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Astringency reduction in red wine by whey proteins ☆

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ABSTRACT

Whey is a by-product of cheese manufacturing and therefore investigating new applications of whey proteins will contribute towards the valorisation of whey and hence waste reduction. This study shows for the first time a detailed comparison of the effectiveness of gelatin and β-lactoglobulin (β-LG) as fining agents. Gelatin was more reactive than whey proteins to tannic acid as shown by both the astringency method (with ovalbumin as a precipitant) and the tannins determination method (with methylcellulose as a precipitant). The two proteins showed similar selectivity for polyphenols but β-LG did not remove as much catechin. The fining agent was removed completely or to a trace level after centrifugation followed by filtration which minimises its potential allergenicity. In addition, improved understanding of protein–tannin interactions was obtained by fluorescence, size measurement and isothermal titration calorimetry (ITC). Overall this study demonstrates that whey proteins have the potential of reducing astringency in red wine and can find a place in enology.

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1. Introduction

Red wine is a beverage that is rich in phenolic compounds, mainly tannins, but also other smaller molecular weight phenolics which have demonstrated to have many health benefits due to several biological activities, such as antioxidant, cancer preventing and anti-inflammatory activity (Middleton, Kandaswami, & Theoharides, 2000). Tannins are high molecular weight (over 500 Da) polyphenols, which have the ability of precipitating with gelatin and other proteins in solution. These proteins are in general rich in proline. The tannins occurring in wine are responsible for the undesired sensorial properties, especially astringency. Astringency is a rough or drying mouth-feel that causes a puckering sensation, associated with interactions between polyphenols from wine and certain proteins from saliva. Mainly the proline rich proteins (PRPs) from saliva are the ones responsible for this sensation (Mehansho, Butler, & Carlson, 1987). According to Charlton et al. (2002), and as described by Dinnella, Recchia, Fia, Bertuccioli, and Monteleone (2009), binding and precipitation of polyphenols by PRPs involves a multi-step mechanism. At first, reversible hydrophobically-driven binding of the polyphenol to the protein takes place to give a soluble complex. Then, more polyphenol is added and cross-linking of peptides occurs, the complex becomes insoluble, and, finally, further aggregation (phase separation) of the insoluble complexes occurs. The intensity of this sensation depends on the polyphenolic composition and concentration in wine, as well as on the palate of the individual tasting it. Some proteins, such as casein, gelatin, egg albumin, and isinglass are known to interact with phenolic compounds in a similar way to saliva proteins (Guerrero, Smith, & Bindon, 2013), improving clarity, sensory characteristics and aging capacity of wines. The model for polyphenol–protein interactions described above is also valid to explain interactions between tannins and these proteins.

When a protein treatment is applied to wine, care must be taken for the molecules used to be selective. Otherwise, most of the beneficial properties from wine phenols could be lost in the process. Moreover, it is important to reduce astringency to a limit where it does not result in extensive precipitation of polyphenols. It is desired that some mild astringent sensation remains, for a winemaker should have the ability to modulate astringency by adjusting the balance accordingly. For all these reasons, the study of the protein–phenolic interactions is very important.

Whey obtained from cheese manufacture has low commercial value and represents an alternative for the obtention of proteins that can interact with phenolics. The addition of milk to tea has shown to result in complexation of milk proteins and tea catechins without impairing the bioavailability of the catechins and improving its sensorial properties (Kanakis et al., 2011; Ye, Fan, Xu, & Liang, 2013). Whey proteins and in particular β-LG (the major whey protein) has also shown to interact with polyphenols in tea (Kanakis et al., 2011) and to complex with particular polyphenols

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(von Staszewski et al., 2012) however, it has never been applied to the reduction of red wine astringency. β-LG is a small globular protein of 18,350 Da which possess a hydrophobic pocket that shows particular affinity for hydrophobic molecules. β-LG has some technological advantages for its application as a fining agent: inexpensive, food grade and non-toxic material, capable of solubilising and protecting hydrophobic biologically active molecules in aqueous media whilst retaining the sensory properties.

Tannin–protein interactions have been investigated by a wide range of physicochemical techniques (Frazier, Papadopoulou, & Green, 2006; McRae, Falconer, & Kennedy, 2010). In this work we used isothermal titration calorimetry (ITC) to gain better understanding of the affinity of β-LG and gelatin for tannic acid, model tannin from wine. ITC provides information not only on thermodynamic parameters and the strengths of the tannin–protein interactions but also on the stoichiometry of the resulting complex. The conformation of the protein is of critical importance as random coil proteins have a higher interaction with tannins than globular proteins. Therefore, the comparison of β-LG with a widely used random coiled protein, gelatin, is included.

The aim of this work was to assess the efficiency of reduction of astringency of whey proteins particularly, β-LG and a mixture of β-LG and caseinomacropeptides (CMP) and to compare them against gelatin. The whey samples were produced by a combination of ion exchange and microfiltration following a method developed in our group (Welderufael, Gibson, & Jauregi, 2012). Astringency was assessed by an analytical method developed by Llaudy et al. (2004) which relies on the precipitation of tannins by ovalbumin. They found good correlation between this analytical method and the sensory evaluation of astringency in a range of wines. Further investigation of the tannin–protein interactions was also carried out by fluorescence, dynamic light scattering (particle size) and ITC.

2. Materials and methods

2.1. Materials

All Chemicals used were of analytical grade. Bovine β-lactoglobulin (β-LG), bovine serum albumin (BSA), bicinchoninic acid solution (BCA), copper sulphate solution, DEAE Sepharose®, rennet, ovalbumin, tannic acid, tartaric acid, potassium monophosphate, potassium diphosphate, sodium hydroxide, sodium chloride, hydrochloric acid (32.5%), trifluoroacetic acid (TFA), methanol, ethanol, methylcellulose, ammonium sulphate, acetonitrile, catechin, epicatechin, gallic acid and type B gelatin from bovine skin were purchased from Sigma–Aldrich (Dorset, UK). Flat sheet microfiltration membranes (0.45 μm), syringe driven PVDF Filters (0.45 μm) were sourced from Millipore Corporation (Bedford, UK). Protease N 'Amano' Enzyme from Bacillus subtilis was purchased from Amano Enzyme Inc. (Nagoya, Japan). Pasteurised Skimmed milk and Merlot red wine, La Chasse Merlot (2012), from France (13% alcohol) were purchased from local stores.

2.2. Preparation of sweet whey from skimmed milk and purification of peptides

Skimmed milk was heated to 35 °C in a water bath. Commercial rennet was added at a concentration of 0.3 mL per litre of milk with gentle stirring for 2 min. Incubation took place for one hour at that temperature and then the casein coagulum was cut in small squares to allow the remaining lactoserum to drain out of it. Incubation was extended for 20 additional minutes and then the coagulum was scooped and filtered to drain the most of the serum with the aid of vacuum. The whey was centrifuged at 3200 RCF and filtered with 0.45 μm syringe driven filter to remove the last of the left over casein curds.

