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Chapter

Salivary Protein-Tannin Interaction: The Binding behind Astringency

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Abstract

Interactions between salivary proteins and tannins are at the basis of one of the main mechanisms involved in the perception of astringency. Astringency is a tactile sensation evoked in the mouth by plant polyphenol-derived products, such as red wine. It is generally recognised that tannins can provoke negative sensations such as shrinking, drawing, or puckering of the epithelium. On the other hand, the astringency of some red wines can be felt as pleasant mouth feelings of richness, fullness, mouth-coating, and velvet in the mouth. In this chapter, an overview of the research concerned with molecular and sensory mechanisms of astringency was updated. Because of many variables influence the perception of astringency, several methods have been developed to measure the intensity of the sensation. In this context, different indirect assessments were critically evaluated considering the pros and contras and correlated with sensory analysis. We focused the attention on the saliva precipitation index (SPI), based on the binding and precipitation of human saliva with grape and wine tannins, because it has been widely used for many applications in winemaking. A current great challenge is to have an *in vitro* measurement of astringency able to provide information on the fate of wine, from grape to bottle.

Keywords: astringency, salivary proteins, polyphenols, precipitation, methods

1. Introduction

The interaction between plant tannins and macromolecules such as proteins is at the basis of many processes involved in the industry, ecological and agricultural systems [1–3], and food and beverage sensory characteristics. The common factor is the binding between macromolecules and tannins that lead to: (i) the conversion of an animal hide into the leather (tanning or *tannage*); (ii) the plant defence strategies against pathogens [1, 4]; (iii) reduced palatability of high tannin feedings to both terrestrial and marine herbivores and then a reduced interference in the process of digestion [2, 5]; (iv) the perception of astringency in tannin-rich food and beverage [6].

In the tanning process, the tannins bind to the hide's matrix, which is composed primarily of the protein collagen ordered in microcrystalline helical units. The purpose of *tannage* is primarily to increase the hydrothermal stability of the structure of collagen, secondarily to increase biological inertness, and finally, to improve the utility of the hide's physical properties [7].

In higher plants, tannins are primarily reserved as a chemical defence against pathogens. The complex with macromolecules such as cellulose and pectin, send out the exo-enzymes capable of utilising cellulose or pectin, either as a carbon source or for branching cell wall barriers to more nutrient-cytoplasm, depriving of the substrate or binding sites to these substrates. Another important function of tannin complexes is to impede the decomposition of plant litter, also when the leaf is fallen. This provides the delay in decomposition, which allows a constant input or seasonally demanding input of nutrients to the soil [1].

In the other processes, proteins of animal or human saliva interact with tannins of the unripe fruit, forages, or vegetable-derivates such as red wine, tea, and chocolate. Tannin molecules can bind proteins or enzymes at the level of specific amino acids, and modify the folding, the molecular weight, and the core binding site, to form soluble complexes or precipitates, which can alter protein function or inhibit enzyme activity [8]. This binding is at the basis of the astringent sensation experienced when tannins precipitate salivary proteins, and as a result, they lose their ability to lubricate the epithelial membranes of the mouth [6]. This sensation in mouth discourages the animal from feeding the unripe fruit or high-tannin forages and determines the unpleasantness of consumers for some tannin-rich products. These are the reason why, in the last decades, the interest in astringency has been constantly increased in different research areas.

2. Perception of astringency

The term astringency derives from the Latin verb, *ad-stringere* that means tightly bind, strongly join. It refers to the propensity of vegetable tannins to complex with macromolecules, such as proteins and polysaccharides, and alkaloids. Bate-Smith [9] first speculated that astringent sensations were caused by the increase in friction between the mucosal surfaces, which resulted from a reduction in lubrication in the oral cavity as astringent compounds bound salivary proteins. The binding between polyphenols/salivary proteins forms soluble complexes and/or precipitates that can cause the rupture of the salivary pellicle [10], interact with oral cells [11], and stimulate and activate mechanoreceptors (MRs) hold in the mouth [12]. MRs are nerve endings that function like those of the skin, except that they have smaller receptive fields and lower activation thresholds [13]. They are selectively sensitive to different stimulus properties, such as particle size and/or mouth movements, and project such information to the central nervous system [14]. Besides, the activation of G-coupled proteins also seems to be involved in the perception of astringency, activating signal transduction pattern as that of taste recognition [15]. Some brain regions (hippocampus and anterior cingulate cortex) that have been shown to respond to basic tastes were activated by the intensity and pleasantness of astringency [16]. In particular, the right ventral anterior insula that responded to astringent stimuli contributed to the ability to recognise the qualitative features of astringency. The activation of the trigeminal nerve, chorda tympani, and brain regions involved in memory and emotions could explain astringency as a multi perceptual phenomenon.

