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Post-Fermentative Addition of Grape Seed Protein Hydrolysates and Their Impact on Wine Colour-Related Polyphenols

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Abstract: This study evaluates the effect of protein hydrolysates, obtained from grape seed meal (industrial waste), as colour stabilisers in red wines from warm climates. Protein hydrolysates were added to the wine after fermentation and maceration. Assays were performed using different types and doses of protein hydrolysates. Two grapevine-growing seasons were monitored over 8 months. Attention was focused on different families of polyphenolic compounds, copigmentation, and polymerisation, and colour changes were assessed by differential tristimulus colourimetry. Regardless of doses and typology, wines with protein hydrolysates suffered a decrease in the amount of phenolics and an increase in % polymerisation. Only wines treated with 3 g/L of hydrolysates showed lower colour intensity and greater clarity and hue after 8 months, while treatment with 0.5 g/L produced brownish wines. The addition of hydrolysates from a low hydrolysis time did not affect the copigmentation balances or produced negative visually perceptible colour differences over time. These results indicate that the post-fermentation addition of protein hydrolysates does not seem to significantly improve the stabilisation of the wine colour, in contrast to the addition at other stages. This information is of great interest to wineries to consider the application of this novel technique at the optimal time.

Keywords: grape seed protein hydrolysates; polyphenolic compounds; CIELAB; differential tristimulus colourimetry; warm climate; wineries

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1. Introduction

The typical sensory characteristics of red wines, such as colour, astringency, and bitterness, are highly valued today for consumers and thus influence the economics of the wine industry. These organoleptic characteristics are mainly influenced by phenolic compounds [1–6], and their red colour is primarily determined by anthocyanins [7,8]. They stabilise by non-covalent bonds with other phenolic compounds (principally colourless phenols) by the copigmentation process [9,10] but are highly susceptible to degradation by different factors such as temperature, oxygen, light, enzymes, and pH [7,11–13].

The degradation of anthocyanin is normally accelerated in areas with warm climates, characterised by high temperatures and scarce rainfall, intensifying it in advance of climate change. As a consequence, the wines elaborated in these areas usually present a chemical imbalance in the pigment–copigment ratio due to a time lag between phenolic maturity and the technological maturity of the grapes at the time of harvest. As a result, wines with an inadequate colour stability are produced, which avoid ageing processes and force them to be consumed as young wines [14,15].

Several studies have demonstrated that the stability of anthocyanins could be improved by interacting with proteins or peptides to form copigment complexes [16–24]. Chung et al. [25] and Zang et al. [26] reported that the colour stability of anthocyanins in model beverages and anthocyanin solution, respectively, improved by adding whey protein isolate. Other studies affirmed that milk proteins improved the stability of grape skin anthocyanin extracts [27]. Chamizo-González et al. [28] described the interactions between 7S and 11S grape seed globulins and malvidin-3-O-glucoside at the wine pH, producing a darker and more vivid bluish colour of a malvidin-3-O-glucoside solution. Based on these research studies, it could be possible to affirm that proteins and peptides establish interactions with anthocyanins and possibly contribute to a colour stabilization of red wines [29–31].

Regarding the wines to be stabilised, previous studies highlight the need to describe the effect of a new oenological strategy in the three different stages in the winemaking process (pre-fermentation, post-fermentation, and the stabilisation stage) [32], considering their diverse chemical composition and colourimetric characteristics [33,34]. Consequently, optimal conditions about the moment of the addition of grape protein hydrolysates will be possible to establish with the broad objective of the colour stabilisation of red wines.

The pre-fermentative addition of grape seed protein hydrolysates resulted in red wines with higher chroma values and percentages of copigmentation, without significant changes in hue [35]. Similarly, the addition of different protein hydrolysates to Syrah wines during the stabilisation stages (when the colour of wines from warmer climates generally begins to fade) helped to prolong the colour of the wine for longer [36]. However, it is necessary and opportune to study the behaviour of the red wine after the post-fermentative addition of the grape protein hydrolysates, which has not previously been reported.

Therefore, this study aims to scrutinise, for the first time, the effects of adding grape protein hydrolysates to Syrah red wines in the post-fermentation stage, as a further step toward stabilising the colour of red wines elaborated in warm climate. In this investigation, two different factors were considered: the hydrolysis time and the dose of protein hydrolysate applied. For this purpose, the total and individual phenolic composition, chromatic characteristics, copigmentation, and polymerisation of the anthocyanins of the red wines during storage were analysed. This is a new advance for the correct application of this technique as a possible oenological strategy and its implementation in the wine industry.

2. Materials and Methods

2.1. Chemicals and Solvents

Ammonia, hydrochloric acid, and sodium carbonate were purchased from Panreac Química S.L.U. (Barcelona, Spain). Folin-Ciocalteau reagent, sodium hydroxide, formic acid, malvidin-3-glucoside, (+)-catechin, (−)-epicatechin, gallic acid, caffeic acid, and quercetin were supplied by Sigma-Aldrich (Madrid, Spain) and acetaldehyde from Merck (Darmstadt, Germany). Ethanol and potassium metabisulphite were acquired by Panreac Química S.L.U. (Barcelona, Spain), and HPLC-grade acetonitrile was provided by VWR International Eurolab S.L. (Barcelona, Spain). Enzymatic protein hydrolysates were produced using the endoprotease Alcalase® (subtilisin A), which was acquired from Novozymes® (Bagsvaerd, Denmark).

2.2. Enzymatic Hydrolysis of Defatted Grape Seed Meal

The protein hydrolysates of the grape seeds were obtained from defatted grape seed meal (DGSM), which is the resulting by-product of the industrial processing of grape pomace. Alvinesa Natural Ingredients, S.A. (Daimiel, Ciudad Real, Spain), supplied the

DGSM, and the industrial process for obtaining DGSM is explained in detail by Mora-Garrido et al. [29].

Protein hydrolysates were obtained by an extraction process followed by an enzymatic hydrolysis of DGSM (derived from Airén/Tempranillo varieties, 75/25). This process of obtaining protein hydrolysates has been extensively described by Mora-Garrido et al. [31]. Briefly, DGSM/water (20:80 *w/v*) was subjected to alkaline extraction of proteins in a bioreactor (Biorreactor Bio Console ADI 1025 Applikon Biotechnology, Inc, Delft, Netherlands) at room temperature and controlled agitation and pH (180 rpm, pH 10.5) for 3 h. The soluble protein concentrate (supernatant) was separated by centrifugation (14,880× *g*, 4 min, 4 °C) and concentrated under vacuum (70 °C, until pH was about 8). Subsequently, once the endogenous enzymes of the soluble protein concentrate were inactivated in a water bath (5 min at 80 °C), the protein concentrate was subjected to enzymatic hydrolysis under alkaline conditions with 0.6% *v/v* Alcalase (50 °C and pH 8.5).