The sweet whey was fractionated to obtain a β-LG rich fraction and a fraction containing CMP and β-LG following method developed in our group (Fig. S1) based on a combination of adsorption and microfiltration (Welderufael et al., 2012). Nitrocellulose microfiltration membrane was cut and placed into the 150 mL ultrafiltration magnetically stirred Amicon cell. To begin the purification process, 100 mL of whey (pH 6.4) and 10 mL of resin were added to the cell and stirred for 10 min. The mixture was filtered through the membrane, with the aid of positive pressure of air. The mixture was micro-filtered to separate the non-adsorbed proteins from the adsorbed proteins. The resin was washed with 10 mM potassium phosphate buffer at pH 6.5. Adsorbed proteins (β-LG and CMP) were desorbed and eluted with known volume of elution buffer, 10 mM potassium phosphate buffer at pH 4.5. For an enriched β-LG fraction without CMP, a hydrolysis step was introduced while proteins were adsorbed to the resin (S1). Hydrolysis started after re-solubilising the adsorbed proteins with a pH 7, 10 mM potassium phosphate buffer, at 45 °C. Then, protease 'N'Amano enzyme was added to the mixture. After 2 h, hydrolysed CMP were microfiltered and finally, the non-hydrolysed protein remaining, β-LG, was desorbed and eluted with 10 mM potassium phosphate buffer at pH 4.5 containing 0.5 M NaCl. Total protein content was analysed by BCA method, and HPLC was used for qualitative analysis of proteins.

2.3. Total protein content

Total proteins were quantified according to the bicinchoninic acid assay (BCA). Briefly, 100 μl of standard or sample was mixed with 2 mL of the BCA working reagent (copper sulphate solution: BCA solution at a ratio of 1:50). The mixture was allowed to stand at 37 °C for 30 min, and then allowed to cool at RT for 5 min. Finally, absorbance was read for each sample/standard, at 562 nm within 8 min with water as a blank. Bovine serum albumin was used as a standard for protein quantification.

2.4. Whey protein analysis by HPLC

The major whey proteins, (β-LG, alpha-lactalbumin, BSA) could be identified using RP-HPLC, with a method adapted from Thoma, Krause, and Kulozik (2006). Samples of known total protein content were filtered through a 0.45 μm PVDF filter and analysed in a Dionex HPLC with a P680 pump, ASI-100 automated sample injector, thermostated column compartment TCC100, PDA-100 photodiode array detector with C-18 column (250 × 4.6 mm). A gradient of solvent A (0.1% TFA in water) and solvent B (0.08% TFA in ACN) was utilised in the following way: B 0–45% in 0–60 min, B 45–70% in 60–65 min, 70% B in 65–75 min, and finally 0% B in 75–90 min. The column temperature was set at 40 °C. The flow rate was 0.8 mL/min, injection volume was 50 μl and the absorbance of the samples was monitored at 214 and 280 nm.

2.5. Particle size measurement

Dynamic light scattering was used to monitor the interaction between tannic acid and protein, and to determine the size of the β-LG nanoparticles, using a Zetasizer Nano S (Malvern instrument, Malvern, UK), equipped with a 4 mW He-Ne laser (633 nm). Before measurements, samples were filtered (0.23 μm) and diluted (1:10 in the corresponding buffer of preparation). In another experiment with the same equipment disposition, the size of complex formed between tannic acid (0.2–1.2 mg/mL) and β-LG (0.1 and 0.5 mg/mL) was monitored. Measurements were

performed in triplicates at 25 °C, at an angle of 90° from the incident beam.

2.6. Protein treatments on wine

4 mL of wine and 1 mL of protein solution were mixed in flasks by vortex; final protein concentrations in the mixture were 0.1 and 0.5 mg/mL. The protein concentrations were chosen based on previous screening (data not shown) and commercially used concentrations in wine fining where for example, maximum recommended dose of caseinate is 0.6 mg/mL (Guerrero et al., 2013). Wine controls were diluted in the same manner as the treated wine samples for their analyses. After 10 min samples were centrifuged at 3200 RCF and their astringency was measured following the analytical method described below and their phenolic chromatographic profiles obtained.

2.7. Astringency assessment experiments

An analytical method was used to determine astringency, based on the precipitation of tannins by ovalbumin, adapted from Llaudy et al. (2004). We modified the method by reducing the volumes of standard needed for the preparation of the calibration curve and wines samples. Solutions of tannic acid at 0.2–1.0 g/L and solutions of ovalbumin at 0.4–3.2 g/L were prepared. To solubilise the standards, a solution similar to wine in composition was prepared using 4.0 g/L tartaric acid, 95 g/L ethanol and 0.1 M sodium hydroxide. For each tannic acid concentration or wine sample, 200 μL of the sample were mixed with increasing concentrations of ovalbumin in Eppendorf tubes and vortexed. After 10 min samples were centrifuged at 11,700*g* for 10 min. The resulting supernatants (0.1 mL) were diluted with distilled water (4.9 mL). Finally, absorbance was measured immediately at 280 nm in a quartz bucket with an optical path of 10 mm; experiments were carried out at room temperature and in triplicates.

2.8. Phenolic profile of wines by HPLC

Prior to analysis, wine samples were filtered through a 0.45 μm pore size nylon membrane. Samples of 30 μL were injected into the chromatographic system consisting of a Hewlett–Packard 1100 series HPLC equipped with a degasser, a quaternary pump and a photodiode array detector model (Agilent Technologies). Separation was performed in a reversed-phase column Nova Pak C18 (250 × 4.6 mm; 100 Å pore size; 5 μm particle size; 30 °C), equipped with a Cyano guard column (4 × 3 mm) (Phenomenex). Two mobile phases were employed for elution: A (water:MeOH:formic acid, 95:4.9:0.1% v/v) and B (acetonitrile:formic acid, 99.9:0.1, v/v), and the gradient was: 5% B (min 0); 31% B (min 26); 50% B (min 34); 90% B (min 38) and 5% B (min 42). The flow rate was 0.8 mL/min. Each sample was run in triplicate and excellent reproducibility was observed between runs. Diode array detection proceeded at 280, 320, 365 and 520 nm. The presence of formic acid in the elution solvents is needed to maintain the pH below 2.5, ensuring that anthocyanins are present as a single species (flavylium cation). Compounds were identified by comparison of their retention times with spectra of pure standards.