Whilst the chemical definition of astringency is related to the ability of tannins to bind proteins, in sensory terms, it is described as different and concomitant feelings of drying, puckering, and roughing [17, 18]. Astringency can be defined as a tactile sensation, because: (i) it is perceived on non-gustatory surfaces such as on the soft palate, gingiva, lips [12], (ii) does not show adaptation but also (iii) increases upon repeated ingestion [19], leading to carry-over effects during the tasting. However, side tastes as bitterness, sourness, and sweetness can highly modulate the overall

astringency [20]. The sensitivity of MRs to astringents as well as basic tastes may elucidate the complexity of red wine astringency, which has been described by 33 different subqualities [21]. Amongst these "hard," "green," and "rich" have been associated with bitterness, acidity, and high flavour concentration, respectively [22], "harsh," "abrasive," and "drying" have been found to define astringency as a negative sensation, whilst the "complex" and "mouth-coat" subqualities have been associated to a positive impact during tasting [21]. These subqualities were also associated with touch standards when utilised to describe the tactile astringent sensations in the mouth elicited by red wines [23, 24]. The qualitative traits of astringency as "soft", "mouth-coat", and "rich" represented the drivers of liking for Sangiovese wine [25]. Similarly, for Tannat [26], and Côtes du Rhône and Rioja appellations wines [27], the attribute "mouth-coat" contributed to the quality of the wine.

It is also true that the perception of astringency is mediated by psychological factors [28], but salivary protein composition [29] and tannin's structure and composition [30, 31] represent the principal factors. In this regard, numerous reviews have been produced during the past years [32–38].

3. Salivary proteins

Saliva is a biological fluid primarily produced by the three pairs of "major" salivary glands (parotid, submandibular, and sublingual glands) in mouth and by the minor ones by 10% [39]. In the whole, saliva are presently more than 2000 different proteins and peptides [40, 41], which are the result of protein post-translational modifications before being secreted in the mouth [42]. Although saliva is predominantly a watery fluid (99.5%) with a complex mixture of proteins (0.3%; 1–2 mg/mL), ions and other organic compounds (0.2%) are also present. The whole saliva continuously baths the oral cavity and having a pH ranging from 6.2 to 7.4 acts as a buffering system. The saliva is continuously secreted (0.3–7 mL/min) and plays a role in protecting the tooth and mucosal integrity, in antibacterial and antiviral activity, digestion of food, speech, lubrication, taste, and represents a biomarker tool for some diseases [41, 43]. The main families of proteins include enzymes (amylase, carbohydrase, lipase), lactoferrin, high (M1), and low (M2) molecular-weight glycoproteins (mucins), peptides as agglutinins, immunoglobulins, proline-rich proteins (PRPs), cystatins, histatins and statherins [44].

There is evidence that saliva may affect the way we perceive the taste and mouth-feel of foods in various ways [45–47]. During the wine tasting, saliva transports and dissolves the stimuli substances [48]. Saliva constituents are of great importance for establishing protein-tannin interactions. In particular, the PRPs, histatins, mucin, amylase are the main salivary proteins involved in the binding with polyphenols eliciting astringency [49]. The differences between the binding of the same polyphenol to different proteins result from differences in the amino acid sequences [50].

The PRPs account for approximately 70% of the total secretory protein and are subdivided into acidic, basic, and glycosylated PRPs. They are characterised by an abundance of proline, glutamic acid/glutamine, and glycine [51]. The presence of these four amino acids, especially proline, which are the so-called alpha-helix breakers, enables the protein to form secondary structures, which assumes a random coils conformation in solution [10, 52]. This feature may allow PRPs to universally bind various types of polyphenols, mainly tannins with different sizes and structures. Some species, such as humans, rats, and mice, produce PRPs containing about 40% proline [53, 54]. However, some species produce salivary proteins, which are rich in proline but do not show a high affinity to tannins due to extensive glycosylation [54].