Two different hydrolysis times (1 h and 4 h) were used, named as low (L) and high (H), respectively; thus, two protein hydrolysates were obtained with different molecular weight distributions [31]. After hydrolysis, the pH was reduced to 3.5 with HCl (37%), separating and discarded non-soluble peptides by centrifugation (33,600× *g*, 20 min, 4 °C). The supernatants were concentrated in a vacuum rotary evaporator and lyophilised for subsequent addition to wine.

2.3. Winemaking

Different winemaking based on the addition of protein hydrolysates was carried out in two different grapevine growing seasons (2020 and 2022). Winemaking was performed with *Vitis vinifera* cv. Syrah grapes cultivated in the "Condado de Huelva" designation of origin in southern Spain, a typical warm climate region according to the Köppen climatic classification [37]. In the 2020 and 2022 vintages, the average temperatures were 26.8 °C (range 34.6 to 19.9 °C) and 25.9 °C (range 33.8 to 19.2 °C), the humidity was 49.3% (range 78.2 to 23.9%) and 51.4% (range 76.1 to 26.4%), and rainfall was 0.11 and 0.48 mm/day, respectively (data provided by Instituto de Investigación y Formación Agraria y Pesquera (IFAPA), Junta de Andalucía, Spain).

Winemaking was performed in the facilities of the Bodegas Nuestra Señora del Socorro (Rociana del Condado, Huelva, Spain) following the methodology described by Mora-Garrido et al. [36]. Grapes were harvested at a good maturity grade (20 °Brix, pH 3.4 and 6.7 g tartaric acid/L of acidity) [38]. After alcoholic fermentation, different experimental microvinifications were performed in triplicate, based on the post-fermentative addition of protein hydrolysates. Malolactic fermentation occurred spontaneously.

During the first grapevine growing season, the two different protein hydrolysates obtained were added to the wines at 0.5 g/L. In the second season (under similar winemaking conditions), the hydrolysates were added at 3 g/L. Two doses were applied to test the importance of the added dose of protein hydrolysate as a relevant factor in the implementation of this new oenological technique. These concentrations were comparable to those used in the stabilisation stage [36] or in the application of other protein sources in winemaking [39].

Six different types of wine were produced: wines with the addition of protein hydrolysates with high and low hydrolysis times, at 0.5 g/L and 3 g/L (HW05, LW05, HW3, and LW3), and the respective two control wines (CW) without adding protein hydrolysates. The wines were monitored at different stages along the stabilisation period after the addition of protein hydrolysates. Special attention was focused on 1 month and 4 months, when important decisions can be made in industrial vinification (start ageing period, adding wood chips, or bottling), and 8 months (normal time to bottling).

2.4. HPLC-DAD Analysis of Polyphenolic Compounds

HPLC separation, identification, and quantification of phenolic compounds was carried out with an Agilent 1200 chromatographic system (Agilent Technologies, Palo Alto, CA, USA), equipped with a quaternary pump, a UV–Vis diode array detector, an automatic injector, and ChemStation software (B.04.03 version) (Agilent Technologies, Palo Alto, CA, USA). Prior to direct injection, the samples were filtered through a 0.45 µm Nylon filter (E0034, Análisis Vínicos, Tomelloso, Spain). All analyses were performed in triplicate.

For the determination of individual anthocyanins, flavonols, flavan-3-ols, hydroxycinnamic acid derivatives (HCAD), and benzoic acids, the methods described in Mora-Garrido et al. [36] were used. The solvents used were acetonitrile–formic acid–water (3:10:87), as solvent A, and acetonitrile–formic acid–water (50:10:40), as solvent B. A quantity of 50 µL was employed as the injection volume. The gradient (0.8 mL/min) used for the determination of anthocyanin was as follows: 0–10 min, 94% A–6% B; 10–15 min, 70% A–30% B; 15–25 min, 60% A–40% B; 25–35 min, 55% A–45% B; 35–40 min, 50% A–50% B; 40–42 min, 40% A–60% B; 42–43 min, 94% A–6% B. Moreover, the gradient (0.63 mL/min) used for the phenolic compounds was: 0–5 min, 94% A–6% B; 5–15 min, 89% A–11% B; 15–20 min, 80% A–20%B; 20–25 min, 77% A–23% B; 25–30 min, 74% A–26% B; 30–35 min, 60% A–40% B; 35–38 min, 50% A–50% B; 38–46 min, 40% A–60% B; and 46 min, 94% A–6% B. UV–Vis spectra were recorded from 200 to 800 nm with a 10.0 nm bandwidth. The quantification of individual anthocyanins was made at 525 nm, at 280 nm for flavan-3-ols and benzoic acids, at 320 nm for hydroxycinnamic acid derivatives (HCAD), and at 360 nm for flavonols, by comparing the areas and retention time with the standards of malvidin 3-glucoside, catechin and gallic acid, and caffeic acid and quercetin, respectively, using calibration curves.

The total content of each phenolic family was calculated as the sum of the individual phenolic compounds identified by HPLC. All measurements were taken in triplicate.

2.5. Spectrophotometric Colour Measurement

The colour analysis was performed by Tristimulus Colourimetry using the CIELAB uniform colour space, recommended by the Commission Internationale de l'Éclairage (CIE) as the most suitable tool for colour specification in industrial applications. From this colour space, colourimetric variables based on both the rectangular (L^*, a^*, b^*) and cylindrical (L^*, C^*_{ab}, h_{ab}) colour coordinates were used.

An Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies, Palo Alto, CA, USA) was used to determine the whole visible spectrum (380–770 nm) at constant intervals ($\Delta\lambda$ = 2 nm). The 2 mm path-length glass cells and distilled water were used as reference. The CIELAB colour parameters (L^*, a^*, b^*, C^*_{ab}, and h_{ab}) were calculated by using the original CromaLab© software (2.0 version) [40], following the recommendations of the CIE 1964 [41]: 10° Standard Observer and the Standard Illuminant D65. In addition, the Euclidean distance between two points in the three-dimensional space defined by L^*, a^*, and b^* was used to calculate the colour differences (ΔE^*_{ab}): $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$.

Additionally, the relative contribution of lightness (% ΔL), chroma (% ΔC), and hue (% ΔH) of a given colour difference (ΔE^*_{ab}), expressed in percentages, was calculated:

$$\% \Delta L = [(\Delta L^*)^2 / (\Delta E^*_{ab})^2] \times 100$$

$$\% \Delta C = [(\Delta C^*_{ab})^2 / (\Delta E^*_{ab})^2] \times 100$$

$$\% \Delta H = [(\Delta H)^2 / (\Delta E^*_{ab})^2] \times 100$$

with ΔH being deduced by the following mathematical equation: $\Delta H = [(\Delta E^*_{ab})^2 - (\Delta L)^2 - (\Delta C)^2]^{1/2}$.