2.9. Isothermal titration calorimetry

To measure enthalpy changes associated with tannin–protein interactions, a microcalorimeter ITC 200 (MicroCal, 22 Industrial Drive East, Northampton, MA 01060 USA) was used. In a typical experiment, buffered gelatin or β-LG solutions (0.2–1.0 mg/mL) were placed in the 206 μl sample calorimetric cell and buffered tannic acid solutions (3–8.8 mg/mL) were loaded into the injection syringe, at 298 K. Tannins were titrated into the sample cell as a sequence of 33 injections of 1.2 μl. To allow equilibration, 250 s separated successive injections. The contents of the sample cell were stirred throughout the experiment at 800 rpm to ensure thorough mixing. The pH was checked after the preparation of the solutions. Both titrate and titrant solutions were degassed for 10 min to prevent bubbles from affecting measurements, the weight of the solutions was controlled after degassing, ensuring acetate buffer did not evaporate. Measurements were carried out in duplicates and raw data were obtained as a plot of heat (μCal/s) against time and featured a series of peaks for each injection. Control experiments included the titration of buffered tannin solutions into buffer, buffer into protein and buffer into buffer. The last two controls resulted in small and equal enthalpy changes for each successive injection of buffer and were neglected. Corrected data refer to experimental data after subtraction of the tannin into buffer control data and, finally, Microcal ORIGIN software was used for the iterative curve fitting of the binding isotherms.

2.10. Methylcellulose assay

The tannin content in untreated and treated red wine was determined using the methyl cellulose precipitation (MCP) assay (Mercurio & Smith, 2008). Red wine was treated with β-LG and a mixture of β-LG and CMP at 0.1 and 0.5 mg/mL, centrifuged and supernatant analysed for tannin concentrations. The treated and untreated wine samples (25 μL) reacted with a solution in water of 300 μL of 0.04% MCP (or pure water for the control mixture), shaken slightly and allowed to stand for 3 min. A saturated ammonium sulphate solution (200 μL) was added to the mixture and water was added to a final volume of 1000 μL. The mixture was shaken slightly, allowed to stand for 10 min at room temperature and finally, centrifuged at 10,000 rpm for 5 min. Then, the absorbance of the supernatant was measured at 280 nm (A280 nm). Aqueous (−)-epicatechin solutions (10, 25, 50, 75, 100, 150, 200, and 250 mg/L) were used to prepare the calibration curve. Tannin concentration was calculated from the difference between the A280 nm of the control and A280 of red wine samples. Total tannin values were reported in mg/L epicatechin equivalents (EE).

$$\mathrm{A280tannin} = \mathrm{A280control} - \mathrm{A280supernatant}$$

$$\text{Tannin concentration}(\text{mg of EE/L}) = [\text{tannin}] \times \mathrm{DF}$$

where [tannin] is the tannin concentration calculated from the epicatechin calibration curve and DF is the dilution factor [DF = 40].

2.11. Total polyphenols determination

Folin–Ciocalteu's micro method as adapted for wine analysis by Waterhouse (2009) using gallic acid as the standard was used to determine the phenolic content. For the analysis, 20 μL of each calibration solution, treated red wine, red wine and blank were placed in a cuvette, and 1.58 mL water and 100 μL of Folin–Ciocalteu reagent were added, thoroughly mixed and allowed to stand between 30 s and 8 min. Then, 300 μL of the saturated sodium carbonate solution was added, mixed well and left at 20 °C for 2 h, after which the absorbance of each solution was read at 765 nm using a spectrophotometer. Results were expressed as gallic acid equivalents (mg GAE/L).

2.12. Fluorescence quenching

The Fluorescence quenching measurements were carried out using a Perkin-Elmer LS 5 fluorometer. Excitation and emission bandwidths were 10 nm. The emission spectra were recorded from 290 to 450 nm. β-LG and tannic acid were dissolved in 10 mM

phosphate buffer, pH 3.5. Tannic acid (0–1.2 mg/mL) and β-LG (0.1 and 0.5 mg/mL) solutions were filtered (0.45 μm) and mixed to measure the quenching of the protein. Each measurement was repeated in triplicate and fluorescence quenching data was plotted as relative fluorescence intensity (RFI = $F/F_0 \times 100$) against tannic acid concentration; where F_0 and F are the fluorescence intensities before and after the addition of the quencher (tannic acid) respectively. For the calculation of quenching constants, data was plotted as a Stern–Volmer plot of F_0/F against [Q] and the quenching constant (Ksv) was calculated by linear regression.

2.13. Statistical analysis

All experimental data were expressed as means ± standard deviations (S.D.) of duplicates or replicates. Statistical differences were evaluated using analysis of variance (ANOVA) while the mean values were compared using a Turkey test. All statistical analysis was performed using SPSS 16.0 (SPSS Inc.) at 95% confidence level.

3. Results and discussions

3.1. Whey proteins production

100 mL of sweet whey contained 970 mg total protein (9.70 mg/mL) as determined by total protein assay. Then 100 mL of whey was processed as described in Fig. S1 and the two separated fractions analysed for total protein and β-LG with the following results: (i) β-LG fraction contained 5.920 mg/mL protein (β-LG purity 90–95%) (ii) β-LG-CMP fraction contained 6.495 mg/mL protein (β-LG 75%).

3.2. Whey proteins and astringency

The ovalbumin precipitation method used for astringency estimation led to a decrease in absorbance at 280 nm due to the precipitation of tannins or tannic acid which, was used as standard. The relationship between absorbance and the ovalbumin concentration was logarithmic in agreement with the work by Llaudy et al. (2004). The slope of the logarithmic equations increased with the tannic acid concentration. The calibration curve obtained by plotting the tannic acid concentration against the slope was linear with a regression coefficient of 0.998. Merlot wine was used for this work because it is one of the most widespread red cultivars and, regarding its sensorial properties, quite an astringent one. The astringency of the untreated red wine (0.156 mg/mL) was within the reported range of values 0.112–0.566 mg/mL by Llaudy et al. (2004). The addition of either the β-LG enriched sample (β-LG) or the CMP containing sample (β-LG-CMP) led to a significant decrease in astringency of the commercial red wine (20–22%) at both protein concentrations studied (Table 1). The β-LG-CMP was as efficient as the β-LG enriched sample which suggests that CMP did not contribute significantly to the reduction in astringency. Gelatin and β-LG reduced astringency similarly. However, at a higher concentration, gelatin reduced astringency more than β-LG. Moreover the astringency reduction observed here was comparable to that reported by Guerrero et al. (2013) for potassium caseinate, a commercial fining agent, which when added at 0.6 mg/mL led to a 19% astringency reduction in wine.

The transparent and bright colour of the red wine became cloudy after the addition of the proteins, indicating complex formation between proteins and polyphenols (Siebert, Troukhanova, & Lynn, 1996). Both hydrophobic interactions and hydrogen bonding have been reported to contribute to complex formation. Hydrophobic interactions were reported to be the driving force for complex formation (Charlton et al., 2002) while hydrogen bonding stabilized the association. The structure and composition of the proteins and polyphenols play a role in their interactions. Previously it has been reported that proteins of different molecular weights interact at different strength and with different selectivity towards different fractions of condensed tannins (Cosme, Ricardo-da-Silva, & Laureano, 2009; Ricardo-Da-Silva et al., 1991). Various polyphenols have also been reported to have different binding affinity for different types of proteins as shown for bovine serum albumin and human α-amylase albumin (Soares, Mateus, & De Freitas, 2007). Tannins have the highest affinity for proteins that are large, high in proline content and open coiled such as gelatin and the lowest affinity for small globular proteins (Bennick, 2002). However, β-LG, a small globular protein, was able to reduce astringency similarly to gelatin (Table 1). In order to get a better insight into the extent of interaction and selectivity of these proteins and polyphenols in the wine the soluble polyphenols were quantified in the treated wines.