Parotid and submandibular secretions also contain several low molecular-weight histidine-rich peptides [55, 56]. Amongst 12 forms, the histatin 1, 3, and 5 are predominant and vary in size from 7 to 38 residues. These peptides show a high content of basic residues, such as lysine, arginine, and histidine [57]. They tightly bind tannins, even if some peptides are devoid of proline [58]. Conversely, others observed high tannin precipitation by histatins thanks to the interactions formed by basic residues and proline [59].

Amongst the low molecular weight salivary proteins, there is a selectivity in binding polyphenols (as PGG, procyanidin trimer, epicatechin, malvidin-3-glucoside): the acidic PRPs considerably form soluble and insoluble complexes with PGG and trimer but not with epicatechin; basic PRPs and glycosylated PRPs seem to not interact with trimer, whilst basic PRPs show a high affinity for epicatechin, malvidin-3-glucoside, and a mixture of both; the statherin shows no selectivity [60, 61].

Mucins are the major constituents of the viscous layer coating hard and soft tissues in the oral cavity. Mucins are generally composed of a peptide core (apomucin) enriched in serine, threonine, and proline residues and carbohydrate side chains (oligosaccharides) that are linked O-glycosidically to threonine or serine. M1 is a polymeric mucin due to the formation of disulfide linkages between cysteine residues in non-glycosylated domains, whilst M2 is a monomer [62]. Average proline content of 10% seems to be responsible for protein-phenol interactions [63].

Amylase is secreted mainly by the parotid gland in both glycosylated and non-glycosylated isoforms [64]. It is an enzyme capable of hydrolysing bonds within amylose and amylopectin and is composed mainly of amino acids like aspartic acid > glutamic acid > arginine [65]. However, amino acids as tyrosine and tryptophan seem to be crucial for interaction with polyphenols [66]. The non-glycosylated form of amylase contains 22 proline and 16 tryptophan amino acid residues in its sequence that enable the binding with polyphenols [50].

4. Tannins

Astringent wines are commonly defined as “tannic” because tannins are the main polyphenolic compounds involved in the sensation of astringency. Swain and Bate-Smith [67] provided the first useful phytochemical definition of tannin, being “water-soluble phenolic compounds, having molecular weights lying between 500 and 3000, which have the ability to precipitate alkaloids, gelatin, and other proteins”. Tannins can be classified in condensed tannins, phlorotannins, and hydrolysable tannins. Condensed tannins are large macromolecules that consist of two or more monomeric (+)-catechin or (−)-epicatechin units called procyanidins, whilst prodelphinidins consist of (+)-gallocatechin or (−)-epigallocatechin units. In plants, condensed tannins are found as oligomers (2–10 monomer units) or polymers (>10 monomer units). The number of monomer units in a polymer may be as high as 83 units [68]. The subunit composition varies amongst tannins from grape skins, seeds, and stems [69–71]. The phlorotannins are present in marine brown algae as polymers of phloroglucinol (1,3,5 trihydroxy-benzene) in different ranges of molecular sizes (126 Da–650 kDa). They are analogous to the terrestrial condensed tannins since they do not contain a carbohydrate core [72]. Hydrolysable tannins, structurally perhaps the most complex tannins, comprise three subclasses such as simple gallic acid, poly-galloyl esters of glucose (gallotannins), and esters of ellagic acid (ellagitannins). Derivatives of gallic acid contain one to five galloyl groups that can be esterified to either glucose (e.g., pentagalloyl glucose) or quinic acid (e.g., monogalloyl quinic acid). Gallotannis can contain six or more galloyl groups and can be characterised by having one or more digalloyl groups

(e.g., hetpagalloyl glucose). Complex gallotannins have a higher capacity for precipitating proteins than simple galloyl glucoses [73].

Ellagitannins may be divided into six subgroups: hexahydroxydiphenoyl esters, dehydro-hexahydroxydiphenoyl esters and their modifications, nonahydroxytriphenoyl esters (e.g., vescalagin), flavonoellagitannins (e.g., acutissimin A), and oligomers with different degrees of oligomerisation and types of linkages [74].

