

# Evaluation of aglianico grape skin and seed polyphenol astringency by SDS–PAGE electrophoresis of salivary proteins after the binding reaction

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## Abstract

SDS–PAGE electrophoresis and densitometry analysis were carried out to evaluate the reactivity of Aglianico red grape skin and seed polyphenols with human salivary proteins in order to find a method able to assess their astringency. Analysis of the supernatant obtained after a tannin/human salivary protein binding assay and sensorial analysis showed that four proteins, lactoferrin, PRPbg1, PRPbg2 and  $\alpha$ -amylase, were the proteins best able to distinguish tannin solutions characterised by different levels of astringency.

A correlation between densitometric data and tannin concentration was plotted in order to give an indirect measure of astringency. The two sources of Aglianico grape polyphenols differed from each other in astringency power; the seed extract solution was about two-fold more tannic than the skin one. The difference in astringency was also perceived by sensorial analysis.

The results from this study show that SDS–PAGE electrophoresis of human salivary proteins after the binding reaction with grape polyphenol extracts, coupled with densitometric analysis and the use of a calibration curve, looks extremely promising as a new approach to evaluate polyphenol astringency.

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## 1. Introduction

Astringency is defined as the complex sensation due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins (ASTM, 1989). The basic mechanism of astringency is not yet well understood. According to some authors, astringency is considered a taste sensation mediated by gustative nerves (Bartoshuk, 1978; Critchley & Rolls, 1996; Shiffman, Suggs, & Simon, 1992), it is also defined as a chemically induced tactile sensation, because it can be evoked from oral areas lacking taste

receptors (Breslin, Gilmore, Beauchamp, & Green, 1993). However, when a *stimulus* is introduced in the oral cavity, the formation of insoluble aggregates and their precipitation with salivary proteins, is observed. This leads to obstruction of palate lubrication, causing an unpleasant sensation of roughness, dryness and constriction (Joslyn & Goldstein, 1964). Regarding wine, this precipitation is due to the interaction of grape tannins with salivary proteins (Bate-Smith, 1954; Clifford, 1997; Green, 1993).

Grape tannins are polymeric flavonoid compounds in which monomer units consist of flavan-3-ols. The B-rings of the monomer units may have different hydroxylation patterns leading to different proanthocyanidin classes (Cheyner, Rigaud, & Silva, 1992). Depending on the source, there is a substantial variation between

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grape tannins in chemical composition and reactivity (Souquet, Cheynier, & Moutounet, 2000). Tannins derived from grape seeds are essentially procyanidins (polymers of catechin and epicatechin), which may present different galloylation degrees; tannins from grape skins, are procyanidins and prodelphinidins (polymers of gallocatechin and epigallocatechin), which have a higher polymerization degree (DP) (Prieur, Rigaud, Cheynier, & Moutounet, 1994; Souquet, Cheynier, Brosaud, & Moutounet, 1996).

During sensory evaluation, the astringency of tannins strongly depends on their polymerization degree and eventually on the presence of ester derivatives on the tannin frame (Brossaud, Cheynier, & Noble, 2001; Francis et al., 2002; Peleg, Gacon, Schlich, & Noble, 1999; Vidal et al., 2003). Moreover, sensorial activity of wine tannins is quite complex, as it is strongly influenced by their chemical nature and quantity, and by the inner balance with the other compounds present in wine (solvent composition, ionic strength, pH and temperature) (Asano, Shinagawa, & Hashimoto, 1982; Asquith & Butler, 1986; Goldstein & Swain, 1965; Hagerman & Butler, 1978; Haslam, 1996; Siebert, Troukhanova, & Lynn, 1996).