Table 1
The effect of whey proteins on the astringency of red wine.

Treatments	Concentration (mg/mL)	Slope	Astringency (mg/mL Tannic acid equivalent)	CV (%)
Control	–	0.032	$0.156 \pm 0.01^{a,A}$	1.5
β-LG	0.1	0.016	0.124 ± 0.01^{c}	0.8
	0.5	0.015	0.122 ± 0.01^{B}	0.7
β-LG-CMP	0.1	0.016	0.124 ± 0.01^{c}	0.4
	0.5	0.017	0.126 ± 0.01^{B}	0.9
Gelatin	0.1	0.022	0.136 ± 0.01^{b}	2.4
	0.5	0.010	0.115 ± 0.01^{C}	7.2

Data were expressed as mean ± SD ($n = 3$).
Different lowercase letters for 0.1 mg/mL protein concentration indicate significant difference ($p < 0.05$) in astringency against untreated wine (control).
Different uppercase letters for 0.5 mg/mL protein concentration indicate significant difference ($p < 0.05$) in astringency against untreated wine.
Coefficient of variation (CV%).

3.3. Total phenolic concentration

The total soluble polyphenols before and after treatment of red wine were expressed as mg of gallic acid equivalent (mg GAE/L). The untreated red wine had a total polyphenol concentration of 2255 mg GAE/L, comparable to those reported for the same variety (Landrault et al., 2001). All proteins at different concentrations decreased significantly the phenols when compared to the untreated red wine (Fig. 1). Increasing the protein concentration had a minimum effect on soluble polyphenols. β-LG reduced soluble polyphenol to a similar extent as gelatin at 0.1 mg/mL. Gelatin reduced the phenol content more than β-LG at the higher concentration studied which is in agreement with the reduction in astringency (Table 1).

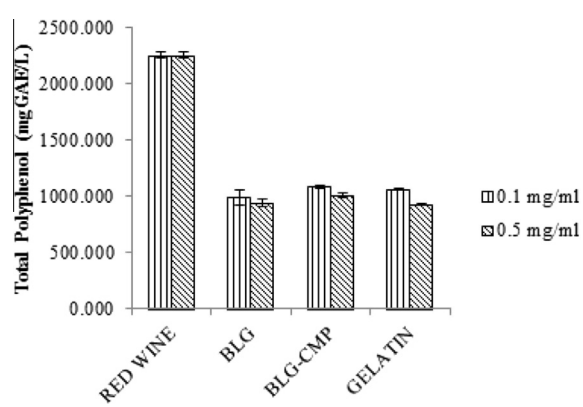

Fig. 1. Phenol content (mg GAE/L) of red wine before and after treatment with β-LG, β-LG-CMP and gelatin. The data represents mean ± SD of three replicates.

3.4. Effect of whey proteins on tannin concentration

Apart from the astringency method, that provides tannic acid equivalents, tannin content was also quantified by a precipitation-based method with methyl cellulose. Tannin concentration of the untreated wine was 1787 mg/L as epicatechin equivalents, within the range (1450–2003 mg/L as epicatechin equivalent) reported by Mercurio and Smith (2008). The same trend was observed as with the total phenols method: at both protein concentrations, the tannin concentration after treatment with β-LG was significantly reduced; the tannin concentration was further reduced by an increase in protein concentration; gelatin on the other hand, reduced tannins more than the whey proteins at the highest concentration. The values estimated by MCP are somewhat higher than the values estimated by the ovalbumin precipitation assay but there was a positive and good correlation between the two methods ($r^2 = 0.921$). Good correlation between the methods was expected as both rely on the precipitation of tannins upon addition of a polymer. The coefficient of variation in both methods was within the normal range (<10%), hence showed good reproducibility.

3.5. Identification of phenolic compounds by HPLC

It is important that in reducing the astringency polyphenol components with health attributes are kept in solution, therefore, the selectivity of polyphenol precipitation was assessed by HPLC. The main groups of phenols and individual phenols identified were: benzoic acids (gallic acid); flavan-3-ols (catechin and epicatechin); and anthocyanins. Figs. 2a and 2b show the HPLC profile of polyphenols in treated and untreated red wine. Interestingly the profile did not change after treatment with proteins, implying that proteins reduced astringency without altering the monomeric polyphenol profiles of the wine. Therefore these results suggest that all the tested proteins interacted and formed complexes with the higher molecular weight or polymeric phenols rather than the monomeric flavan-3-ols; similar result was previously reported for gelatin (Ricardo-Da-Silva et al., 1991). Further analysis of the data of gallic acid, catechins and epicatechins (Table 2) show that β-LG did not reduce as much as gelatin the catechin in solution; gelatin at both concentrations reduced catechin concentration in solution significantly. All proteins at the highest concentration reduced significantly the concentration of epicatechin in solution. Thus although gelatin and β-LG show similar selectivity for polyphenols the latter did not remove as much catechin, an important polyphenol with beneficial health attributes, from solution.

3.6. Particle size determination

DLS provides information on the particle size and the polydispersity of the samples. Increasing concentration of tannic acid (0.2–1.2 mg/mL) to β-LG (at pH 3.5) led to an increase in particle size (10–500 nm) (Table 3). At tannic acid concentration of about 0.3 mg/mL (the equivalent concentration measured in the wine) particles of about 200 nm were formed Table 3. However wine contains larger MW tannins which will be more reactive than tannic acid and larger particles would be formed which resulted in precipitation. The above observations on particle sizes are in agreement with the model postulated by Siebert et al. (1996); a protein is considered as having a fixed number of sites to which a tannin can bind and a tannin is thought as having two (or more) ends that can bind to protein. When there is equal protein binding sites as polyphenol binding ends, large colloidal particles will form and maximum precipitation will occur; in the case of excess protein to tannin, small particles and less haze will occur because of insufficient tannin to bridge many protein dimers and in the case of excess tannin to protein, small particles and less haze will also occur because all sites of the protein are occupied hence at one end polyphenol finds a site to attach but at other end no protein is available for attachment.