Tannins are the main responsible for the qualitative aspects of astringency as well for the intensity of the sensation. Grape seed and skin tannins are felt astringent as the mean degree of polymerisation (mDP), and galloylation increased [75]. Their ability to precipitate proteins also increases with mDP up to a given degree of polymerisation [34, 76]. However, monomeric and dimeric flavan-3-ols can induce astringent and bitter sensations [77]. Galloylation of monomers/oligomers and polymers enhances protein precipitation, and its extent depends on the grape variety [78]. The presence of high galloylation seems to be responsible for the coarse perception [75], which in turn can be decreased by a high content of epigallocatechin units on the tannin molecule. On the contrary, it seems that the hydroxylation of B-ring seems to decrease velvety astringency and increase the perception of puckering and drying astringency of wine fractions [79]. Salivary proteins seem to have a higher affinity for condensed tannins than for hydrolysable tannins because of different structural flexibility, size, polarity, affinity constants, and presence of free galloyl groups [80–84]. Oakwood tannins were mainly associated with smooth and mouth-drying sensations at low concentrations [85]. Astringency subqualities such as mouth-coat, full-body, persistent were mainly associated with oak-derived tannin, whilst the velvet, soft, and satin terms were associated with the exotic wood-derived tannin [25].

4.1 Other stimuli

Compounds able to elicit sensations as tastes and mouth feelings are called *stimuli*. Chemically diverse astringents such as complex salts such as aluminium sulfate (alum), acids, and other phenolics, have also been shown to evoke astringency [17, 86]. Five organic acids and one inorganic elicited astringency and astringent subqualities [87], and dryness has also been reported [86, 88]. The addition of malic and lactic acid in red wine at the same pH did not differ significantly in astringency despite the difference in titratable acidity [89]. However, these acids were defined astringent in addition to their sour taste [90]. Wines more abundant in malic acid showed higher reactivity towards saliva proteins and then higher potential astringency than tartaric acid-rich wines at the same pH, probably due to different buffer capacities [91]. The astringency of acids is attributed either to the direct contribution of H^+ ions or to the hydrogen bonding capabilities of the hydroxyl groups on the anion or un-dissociated acid [17]. Denaturation of proteins in the saliva could also affect the binding and dissociation of phenolic compounds and their precipitation. The intensity of astringency linearly increases as a function of pH reduction [19], implying significant precipitation of salivary proteins [92].

Anthocyanins, composed of a sugar bound to the anthocyanidin moiety (cyanidin, peonidin, delphinidin, petunidin, and malvidin), impart colour to the grapes and red wine and can be modified by different enological practices [93]. Controversial is the studies of the influence of anthocyanins on astringency. An anthocyanin fraction added in model wine solution was felt as "rough and chalk," and slightly contributed to the overall astringency probably for contamination of the fraction with unknown phenolic compounds [94]. Successively, the isolated fractions of anthocyanidin–glucosides and anthocyanin coumarates did not influence astringency of wine solutions either the "coarse," "chalk," or "dry" astringent subqualities [95]. However, anthocyanins were able to interact with human salivary

proteins forming soluble aggregates [96], and even precipitates, being the cinnamoylated the most reactive fraction (precipitation between 6.5 and 17.5%), also influencing the astringency perception [97]. Pyranoanthocyanins, anthocyanin-derived pigments that can form during red wine ageing, seems to be involved in astringency, since they are able to interact with salivary proteins by phenol, catechol or even flavanols structures, similarly to procyanidins [98].

Flavonols (kaempferol, quercetin, and myricetin) are present in grapes and wine as glycosides (sugar attached). In the plant, they act as a natural sunscreen in the skin of grape berries. In wine, they can be hydrolysed and act as cofactors for colour enhancement. Flavonol glycosides, such as 3-O-glucosides and 3-O-galactosides of quercetin, syringetin, and isorhamnetin, have been reported to be astringent at low detection threshold levels and characterised by a velvety astringency [99]. The addition of quercetin 3-O-glucoside (2 g/L) to wine increased astringency, leading to the formation of complexes with saliva at 200 μM [100]. However, such concentrations are not naturally present in red wine, in which quercetin 3-O-glucoside can range from 2 up to 34 mg/L, depending on the cultivar [101].

Many sensory active non-volatile compounds comprising hydroxybenzoic acids, hydroxycinnamic acids, flavon-3-ol glycosides, and dihydroflavon-3-ol rhamnosides were identified as the key inducers of the astringent mouthfeel of red wines using a molecular sensory approach [99]. The phenolic acids in wines, especially hydroxycinnamic and benzoic acid derivatives, have been reported to be more puckering astringent. These compounds have also been correlated with astringency in free-run and pressed wine [102]. The trans-*p*-coumaric, cis-aconitic, and trans-caftaric acids seem to participate in the astringency of Spanish wines [103].