Although several studies have been carried out to evaluate interactions between polyphenols and salivary proteins using SDS–PAGE (Bacon & Rhodes, 2000; De Freitas & Mateus, 2001; Sarni-Manchado, Cheynier, & Moutounet, 1999), viscosity (Prinz & Lucas, 2000), spectrophotometry (Llaudy et al., 2004) and nephelometry (Asano et al., 1982; Horne, Hayes, & Lawless, 2002; Mateus, Pinto, Ruão, & De Freitas, 2004), there are some difficulties in correlating the perceived sensation to a single phenomenon which describes astringency. Even though the involvement of proline-rich proteins (PRPs) (Bate-Smith, 1954; Baxter, Lilley, Haslam, & Williamson, 1997; Haslam, 1981; Haslam & Lilley, 1988; Kallithraka, Bakker, & Clifford, 1998; Luck et al., 1994; Sarni-Manchado et al., 1999) and histatins (Naurato, Wong, Lu, Wroblewski, & Bennick, 1999; Yan & Bennick, 1995) in tannin binding is well known, not many attempts have been made to study the correlation between the binding phenomenon and perceived astringency. Kallithraka, Bakker, Clifford, and Vallis (2001), by means of HPLC analysis, found a positive correlation between the decrease of some proteins in the salivary pattern after wine tasting and sensory data indicating that particular proteins in saliva may be more important in astringency than total proteins. However, the nature of these proteins remain uncertain.

Since SDS–PAGE electrophoresis has previously been used to evaluate salivary protein-tannin binding (Bacon & Rhodes, 2000; Sarni-Manchado et al., 1999), the potentiality of this analytical technique was evaluated in this study in order to investigate the salivary proteins principally involved in astringency and to obtain a measure of the perceived sensation.

The study was focused on *Vitis vinifera* L. cv Aglianico, an important ancient native grape from southern Italy. Red wine obtained from the Aglianico grape is popular in South Italy, but often it has a very bitter and/or astringent character that can limit its consumer acceptance (Moio, Romano, Cirella, & Fuschino, 1999). For this reason, Aglianico wine is a good study model of astringency.

In the present study, the SDS–PAGE electrophoresis technique was applied to evaluate the reactivity of Aglianico red grape skin and seed polyphenols towards human salivary protein. This approach has allowed us to develop a simple and accurate method for the quantitative determination of perceived astringent sensation.

## 2. Materials and methods

### 2.1. Reagents

All solvents and acids used were of HPLC grade and were purchased from J.T. Baker [Levanchimica, (Bari, Italy)].

### 2.2. Grape (*Vitis vinifera*) seed and skin tannins

About 100 berries of the Aglianico variety, harvested and frozen at technological maturity, were defrosted before the analysis; seeds and skins were manually separated from the pulp and then collected. Grape seeds were ground under liquid nitrogen. The resulting powder and grape skins were stirred with acetone/water (60:40 v/v) for 30 min. The solution was filtered through paper filters (Albet 400) to eliminate particle residues. The tannin extract was evaporated under vacuum. The dried extract was dissolved in distilled water to obtain the tannin solution (TS) at a concentration of 50 g/l. The TS was diluted to obtain the different concentrations used in the SDS–PAGE experiments.

### 2.3. Commercial tannins (CT)

The commercial tannins, composed of tannic acid; purchased by Extrasynthèse (Lyon, France), were used to prepare solutions at different tannin contents, for use in both sensorial analysis and in SDS–PAGE.

### 2.4. Human saliva (HS)

Because of substantial individual-to-individual variation in the proteic pattern of parotid saliva (Beeley, Sweeney, et al., 1991), representative saliva (HS) was obtained by mixing saliva samples from different individuals. Saliva was spontaneously collected from six non-smoking volunteers (3 males and 3 females) by expectorating saliva into an ice-cooled tube. The resulting mix

was centrifuged at 10,000g for 10 min. to remove any insoluble material, and the supernatant was referred to as whole saliva (Madapallimattam & Bennick, 1990).

### 2.5. Binding assay

Interaction mixtures (80  $\mu$ l final volume) contained 40  $\mu$ l of saliva (HS) and 40  $\mu$ l of TS or CT. Binding assays were performed in Eppendorfs maintained at 25 °C for 5 min. The mixture was then centrifuged for 10 min at 10,000g. The analyses were performed on the resulting supernatant.

### 2.6. SDS-PAGE electrophoresis

Samples mixed with an equal volume of 2 $\times$  electrophoresis sample buffer (0.125 M Tris-HCl, 4% SDS; 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8), and heated at 95 °C for 4 min, were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) using 14% acrylamide resolving gels. The stacking gel was 4% acrylamide (Bio-Rad). Electrophoresis was performed on a Bio-Rad MiniProtean Cell electrophoresis apparatus (Bio-Rad catalog #1653301), using a PowerPac Junior power supply set a 150 V/gel for the stacking gel and 180 V/gel for the resolving gel. The gels were fixed with a mixture of ethanol, acetic acid, and deionised water (40:10:50) for 1 h. After washing in water for 5 min, the gels were stained with Coomassie Brilliant Blue R250 (0.1% in 25% methanol, 10% acetic acid). The Coomassie Blue destaining step by incubation in 30% acetic acid (methanol or ethanol was omitted) distinguishes PRPs, which stain pink-violet, from other proteins, which stain blue. Molecular weights were estimated by comparison with the migration rates of standard protein (BioRad, Rome Italy).

