (peak 10), the adduct resulting from the direct reaction between Mv 3-glc and acetaldehyde, was identified by its MS spectrum that showed different fragments at *m/z* 517, 355, and 339, and eluting with a shorter retention time than Vitisin A. The DAD-vis analysis confirmed its identity by a characteristic hypsochromic shift of the λ_{max} at about 490 nm [21]. Other minor B-type vitisins derived from Pn 3-acetyl-glucoside, Mv 3-acetyl-glucoside, and Mv 3-coumaroyl-glucoside (peaks 12, 14, and 23, respectively) characterized by the same UV spectrum and the presence of mass ions at *m/z* 529, 559 and 663 were also detected.

Additional orange pyranoanthocyanin pigments exhibiting lower polarity and high factor response were detected in the last part of the HPLC chromatogram (peaks 27, 30, 31 and 32). Their chemical structures were consistent with a group of pyranoanthocyanins linked to a 4-vinylphenol group

271 or directly to flavanols. The UV–vis spectrum features corresponding to each
272 one of these peaks (from 505 to 515 nm) was very useful in the assignment of
273 their identities because they presented λ_{max} values significantly lower than that
274 of free anthocyanins [24]. The molecular ion of peak 27 (*m/z* 625) and its major
275 ion fragment (*m/z* 463) was confirmed to be malvidin 4-vinylcatechol (known as
276 Pinotin A), while its 4-vinylphenol adduct (malvidin 4-vinylphenol) was deduced
277 from its molecular ion at *m/z* 609 and a main ion fragment at *m/z* 447. On the
278 other hand, peaks 30 and 31 were identified as pyranoanthocyanins directly
279 linked to flavanols. The molecular masses of 805 (peaks 30) and 847 (peaks
280 31) are, respectively, attributed to Mv 3-glc and Mv 3-acetylglucoside linked
281 through a vinyl bridge to a catechin monomer. These pigments yielded the
282 same ion fragments MS^2 at *m/z* 643 (aglycone moiety: mv-4-vinyl-cat) and MS^3
283 at *m/z* 491corresponding to a retro-Diels-Alder product from the catechin unit
284 [25].

285 Concerning the new pigments resulting from the reaction between
286 anthocyanins and flavanols mediated by acetaldehyde, several compounds
287 were detected (peaks 16, 24, 25 and 26). Compound 16 showed a
288 characteristic maximum UV-Vis spectrum with λ_{max} in the visible region at 532
289 nm. MS^2 fragmentation of the molecular ion at *m/z* 809 produced a major
290 product ion at *m/z* 357, corresponding to the loss of a terminal unit of flavanol
291 together with a glucose moiety (452 Da), indicating that this compound is a
292 dimer Mv 3-glc ethyl-catechin [26]. Compounds 24 and 26 also showed a
293 characteristic fragmentation pattern of pigments with ethyl linkages. Their
294 molecular ions presented, respectively, 42 and 146 additional amu by
295 comparing them to the mass of compound 16; so they were assigned to

malvidin-3-acetyl and malvidin-3-p-coumaroyl ethyl-catechin dimers. Peak 25 gave a molecular ion at *m/z* 817 and a main fragment at *m/z* 655, which was identified as petunidin-3- glucoside-py-ethyl-catechin.

Moreover, Syrah wines studied also showed to contain a few minor direct anthocyanin–flavanol condensation products (peaks 1, 7 and 15). Their UV–Vis spectra are characterised by a λ max near 535 nm and a shoulder at 280 nm, which can only be confirmed for peak 1, the main compound of this pigment family. However, their retention times, *m/z* ratio of the molecular ions and fragmentation pattern allowed a decisive identification as direct condensation products between catechin with Mv 3-gl (peak 1, *m/z* 781, MS^2 619), Mv 3-glc acetylglucoside (peak 7, *m/z* 823, MS^2 619) and Pn 3-p-coumaroyl glucoside (peak 15, m/z 897, MS^2 589).

3.2. Effect of ageing time on changes in the main phenolic families

The occurrence of each compound identified was variable along the ageing period studied (Table 1), which determined that the anthocyanin profile of the Syrah wines changed progressively toward more chemical complexity (Figure 1). Thus, the influence of the ageing time on the relevance and evolution of the anthocyanic pigments was studied.

The changes in the levels (mgL^{-1}) of the main pigments families are showed in Figure 2. As can be observed, the ageing time had a significant influence over all the pigments classes studied. However, some differences were found concerning their development during maturation in oak barrels.

In general, the highest concentration of the total anthocyanin was found in the 3 months aged wines (Sum_TA=455.8±6.12 mgL^{-1}), from which an important decrease of 84% was produced along the aging process resulting in a

remained content of 73.7±8.4 mgL^{-1} in 12 months samples. The major pigments responsible for the red wine colour, mainly anthocyanins monoglucosides, were the compounds which more clearly contributed to the global pigment loss, followed by their respective acetylated and *p*-coumarylated derivatives. Although the pattern evolution of the diverse monomeric anthocyanin was similar, the rate of decrease was different for each group of compounds. This observation agrees with those reported by Del Álamo [27], which also observed particularly lower decreases of acetylated than non-acetylated anthocyanins content. This fact is explained because acetylated anthocyanins normally combine more quickly with tannins and non-acetylated anthocyanins are more implied in hydrolysis processes. However, in the Syrah wines studied, as ageing process advanced from 3 to 9 months, both monoglucosides and acylated derivatives experimented intense and significant decreases, being more marked for *p*-coumaroyl anthocyanins (sum_GL=80%, sum_AC=85% and sum_COUM=90%). As also reported in literature [28], several simultaneous reactions take place over the shelf-life of red wines in contact with oak wood (oxidation, absorption and precipitation or polymerisation), which additionally are influenced by large number of factors (genetic, agronomical, or oenological). Thus, the final concentration of phenolic compounds can be quite variable in red wines.

After 9 months in barrels, the content for the most abundant anthocyanins remained quite stable in low levels until the end of the aging period, and not significant differences were found among the levels respecting to12 month-old samples.