After adjustment to pH 3.6 using 1 M NaOH and HCl, the percentage contributions of copigmented anthocyanins (% copigmented pigments) to the total colour of the wine and the degree of anthocyanin polymerisation (% polymeric pigments) were determined [36,42]. The Folin–Ciocalteau method was used for the analysis of total phenolics [43]. All measurements were made in triplicate.

2.6. Visual Sensory Evaluation

A visual evaluation of the studied wines was carried out 8 months after the addition of the protein hydrolysates to assess the stabilisation of colour, which was the main objective of this investigation. The samples were evaluated in triplicate. Prior to the sensory analysis, participants were informed and asked to bring the signed consent to participation form.

The evaluation was performed following the specifications described by Mora-Garrido et al. [36] (UNE-ISO 4121:2006 standard [44]). A panel of 14 judges formed in the sensory analysis of wines (nine women and five men, aged between 28 and 54 years) attended the wine tasting. A Colour Assessment Cabinet (CAC_Portable DL041010, VeriVide, Leicester, UK) was used. The colour of the wines was visually evaluated according to 5 descriptors: lightness, colour intensity, tonality, limpidity, and acceptability. The panellists identified the descriptors by rating them on a 9-point scale, where 1 was not perceived (absence) and 9 was the maximum perceived attribute. For tonality, a gradual scale of hues from purple (lower values) to brownish (higher values) was used. The acceptability of the wines was evaluated according to the judges' colour preference criteria.

2.7. Statistical Analysis

For all statistical analyses, Statistica v.8.0 software was used [45]. Univariate analysis of variance (ANOVA) was applied to establish the statistical differences between the samples studied. Furthermore, principal component analysis (PCA) was implemented to highlight the main contributors to variance. Throughout the text, the term significant means p values less than 0.05 ($p < 0.05$) for the statistical differences.

3. Results and Discussion

3.1. Phenolic Profile Evolution

The mean values of the phenolic content (total glucoside, acetyl and *p*-coumaroyl anthocyanin derivatives and anthocyanins, benzoic acids, hydroxycinnamic acid derivatives (HACD), flavan-3-ols, flavonols, and total phenolic content) and the percentages of copigmentation and polymerisation in the wines (control and assayed) at 1, 4, and 8 months after the addition of high- (HW) and low- (LW) protein hydrolysates, at 0.5 g/L and 3 g/L, are summarized in Tables 1 and 2, respectively. Moreover, Tables S1 and S2 show the quantification of individual anthocyanins and polyphenolic compounds (benzoic acids, HCAD, flavan-3-ols, and flavonols) in all the wines.

Over time, the polyphenolic compounds evolved similarly in all wines tested, that is, the amount of most of the phenolic compounds decreased, especially monomeric flavan-3-ols, flavonols, and anthocyanins, in parallel with the increase in the percentage of polymerised anthocyanins [46] (Tables 1 and 2).

As flavonols have been shown to behave as a powerful anthocyanin copigments due to their planar structure, allowing a closer approach to anthocyanin [47,48], the decrease in their content could be related to the diminution of the percentage of copigmentation [9]. However, after 8 months of stabilisation, control wines and those with the addition of 3 g/L of protein hydrolysates experienced an increase in copigmentation, with percentages nearly

the same as the initial ones, at the beginning of treatment, in parallel with the increase in *t*-GRP over time (Tables 2 and S2).

Other substances apart from flavonols, such as hydroxycinnamic acids, could positively contribute to the construction of new products with anthocyanins that help to maintain colour [49]. In contrast, an increase in the content of acids (caffeic and gallic acids) was observed in control wines and wines with 0.5 g/L of protein hydrolysates, probably due to hydrolysis phenomena of the respective tartaric esters of hydroxycinnamic acids or, to a lesser extent, by the cleavage of acylated anthocyanins [9] (Tables 1 and S1), while keeping their content constant and less hydrolysed over time in wines with 3 g/L of protein extract.

Table 1. Mean values of concentrations (mg/L) and standard deviations (n = 3) of total glucoside, acetyl, and *p*-coumaroyl derivatives of anthocyanin compounds, anthocyanins, percentages of copigmentation and polymerisation, total benzoic acids, hydroxycinnamic acid derivatives (HACD), flavan-3-ols, flavonols, and total phenolics (mg GAE/L) of control wines (CW) and wines with the addition of 0.5 g/L protein hydrolysates from defatted grape seed meal with high (HW05) and low (LW05) hydrolysis time after one, four, and eight months of post-fermentation and stabilisation (1, 4, and 8 m).