### 2.7. Densitometry

Densitometric tracing of minigels was performed with Biorad GS800 Densitometer.

### 2.8. Sensorial analysis

A triangular test was also performed in order to evaluate the difference in astringency sensation between solutions containing Aglianico grape skin and seed extracted tannins. This method of testing differences involves the simultaneous presentation of three coded samples, two of which are identical. The 8 assessors were asked to select the sample perceived as different. To achieve a statistically significant difference between the samples, the number of correct responses had to be equal to or higher than six.

CT samples at different concentrations (0–2–4–6–10 g/l) were presented to eight expert judges, all working in the wine field. Each sample (20 ml) was served in black glasses with a 3-digit random number code. Samples were presented in random order to the judges. Between samples, judges rinsed their mouths with a tasteless mineral water and ate a bite of bread in order to remove the lingering astringency. The panel evaluated the perceived astringency of solutions containing water and different amounts of CT. The panel was repeated three times. Panellists had to rank samples according to the increase of perceived astringency in the mouth. According to the order of each sample, a score was assigned for the right position, from 0 to 4, 4 being the most astringent. ANOVA statistical analyses were performed using Statgraphics 5.0 Plus-PC (Manugistics, Inc.).

## 3. Results and discussion

Fig. 1 shows SDS-PAGE patterns of HS (lane 2), HS after interaction with grape skin tannins (lane 3), and HS after interaction with seed tannins (lane 4).

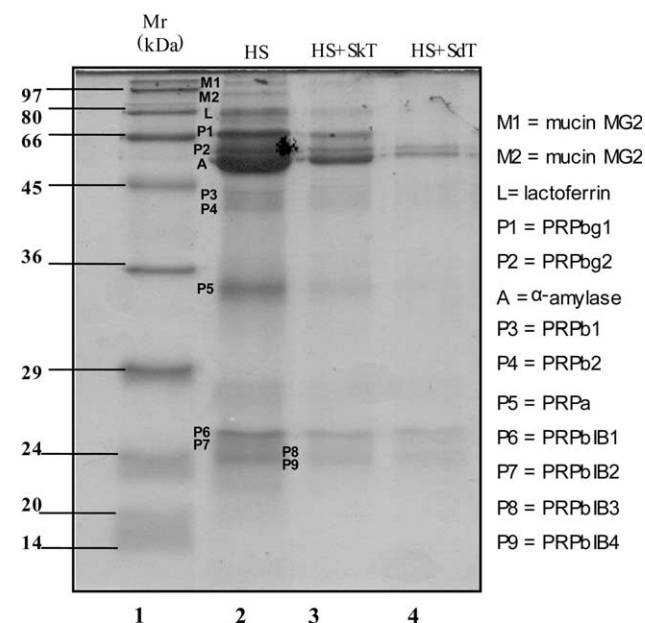


Fig. 1. SDS-PAGE of saliva supernatant after the binding reaction with skin tannin solution (SkT) and seed tannin solution (SdT) from Aglianico grape: (lane 1) Mr markers [molecular mass expressed in kDa as marked on the left side]; (lane 2) HS (human saliva); (lane 3) HS + SkT: saliva proteic pattern (HS) after its interaction with skin tannin solution at 1 g/l (SkT); (lane 4) HS + SdT: saliva proteic pattern (HS) after its interaction with seed tannin solution at 1 g/l (SdT). Letters, near bands, indicated proteins as follows: M1, mucin MG2; M2, mucin MG2; L, lactoferrin; P1, PRPbg1; P2, PRPbg2; A,  $\alpha$ -amylase; P3, PRPb1; P4, PRPb2; P5, PRPa; P6, PRPbIB1; P7, PRPbIB2; P8, PRPbIB3; P9, PRPbIB4.