Among the newly formed red wine pigments, whereas some groups of compounds showed similar behaviour, others seemed to be inversely affected, which resulted in a lower decrease than monomeric anthocyanins. The total amounts of the minor anthocyanins diminished from 34.9±1.3 mgL^{-1} to 22.6±0.3 mgL^{-1} during the whole ageing process (35%); but from 6 months only a slightly decrease was globally produced. The minor pigments content of the aged Syrah wines studied were similar to those described in aged Monastrell wines by Cano-López [13], but it was significant lower than those reported in aged Tempranillo/Graciano wines [29]. These differences can be explained by a dual effect: the warm climate (the case of Monastrell and Syrah wines), which can exert a negative influence on the phenolic content and copigmentation process [30], and the grape variety, since it is well known that Graciano has high phenolic content which improve the copigmentation reactions.

During the first stage of ageing (from 3 to 6 months), together with monomeric anthocyanins, the predominant minor anthocyanins corresponded to the vitisin-like pyranoanthocyanins (A and B type), contributing for 60-69% to the total derived pigments. Their presence in wine is related to the secondary metabolic products (pyruvate and acetaldehyde) mainly released by yeast during alcoholic fermentation, and because of their slow degradation rate, they could become the main derived pigments in young and early aged wines [31]. During ageing, since the formation of vitisins is not strongly favoured, the proportion of both kinds of compounds naturally diminished with time. However, although the concentration of the B-type vitisins was higher at the beginning of the ageing process, A-type vitisins were the most abundant member of this sub-family as wine became older. Specifically, the remaining amount for the B-type

vitisins at the end of the ageing process was only of 10% (1.4±0.02 mgL^{-1}) while for the A-type vitisins was of 40% (5.8±0.2 mgL-1); indicating that the last ones were also more resistant towards the conditions undergone during ageing. This is probably due to the fact that, unlike acetaldehyde, pyruvic acid cannot be used as an intermediate in polymerisation processes [24]. The occurrence of these anthocyanin derivatives is of special interest in red wines typical from warm climates because these relatively small molecules remain in solution, in contrast to the classical flavanol–anthocyanin polymeric pigments that tend to precipitate. Oppositely, in the last three months of ageing (from 9 and 12 month) a progressive increase on the content of the lower polar pyranoanthocyanins took place, being the predominant derived pigments in 12 months wines (63.8%). The occurrence of 4-vinylcatechin and 4-vinylphenol was confirmed at every moment of ageing showing similar behaviour (Table 1). Their concentration tended to increase from 0.2 to 0.9 and from 5.3 to 7.4 mgL^{-1}, respectively, being the increases significant for both compounds. The occurrence of 4-vinylcatechol adduct was detected only from 9 months, reaching the highest levels in the last stage of ageing in the barrel (6.2±0.1 mgL^{-1}). The difference found about the occurrence of those type of pyranoanthocyanins is explained because the reaction between 4-vinylcatechol and Mv 3-gl to produce Pinotin A is reported to proceed rather slowly and requires years of storage to complete [32], while the formation of the Mv 3gl-4-vinylphenol or vinylcatechin adducts take place during fermentation [10].

Regarding acetaldehyde mediated derived anthocyanins; the main member of this family (Mv 3-glc-ethyl-cat) was present during the whole process of ageing. Nevertheless, after 9 months, the global content decreased

395 significantly from 4.4±1.2 to 0.7±0.01 mgL^{-1}, which might indicate a lower
396 resistance against the ageing process of this pigment family.

397 Finally, it is worth mentioning the lower importance of direct condensation
398 adducts over the pigment profile of aged Syrah wines during the first and the
399 last stages of ageing, where practically were quantified as very low
400 concentrations. The highest contents of this sub-family of pigments were found
401 at intermediate stages of ageing (from 6 to 9 months), reaching concentrations
402 around 3.0±0.3 mgL^{-1}. Despite the lower amounts of this anthocyanin-derived
403 pigments comparatively to pyranoantocyanins or ethyl mediated adducts, it was
404 shown that they can induce significant modification in the colour of the genuine
405 anthocyanins at concentrations similar to those that can exist in red wines [33].
406 Thus, they could also be considered as compounds with sensorial impact in
407 Syrah aged wines.

408 To summarize, as wine became older, the proportion of the original grape
409 anthocyanins (monomeric anthocyanins) tended to decrease from 92% to 70%
410 (Figure 3). On contrast, the anthocyanin-derived pigments played an increasing
411 role (from 8% to 30%), being mainly due to compounds providing the wine with
412 orange hues (pyranoanthocyanins). This gradual chemical transformation is in
413 accordance with the positive maturation effects attributed to barrels [34]. As
414 reported in literature, most of the anthocyanin derived pigments have a greater
415 colour expression and stability than monomeric anthocyanins at wine pH [8].
416 Thus, despite being minor pigments, is expected they can contribute
417 substantially to the overall colour of the aged Syrah wines.

418

419

420 *3.3. Effect of ageing time on copigmentation, polymerisation and wine colour*

421 Although copigmentation is a phenomenon mainly described in young
422 red wines, the results showed that it occurred in the Syrah wines during the
423 whole process of ageing in oak barrels (Figure 4), which was in accordance with
424 the results of some authors [35,36]. Notwithstanding, the general values
425 obtained were lower than those reported for young red wines, were
426 copigmented anthocyanins can even reach up to 50% of total colour [7]. The
427 highest value of the magnitude of copigmentation was found in the three
428 months samples (28.4%±0.5), but as the ageing advanced, the degree of
429 copigmentation significantly decreased reaching a minimum value of 9.14%±2.6
430 in 12 months samples.

431 Tristimulus Colorimetry was also applied to asses the colorimetric effect
432 of copigmentation on the total colour of the aged Syrah wines as reported in
433 [14]. Thus, the wine colour with and without copigmentation effect was
434 calculated at each stage of ageing, and was represented graphically in the
435 CIELAB (a*b*) diagram (Figure 5). The CIELAB differences (ΔE^*_{ab}, ΔL, ΔC^*_{ab},
436 and Δh_{ab}) for every pair of wines were also calculated to follow the trend of the
437 changes. As observed, the different location of the copigmented and no
438 copigmented wines in the (a*b*) diagram confirms that not only the magnitude
439 of copigmentation was influenced by the ageing process but also the colour
440 changes induced by this phenomenon.