	Stage	CW	HW05	LW05
Total glucoside derivatives of anthocyanins	1 m	246.99 ± 7.55 ^{b}	192.76 ± 8.89 ^{a}	189.91 ± 2.37 ^{a}
	4 m	107.38 ± 11.53 ^{b}	60.46 ± 3.73 ^{a}	76.79 ± 5.22 ^{a}
	8 m	77.37 ± 5.64 ^{b}	50.81 ± 3.65 ^{a}	60.17 ± 4.45 ^{a}
Total acetyl derivatives of anthocyanins	1 m	127.94 ± 3.08 ^{b}	105.02 ± 3.99 ^{a}	101.69 ± 0.40 ^{a}
	4 m	59.33 ± 6.97 ^{b}	34.96 ± 1.67 ^{a}	43.24 ± 3.15 ^{a}
	8 m	41.33 ± 2.52 ^{b}	29.31 ± 1.64 ^{a}	33.93 ± 2.10 ^{a}
Total *p*-coumaroyl derivatives of anthocyanins	1 m	74.34 ± 3.08 ^{b}	59.02 ± 2.81 ^{a}	57.49 ± 0.89 ^{a}
	4 m	37.08 ± 4.30 ^{b}	26.12 ± 0.64 ^{a}	29.02 ± 1.43 ^{a}
	8 m	28.68 ± 1.18 ^{c}	16.95 ± 0.45 ^{a}	26.37 ± 0.77 ^{b}
Total anthocyanins	1 m	449.31 ± 11.79 ^{b}	356.79 ± 15.23 ^{a}	349.08 ± 2.58 ^{a}
	4 m	203.78 ± 22.75 ^{b}	121.53 ± 6.01 ^{a}	149.05 ± 9.78 ^{a}
	8 m	147.39 ± 9.35 ^{b}	96.36 ± 6.51 ^{a}	120.47 ± 7.32 ^{a}
% copigmented anthocyanins	1 m	12.67 ± 0.51	15.05 ± 0.81	12.86 ± 1.18
	4 m	11.72 ± 0.34 ^{c}	7.91 ± 0.28 ^{a}	9.93 ± 1.17 ^{bc}
	8 m	5.01 ± 0.77	4.07 ± 1.29	4.86 ± 0.99
% polymerised anthocyanins	1 m	71.58 ± 1.48 ^{a}	77.56 ± 1.64 ^{b}	79.27 ± 2.08 ^{b}
	4 m	98.80 ± 0.09 ^{a}	99.92 ± 0.40 ^{ab}	100.50 ± 0.95 ^{b}
	8 m	81.58 ± 1.08 ^{a}	89.02 ± 1.72 ^{b}	85.85 ± 0.47 ^{b}
Total benzoic acids	1 m	27.90 ± 0.86	31.05 ± 4.94	25.00 ± 1.10
	4 m	46.10 ± 2.31	44.02 ± 1.73	44.19 ± 2.73
	8 m	45.13 ± 4.73 ^{b}	38.14 ± 0.83 ^{ab}	36.91 ± 0.57 ^{a}
Total hydroxycinnamic acid derivatives	1 m	62.91 ± 0.75	62.84 ± 1.07	62.79 ± 0.32
	4 m	66.32 ± 0.06 ^{c}	64.15 ± 0.34 ^{a}	64.72 ± 0.11 ^{b}
	8 m	60.32 ± 0.37 ^{b}	59.41 ± 1.07 ^{a}	59.78 ± 0.35 ^{ab}
Total flavan-3-ols	1 m	64.24 ± 2.49	67.49 ± 2.36	67.33 ± 1.23
	4 m	82.93 ± 6.83 ^{b}	57.89 ± 5.43 ^{a}	65.25 ± 12.79 ^{ab}
	8 m	55.31 ± 0.94 ^{b}	40.88 ± 4.13 ^{a}	46.91 ± 2.30 ^{a}
Total flavonols	1 m	19.60 ± 1.00	21.06 ± 1.64	19.98 ± 0.58
	4 m	29.36 ± 2.03 ^{b}	24.09 ± 1.80 ^{a}	25.28 ± 1.22 ^{ab}
	8 m	11.62 ± 0.48	10.17 ± 1.11	11.98 ± 0.96
Total phenolics (Folin–Ciocalteau)	1 m	2308.85 ± 48.05	2459.03 ± 88.38	2505.70 ± 120.70
	4 m	2430.98 ± 93.82 ^{ab}	2247.55 ± 65.86 ^{a}	2640.28 ± 117.33 ^{b}
	8 m	2578.40 ± 160.15	2382.20 ± 73.90	2509.28 ± 44.88

Different letters in the same row denote significant differences ($p < 0.05$) according to the Tukey test.

Table 2. Mean values of concentration (mg/L) and standard deviations ($n = 3$) of total glucoside, acetyl and *p*-coumaroyl derivatives of anthocyanin compounds, anthocyanins, percentages of copigmentation and polymerisation, total benzoic acids, hydroxycinnamic acid derivatives (HACD), flavan-3-ols, flavonols, and total phenolics (mg GAE/L) of control wines (CW) and wines with the addition of 3 g/L protein hydrolysates from defatted grape seed meal with high (HW3) and low (LW3) hydrolysis time after one, four, and eight months of post-fermentation and stabilisation (1, 4, and 8 m).

	Stage	CW	HW3	LW3
Total glucoside derivatives of anthocyanins	1 m	332.80 ± 41.65	346.30 ± 6.67	325.37 ± 41.15
	4 m	133.91 ± 0.38 ^{c}	75.97 ± 6.64 ^{a}	95.45 ± 3.81 ^{b}
	8 m	45.76 ± 1.38	44.46 ± 0.79	44.21 ± 0.89
Total acetyl derivatives of anthocyanins	1 m	145.47 ± 11.69	159.90 ± 7.20	139.85 ± 6.99
	4 m	98.68 ± 9.12 ^{b}	47.01 ± 2.39 ^{a}	49.74 ± 2.53 ^{a}
	8 m	25.47 ± 0.32	25.49 ± 0.57	25.60 ± 0.26
Total *p*-coumaroyl derivatives of anthocyanins	1 m	99.99 ± 13.36	93.54 ± 10.17	74.39 ± 11.22
	4 m	44.13 ± 2.51 ^{b}	31.24 ± 1.99 ^{a}	31.10 ± 3.27 ^{a}
	8 m	27.45 ± 1.16 ^{b}	24.12 ± 0.08 ^{a}	24.24 ± 0.21 ^{a}
Total anthocyanins	1 m	578.26 ± 66.65	599.75 ± 23.19	539.61 ± 38.20
	4 m	276.72 ± 9.24 ^{c}	154.22 ± 9.12 ^{a}	176.29 ± 7.95 ^{b}
	8 m	98.68 ± 1.59 ^{b}	94.07 ± 1.37 ^{a}	94.05 ± 1.03 ^{a}
% copigmented anthocyanins	1 m	7.77 ± 0.05	6.46 ± 0.65	7.93 ± 1.44
	4 m	1.53 ± 0.18	1.29 ± 0.43	1.13 ± 0.06
	8 m	6.14 ± 0.36 ^{b}	4.50 ± 0.51 ^{a}	5.20 ± 0.61 ^{ab}
% polymerised anthocyanins	1 m	82.57 ± 1.43	82.22 ± 2.28	80.54 ± 2.78
	4 m	90.92 ± 1.13 ^{a}	92.67 ± 1.19 ^{ab}	93.64 ± 0.22 ^{b}
	8 m	95.04 ± 0.86	96.27 ± 0.92	95.91 ± 0.72
Total benzoic acids	1 m	210.69 ± 8.92	184.04 ± 32.44	165.56 ± 25.84
	4 m	142.21 ± 6.04 ^{a}	155.05 ± 3.04 ^{b}	149.80 ± 4.65 ^{ab}
	8 m	131.86 ± 13.08	126.93 ± 2.20	127.31 ± 3.60
Total hydroxycinnamic acid derivatives	1 m	80.56 ± 2.92	82.32 ± 3.43	81.65 ± 6.76
	4 m	71.60 ± 1.18	72.82 ± 0.60	71.71 ± 1.76
	8 m	66.94 ± 1.58	65.53 ± 0.81	65.97 ± 0.70
Total flavan-3-ols	1 m	64.08 ± 6.67 ^{b}	43.46 ± 4.86 ^{a}	88.06 ± 2.79 ^{c}
	4 m	41.66 ± 0.69	38.35 ± 1.53	38.71 ± 2.47
	8 m	38.65 ± 6.54 ^{b}	25.49 ± 0.22 ^{a}	34.57 ± 0.74 ^{ab}
Total flavonols	1 m	69.40 ± 1.76	65.66 ± 2.84	65.52 ± 1.06
	4 m	57.83 ± 2.79	56.75 ± 2.09	52.24 ± 3.29
	8 m	43.04 ± 3.69	40.96 ± 1.24	41.19 ± 2.00
Total phenolics (Folin–Ciocalteau)	1 m	3119.83 ± 572.82	3428.83 ± 332.64	2723.97 ± 516.51
	4 m	3467.05 ± 220.55	3455.37 ± 572.40	3203.50 ± 397.34
	8 m	2954.33 ± 406.04	2396.57 ± 210.08	2653.63 ± 202.19

Different letters in the same row denote significant differences ($p < 0.05$) according to the Tukey test.