The number of proteic bands on the HS profile was about 13.

Based on molecular weight and literature, the band at about 100 kDa, is due to mucin protein and seems to be resolved into two bands (M1 and M2, lane 2), which may correspond to two genetically distinct mucin types (Levine, Reddy, & Tabak, 1987). The protein band visible at about 80 kDa (L, lane 2) is probably lactoferrin (Schwartz, Zhu, & Sreebny, 1995). According to Beeley, Sweeney, et al. (1991), molecular masses of about 69 and 66 kDa correspond to the major basic glycosylated PRP (P1 = PRPbg1) and to another basic glycosylated PRP (P2 = PRPbg2), respectively. The proteic band A, at about 62–59 kDa, represents  $\alpha$ -amylase, a major protein component of the HS pattern (Beeley, Sweeney, et al., 1991). At about 43 kDa, there is a series of bands (P3, P4) that can be attributed to basic PRPs (Beeley, Khoo, & Lamey, 1991), while the acidic PRPs may be responsible for bands at molecular masses of 30–33 (P5) (Beeley, Khoo, et al., 1991). At 25–22 kDa, there are the lower molecular weight basic PRPs (P6–9) (Beeley, Sweeney, et al., 1991).

All salivary protein bands in lane 3 were more intense than their counterparts in lane 4, indicating a more extensive binding of salivary proteins to seed tannins than to skin tannins (Fig. 1).

This indicated that seed polyphenols showed a stronger reactivity toward salivary proteins. The higher reactivity of seed tannins can be attributed to their chemical nature, characterized by a high galloylation degree and by a lower medium polymerization degree than skin tannins (Cheynier, Prieur, Guyot, Rigaud, & Moutounet, 1997; Drownowski & Gomez-Carneros, 2000; Ricardo-da-Silva et al., 1991). Moreover, the molecular mass of skin tannin may also be increased by protein (Yokotsuka & Singleton, 1987), polysaccharides (Guaita & Di Stefano, 2001; Waters, Wallace, & Williams, 1991) and anthocyanin complexation (Es-Safi, Cheynier, & Moutounet, 2003; Saucier, Bourgeois, Vitry, Roux, & Glories, 1997; Saucier, Guerra, Pianet, Laguerre, & Glories, 1997).

Fig. 2 shows the difference in density of the proteic bands that represent human saliva (HS) before and after interaction with seed (HS + Sd) and skin (HS + Sk) polyphenols. For almost all proteins, there is substantial reactivity towards polyphenol sources, denoted by the reduction in band density. This is more marked for seed tannins which are more reactive toward salivary proteins.

The bands of some proteins, such as the two isoforms of mucin, totally disappeared after interaction with both polyphenol solutions (1 g/l). Instead, other proteins seem to be implicated in differentiating the two sources of tannin since they show a different reactivity. It is possible that each protein, interacting with a different intensity, discriminates between polyphenols in a qualitative manner.

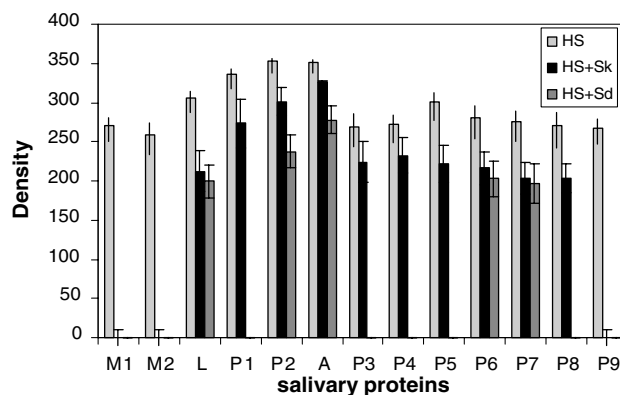


Fig. 2. Density (provided by densitometry) of salivary proteic bands after reaction of HS (human saliva) with tannins extracted from grape skins (HS + Sk) and seeds (HS + Sd). Standard deviations have been considered.

Table 1  
Triangular test results

	Number of correct answers	Significance
Seed extract in water vs. skin extract in water	6/8	$p < 0.05$

Sensorial analysis was in accordance with these results. Triangular sensory tests indicated that there were significant astringency differences between the grape skin tannin solution and the seed tannin one, the latter being perceived as more astringent (Table 1).