441 At shorter ageing times (3 and 6 months), copigmentation induced the
442 greatest amplitude of the colour effect (ΔE^*_{ab}=11.1±0.35 and 8.24±0.37,
443 respectively) contributing to the total colour mainly with quantitative changes,
444 which consisted in strong increases of chroma and decreases of lightness

445 (ΔC_{ab}=+8.6±0.2 and +5.4±0.1; ΔL=-7.0±0.3 and -6.2±0.3, respectively). On
446 contrast, at longer ageing times (9 and 12 months), although the colour effects
447 were significantly lower (ΔE^*_{ab}=6.35±0.8 and 3.9±0.7, respectively) the most
448 important contribution of the copigmentation was qualitative, leading to a more
449 bluish hues (Δh_{ab}=-4.9 ±1.1, respectively). In any case, all the colour differences
450 calculated were higher than 3.0 CIELAB u. (visually perceptible by the human
451 eye) [37], which confirms the relevance of this physicochemical phenomenon on
452 the quality colour of aged wines, mainly at earlier stages.
453 Simultaneously to the copigmentation evolution, the degree of
454 anthocyanin polymerisation increased during ageing, being maximum at 9
455 months old wines (%PP=77.0%±3.6) (Figure 4). This significant increase
456 coincided with the observed losses of monomeric anthcoyanins and gradual
457 formation of new derived pigments, which reflects the complex pigment
458 transformation described in the previous section. However, after 9 months of
459 ageing, a slightly decrease of the polymerisation occurred (%PP=70.7%±3.6),
460 evidencing that extended ageing times affected negatively the chemical stability
461 of pigments in the Syrah wines studied.
462 Regarding the colour evolution, the changes on the colorimetric
463 parameters are shown in Figure 6. The results showed that the evolution of the
464 anthocyanins and the copigmentation/polymerisation processes resulted in
465 important changes both on quantitative (L* and C^*_{ab}) and qualitative (h_{ab})
466 psychophysical components of the colour. As expected, the ageing time
467 influenced significantly the trend of the changes.
468 From 3 to 9 months of ageing, the quantitative attributes of the wine
469 colour evolved progressively in a positive way. Specifically, the lightness of the

470 samples decreased by 6% (from 80.4±0.6 to 75.6±0.1 CIELAB u.), while
471 chroma increased by 13% (from 21.4±0.4 to 24.7±0.4 CIELAB u.), that is, the
472 wines became darker, with a more vivid colour. This colorimetric evolution
473 indicates that no colour fall was produced in this period of time. Thus, it can be
474 said that the significant loss of pigment and %copigmentation observed was
475 due more to their transformation into derived pigments than to degradation,
476 which also was confirmed by the increase in the %PP. However, from 9 to 12
477 month of ageing, the lightness and chroma of samples were negatively affected.
478 As observed, the lightness increased by 4% (from 75.6±0.1 to 78.7±0.5 CIELAB
479 u.) and the chroma decreased by 13% (from 24.6±0.4 to 21.3±0.4 CIELAB u.),
480 that is, wines became lighter and with less vivid colour. Thus, despite not being
481 especially significant the loss of anthocyanins in the last period of ageing, it
482 seems to be mainly responsible for the colour loss since the polymerisation
483 process tended also to decrease. It is well-known that the partial elimination of
484 new polymeric pigments by precipitation is a usual chemical transformation of
485 more matured red wines, which additionally can be intensified in warm climate
486 regions. The colour difference (ΔE^*_{ab}) calculated between the colour of the 9
487 and 12 months samples revealed that this colour variation was of 5.1±0.8
488 CIELAB u., thus, visually detectable by the human eye.

489 As far as qualitative attribute of colour (h_{ab}), it was remarkably affected,
490 showing a clear tendency during the whole ageing process. It increased with
491 increasing ageing time from 0.9º±0.8 to 11.3º±0.1, that is, from redness toward
492 more red-orange hues, which is expected according to the increasing role of
493 anthocyanin derived pigments over the total pigment content.

494 *3.4. Differentiation of aged Syrah wines based on the pigment and colorimetric*
495 *characteristics*

496 By comparing the four groups of wines (3, 6, 9 and 12 months) on the
497 basis of their pigment composition and colorimetric characteristics, a PCA
498 analysis was performed in order to examine which of these variables are more
499 involved in the differentiation of aged Syrah wines from Condado de Huelva
500 D.O. region, with different wood contact period. Figure 7 shows the distribution
501 of the samples along the first two principal components, where an accurate
502 differentiation and classification of wines into each of their respective groups
503 was obtained. The two principal components, with eigenvalues>1, explained
504 91.3% of the variability in the original data.

505 The first factor, which explained 72.5% of the variance, clearly separates
506 Syrah wines aged for 3 and 6 months, grouped in the negative area of PC1,
507 from the respective ones aged for 9 and 12 months, located separately in the
508 positive area of the graphic. Results showed that several compounds were
509 useful for the differentiation between shorter and longer aged Syrah wines,
510 including not only monomeric but also derived anthocyanic pigments. In
511 particular, significant negative correlations were obtained for Glucosides,
512 Acetates, Coumarates, Vitisin A dvs., Vitisin B dvs., and acetaldehyde mediated
513 derived anthocyanins, which generally are more characteristics of earlier
514 maturation stages in red wines. On contrast, significant positive correlations
515 were obtained for minor anthocyanic compounds Mv 3-glc 4-vinylcatechol, Mv
516 3-glc-4-vinyl-catechin and Mv 3-glc 4-vinylphenol, usually associate with more
517 extended ageing periods. Regarding the colour variables, shorter aged wines
518 were significant correlated with lighter colour and higher levels of

519 copigmentation (negative correlations), while longer aged wines with re-orange
520 hues and higher degree of polymerisation (positive correlations).
521 On the other hand, the second factor which explained 18.8% of the
522 variance, showed that 3 and 12 aged wines could be also differentiated from 6
523 and 9 aged wines on the basis of the direct condensation adducts content and
524 chroma, both of them being significant positive correlated. This meant that
525 intermediate ageing periods were characterised by more intense colours and
526 higher content of direct condensation anthocyanin adducts.
527
528 **4. Conclusions**
529 This work provide a better knowledge of the ageing aptitude of Syrah wines
530 elaborated in warm climate based on the evolution of its detailed pigment
531 composition and colorimetric behaviour. HPLC–DAD–MS analysis permitted the
532 identification of several major and minor anthocyanic pigments. Although the
533 total pigment content decreased during the whole ageing process, as wines
534 became older, the relative amounts of compounds which give higher chemical
535 stability and orange hue increased and that of compounds less stable that gives
536 blue hues decreased. With respect to the colorimetric characteristics, the results
537 showed that wines summited to shorted or intermediate ageing times (between
538 3 to 9 months) exhibited most desirable global characteristics as required
539 nowadays to produce high quality wines, that is, darker and more redness vivid
540 colour. On contrast, those submitted to longer ageing times (between 9 to 12
541 months) finally showed lighter and less vivid colour with more red-orange hues.
542
543

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632

FIGURE CAPTIONS

Figure 1. Chromatograms recorded at 520 nm corresponding to (a) wine sample of 3 months, (b) wine sample of 12 months. The compound numbers correspond to those in Table 1.

