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Influence of the addition of grape seed procyanidins to Port wines in the resulting reactivity with human salivary proteins

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Abstract

The tannin specific activity of several Port wines was evaluated through the reaction with bovine serum albumin (BSA) and two groups of previously extracted human salivary proteins: α-amylase and proline-rich proteins. The wine reactivity towards BSA and salivary proteins was studied by nephelometry. The tannin specific activity of the wine increased progressively with incremental addition of grape seed procyanidins. α-Amylase was shown to be the best protein to distinguish wines containing different amounts of procyanidins regarding their polyphenol reactivity.

Keywords: Procyanidin; Grape seeds; Port wine; Salivary protein; Astringency

1. Introduction

The role of tannins in winemaking has been reported during recent years, and is one of the major interests of modern oenology. Tannins constitute a large family of polyphenols and are associated to some organoleptic properties of the wine, such as astringency and bitterness. Effectively, these compounds are thought to react with salivary proteins (namely proline rich proteins), causing a sensation of dryness in the palate, known as astringency (Bate-Smith, 1954, 1973; Haslam, 1981; Joslyn & Goldstein, 1964; Kallithraka, Bakker, & Clifford, 1998; Noble, 1990; Sarni-Manchado, Cheynier, & Moutounet, 1999). Although this feature may be considered unpleasant in young wines, it predicts a good wine potential by ability to age. Indeed, these polyphenols undergo several structural transformations with time due to oxidation reactions and interactions with other compounds (e.g. anthocyanins), progressively changing the organoleptic properties of the wine. Sensorial analysis has been widely used to assess the flavour of many foods and beverages, including wine (Arnold, Noble, & Singleton, 1980; Lea & Arnold, 1978; Robichaud & Noble, 1990). Nevertheless, due to the inherent subjectivity of such analysis, new physical-chemical methods have been developed, aiming to evaluate the interactions between polyphenols and proteins more objectively. The concept of relative astringency was previously introduced by Bate-Smith (Bate-Smith, 1973). Since then, the interactions between polyphenols and proteins have been studied by several authors, using different approaches. Indeed, the complexation between proteins and polyphenols has been studied in solution using colorimetric methods (Bate-Smith, 1973; Bradford, 1976; Porter & Woodruffe, 1984), NMR techniques (Baxter, Lilley, Haslam, & Williamson, 1997; Murray, Williamson, Lilley, & Haslam, 1994; Richard, Verge, Berke, Vercauteren, & Monti, 2001), microcalorimetry (Beart, Lilley, & Haslam, 1985), enzymatic inhibition (Goldstein & Swain, 1965), nephelometry (Chapon, 1993; Coupois-Paul, 1993; De Freitas & Mateus, 2001, 2002; Horne, Hayes, & Lawless, 2002), affinity chromatography (Oh, Hoff, Armstrong, & Haff, 1980), and competition assays (Bacon & Rhodes, 2000). Bovine serum albumin (BSA) has often been used as a model protein to measure the reactivity of tannins although its globular conformation is quite different from that of the linear proline rich proteins (Asquith &

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Butler, 1985; Bradford, 1976; Hagerman & Butler, 1980; Hagerman & Robins, 1987; Martin & Martin, 1982), which represent about 70–80% of the total human salivary protein content (Beeley, 1993). Nephelometry is one of the easiest methods that allow direct estimation of the amount of protein/tannin complexes in solution. The present work was aimed to study the reactivity of wines containing different amounts of grape seed procyanidins, towards human salivary proteins, using nephelometry.

2. Material and methods

2.1. Grape seed extract

Grape seeds were removed from Touriga Nacional grapes and crushed in a hydroalcoholic solution. Ethanol was removed by rotator evaporation at 35 °C and the resulting aqueous solution was extracted with ethyl acetate. The procyanidin oligomers were extracted from the organic phase by precipitation with hexane according to the procedure described in the literature (Michaud, Lacaze, & Masquelier, 1971).

2.2. Wine samples

The Port wine (*Vitis vinifera*, Touriga Nacional cv.) was provided by Adriano Ramos Pinto, Vinhos S. A.