Compared among wines, the content of anthocyanins decreased more over time in all treated wines, being significantly higher than the content of all individual anthocyanins in control wines (Tables 1, 2, S1 and S2), regardless of the typology and the dose of protein hydrolysates. Similar behaviour was already observed in wines after the addition of different quantities of grape seed protein hydrolysates during the stabilisation stage [36]. Other authors have described interactions between proteins or their derivatives and anthocyanins [27,28,50,51].

On the other hand, flavonol contents did not experience differentiation over the initial months from the addition of protein hydrolysates, similar to that observed when the products were added to wines during the stabilisation stage [36]. However, the changes in flavonols were punctual (myricetin-3-glucoside, quercetin-3-glucoside, and isorhamnetin-3-glucoside) in the latest months of evolution only in wines with the addition of 0.5 g/L high-

hydrolysed proteins (HW05), evidencing in virtually all individual flavonols a significantly lower content (Tables 1 and S1).

This fact was in contrast to the findings of Cejudo-Bastante et al. [35] when a pre-fermentative addition of protein hydrolysates was conducted. No significant ($p > 0.05$) differences were observed when a higher quantity of protein hydrolysates was added to the wine, for both L and H protein hydrolysate (Tables 2 and S2), in agreement with the results described by Mora-Garrido et al. [36] and Cejudo-Bastante et al. [35]. Although some interactions have been reported between the flavonol quercetin-3-glucoside with salivary proteins [52], grape seed-derived peptides did not appear to have a significant effect on the flavonols of red wines, at least at a high dose. Furthermore, regarding the other individual phenols, the high dose of protein hydrolysates seemed to offer advantages, exerting a positive effect on the concentrations of benzoic acids, HCAD, and flavan-3-ols, for both types of hydrolysates (H and L) (Table S2). Lower doses of protein hydrolysate had no significant ($p > 0.05$) effect on these compounds (Table S1). In that sense, significantly higher contents of t-GRP and p-coumaric acid were observed in wines treated with 3 g/L protein hydrolysates (HW3 and LW3), as is the case when grape seed protein hydrolysates are added to the wine in its pre-fermentation phase [35]. In addition, higher contents of gallic acid and (−)-epicatechin were found in HW3, and higher contents of (+)-catechin were found in LW3 (Table S2). It was noticeable that an increase in the content of gallic acid and (+)-catechin in wines was already reported when a high quantity of protein hydrolysates was added during the stabilisation stage [36]. This could be due to the presence of remaining enzymatic activity (hydrolases or proteases and pectinases), provoking the liberation of gallic acid and the excision of the bond among hydroxycinnamic acids and the respective sugars.

On the other hand, regardless of the dose, the type of protein hydrolysate influenced copigmentation, showing significantly lower percentages in HWs (Tables 1 and 2). This fact could be related to the significantly lower content of some copigments that favoured this phenomenon, such as flavonols, (−)-epicatechin, and caffeic acid in HW05 and (+)-catechin or *c*-GRP in HW3 (Tables S1 and S2). Most likely, the significant low content of anthocyanins and phenolic compounds was not sufficient to achieve higher chemical stability during stabilisation [32]. As affirmed by Escribano-Bailón et al. [9], a wide variety of substances can act as copigments, and the lower presence of other compounds with planar polarisable nuclei in the high-hydrolysed protein hydrolysates from DGSM could negatively influence the copigmentation equilibrium [53,54]. Mora-Garrido et al. [31] displayed slightly lower concentrations of aromatic amino acids in protein hydrolysates derived from white DGSM with a high hydrolysis time, mainly tyrosine, proline, and histidine, related to the protection of the flavylium cation [28]. Therefore, the decrease in the percentage of copigmentation in wines with hydrolysates could be due to the low concentrations of these aromatic amino acids. These findings in copigmentation contrasted with the positive behaviour observed when adding protein hydrolysate in the pre-fermentative stage [35] and during the stabilisation stage [36].

Furthermore, the lower quantity of individual flavan-3-ols ((+)-catechin and (−)-epicatechin) (Tables S1 and S2) could be related to the significantly higher percentage of polymerisation of treated wines, regardless of the hydrolysis grade and the dose of protein hydrolysates (Tables 1 and 2). The significantly lower percentage of polymerisation in control wines led us to think that despite having important amounts of copigments, these wines showed difficulty in converting the earlier copigmentation complexes into more stable pigments (Tables 1 and 2).

3.2. Colour Evolution

The evolution of the CIELAB colour parameters (L^*, C^*_{ab}, and h_{ab}) along post-fermentation and stabilisation for control wines (CWs) and for wines treated with 0.5 and 3 g/L protein hydrolysate (LW and HW) is shown in Figure 1.