Figure 2. Evolution of the anthocyanin content (mgL^{-1}) during the aging of Syrah wines, grouped as (a) Monoglucosides and acyl derivatives, b) main minor anthocyanin-derived pigments.

Figure 3. Relative abundance (%) of the anthocyanin pigment families during ageing.

Figure 4. Evolution of the copigmented anthocyanins (%CA) and polymeric pigment (%PP) during Syrah wine ageing.

Figure 5. Colour of the copigmented and no copigmented aged wines at (a) 3 and 6 months, (b) 9 and 12 months.

Figure 6. Evolution of colour parameters: (a) L* (lightness), (b) C^*_{ab} (chroma), and (c) h_{ab} (hue angle), during ageing.

Figure 7. Distribution of the Syrah wines in the two dimensional coordinates system defined by the first two principal components according to different wood contact periods (3, 6 , 9, and 12 months).

Table 1. Retention times, mass spectra details and chromatographic UV-vis spectrum data of anthocyanins and anthocyanin-derived pigments of aged Syrah wines found during ageing period.

Peak	Compound	$Rt_{(min)}$	M^+	M^2	M^3	$\lambda_{max(nm)}$	Pigment Family	Samples (months)			
								3	6	9	12
1	Mv 3-glc-cat	19.04	781	619	467	280,535	Direct condensation	nd	*	*	*
2	Dp 3-glc	19.49	465	303		277,523	Anthocyanidin- 3-*O*-gluc	*	*	nd	nd
3	Pt 3-glc	26.58	479	317	302	277,526	Anthocyanidin- 3-*O*-gluc	*	*	*	*
4	Mv 3-cafglc	29.80	655	331	315	277,526	Caffeoyl anthocyanins	*	nd	nd	nd
5	Pn 3-glc	31.80	463	301	286	280,517	Anthocyanidin- 3-*O*-gluc	*	*	*	*
6	Mv 3-glc	33.01	493	331		280,526	Anthocyanidin- 3-*O*-gluc	*	*	*	*
7	Mv 3-acetylglc-cat	36.86	823	619	467	-	Direct condensation	*	nd	nd	nd
8	Dp 3-acetylglc	38.29	507	303	257	280,526	Acetylated anthocyanins	*	*	nd	nd
9	Vitisin A	38.60	561	339	338	277,511	Pyranoanthocyanins	*	*	*	*
10	Vitisin B	40.07	517	355	339	274,490	Pyranoanthocyanins	*	*	*	*
11	A-type vitisin of Mv-3-acetylglc	43.02	603	339	388	277,517	Pyranoanthocyanins	*	*	*	*
12	B-type vitisin of Pn-3-acetylglc	44.85	529	325	355	277,480	Pyranoanthocyanins	nd	*	nd	nd
13	Pt 3-acetylglc	45.51	521	317	302	268,529	Acetylated anthocyanins	*	*	nd	nd
14	B-type vitisin of Mv-3-acetylglc	45.61	559	355	339	268,499	Pyranoanthocyanins	nd	*	nd	nd
15	Pn 3-*p*-coumglc-cat	48.26	897	589	437	-	Direct condensation	nd	*	nd	nd
16	Mv 3-glc-ethyl-cat	50.00	809	357	342	280,532	Acetaldehyde-mediated	*	*	*	*
17	Pn 3-acetylglc	50.18	505	301	286	280,529	Acetylated anthocyanins	*	*	*	*
18	Mv 3-acetylglc	51.63	535	331	315	277,529	Acetylated anthocyanins	*	*	*	*
19	Dp 3-*p*-coumglc	53.85	611	303	257	280,532	*p*-coumaroyl anthocyanins	*	*	nd	nd
20	A-type vitisin of Mv-3-*p*-coumglc	56.27	707	339	383	280,518	Pyranoanthocyanins	*	*	*	*
21	Cy 3-*p*-coumglc	58.93	595	287	175	280,526	*p*-coumaroyl anthocyanins	*	nd	nd	nd
22	Pt 3-*p*-coumglc	59.97	625	317	302	280,532	*p*-coumaroyl anthocyanins	*	*	nd	nd
23	B-type vitisin of Mv 3-*p*-coumlglc	60.59	663	355	340	286,532	Pyranoanthocyanins	nd	nd	*	nd
24	Mv-3-acetylglc-ethyl-cat	61.63	851	357	432	280,520	Acetaldehyde-mediated	nd	*	*	nd
25	Pt 3-glc-py-ethyl-cat	64.25	817	655	531	280,520	Acetaldehyde-mediated	nd	nd	*	nd
26	Mv 3-*p*-coumlglc-4-ethyl-cat	64.52	955	665	357	280,532	Acetaldehyde-mediated	*	*	*	*
27	Mv 3-glc 4-vinylcatechol	64.70	625	463	447	280,511	Pyranoanthocyanins	nd	nd	*	*
28	Pn 3-*p*-coumglc	64.97	609	301	286	280,526	*p*-coumaroyl anthocyanins	*	*	*	*
29	Mv 3-*p*-coumglc	65.43	639	331	315	283,529	*p*-coumaroyl anthocyanins	*	*	*	*
30	Mv 3-glc-4-vinyl-cat	67.61	805	643	491	280,508	Pyranoanthocyanins	*	*	*	*
31	Mv-3-acetylglc-4-vinyl-cat	69.17	847	643	491	280,508	Pyranoanthocyanins	*	*	nd	*
32	Mv 3-glc 4-vinylphenol	69.66	609	447	431	280,505	Pyranoanthocyanins	*	*	*	*

Rt: retention time (min); M^+: positive charged molecular ion; MS^2, MS^3: fragmentation of M^+ and M^2
*Detected; n.d. – not detected