5. Polyphenol-protein interactions

Given that the carbonyl function of salivary proteins is a very effective hydrogen bond acceptor [104], it would appear that it would play a significant role in bonding to polyphenols hydroxyls [10, 105]. Nowadays, the interaction between proteins and proanthocyanidins is widely recognised to be a combination of hydrogen bonding and hydrophobic effects in the acidic wine matrix. However, covalent bonding is also possible between proteins and polyphenols during oxidation [106] and nucleophilic addition processes [107]. In this chapter, we focused on the non-covalent binding involved in the astringent sensation.

Physico-chemical quantities (binding constants, stoichiometry, and atomic structure of complexes, driving forces for the association) have been utilised to understand the multifaceted sensation of astringency. Many techniques including circular dichroism (CD) [108], isothermal titration microcalorimetry (ITC) [109], fluorescence spectroscopy [50], dynamic light scattering (DLS) [110], and nuclear magnetic resonance (NMR) [111] have been employed to understand the formation mechanism of protein/polyphenol aggregates in solution. Generally, these studies focused on interactions between protein segment from human saliva PRPs proteins family and selected procyanidins, because it represents the easiest way to simulate such a complex phenomenon. They can reveal the hydrophobic interactions formed between the phenolic rings of the procyanidins and proline residues, and the hydrogen bonding between the hydroxyl groups on the phenolic B-ring and hydrogen acceptor sites of the peptide bond [52, 112]. The aggregation of procyanidin with peptide seems to be firstly mediated by hydrophobic forces, and then hydrogen bonding has been postulated to provide directional and robust bonding that stabilises the complex. The peptide is coated by polyphenols, which provides a crosslink between two or more peptides up to a critical point, after which precipitation begins.

The stability of these complexes depends on the tannin dimension and number of free phenolic groups, as well as the nature of the protein involved [81, 109].

The driving factors that determine the binding between tannins and salivary proteins were identified to be the critical micelle concentration value (CMC), tannin structure preferences, and tannin colloidal state [113]. Below the values observed in wine (from 1.5 to 2.9 mM), procyanidins specifically interacted with peptide through hydrophilic recognition. A network of interactions can be formed depending on tannin conformation, and precipitation of the complex can occur, or if an intramolecular staking Π-Π of phenolic groups is preferred, the precipitation is not observed. Above these values, tannins spontaneously tend to form aggregates that, at first through specific interactions bind proteins, and then surrounded by the hydrophobic residues, stabilise the complex by hydrophobic bonding. To summarise, both hydrophilic and hydrophobic interactions contribute to form a complex network, which determines the precipitation of salivary proteins with tannins.

6. Assessments of astringency

A method for measuring astringency remains one of the great analytical challenges in wine chemistry and oenology. The interest in investigating the mechanisms and interactions between polyphenols and proteins can allow us to find the optimal way to simulate and evaluate what happens during the red wine tasting. Quite often, sophisticated techniques rely on the purification of both tannin and protein fractions, the extrusion from the wine content, and the omission of matrix components during reactions, and all contribute to send away astringency from the reality that is: wine polyphenols interacting with salivary proteins in mouth, causing drying sensations.

Several procedures have been carried out during the last decades for measuring tannins. Additionally, analyses of soluble (turbidimetric analysis) and insoluble (precipitation protein assays) protein-polyphenols complex have been developed for assessing astringency. The sensory analysis represents the human response as an analytical tool to evaluate wine perception. Many training and tasting sections are necessary over a long period involving a high number of tasters to form a reliable panel. In the case of astringency, it is complicated to discern amongst tastes and brings on fatigue. A method capable of estimating tannin palatability has to be the most objective as possible and must correlate with sensory data in order to reflect the real phenomenon of wine tasting.

6.1 *Stimuli* analysis: pros and contras

Amongst *stimuli* able to elicit astringency, tannins are the main compounds responsible for this sensation. Tannins are intrinsically amphiphilic molecules with high reactivity, have a diverse range of structures, and are often found in matrices with other phenolic molecules containing similar functional groups. Besides using sophisticated equipment and analytical techniques, there is also a great interest in a relatively simple method.

