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 ME 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 levelafter 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).Overal this

study demonstrates that whey proteins have the potential of reducing astringency in red wine and can find a place in enology.

Keywords: beta-lactoglobulin, astringency, wine, gelatin, ITC, fluorescence, tannins

1. Introduction

Red wine is a beverage that is rich in phenolic compounds, mainly tannins, but also other smaller molecular weight phenolicswhich have demonstrated to have many health benefits due to several biological activities, such as antioxidant, cancer preventing and anti-inflammatoryactivity(Middleton, Kandaswami, & Theoharides, 2000). Tannins are high molecular weight (over 500 Da) polyphenols, which have the ability of precipitatingwith gelatin and other proteins in solution. These proteins are in generalrich in proline. The tannins occurring in wine are responsible for the undesired sensorial properties, specially 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., (Charlton, Baxter, Khan, Moir, Haslam, Davies, et al., 2002) and as described by Dinnella et al (Dinnella, Recchia, Fia, Bertuccioli, & 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 aboveis 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, fora 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 manufacturehas 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, Hasni, Bourassa,

Tarantilis, Polissiou, & Tajmir-Riahi, 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, Hasni, Bourassa, Tarantilis, Polissiou, & Tajmir-Riahi, 2011) and to complex with particular polyphenols (von Staszewski, Jara, Ruiz, Jagus, Carvalho, & Pilosof, 2012)however, it has never been applied to the reduction of red wine astringency. β-LG is a small globular protein of 18350 Da which possess a hydrophobic pocket that shows particular affinity for hydrophobic molecules. β-LGhas some technological advantages for its application as a fining agent: inexpensive, food grade and non-toxic material, capable of solubilizing and protecting hydrophobic biologically active molecules in aqueous media whilst retaining thesensory 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, a 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 ofβ-

LGandcaseinomacropeptides (CMP)and to compare them againstgelatin. 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 (Llaudy, Canals, Canals, Rozes, Arola, & Zamora, 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 βeta-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). Pasteurized Skimmed milk and Merlotred 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℃ in a water bath. Commercial rennet was added at a concentration of 0.3 ml per litre of milk with gentle stirring for 2 minutes. Incubation took place for onehour 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 0.45μm 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, Gibson, & Jauregi, 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 10mM potassium phosphate buffer at pH 6.5. Adsorbed proteins (β-LG and CMPs) were desorbed and eluted with known volume of

elution buffer, 10mM 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 startedafter re-solubilising the adsorbed proteins with a pH 7,10mM potassium phosphate buffer, at 45°C. Then, protease 'N'Amano enzyme was added to the mixture. After 2hrs, hydrolysed CMPs were micro-filtered and finally, the non-hydrolysed protein remaining, β-LG, was desorbed and eluted with 10mM potassium phosphate buffer at pH 4.5 containing 0.5M 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 ul 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 ℃ 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 minutes 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 et al.,(2006). Samples of known total protein contentwere filtered through a 0.45 um 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 x 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℃. 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 ZetasizerNano S (Malvern instrument, Malvern, UK), equipped with a 4mW He-Ne laser (633nm). 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 ^{O}C, at an angle of 90^{O} from the incident beam.

2.6 Protein treatments on wine

4 mL of wine and 1 mL of protein solutionwere 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 finingwhere for example, maximum recommended dose of caseinate is 0.6 mg/ml (Guerrero, Smith, & Bindon, 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.(Llaudy, Canals, Canals, Rozes, Arola, & Zamora, 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 wereprepared. 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 0.1M sodium hydroxide. For each tannic acid concentration or wine sample, 200 µL of the sample were mixed with increasing concentrations of ovalbumin inEppendorf tubes and vortexed.After 10 mins samples were centrifuged at 11700g for 10 mins. 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 30µLwereinjected 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 ina 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 (flavyliumcation). Compounds were identified by comparison of their retention times withspectra of pure standards.

2.9 Isothermal titration calorimetry

To measure enthalpy changes associated with tannin–protein interactions, a microcalorimeterITC 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 33injections of 1.2 μl. To allow equilibration, 250 sec 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/sec) 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 CMPs 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 sulfate solution (200 uL) was added to the mixture and water was added to a final volume of 1000 uL. The mixture was shaken slightly, allowed to stand for 10 mins at room temperature and finally, centrifuged at 10000 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) wereused to prepare thecalibration 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).