Figure 1

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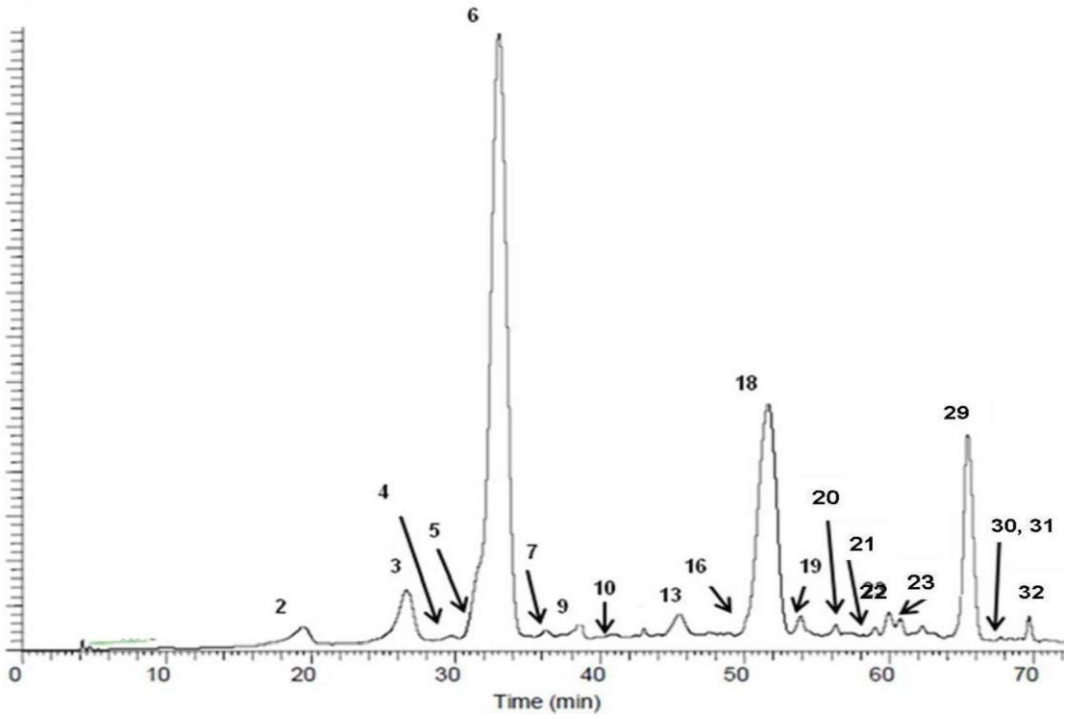
Figure 1.

(a)



(b)