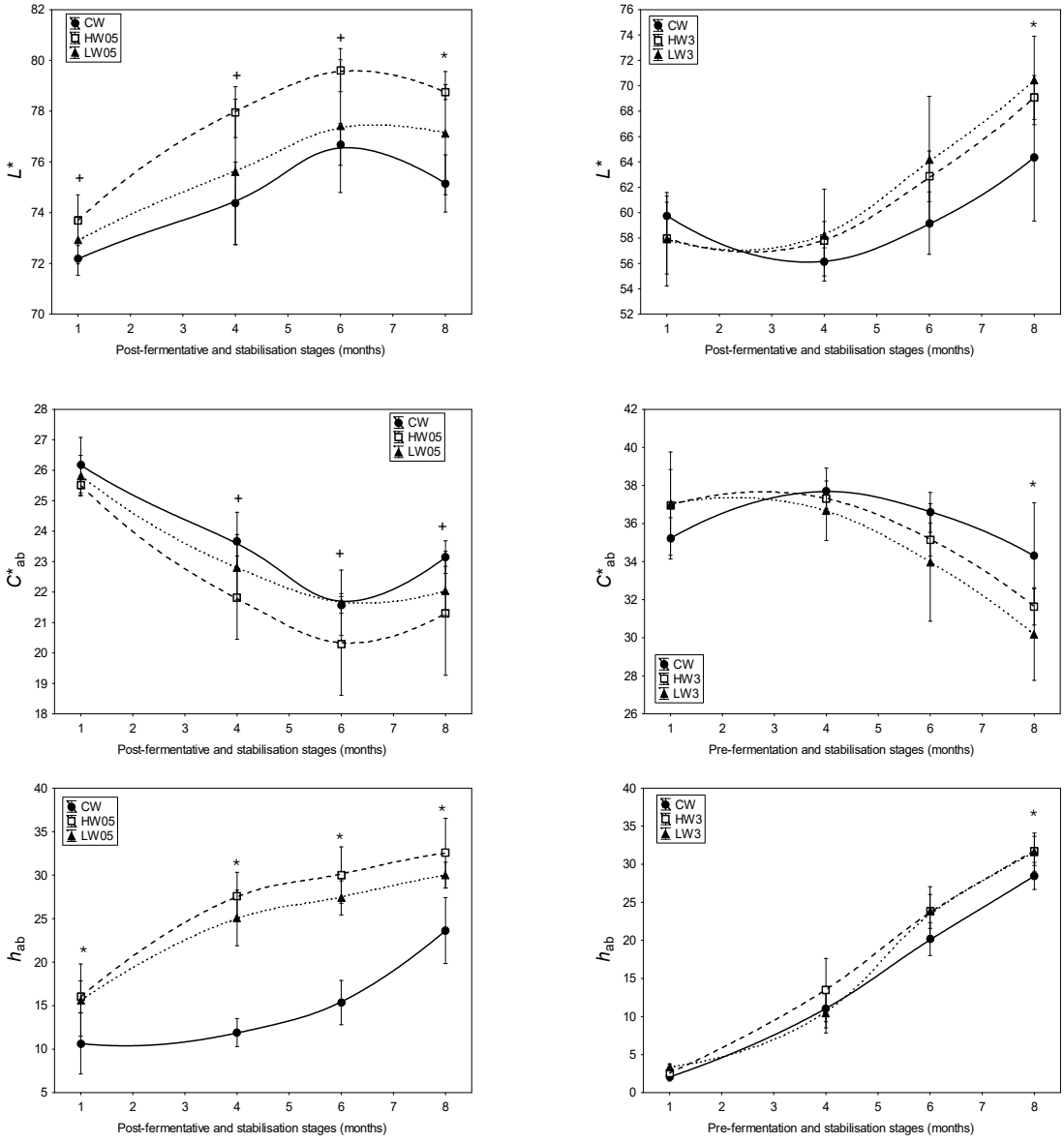

Figure 1. Evolution of CIELAB parameters along post-fermentative and stabilisation stages of control wines (CWs) and wines with the addition of protein hydrolysates from defatted grape seed meal with high and low hydrolysis time at 0.5 g/L (**a**) and 3 g/L (**b**) (HW05, LW05, HW3, and LW3). Significant differences ($p < 0.05$) according to the Tukey test were expressed among CW vs. LW and HW (*) and HW vs. CW and LW (+).

The colour modification was the same for all wines showing the same evolution over time for all CIELAB parameters: a decrease in chroma (C^*_{ab}) and increases in lightness (L^*) and hue (h_{ab}), mainly during the last stabilisation months.

Adding 0.5 g/L protein hydrolysates to wine after alcoholic fermentation strongly influenced CIELAB parameters. Regardless of the type, the addition of the protein hydrolysates after fermentation/maceration provoked a significant increase in hue toward a brownish tonality. This behaviour was in concordance with the higher per-

centages of polymerisation reported in these wines (Table 1). This was consistent with Mora-Garrido et al. [36], where protein hydrolysates were added after the stabilisation stage but differed from those added in the pre-fermentative stage [35]. Moreover, treated wines, especially HW05, showed a significant increase in L^* and a diminution of C^*_{ab}, maintained during the subsequent months of stabilisation, compared with CW and LW05, which showed no significant differences in colour intensity and lightness (Figure 1a).

However, compared with CW, the wines elaborated with the addition of 3 g/L protein hydrolysates did not vary significantly until 8 months of the stabilisation period, becoming brownish, lighter, and less intense (Figure 1b). This loss of colour could be due to the formation of brown pigments or the co-precipitation of proteins and phenolic compounds [55]. In fact, significantly lower levels of HCAD and flavan-3-ols were reported in the first stages when adding 0.5 g/L of hydrolysates and only after 8 months of storage for the addition higher amounts (Tables 1 and S1).

The assessment of the colour differences (ΔE^*_{ab}) occurring from the end of alcoholic fermentation to the end of the stabilisation period (8 months) allowed visual differentiation between pairs of wines (Figure 2). The role of each colour attribute on ΔE^*_{ab} was calculated as a percentage of the quadratic increase in lightness, chroma, and hue. Although some visual colour differences were observed between the control and treated wines, low values of ΔE^*_{ab} were found, indicating low colour differences, that is, similar colour changes of the wines. Visually perceptible colour differences ($\Delta E^*_{ab} > 4$), maintained during the stabilisation time, were only observed in HW05 compared with the respective CW, mainly qualitative and due to higher tonality values (h_{ab}). Negligible quadratic variations in the chroma were detected. The values of perceptible colour differences were also observed between HW3 and LW3 and the respective CW only after 6 and 8 months of stabilisation, respectively and mainly due to lightness (L^*). Quadratic variations in hue and chroma were negligible.