A280tannin = A280control- A280supernatant

tannin concentration (mg of EE/L) = [tannin] X 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 seconds and 8 minutes. Then, 300 μL of the saturated sodium carbonate solution was added, mixed well and left at 20 ºC 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-LGand 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)solutionswere 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 x100) against tannic acid concentration; where $F_o$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 contained5.920 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

Theovalbumin precipitation method used for astringency estimation led to a decrease in absorbance at 280nm due to the precipitation of tannins or tannic acid which, wasused 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 values0.112-0.566 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 astringencyof thecommercial 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 addedat 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 interactionswerereported to be the driving force for complex formation (Charlton, et al., 2002)while hydrogen bonding stabilized the association. The structure andcomposition of the proteins and polyphenols play a role in their interactions. Previously it has been reportedthat 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, Cheynier, Souquet, Moutounet, Cabanis, & Bourzeix, 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(Susana Soares, Mateus, & De Freitas, 2007) . Tannins have the highest affinity for proteins that are large, high in proline content and open coiledsuch 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.

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, Poucheret, Ravel, Gasc, Cros, &

359 Teissedre, 2001). All proteins at different concentrations decreased
360 significantly the phenols when compared to the untreated red wine (Fig 1).
361 Increasing the protein concentration had a minimum effect on soluble
362 polyphenols. β-LG reduced soluble polyphenol to a similar extent as gelatin at
363 0.1 mg/ml. Gelatin reduced the phenol content more than β-LG at the higher
364 concentration studied which is in agreement with the reduction in astringency
365 (Table 1).

366

367 **3.4 Effect of whey proteins on tannin concentration**

368 Apart from the astringency method, that provides tannic acid equivalents,
369 tannin content was also quantified by a precipitation-based method with
370 methyl cellulose. Tannin concentration of the untreated wine was 1787 mg/l as
371 epicatechin equivalents,within the range (1450-2003 mg/L as epicatechin
372 equivalent) reported by Mercurio et al.(2008).The same trend was observed as
373 with the total phenols method: at both protein concentrations, the tannin
374 concentration after treatment with β-LG was significantly reduced;the tannin
375 concentration was further reduced by an increase in protein concentration;
376 gelatin on the other hand, reduced tannins more than the whey proteins at the
377 highest concentration. The values estimated by MCP are somewhat higher
378 than the values estimated by the ovalbumin precipitation assay but there was a
379 positive and good correlation between the two methods(r^2= 0.921).
380 Goodcorrelation between the methods was expected as both rely on the
381 precipitation of tannins upon addition of a polymer. The coefficient of

variation in bothmethods waswithin the normal range (< 10%),henceshowed 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 identifiedwere:benzoic acids (gallic acid);flavan-3-ols (catechin and epicatechin); and anthocyanins. Fig 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 thatall the tested proteins interacted and formed complexes with the higher molecular weight or polymeric phenols rather than the monomeric flavan-3-ols; similarresult was previously reported for gelatin(Ricardo-Da-Silva, Cheynier, Souquet, Moutounet, Cabanis, & Bourzeix, 1991). Further analysis of the data ofgallic acid, catechins and epicatechins(Table2) 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 siginificantly the concentration of epicatechin in solution. Thus although gelatinand β-LG show similar selectivity for polyphenols the latter did not remove as much cathechin, an important polyphenol with beneficial health attributes, from solution.

406 **3.6 Particle size determination**

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

425

426 **3.7 Analysis of protein traces in wines after treatments**

427 The presence of residual protein wasassessed to avoidany risks posed to
428 allergenic individuals. This is particularly important when using fining agents
429 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, Carstens, Webber-Witt, Schaefer, Eichhorn, Schroeter, et al., 2014).β-LG was dissolved in synthetic wineat0.4 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 time 7 - 45 min) while using the proposed method (Fig S2). Finally, in thetreated sampleno 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 withovalbumin 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 itbinds to the protein, which is in agreement with our findings on astringency reduction. There was no

454 significant shift in the emission spectra. This implies that whatever the
455 mechanism of interaction, the molecular conformation of the protein was not
456 altered and no other change in the immediate environment of the tryptophan
457 residues occurred (Papadopoulou, Green, & Frazier, 2005).