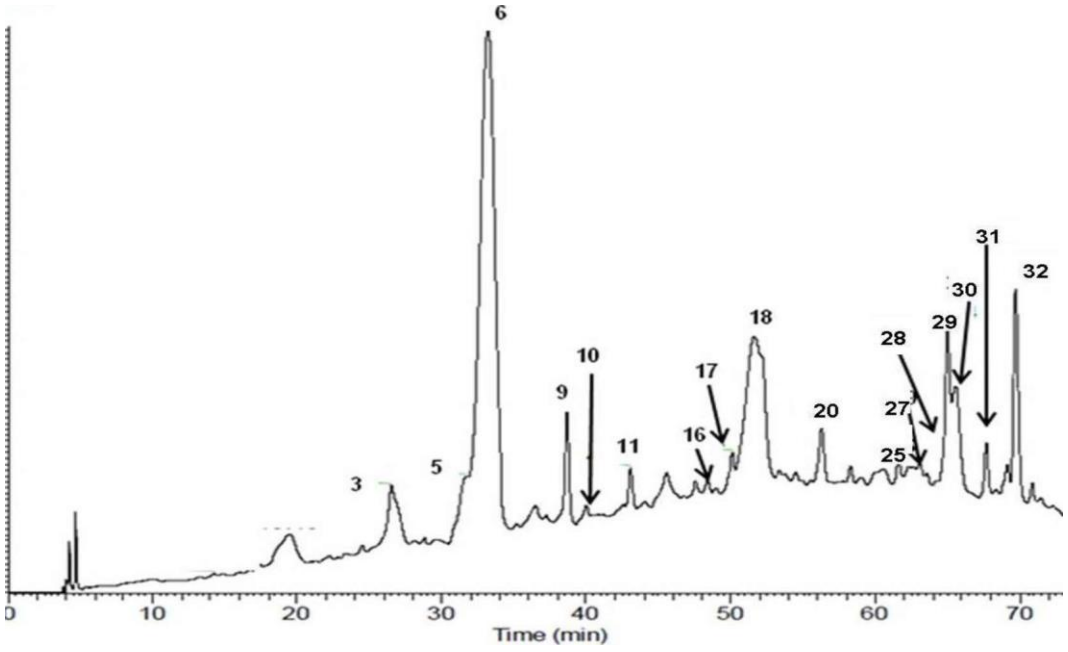


Figure 2
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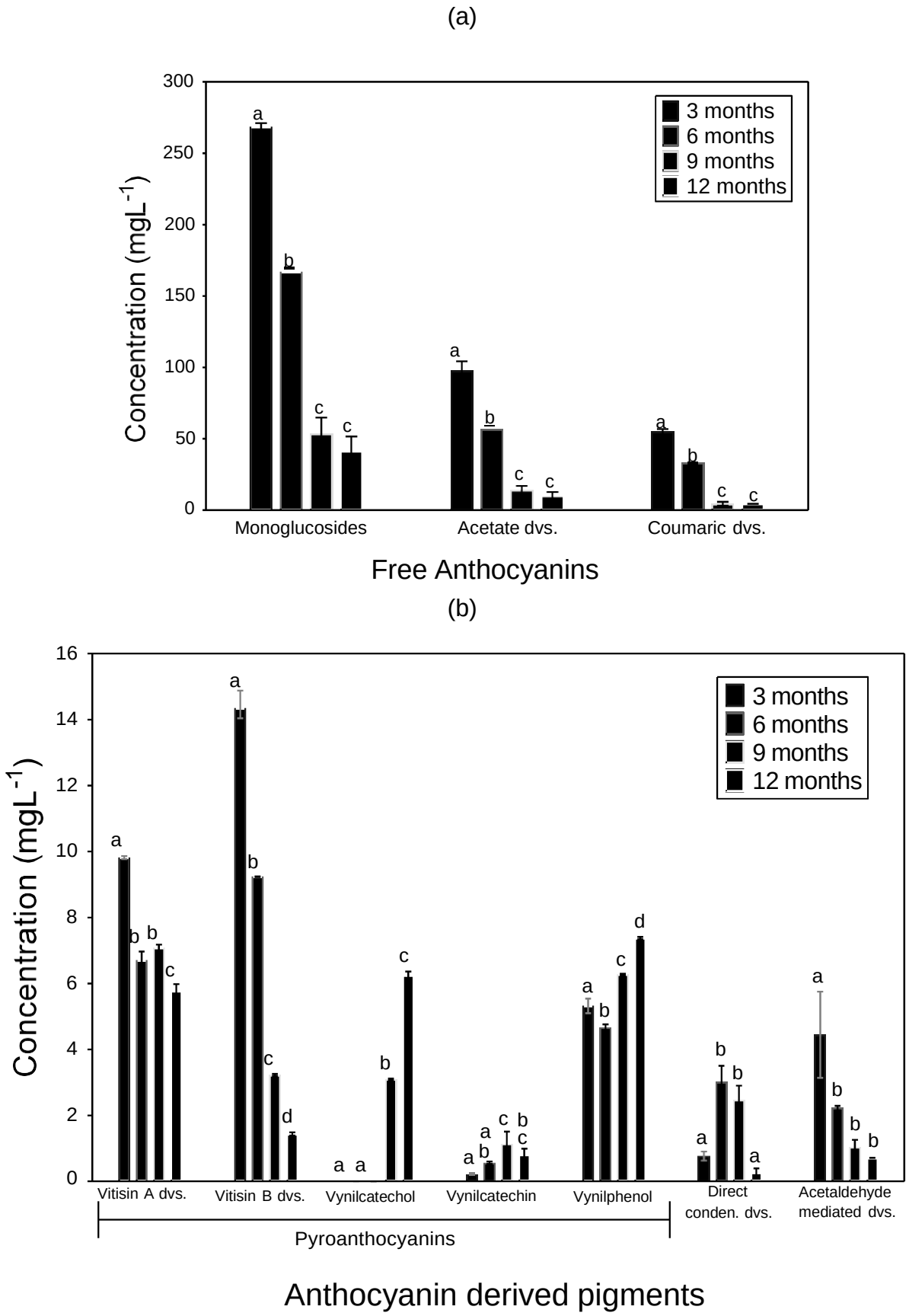
Figure 2.



Different letters for each group of pigments among successive ageing stages mean significant differences ($p < 0.05$).

Figure 3

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Figure 3.