3.7. Analysis of protein traces in wines after treatments

The presence of residual protein was assessed to avoid any risks posed to allergenic individuals. This is particularly important when using fining agents with allergic potential as under a new EC directive since 2012 wine manufacturers must label wines containing more than 0.25 mg/L of potentially allergenic residues of fining agent proteins (Deckwart et al., 2014). β-LG was dissolved in synthetic wine at 0.4 mg/mL and analysed by HPLC. There was no interference in the zone of the β-LG (genetic forms A and B, retention time 61 min) caused by wine's polyphenols (retention

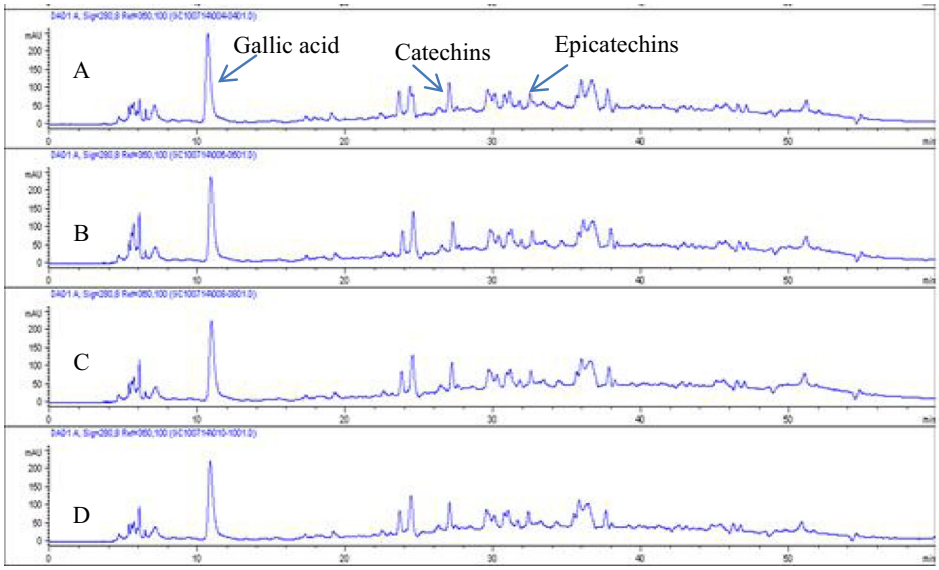

Fig. 2a. Representative HPLC Profiling of gallic acid, catechins and epicatechins as detected by UV at 280 nm of red wine treated with proteins: (A) untreated red wine (control) (B) β-LG (C) β-LG-CMP and (D). Gelatin. The data represents mean ± SD of three replicates.

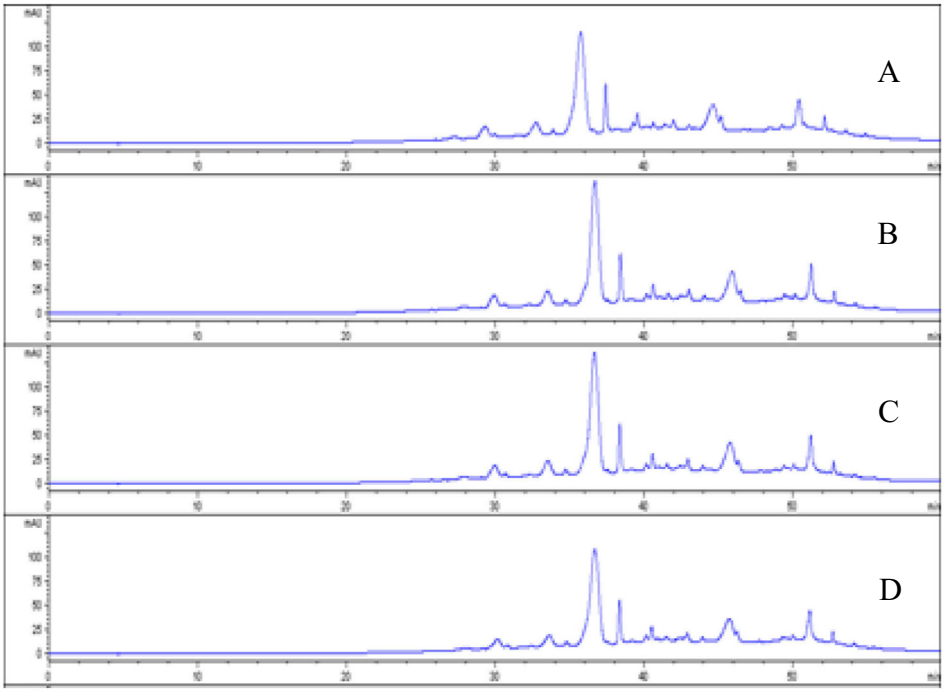

Fig. 2b. Representative HPLC Profiling of anthocyanin as detected by UV at 520 nm of red wine treated with proteins: (A) untreated red wine (control) (B) β-LG (C) β-LG-CMP and (D). Gelatin. The data represents mean ± SD of three replicates.

Table 2
The effect of Whey proteins on selected phenolic in red wine.

Treatments	Concentration (mg/mL)	Gallic acid (mg/mL)	Catechin (mg/mL)	Epicatechin (mg/mL)	Anthocyanins (%)
Control	–	$31.1 \pm 0.8^{b,A}$	$15.75 \pm 1.39^{b,A}$	$23.25 \pm 3.35^{a,A}$	$100^{c,B}$
β-LG	0.1	32.08 ± 0.40^{a}	16.33 ± 0.46a	21.25 ± 1.87^{a}	104.76 ± 3.60^{ab}
	0.5	30.96 ± 0.10^{A}	13.82 ± 0.50^{A}	19.29 ± 3.39^{B}	112.69 ± 5.71^{A}
β-LG-CMP	0.1	31.21 ± 0.33^{ab}	16.60 ± 1.32^{a}	21.61 ± 1.96^{a}	105.05 ± 3.98^{ab}
	0.5	30.67 ± 0.25^{A}	14.38 ± 0.13^{A}	20.88 ± 0.47^{B}	111.23 ± 3.32^{A}
Gelatin	0.1	30.24 ± 0.44^{b}	11.86 ± 0.17^{c}	21.99 ± 0.89^{a}	111.476 ± 4.57^{a}
	0.5	30.65 ± 0.20^{A}	12.70 ± 0.33^{B}	16.44 ± 0.24^{B}	106.18 ± 2.34^{AB}

Data were expressed as mean ± SD ($n = 3$).
Different lowercase letters for 0.1 mg/mL protein concentration indicate significant difference ($p < 0.05$) polyphenol against untreated wine (control).
Different uppercase letters for 0.5 mg/mL protein concentration indicate significant difference ($p < 0.05$) polyphenol against untreated wine.

Table 3
Influence of tannic acid concentration on the average particle size of β-LG-tannic acid complex; the tannic acid to protein molar ratio ranged from 4 to 25. The data represents mean ± SD of three replicates.