In agreement with our findings, earlier sensorial studies (Brossaud et al., 2001) have already denoted differences between seed and skin tannin astringency. In order to give a measure of the astringency intensity of skin and seed tannins, an astringency calibration curve was realized, using solutions at increasing CT concentrations.

Fig. 3 shows the electrophoretic patterns of the supernatant after the interaction of human salivary proteins with CT at different concentrations. As the amount of tannins increased (as shown in lanes 3–4–5–6; 2–4–6–10 g/l, respectively), a decrease of salivary protein abundance was observed. The percentual decrease in density of the most abundant proteic bands, provided by densitometric analysis, is reported in Table 1. The mucin (M1) band, after CT (2 g/l) binding, showed a total reduction with respect to the relative band in HS, and it already disappeared at CT concentration up to 2 g/l.

Therefore, since M1 was totally precipitated at lower CT concentrations, it was not suitable as a reference protein for quantitative differentiation of tannin binding. Instead, four proteins L, P1, P2 and A, not only gave positive responses to the reaction with increasing CT, but their bands showed a progressive reduction (Table 2).



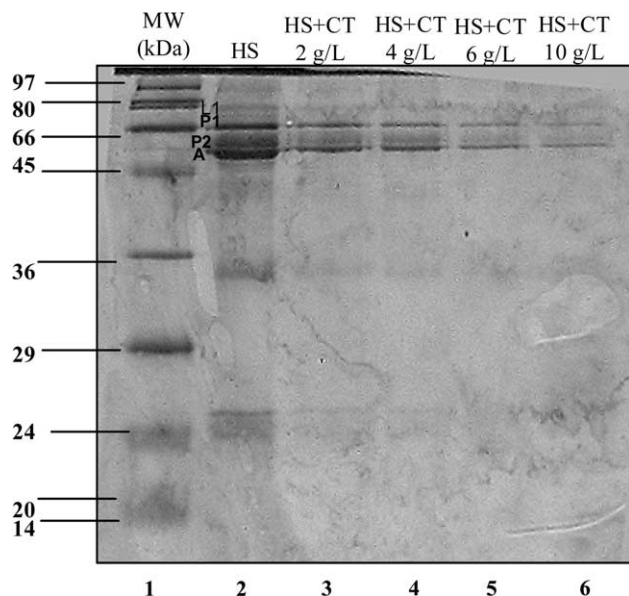


Fig. 3. SDS-PAGE of saliva supernatant after the binding reaction between HS and CT at different concentrations. (lane 1) Mr markers [molecular mass (kDa) as marked on the left side]; (lane 2) HS (human saliva); (lane 3) HS + CT 2 g/l: saliva proteic pattern (HS) after its interaction with CT 2 g/l; (lane 4) HS + CT 4 g/l: saliva proteic pattern (HS) after its interaction with CT 4 g/l; (lane 5) HS + CT 6 g/l: saliva proteic pattern (HS) after its interaction with CT 6 g/l; (lane 6) HS + CT 10 g/l: saliva proteic pattern (HS) after its interaction with CT 10 g/l. Letters near bands indicate proteins as follows: L, lactoferrin; P1, PRPbg1; P2, PRPbg2; A,  $\alpha$ -amylase.

Before searching for a correlation between sensorial data and SDS-PAGE results, the relationship between the intensity of perceived astringency and the CT concentration in water was checked. Data shown in Fig. 4 show, in agreement with Lea and Arnold (1978) and Robichaud and Noble (1990), a direct dependence of astringency sensation on tannin concentration.

Correlation of the perceived astringency, expressed as mean rank sum, with the percentage of band proteic reduction of the four salivary proteins progressively involved (L, P1, P2 and A), gave the  $R^2$  values shown in Table 3. Correlation coefficient of M4P for increased

Table 2

Percentages of density reduction of salivary protein bands (obtained by densitometric analysis) after tannin binding assay between saliva and different CT concentrations (2–4–6–10 g/l); M4P refers to the mean of the values for the four proteins: L, lactoferrin; P1, PRPbg1; P2, PRPbg2; A,  $\alpha$ -amylase

Protein	Density band reduction (%)			
	CT 2 g/l	CT 4 g/l	CT 6 g/l	CT 10 g/l
L	12	16	37	41
P1	3	17	30	33
P2	3	13	17	29
A	4	9	23	32
M4P	6	14	27	34