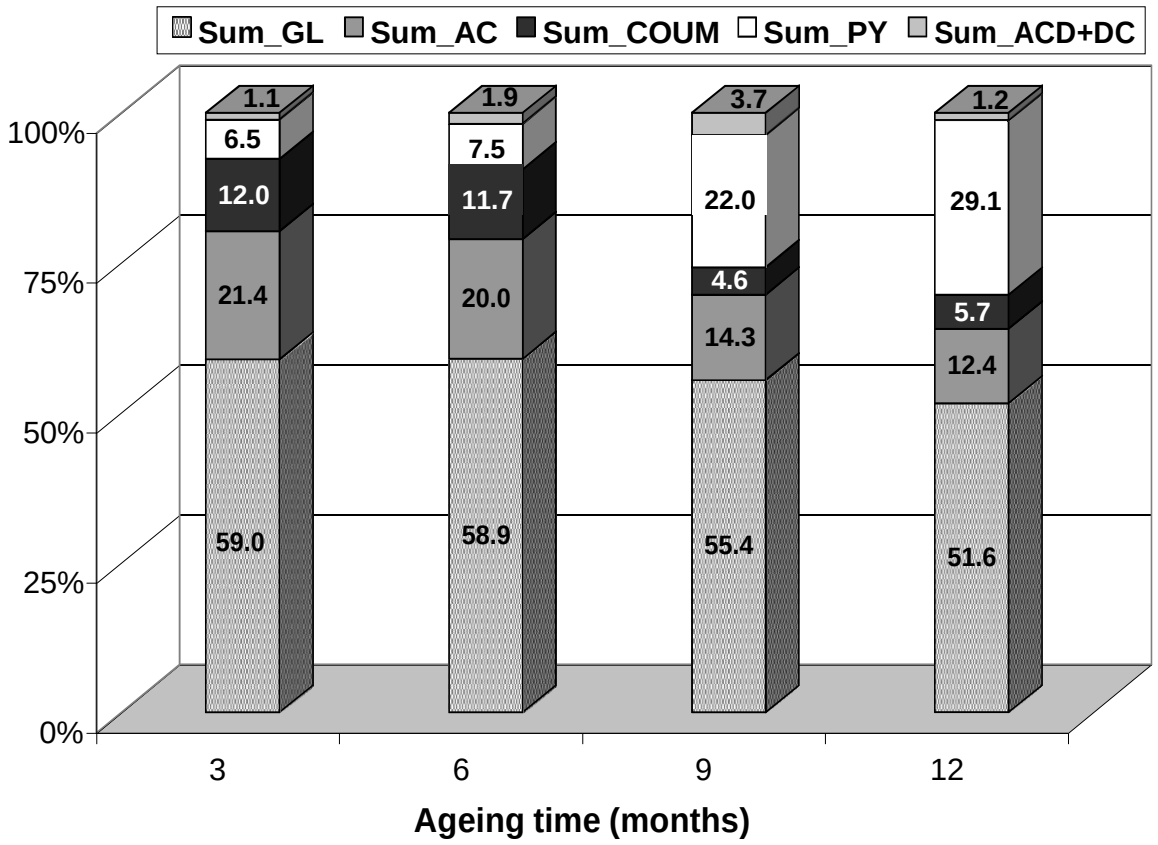

Figure 4

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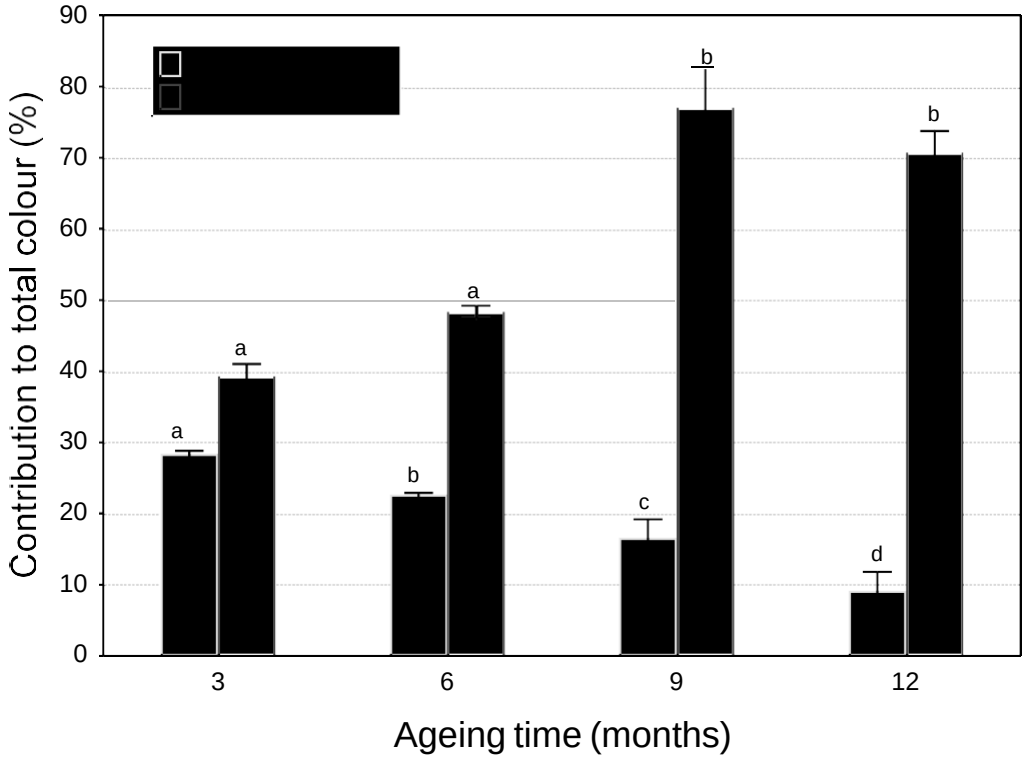
Figure 4.



Different letters for the %Copigmentation and %Polymerization among successive ageing stages mean significant differences ($p < 0.05$).

Figure 5
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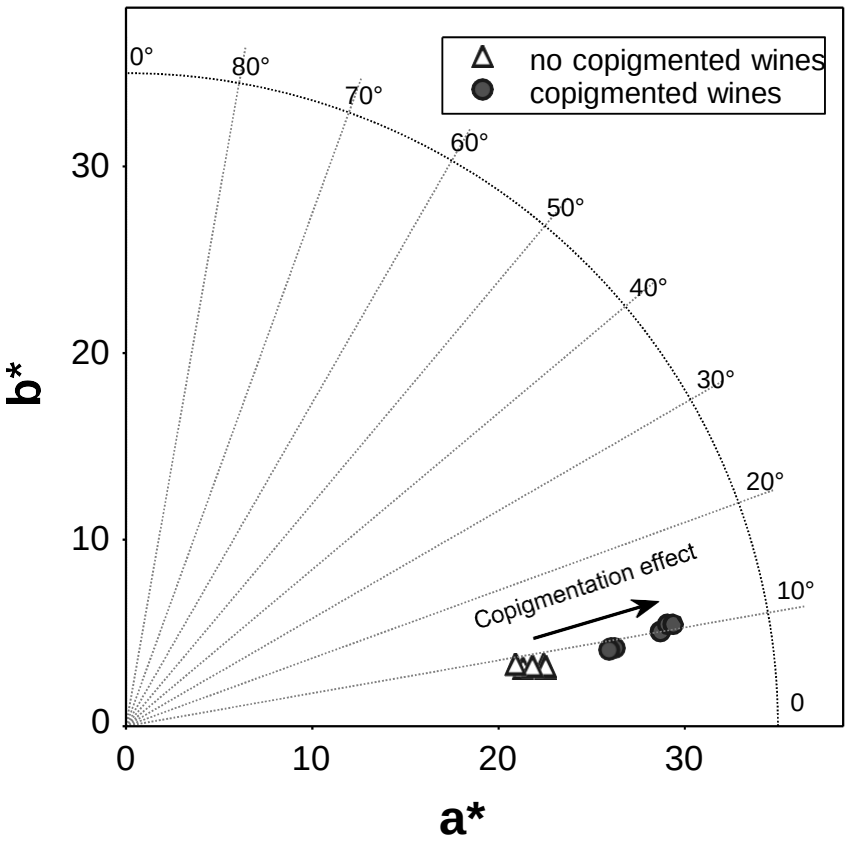
Figure 5.