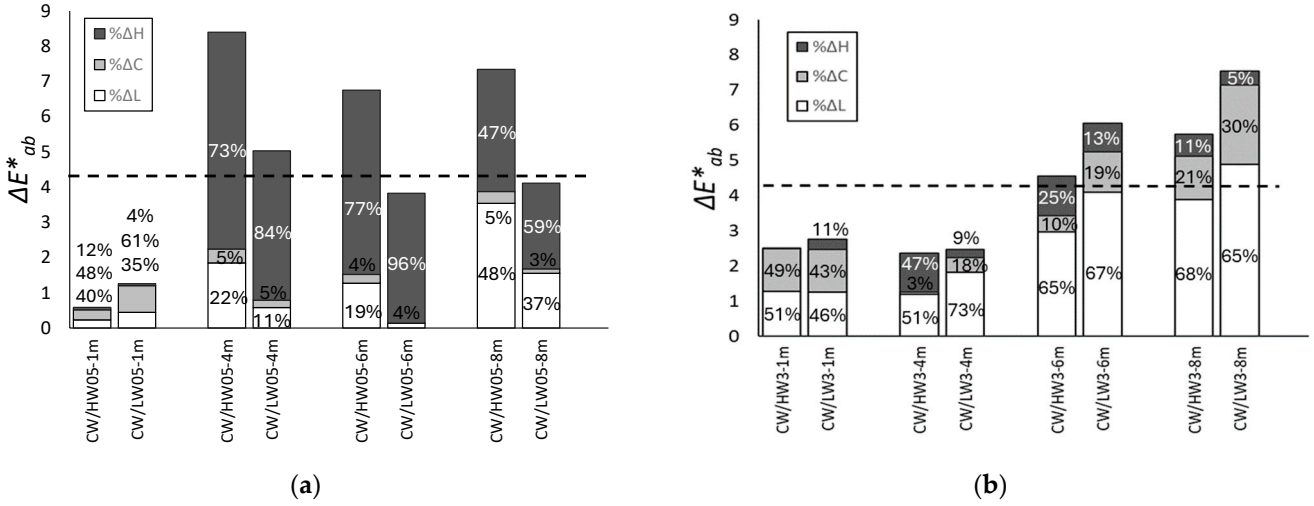

Figure 2. Colour differences (ΔE^*_{ab}) and percentages of the relative contributions of lightness, chroma, and hue (%Δ*L*, %Δ*C*, and %Δ*H*) during post-fermentative and stabilisation stages (1, 4, 6, and 8 months) of control wines (CWs) and wines with the addition of protein hydrolysates from defatted grape seed meal with high and low hydrolysis time, at 0.5 g/L (**a**) and 3 g/L (**b**) (HW05, LW05, HW3, and LW3).

Therefore, in contrast to the results found when protein hydrolysates were added before alcoholic fermentation [35] and during the stabilisation period [36], the addition of low quantities of protein hydrolysate after alcoholic fermentation had either no visual impact or a perceptible but acceptable impact on the chromatic stability of red wines, depending on the type of hydrolysate, L and H, respectively. The use of higher amounts

of protein hydrolysates, regardless of their type, did not produce a visual impact on the CIELAB parameters, being slightly perceptible only in the advanced stages of storage.

3.3. PCA Analysis

An unsupervised pattern recognition statistical analysis (principal component analysis, PCA) was performed to determine the main parameters responsible for the differences between control wines and wines with the addition of 0.5 and 3 g/L protein hydrolysates from defatted grape seed meal with high and low hydrolysis time (HW05, LW05, HW3, and LW3). Figure 3 shows the samples in the plane defined by the two first PCs, which explained 87% and 74% of the total variance, using a Kaiser's criterion (eigenvalues > 1).

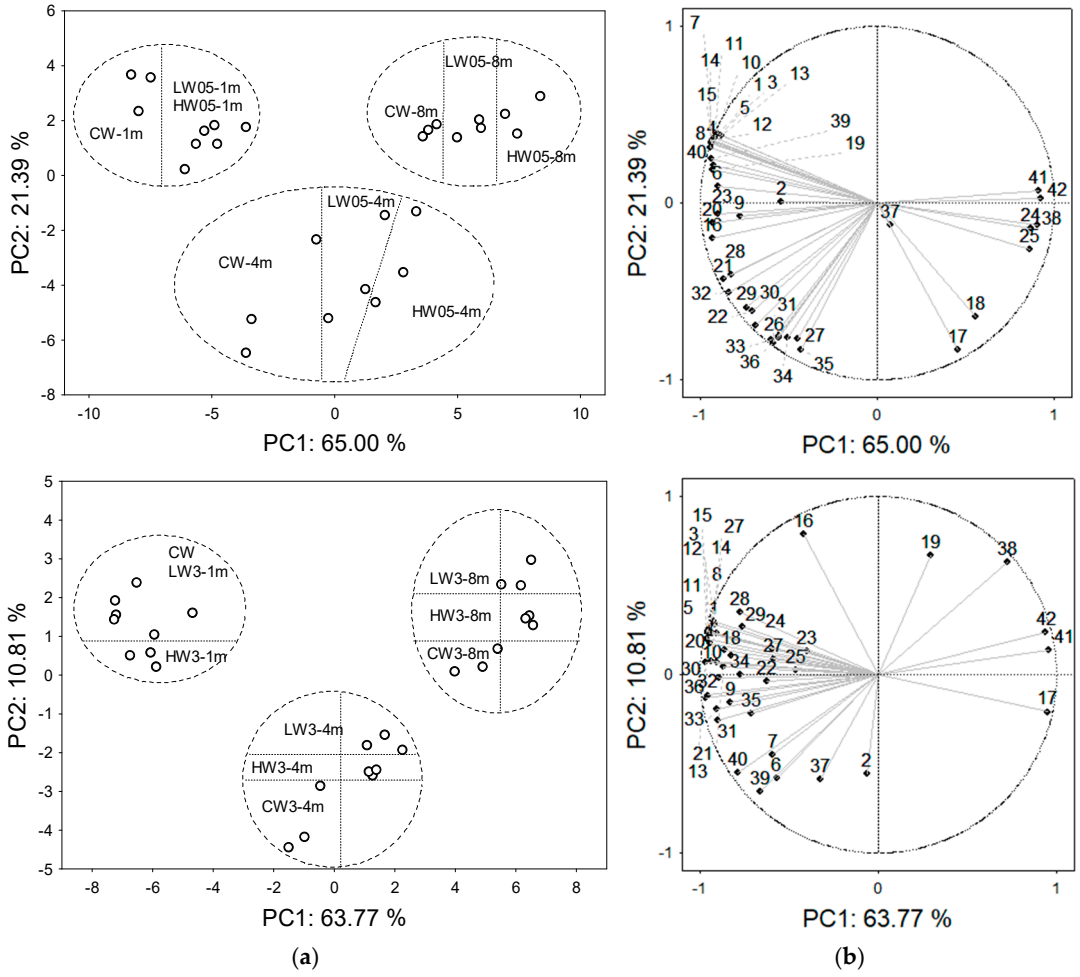

Figure 3. Distribution of samples in the plane defined by the two first discriminate functions of control wines (CWs) and wines with the addition of protein hydrolysates from defatted grape seed meal with high and low hydrolysis time, at 0.5 g/L (**a**) and 3 g/L (**b**) (HW05, LW05, HW3, and LW3) over time (1, 4, and 8 months). 1, delphinidin-3-glucoside; 2, cyanidin-3-glucoside; 3, petunidin-3-glucoside; 4, peonidin-3-glucoside; 5, malvidin-3-glucoside; 6, petunidin-3-acetyl-glucoside; 7, peonidin-3-acetyl-glucoside; 8, malvidin-3-acetyl-glucoside; 9, petunidin-3-p-coumaroyl-glucoside; 10, peonidin-3-*p*-coumaroyl-glucoside; 11, malvidin-3-*p*-coumaroyl-glucoside; 12, sum of glucoside derivatives; 13, sum of acetyl derivatives; 14, sum of *p*-coumaroyl derivatives; 15, sum of anthocyanins; 16, % copigmented anthocyanins; 17, % polymerised anthocyanins; 18, gallic acid; 19, *t*-GRP; 20, *c*-GRP; 21, *t*-caftaric acid; 22, *t*-coutaric acid; 23, *c*-coutaric acid; 24, *p*-coumaric acid; 25, caffeic acid; 26, sum of HACD; 27, (+)-catechin; 28, (−)-epicatechin; 29, sum of flavan-3-ols; 30, myricetin-3-glucoside; 31, quercetin-3-glucuronide; 32, quercetin-3-glucoside; 33, laricitrin-3-glucoside; 34, isorhamnetin-3-glucoside; 35, syringetin-3-glucoside; 36, sum of flavonols; 37, total phenolics (Folin–Ciocalteau); 38, L^*; 39, C^*_{ab}; 40, a^*; 41, b^*; 42, h_{ab}.