2.3. Purification and analysis of 3-flavanols

The low molecular weight proanthocyanidins were first extracted from the wines by ethyl acetate. The extract was then applied on a Toyopearl Gel HW-40(s) column (250×25 mm i.d.) using methanol as eluant at 0.8 ml min^{-1} (De Freitas, Glories, Bourgeois, & Vitry, 1998). Two fractions were obtained: the first (120 ml) contained (+)-catechin and (−)-epicatechin, and the second (100 ml) contained (−)-epicatechin *O*-gallate, procyanidin dimers (B1 to B8), B2-3′-*O*-gallate and trimer C1. These compounds were detected and quantified by HPLC-DAD, using two connected 250×4.6 mm i.d. reversed-phase C18 columns (Merck®, Darmstadt) protected with a guard column packed with the same packing. The elution system consisted of two solvents, A: 2.5% acetic acid in water, and B: 80% acetonitrile in A. Gradient elution consisted of: 7% B with isocratic elution for 5 min; 7–20% B, from 5 to 90 min; 20–100% B, from 95 to 100 min; 100% B, from 100 to 110 min (isocratic), followed by washing with acetonitrile during 10 min and reconditioning of the column with 7% B during 10 min. The analysis was carried out at 25 °C and at a flow rate of 1.0 ml/min. Procyanidin dimers B1 to B8 and trimer C1, used as standards, were synthesised following the reported methods (Geissman & Yoshimura, 1966; Michaud, Masquelier, & Roudge, 1973). Catechins and procyanidin dimers in wines were identified by analytical HPLC, by comparison with authentic standards, as described elsewehere (Mateus, Marques, Gonçalves, Machado, & De Freitas, 2001).

2.4. Polyphenol molecular weight index

This assay was performed according to the procedure described in the literature (Glories, 1978). Wine (5.0 ml) was placed in a dialysis tubing (cellulose; 6 mm i.d.; nominal molecular weight cut-offs of 12,000–16,000 kDa; average porous radius of 25 angstroms) which was then placed in a vial with 50.0 ml of 12% aqueous ethanol solution (5 g/l tartaric acid, pH 3.2). In a second vial, 5.00 ml of wine were diluted directly with the same hydro alcoholic solution up to 50.0 ml (reference solution- do). Both vials were closed and stored at room temperature for 24 h. The difference between the two absorbance values at 280 nm corresponded to the higher phenolic compounds which were retained inside the dialysis tubing [Index = (do-d)/do]. The membrane permits the diffusion of lower molecular weight compounds in the hydro alcoholic solution through the walls but retain higher molecular weight compounds.

2.5. Bovine serum albumin (BSA)

BSA was purchased from Sigma Chemical Co. (St Louis, USA).

2.6. Saliva collection

Saliva was collected from a volunteer by expectorating saliva into an ice cooled tube. Saliva flow was induced by applying small quantities of lemon juice onto the volunteer's tongue. After collection, EDTA was added to a final concentration of 5 mM and the saliva samples were bulked and stored at −20 °C.

2.7. Salivary proteins

Salivary proteins were fractionated using a combination of methods described in the literature with some minor modifications (Kauffman & Keller, 1979; Levine & Keller, 1977). All operations were performed at 2 °C. The whole saliva was dialysed overnight against 50 mM KH_2PO_4 buffer, pH 6.8. Ammonium sulphate (biochem. Grade) was added slowly to the dialysed solution with stirring to achieve 45% saturation. The resultant protein suspension was stirred overnight and then centrifuged at 20,000×*g* for 30 min. The supernatant (from which the PRPs were purified) was dialysed against several changes of 50 mM Tris–HCl buffer, pH 8.6 and then applied to a 17×1.6 cm column of DEAE Sephadex A25 previously equilibrated with the same buffer.

The column was eluted with starting buffer at 15 ml h^{-1}. The absorbance of the eluate was monitored at 230 nm. This first fraction yielded the unbound total basic fraction. After collecting 250 ml, when all unbound protein had been eluted, 50 mM Tris–HCl, 0.5 M NaCl buffer, pH 8.6 was applied to the column to yield the acidic fraction. The unbound basic fraction was concentrated to 5 ml using a 50 ml Amicon stirred ultrafiltration cell with a 3 kD cut-off membrane. The pellet from the ammonium sulphate precipitation described above was resuspended, dialysed exhaustively against water and lyophilised. The residue was dissolved in 50 mM KH_2PO_4 buffer, pH 8.0, and applied to a 17×1.6 cm column of DEAE Sephadex A50 equilibrated in the same buffer. The column was eluted with starting buffer at 15 ml h^{-1} and the absorbance was monitored at 230 nm. The eluate yielded the unbound α-amylase fraction. All the final fractions from all the columns were dialysed exhaustively against water and freeze-dried. Polyacrylamide gel electrophoresis was then performed over the resulting fractions of PRPs and α-amylase, as described elsewhere (De Freitas & Mateus, 2001).