(a) 3-6 months



(b) 9-12 months

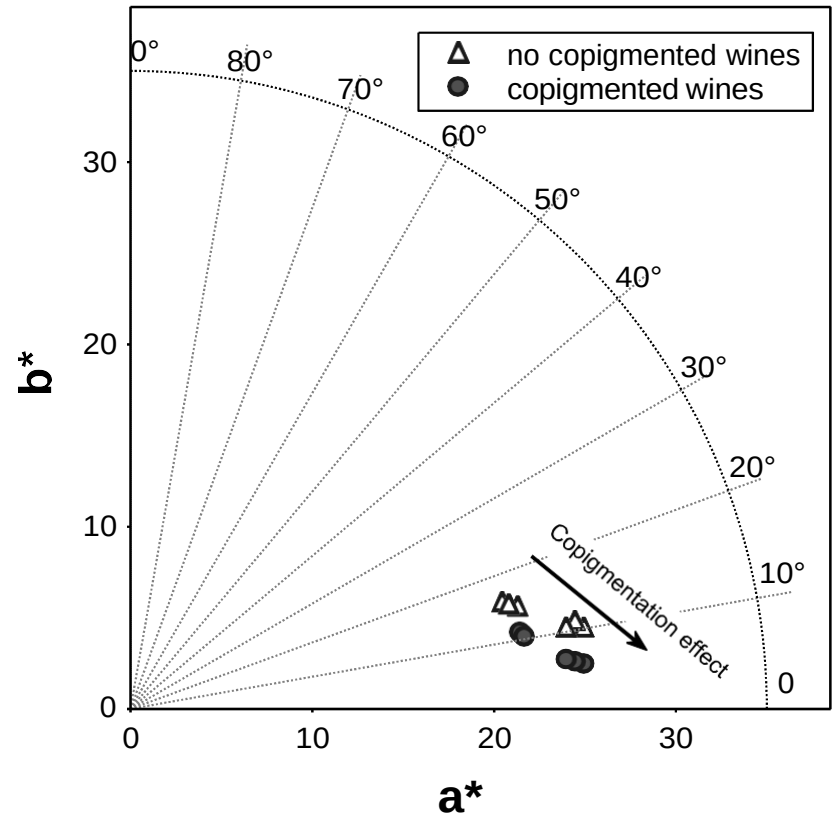


Figure 6
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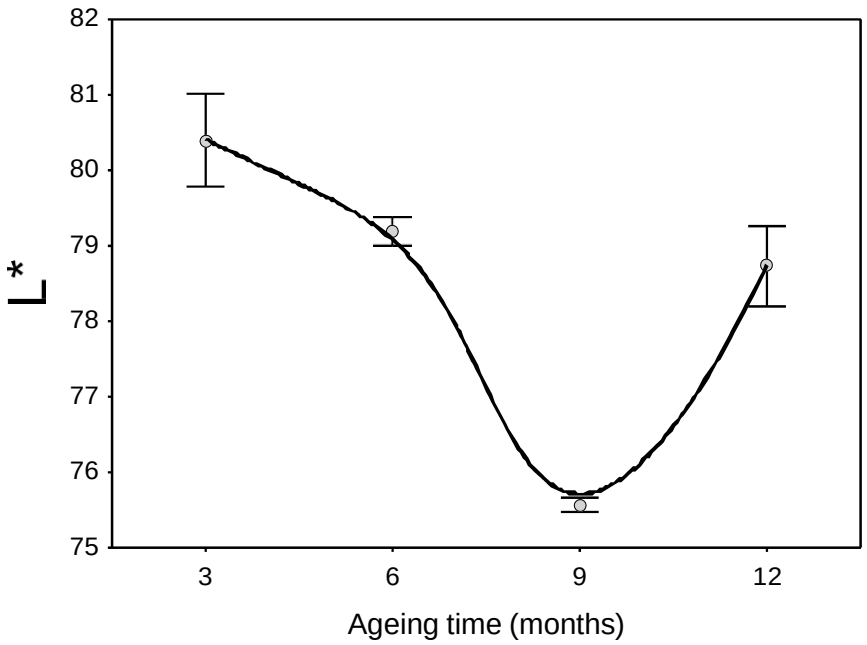
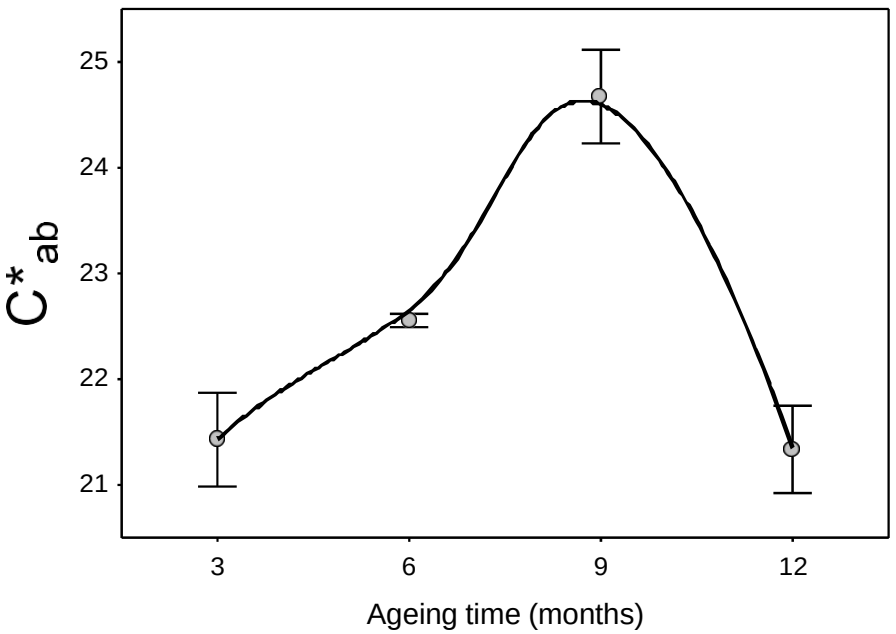
Figure 6.

(a)



(b)



(c)

(c)

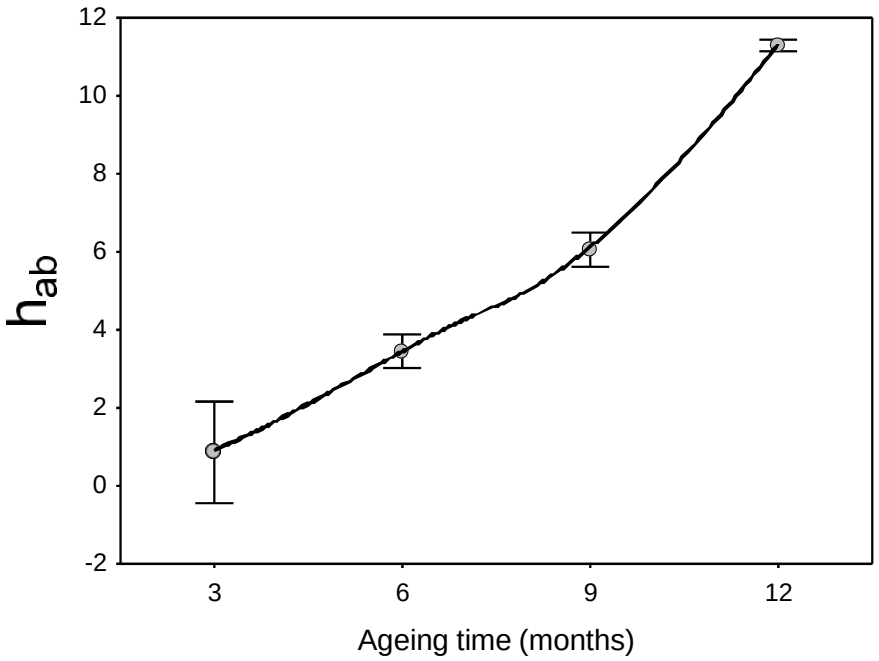


Figure 7
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Figure 7.