Tannic acid (mg/mL)	Average particle size (nm)
0.2	32 ± 12
0.4	168.67 ± 4.16
0.6	198.00 ± 4.36
0.8	328.00 ± 19.52
1.0	409.00 ± 12.12
1.2	493.33 ± 4.73

time 7–45 min) while using the proposed method (Fig. S2). Finally, in the treated sample no signal at all could be detected around the 61 min retention time of the protein. This is important as it shows that after fining of the wine with the protein and subsequent filtration no allergenic residues remain which, is in agreement with findings by Deckwart et al. (2014) where they found that treatment of wine with ovalbumin and subsequent filtration resulted in ovalbumin concentration lower than the threshold level (0.25 mg/L).

3.8. Interactions of model tannins with proteins

To gain a better insight into the protein–phenolic interactions that produce the observed astringency reducing effects and to try to better assess the selectivity of our treatment towards phenolics, fluorescence and isothermal titration calorimetry techniques were applied. The fluorescence emission spectrum was obtained for a β-LG solution (0.1 mg/mL) at pH 3.5 (Fig. S3), and it was found that increasing the tannic acid concentration decreased the fluorescence intensity of the protein. Therefore, there is a significant quenching effect caused by tannic acid, indicating that it binds to the protein, which is in agreement with our findings on astringency reduction. There was no significant shift in the emission spectra. This implies that whatever the mechanism of interaction, the molecular conformation of the protein was not altered and no other change in the immediate environment of the tryptophan residues occurred (Papadopoulou, Green, & Frazier, 2005).

The raw data for quenching of β-LG by addition of tannic acid is shown in Fig. 3, plotted as RFI (the ratio of fluorescence intensity, F/F_0; where F_0 and F are the fluorescence intensities before and after addition of the tannic acid respectively) against tannic acid concentration. At the maximum tannic acid concentration studied

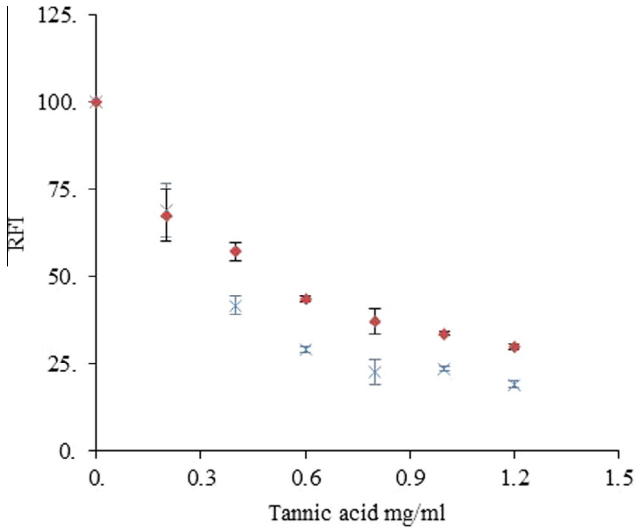

Fig. 3. Tryptophan fluorescence quenching of β-LG fraction at pH 3.5 for 0.1 mg/mL (o) and 0.5 mg/mL (x) plotted as RFI = ($F/F_0 \times 100$) against tannic acid concentrations. Fluorescence emission intensity was recorded at excitation wavelength 280 nm and emission wavelength 354 nm.

protein fluorescence quenching was 70% and 80% approximately at 0.1 and 0.5 mg/mL β-LG respectively. Interestingly at the equivalent tannic concentration in the wine (about 0.3 mg/mL) a very significant quenching was observed at both protein concentrations.

The rate of quenching observed here is in agreement with that reported by Soares et al. (2007), who discovered a rapid quenching of BSA by tannic acid and suggested that this might be due to the high molecular weight of tannic acid components (commercially, tannic acid is acquired as a mixture of compounds as we could prove by HPLC analysis in previous work, unpublished) which is an attribute for good affinity for complex formation with proteins.

The Stern–Volmer plots (F_0/F vs [TA]) show a linear relationship between tannic acid concentration and fluorescence data for both β-LG concentrations: (i) 0.1 mg/mL: $y = 1.9598x + 1.0465$, $R^2 = 0.9938$; (ii) 0.5 mg/mL: $y = 3.6253x + 1.0014$, $R^2 = 0.9615$; where the slopes are equal to Stern–Volmer constant, Ksv. As expected the quenching constant was higher for β-LG 0.5 mg/mL. The linear relationship implies a simple collision quenching mechanism (Papadopoulou et al., 2005). Overall the fluorescence results show that there was high binding affinity for β-LG and the drastic reduction in RFI at tannic acid to protein ratio equivalent to those in the treated wines confirms the mechanism of complex formation of tannins with protein and their subsequent precipitation.