458 The raw data for quenching of β-LG by addition of tannic acid is shown in
459 Fig.3, plotted as RFI (the ratio of fluorescence intensity, F/F_o; where F_o and F
460 are the fluorescence intensities before and after addition of the tannic acid
461 respectively)against tannic acid concentration. At the maximum tannic acid
462 concentration studied protein fluorescence quenching was 70% and 80%
463 approximately at 0.1 and 0.5 mg/ml β-LG respectively.Interestingly at the
464 equivalent tannic concentration in the wine (about 0.3 mg/ml) a very
465 significant quenching was observed at both protein concentrations.

466 The rate of quenching observed hereis in agreement with that reported
467 bySoares, Mateus and De freitas (2007), who discovered a rapid quenching of
468 BSA by tannic acid and suggested that this might be due to the high molecular
469 weight of tannic acid components (commercially, tannic acid is acquired as a
470 mixture of compounds as we could prove by HPLC analysis in previous work,
471 unpublished) which is an attribute for good affinity for complex formation
472 with proteins.

473 The Stern-Volmerplots (F_0/F vs [TA])show a linear relationship between
474 tannic acid concentration and fluorescencedata for both β-LG concentrations:
475 (i) 0.1 mg/ml: $y = 1.9598x + 1.0465$, $R^2 = 0.9938$; (ii) 0.5mg/mL: $y =$
476 $3.6253x + 1.0014$, $R^2 = 0.9615$; wherethe slopesare equal to Stern-Volmer
477 constant, Ksv. As expected the quenching constant was higher for β-LG0.5

mg/ml . The linear relationship implies a simple collision quenching mechanism(Papadopoulou, Green, & Frazier, 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.

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 moleculeshas been previously used for measuring interactions between proteins and tannins (McRae, Falconer, & Kennedy, 2010; Prigent, Gruppen, Visser, van Koningsveld, de Jong, & Voragen, 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 (3mg/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 flavanolswhen compared againstBSA, 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 gelatininteracted 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 (3mg/mL) with gelatin (0.2mg/mL) was about 2.2×104M-1, Figs 4A 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, Fig4B 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 et al. (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 (Table2) where it was found no significant reduction of catechin by whey protein samples although a small reduction by gelatin.

Conclusions

Whey proteins (β-LG and CMP-β-LG) 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 cathechin 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 imporoved understanding of the mechanism of interactions of these proteins with tannins was obtained which suggested thatβ-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

549 unchanged.Ultimately sensory analysis will have to be carried out to confirm
550 these results.

551

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Tables

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±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 %).

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	-	$31.06\pm0.78^{b,A}$	$15.75\pm1.39^{b,A}$	$23.25\pm3.35^{a,A}$	$100^{c,B}$
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
	0.5	30.65±0.20^{A}	12.70±0.33^{B}	16.44±0.24^{B}	106.18±2.34^{A}

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 BLG-tannic acid complex; thetannic acid to protein molar ratio ranged from 4 to 25. The data represents Mean ± SD of three replicates.