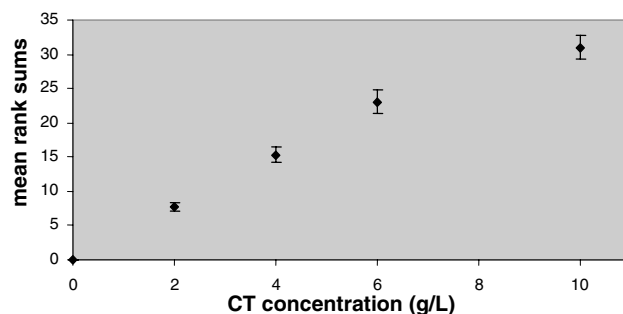


Fig. 4. Correlation between the perceived astringency by a panel test (expressed as mean of sums of ranks) and CT concentration (g/l) ( $p < 0.05$ ). Standard deviations have been considered.

Table 3

$R^2$  values obtained correlating the perceived astringency (expressed as mean rank sum) and percentual density reduction of salivary protein bands (obtained by densitometric analysis) after tannin binding assay between saliva and different CT concentrations (2–4–6–10 g/l); M4P refers to the mean of the values for the four proteins: L, lactoferrin; P1, PRPbg1; P2, PRPbg2; A,  $\alpha$ -amylase

Protein	$R^2$ values
	CT (2–4–6–10 g/l)
L	0.91
P1	0.90
P2	0.93
A	0.91
M4P	0.96

CT, shows the best correlation between sensorial and analytical data.

In order to obtain a useful calibration curve to evaluate astringency power of grape skin and seed tannins, the values of percentual density reduction of M4P bands were plotted against the CT concentration, ranging from 0 to 10 g/l. A regression line was obtained ( $R^2 = 0.9252$ ), as shown in Fig. 5.

It was possible to assign, by means of the equation obtained from this calibration line, to 1 g/l skin polyphenol solution, an astringency power of 4 g/l of

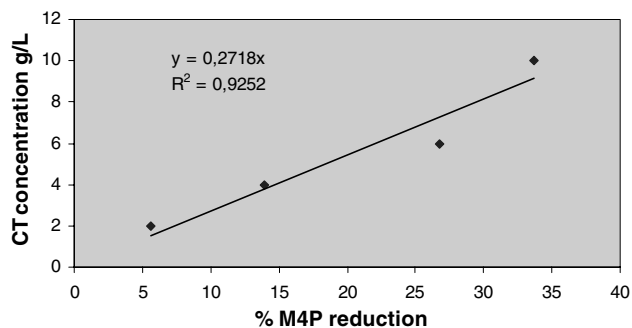


Fig. 5. Correlation line between the percentual density reduction of M4P bands (related to the mean of L, P1, P2 and A proteins) and CT concentration (g/l). The line equation is also shown.

commercial tannins, and to 1 g/l of seed polyphenol solutions, a value of 7.7 g/l of commercial tannins.

Considering this evaluation scale, which is based on perceived astringency of commercial tannin solution (in water), the astringent power of grape seed polyphenols is about two-fold greater than skin polyphenols.

SDS-PAGE, coupled with densitometric analysis is, therefore, a useful tool for evaluating the reactivity of different sources of polyphenols from Aglianico grapes with salivary proteins, and to distinguish those mainly implicated. Four proteins, lactoferrin, PRPbg1, PRPbg2 and  $\alpha$ -amylase, seemed to be preferentially involved in causing astringency, since they showed a qualitative and quantitative selectivity in polyphenol binding and good correlation with sensorial data. By these findings, a correlation line between densitometric data and tannin concentration was constructed to assign an astringency power to tannic solutions. The correlation line represented an indirect evaluation of astringency, so it was possible to differentiate Aglianico grape skin polyphenol extract from the seed one as had already been revealed by sensorial analysis.

New experiments with red wine are underway to extend the application of this method. This correlation may be applied to red grape skins and seeds, and to red wines in order to classify different grapes and wines on the basis of their astringency power. Moreover, SDS-PAGE analysis after the binding reaction of M4P proteins, may represent a useful tool for investigating behaviour of salivary proteins involved in astringency.

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