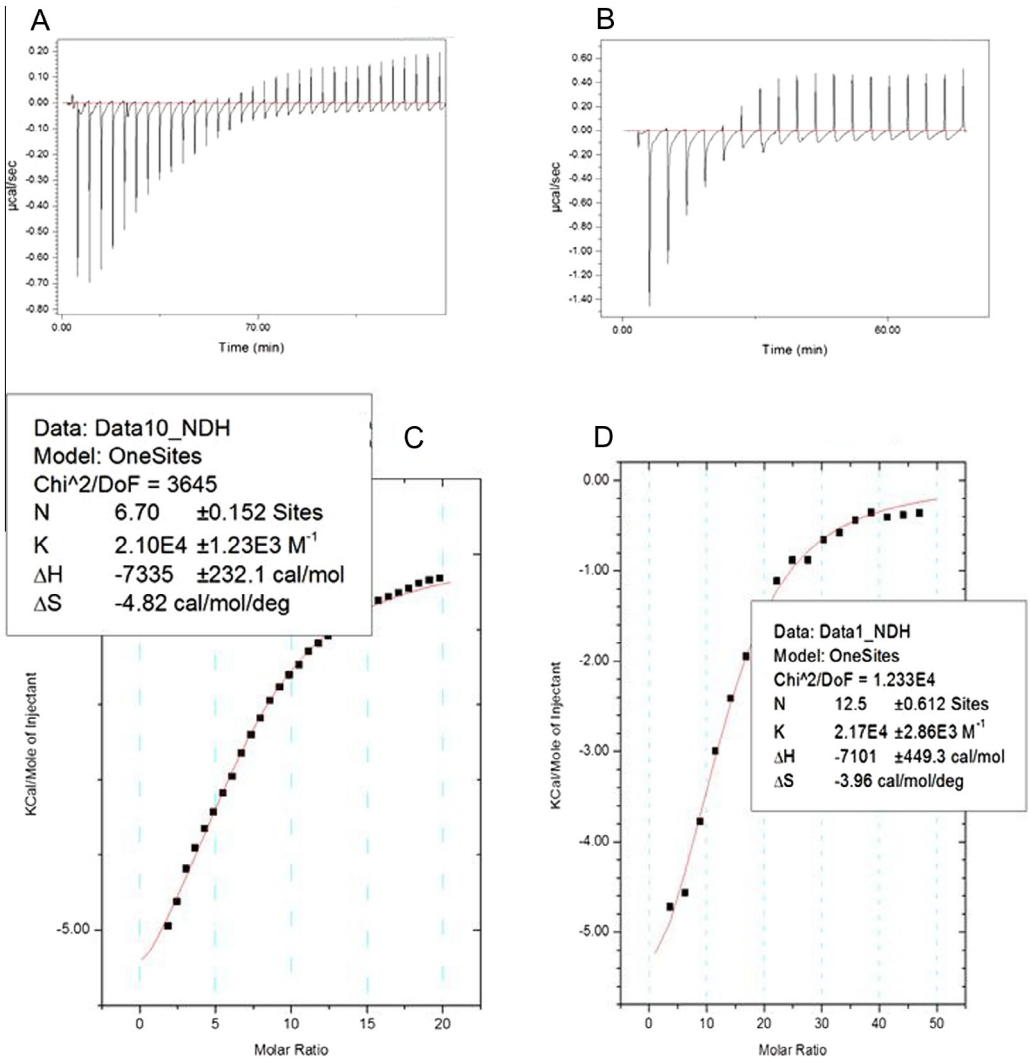

Fig. 4. Thermogram A and fitting C correspond to titrations of tannic acid (3 mg/mL) into gelatin (0.2 mg/mL). Thermogram B and fitting D correspond to titrations of tannic acid (8.8 mg/mL) into gelatin (0.2 mg/mL).

Further evaluation of the interactions of tannic acid with β-LG and with gelatin was carried out by ITC. This calorimetry technique that measures the heat produced/used upon binding of molecules has been previously used for measuring interactions between proteins and tannins (McRae et al., 2010; Prigent et al., 2003; Yuksel, Avci, & Erdem, 2010) as it is extremely sensitive and the detection it provides is universal. The binding isotherm for the interaction of tannic acid with β-LG was plotted as changes in enthalpy versus tannin:protein molar ratio. Interestingly, in spite of the appreciable interaction that could be measured by fluorescence, no clear heat signal related to binding could be observed for β-LG (0.2 mg/mL) titrated with different concentrations of tannic acid (3 mg/mL, 8.8 mg/mL), which indicates a low binding affinity. Being a globular protein, β-LG represents proteins displaying low binding affinity for monomeric flavanols when compared against BSA, gelatin B, and β-casein. From the fluorescence and ITC results of β-LG it can be deduced that tannins coat the surface of the protein which promotes aggregation of the protein molecules and their subsequent precipitation.

On the other hand gelatin interacted strongly with tannic acid (Fig. 4). The *n*-values obtained when data was fitted to a binding model of one set of sites suggested a high stoichiometry of tannin to protein. The binding stoichiometry of (tannic:gelatin) was in the range of 7:1 and the equilibrium binding constants (K) for the interactions of tannic acid (3 mg/mL) with gelatin (0.2 mg/mL) was about $2.2 \times 10^4\ M^{-1}$, Fig. 4 A and C. These values were of the same order as those obtained when the gelatin solution was titrated with a solution of tannic acid of higher concentration (8.8 mg/mL). In this case, the mixture reached the equilibrium faster, Fig. 4B and D. However, the amount of tannin needed to titrate the same quantity of available protein in the cell, was the same, about 53.4 mg of tannin per 41.2 mg of protein. Because of the high viscosity brought on by the gelatin in solution and the one caused by the tannin–gelatin complexation, the thermodynamic equilibrium between injections was slowed, and equilibration times had to be increased.

Finally, the interactions of (+)-catechin, with both proteins were measured. Again, no clear heat signals related to binding could be observed for β-LG or gelatin, which indicates low binding affinity.

The difference in affinity between gelatin and catechin and tannic acid is likely to be due to a molecular weight effect. Sarni-Manchado, Cheynier, and Moutounet (1999) reported that protein binding of polymeric condensed tannins was stronger than that of low molecular weight oligomers and monomers. Indeed, higher molecular weight tannins precipitate proteins more readily than monomeric flavonoids, because they are able to bind more strongly to them. These findings are in agreement with those obtained by the HPLC analysis of wines where it was found no significant reduction of catechin by whey protein samples although a small reduction by gelatin (See Table 2).

4. Conclusions

Whey proteins (β-LG and β-LG-CMP) reduced astringency in wine as efficiently as gelatin which is commercially used in the astringency treatment of wine. Only at the highest concentration gelatin was more efficient than β-LG. The molecular weight, and the proline concentration at an increased protein content probably accounted for the greater reduction in astringency by gelatin. Similar selectivity for polyphenols was observed for β-LG and gelatin but β-LG treatment did not lead to a significant reduction of catechin whereas gelatin reduced it significantly. The fluorescence, size and ITC measurements confirmed that β-LG interactions with tannins were not as strong as with gelatin. From these results an improved understanding of the mechanism of interactions of these proteins with tannins was obtained which suggested that β-LG interacted mainly via hydrophobic interactions and hydrogen bonding with tannins; tannins covered exposed hydrophobic areas of the protein and this led to its aggregation and precipitation. Overall these results showed whey proteins could be a potential treatment agent for red wines in the winery as they are as efficient as gelatin but are slightly more selective towards polymeric polyphenols which results in a wine that maintains some of the main monomeric polyphenols with health attributes unchanged. Ultimately sensory analysis will have to be carried out to confirm these results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.12.052.