Tannic acid	Average Particle size

mg/ml	nm
0.2	32.43±11.67
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

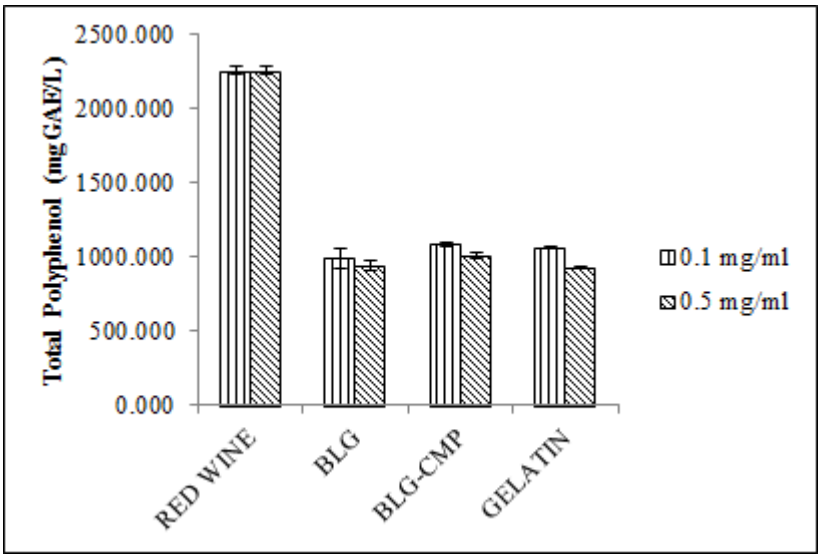
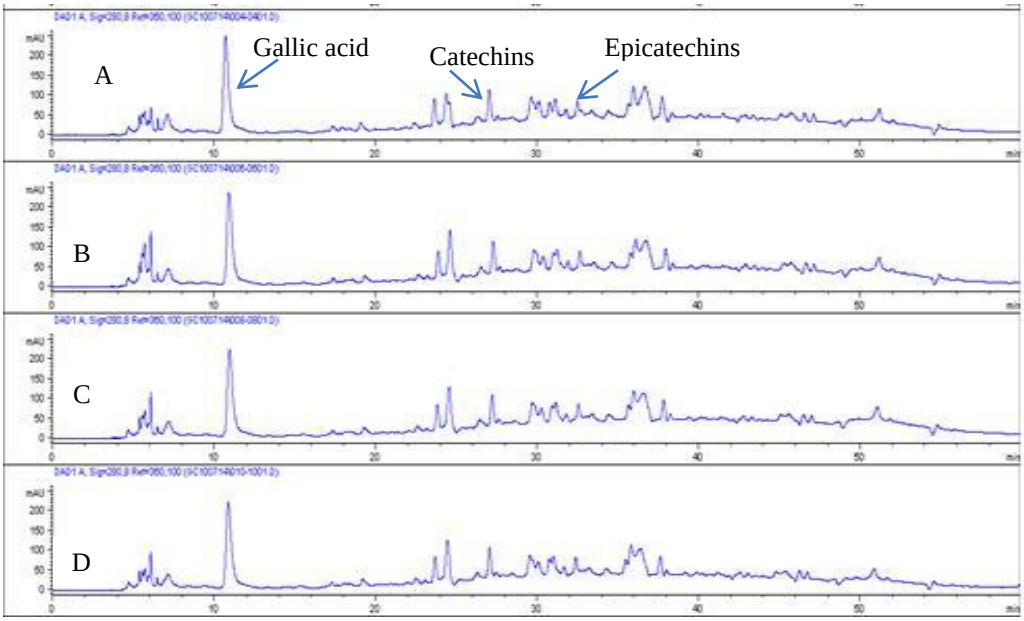


Fig 1. Phenol content (mg GAE/L) of red wine before and after treatment with BLG, BLG-CMP and Gelatin. The data represents Mean±SD of three replicates.



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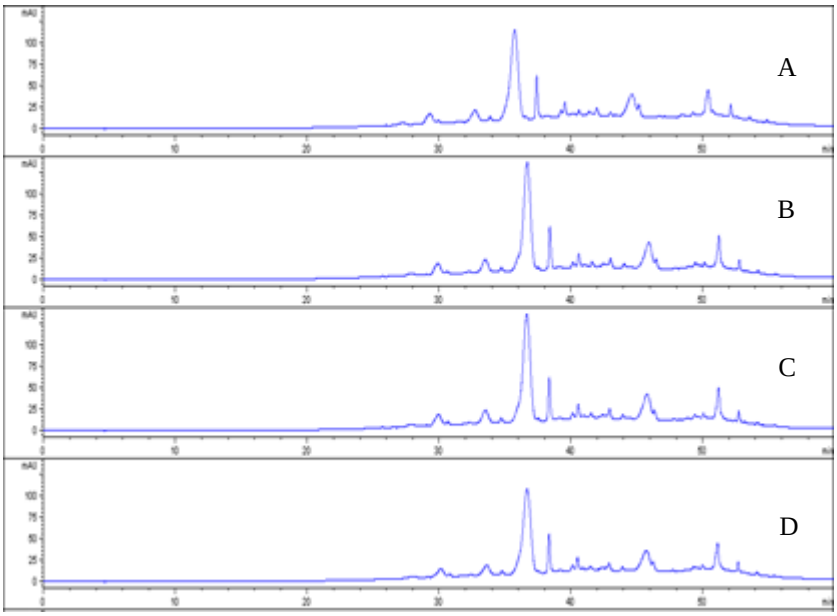
722 **Fig 2a**.Representative HPLC Profiling of gallic acid, catechins and
723 epicatechins as detected by UV at 280nm of Red wine treated with proteins:
724 (A) Untreated Red wine (control) (B) BLG(C) BLG-CMP and (D).
725 Gelatin.The data represents Mean±SD of three replicates.