2.8. Tannin specific activity (TSA)

The tannin specific activity (TSA) of the wines towards bovine serum albumin and the salivary proteins was assessed using nephelometry (De Freitas & Mateus, 2001). Nephelometry experiments were performed in a HACH 2100N Laboratory Turbidimeter. The optical apparatus was equipped with a tungsten-filament lamp with three detectors: a 90 degree scattered-light detector, a forward-scatter light detector and a transmitted light detector. Previous calibration was performed using Gelex© Secondary Turbidity Standard Kit (HACH, Loveland, US), which consists of stable suspensions of a metal oxide in a gel. This analytical method requires ideal conditions where all particles are small and identical (Chapon, 1993). Each wine was diluted to 1/50 with a 12% aqueous ethanol solution at two different pH values (pH 4.0 and 5.0). Furthermore, an excess of protein solution was added to 4 ml of the wine solution in a test tube for each assay; the mixture was shaken and stored at room temperature, according to the procedure previously developed (De Freitas & Mateus, 2002). The haze formation of the solution increased with time and, after 40 min, the formation of the complexes stopped and the haze stabilised. The tannin-specific activities (TSA) were expressed in Nephelos Turbidity Units (NTU) per l of wine. Previous experiments revealed that the proteins were not precipitated by the solvent in the absence of tannins and no precipitate was observed in the wine solutions without any protein. All experiments were performed in quadruplicate. For statistical analysis, T test experiments were performed for every mean in Table 2 (see below) using the SPSS computer package ($P<0.05$).

3. Results and discussion

Oligomeric procyanidins extracted from grape seeds, were added in different quantities (0.5, 1.0, 1.5, 2.0 g l^{-1}) to a Port wine with a low polyphenol content. After eight months of bottle ageing, the wines were analysed in terms of procyanidin oligomers and polyphenol molecular weight index (Table 1). As expected, the concentration of procyanidin oligomers increased systematically with the addition of grape seed procyanidins. The wine polyphenol average molecular weight was also shown to increase with the addition of grape seed procyanidins, suggesting an increasing molecular structure complexity of the polyphenols present in the wine.

Table 1
Polyphenol analysis of the Port wines enriched with different amounts of grape seed procyanidins after 8 months of bottle ageing

	Wine	Wine + procyanidins			
		0.5 g l^{-1}	1.0 g l^{-1}	1.5 g l^{-1}	2.0 g l^{-1}
3-Flavanols (mg l^{-1})	54.6	67.5	99.9	130.5	137.4
Polyphenol MW Index	0.405	0.455	0.517	0.527	0.564

Table 2
Tannin specific activities (NTU ml^{-1} wine) of the Port wines towards BSA and salivary proteins at pH 4.0 and 5.0

	pH	Wine	Wine + procyanidins			
			0.5 g l^{-1}	1.0 g l^{-1}	1.5 g l^{-1}	2.0 g l^{-1}
BSA	5.0	27.5±1.3	53.8±1.5	65.0±1.9	65.0±1.4	65.0±1.5
	4.0	82.5±1.3	85.0±1.6	87.5±2.5	95.0±3.0	95.0±2.8
α-amylase	5.0	30.0±0.5	60.0±1.3	87.5±2.3	133±4.2	153±5.0
	4.0	55.0±1.3	75.0±1.3	103±3.6	157.5±5.0	185±6.3
PRP	5.0	35.0±1.4	50.0±0.8	62.5±1.3	87.5±2.3	92.5±2.9
	4.0	113±3.1	133±4.1	135±1.3	153±4.3	160±6.5

The reactivity of the wine polyphenols towards proteins (BSA, PRP and α-amylase) was assessed by nephelometry. The turbidity of the solution, resulting from the formation of tannin-protein aggregates, increased regularly right after the addition of protein, as previously reported (Chapon, 1993; Coupois-Paul, 1993; De Freitas, 1995; De Freitas & Mateus, 2001). The amount of these aggregates increased up to a maximum value of turbidity that remained practically constant approximately 40 min after the addition of the protein. Bearing the concept of "tanning power" previously introduced and developed by other authors (Chapon, 1993; Coupois-Paul, 1993; De Freitas, 1995), the maximum turbidity expressed in NTU per litre of wine was defined as the tannin specific activity (TSA) of the wine. The definition of TSA was previously introduced in order to characterise the reactivity of different classes of polyphenols towards proteins (De Freitas & Mateus, 2001, 2002). The TSA depends on several factors, such as pH, relative concentrations of tannins and proteins, temperature, ionic strength and the structures of both tannin and protein. Furthermore, the TSA values have been shown to be influenced by the presence of other wine constituents such as carbohydrates (De Freitas, Carvalho, & Mateus, 2003).