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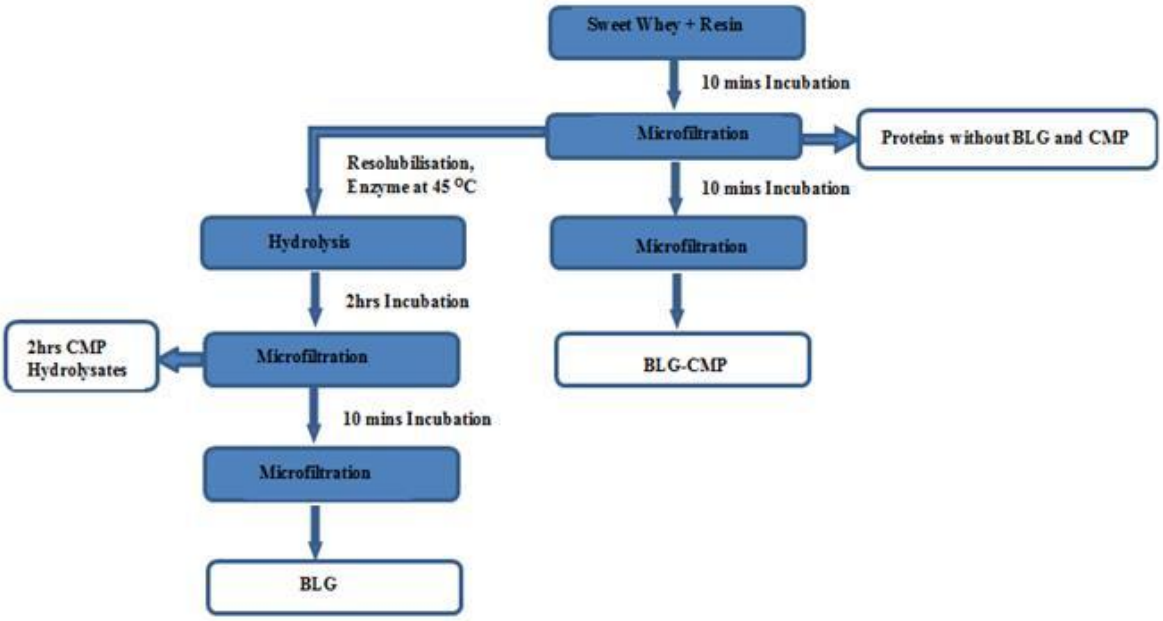
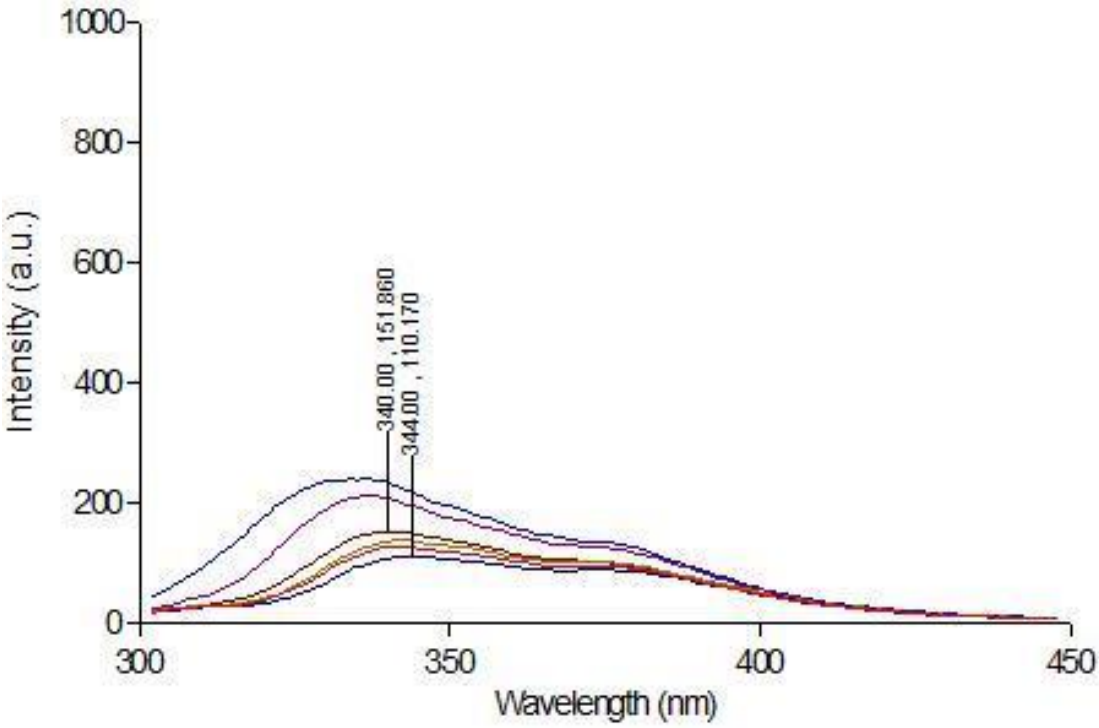
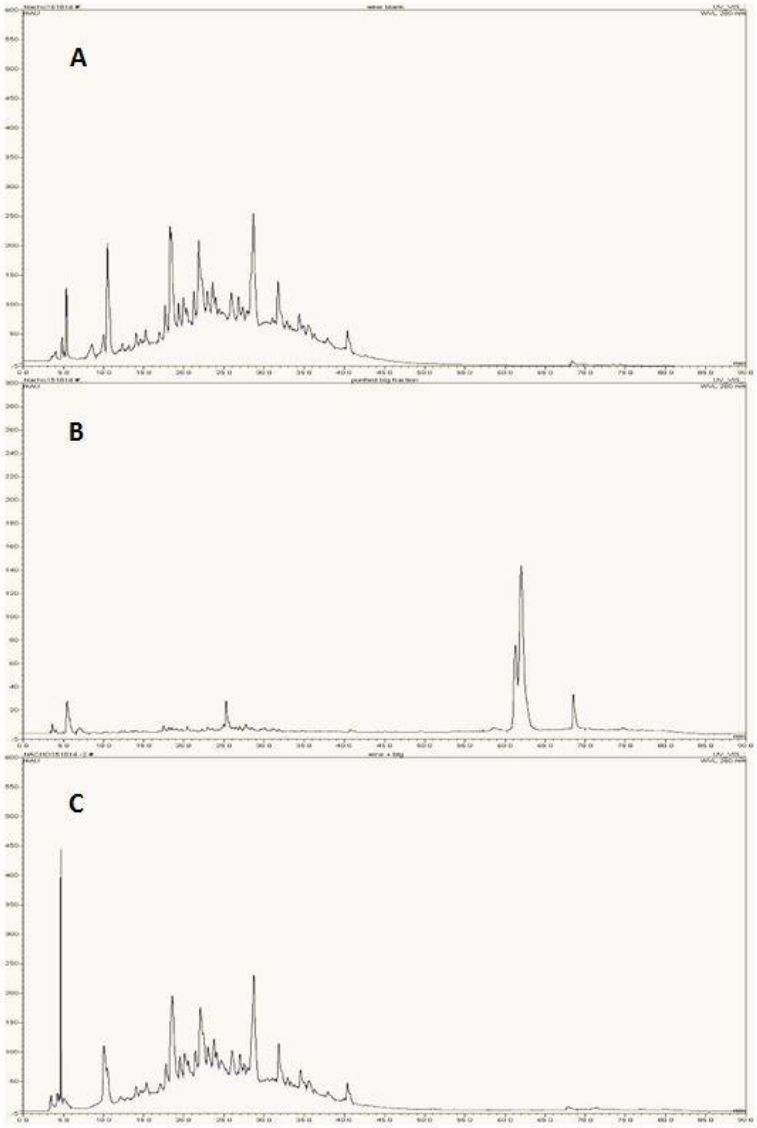
Supplemental material:

S1- Flow chart of the integrative process for the BLG AND BLG-CMP production using sweet whey of pH 6.4±0.2 and at 20 ^{O}C as the starting material.



S2**.** Emission spectra of BLG at 0.1 mg/mL at excitation wavelength = 280nm in the presence of different concentration of tannic acid (0.2 – 1.2 mg/ml).



S3- Chromatograms of the wine control and treated wines with BLG (A) wine (blank), (B) A solution of BLG (0.4 mg/ml) in synthetic wine and (C) the wine after protein treatment and filtration.