726

727 **Fig 2b.**Representative HPLC Profiling of anthocyaninas detected by UV at
728 520nm of Red wine treated with proteins: (A) Untreated Red wine (control)

(B) BLG (C) BLG-CMP and (D). Gelatin.The data represents Mean±SD of three replicates.

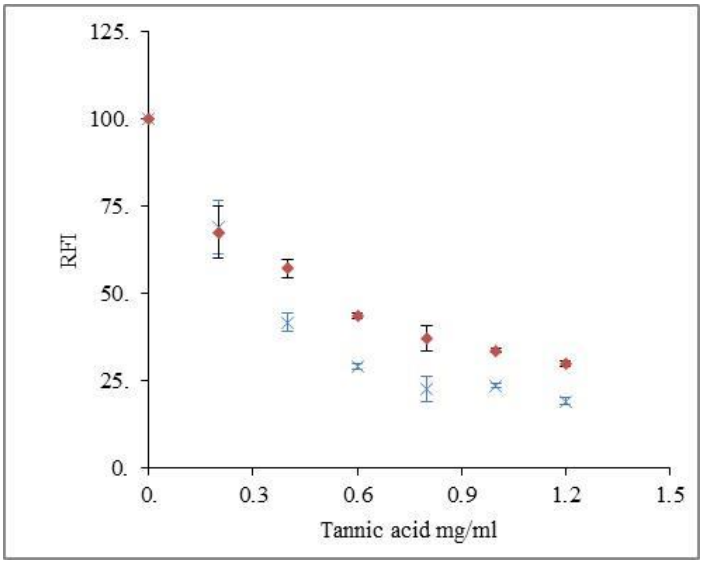


Fig 3. Tryptophan fluorescence quenching of BLG fraction at pH 3.5 for 0.1mg/mL (o) and 0.5mg/mL (x) plotted as RFI = (F/F_0 X 100) against Tannic acid concentrations. Fluorescence emission intensity was recorded at excitation wavelength 280 nm and emission wavelength 354 nm.

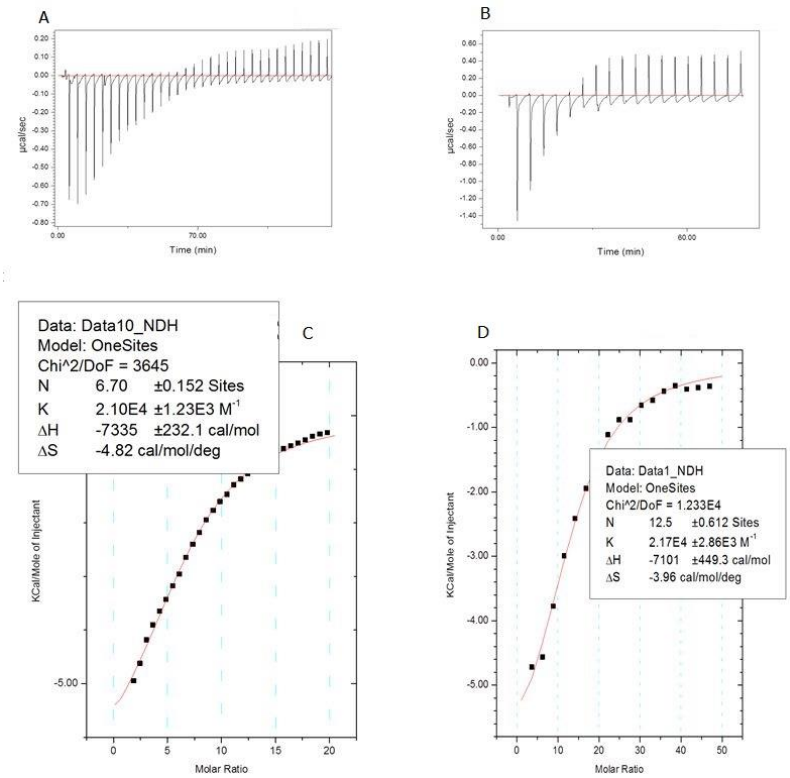


Fig 4. Thermogram A and fitting C correspond to titrations of tannic acid (3 mg/mL) into gelatin (0.2 mg/mL). Thermogram B and fitting D correspond to titrations of tannic acid (8.8 mg/mL) into gelatin (0.2 mg/mL).

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