For the present work, human saliva was purified and separated into two fractions: one was mostly α-amylase and the other essentially proline-rich proteins (PRP). The TSA values of the wines towards BSA and salivary proteins (PRP and α-amylase), are shown in Table 2. The turbidity measures were performed at pH 4.0, which is close to the pH of Port wines, and at pH 5.0, which is close to the pH of the mucous secretion salivary glands (pH 5.6–7.9) and corresponds to a maximum ability of these proteins to form insoluble aggregates with tannins (Fig. 1). Small variations of pH, especially in the case of BSA, may induce significant differences in the turbidity of the solution, as seen from Fig. 1. The proteins and the polyphenols were shown to be stable at these pH values during the period of time of the assays. The evaluation of the TSA of the different wines, using human salivary proteins at a pH close to the one of saliva, is aimed to reproduce the phenomena occurring in the oral cavity. Therefore, this approach provides useful information to better understand the physical-chemical features involved in these polyphenol-protein interactions that are believed to be responsible for astringency.

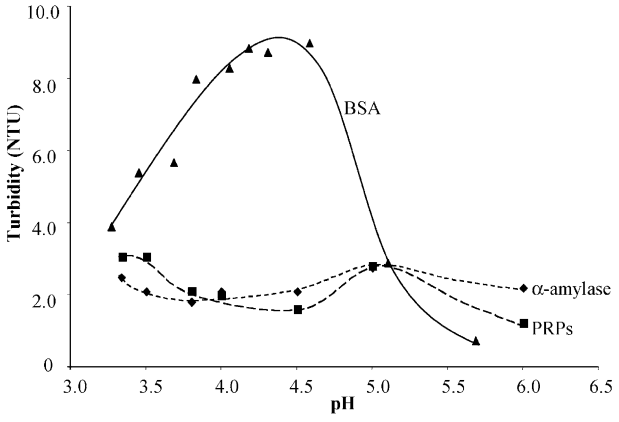

Fig. 1. Influence of pH in the tannin specific activity (TSA) of proline rich proteins (PRPs), α-amylase and bovine serum albumin (BSA) in a solution of oligomeric procyanidins (0.2 g l^{-1}, water/ethanol 12%, v/v) (De Freitas & Mateus, 2002).

In general, the TSA of the wines increased systematically with the addition of grape seed procyanidins, irrespective of the protein used. It is known that the increase of polyphenol concentration favours the formation of polyphenol-protein insoluble aggregates and the TSA values (De Freitas & Mateus, 2002). Curiously, the TSA of the wines towards BSA did not reveal great differences among the wines with different amounts of grape seed procyanidins; the TSA values were similar in the three wines with the higher quantities of grape seed procyanidins, suggesting that this protein is not very selective in the evaluation of the reactivity of the wine polyphenols (Table 2). Although BSA has been often used as a model protein to study the interactions between polyphenols and proteins, these results show that this protein could not be fully reliable for this purpose. By contrast, salivary proteins are likely to distinguish the specific activity of polyphenols in different wines. It is noteworthy that the differences of TSA values between the wines were much greater with α-amylase, suggesting that this protein is the most selective in order to distinguish the polyphenol reactivity or "tanning" capacity of different wines comparatively to PRP. The reactivity of the salivary proteins was found to be higher at pH 4.0. This feature is unexpected, since previous studies showed that the interactions between procyanidins and these proteins were favoured at pH 5.0 (Fig. 1). Nevertheless, it should be taken into account that the chemical composition of the wine is much more complex than that of the procyanidin solutions used to set the pH of the reaction (Fig. 1), and the reactivity of some compounds, can be higher at pH 4.0. Additionally, the colloidal properties of the wine and the presence of other compounds such as carbohydrates should also be determinant in the interactions between polyphenols and proteins, as already reported (De Freitas, Carvalho & Mateus, 2003). Overall, the relative TSA values, within all the wines at pH 4.0, were similar to those obtained at pH 5.0. Although it was not an initial objective of this work, a sensorial evaluation of these wines was performed by a taste panel comprised of 10 tasters with large experience in Port wine tasting. As expected, wine astringency tended to increase with the addition of grape seed procyanidins (data not shown). This outcome seems to indicate a

positive correlation with the wine TSA values previously determined.

In conclusion, α-amylase was shown to be very selective and appropriate for distinguishing wines in terms of their polyphenolic composition, and nephelometry was shown to be a very reliable method. Moreover, these results clearly underline the crucial role of condensed tannins in the sensory features associated with flavour of the resulting wines.

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