As can be seen, PC1 grouped the samples according to the stabilisation time in both types of wines. Samples with a longer stabilisation period (8 months) were located on the positive side of the axis, which contained mainly the decrease in most phenols (negative sign) and the increase in parameters such as % polymerisation and h_{ab} and the content of some hydroxycinnamic and benzoic acids in control wines and wines with 0.5 g/L protein hydrolysate (positive sign). PC1 also helped to differentiate after some months of stabilisation untreated wines from those with added protein hydrolysates, with a higher contribution of those parameters in HW and LW, especially HW05. Thus, treated wines (LWs and HWs) were located toward the positive side of the axis, with higher values of % polymerisation, L^*, and h_{ab} and lower content of virtually all of the studied phenolic compounds. PC2 achieved differentiation by treatment in wines with the highest addition of protein hydrolysates (3 g/L), with samples corresponding to advanced months of stabilisation toward the positive side of the axis, mainly LW3, which showed the highest percentage of L^* and *t*-GRP.

3.4. Visual Sensorial Evaluation

The attributes selected in the sensory evaluation of samples and the mean scores for each are shown in Figure 4.

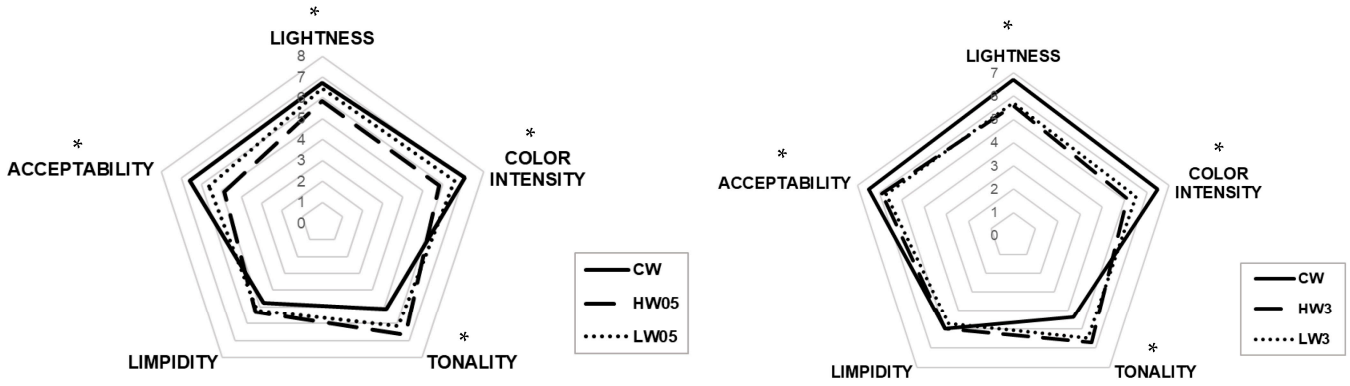

Figure 4. Visual sensory evaluation of control wines (CW) and wines with the addition of 0.5 g/L and 3 g/L protein hydrolysates from defatted grape seed meal with high and low hydrolysis time (HW05, LW05, HW3, and LW3) after eight months of post-fermentation and stabilisation. Asterisks denotes significant differences ($p < 0.05$) according to the Tukey test.

In both seasons, the CWs displayed significantly higher scores of lightness and colour intensity, compared with wines with protein hydrolysates (HW05, HW3, and LW3). CWs also displayed lower values of tonality (a more purple colour). These data are related to the CIELAB colour parameters as wines with protein hydrolysates displayed higher hue (h_{ab}) and lower chroma (C^*_{ab}) values compared with CWs (Figure 1). Finally, the CWs had significantly higher acceptability than wines with protein hydrolysates (HW05, LW05, and LW3). All wines showed similar limpidity values.

4. Conclusions

Compared with the addition in the pre-fermentation and stabilisation stages, where there was a colour stabilising effect on red wine, the post-fermentation addition of DGSM protein hydrolysates to Syrah red wine at concentrations of 0.5 and 3 g/L did not appear to significantly improve the colour stabilisation of wines made in warm climates, regardless of the typology or quantity of protein hydrolysates. In particular, the wines with the addition of protein hydrolysates showed a decrease in the amount of phenols and an increase in the percentage of polymerised anthocyanins. The wines to which a concentration of 3 g/L protein hydrolysates was added showed a lower colour intensity and a higher clarity and

hue over time. On the other hand, treatment with 0.5 g/L protein hydrolysates produced brownish wines. The addition of protein hydrolysates from a low hydrolysis time did not affect the co-pigmentation balances and did not produce visually perceptible negative colour differences over time. This study may be interesting for wineries to consider the application of this novel technique for colour stabilisation at the optimum step, which, based on these results, is not after fermentation and maceration. The post-fermentative addition of protein hydrolysates seems to produce a fining effect in terms of colour, which opens the door to other applications in winemaking, for which more research is needed. This study could be another step forward to improve the production of high-quality red wines in a warm climate region.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/beverages11010005/s1: Table S1: Mean values of concentration (mg/L) and standard deviations (n = 3) of individual anthocyanin compounds, benzoic acids, hydroxycinnamic acid derivatives, flavan-3-ols, and flavonols of control wines (CW) and wines with the addition of 0.5 g/L protein hydrolysates from defatted grape seed meal with high (HW05) and low (LW05) hydrolysis time after one, four, and eight months of post-fermentation and stabilisation (1, 4, and 8 m). Table S2: Mean values of concentration (mg/L) and standard deviations (n = 3) of individual anthocyanin compounds, benzoic acids, hydroxycinnamic acid derivatives, flavan-3-ols, and flavonols of control wines (CW) and wines with the addition of 3 g/L protein hydrolysates from defatted grape seed meal with high (HW3) and low (LW3) hydrolysis time after one, four, and eight months of post-fermentation and stabilisation (1, 4, and 8 m).

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the University of Seville Investigation Ethics Committee (favourable report, code 2024-1372, Act 09/24, dated 17 July 2024).

Informed Consent Statement: Informed consent for participation was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

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