In the past, many colourimetric techniques were developed to analyse phenolics compounds spectrophotometrically. The first one used the Folin-Denis reagent [114], which was successively modified [115, 116], and lastly into the Folin-Ciocalteau assay [117]. However, they were not specific for tannins but detected any phenolic compound. More specific colour reactions were used to measure condensed tannins and their precursors. Depolymerisation in HCl and n-butanol of proanthocyanidins yield anthocyanidins that can be quantified spectrophotometrically [118, 119]. Others used vanillin reagent for flavanols [6, 120],

or *p*-dimethylaminocinnamaldehyde for a more specificity and colour stability [121, 122]. Only the flavonoid-based condensed tannins can be detected with these reagents. As tannins can inhibit the catalytic activity of enzymes [6], many methods used the interaction with proteins in solution to measure the inhibition of different enzymes spectrophotometrically [123].

Other methods, based on the acid-catalysed condensation reactions with benzyl mercaptan (thiolysis) and phloroglucinol (phloroglucinolysis), can determine both the chain length (mDP) and composition by HPLC [124, 125]. Most of our current knowledge about the general composition and structure of grape and wine tannins have been obtained by depolymerisation [126]. Poor yields due to reaction product instability, reactions with non-proanthocyanidin compounds, and side reactions also contribute negatively to the utility of thiolytic methods [124]. The problem with phloroglucinolysis, on the other hand, is that it produces low yields, and only a fraction of the tannin is converted to known flavan-3-ol products [127]. Normal-phase HPLC (NP-HPLC) method has also been developed to quantify the proanthocyanidins into low and high molecular-weight polymers [128]. A simple method based on Fourier transform mid-infrared (FT-MIR) spectroscopy combined with multivariate data analysis, was successfully used to measure the tannin concentration of 86 red wines, previously purified by solid-phase extraction (SPE) [129].

6.2 Precipitation assays: pros and contras

Protein precipitation assays are of particular interest because the interaction of proteins with tannins can be used to model astringency perception [130]. The ability of gelatin to precipitate phenols, including tannins, has been observed since 1934 [131]. The same phenomenon was observed when hide powder or polyvinylpyrrolidone were used in high concentrations [132]. Bate-Smith [130] noted that protein of skin differed from proteins of saliva, which caused the "puckery" sensation induced by tannin. For measuring the relative astringency of tannins, a spectrophotometric technique based on the precipitation of the haemoglobin with tannin was then introduced [130]. Similarly, another spectrophotometric technique measured the inhibition of β-glucosidase after the precipitation with tannic acid and condensed tannins [133]. Alternatively, Hagerman and Butler [134] used bovine serum albumin (BSA) as a precipitant agent, which was successively taken by Harbertson et al. [135] for wine analysis. Glories [136] proposed the gelatin index, in which tannins were precipitated by gelatin protein. This procedure required the measure of proanthocyanidin concentration before and after precipitation with an excess of gelatin. Besides, gelatin is a heterogeneous mixture of proteins, and its composition may change amongst the different commercial products, leading to a source of variability and imprecision of data. For this, some researchers replaced gelatin with ovalbumin [137]. Another tannin assay used the methylcellulose to precipitate tannin (MCP) [138, 139]. The MCP tannin assay is based on the formation of an insoluble polymer-tannin complex, which can be separated by centrifugation. The total phenolic content (absorbance at 280 nm) is measured in control and treated samples. However, if the assays utilise synthetic agent or protein different from saliva, the binding reaction seems not to reproduce the physiological conditions during the wine tasting, because the binding affinity of the protein is not comparable to that of salivary protein. In the case of bovine serum albumin, it has been shown that the salivary protein has a higher affinity for tannin than BSA. In fact, in the presence of an excess of BSA, the tannin preferentially bound the salivary protein. Other proteins, including dietary proteins, may not complex any tannin in the presence of the salivary tannin-binding protein [8]. The use of salivary proteins has been proposed to represent the model system for astringency better. In precipitation

assays, fractionated [8, 140] or whole [141, 142] human saliva has been used. Mixing whole saliva and grape polyphenols give rise to a "soft cloudy" precipitate, which gathered after centrifugation on the bottom of the tube so that the supernatant was easily recovered without disturbing this pellet. The binding reaction was performed at 25°C, and the complex formed was successively precipitated by centrifugation at 4°C in order to stop further reactions. The induced precipitation allowed to separate the proteins bound to polyphenols from whose remained in the solution that not reacted with them. Both the nature of condensed tannin [141] and salivary proteins [142] involved in the precipitation were analysed. In both works, the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of human saliva was carried out, Sarni-Manchado et al. [141], analysed the tannins in the supernatant and pellet. In contrast, Gambuti et al. [142], analysing the supernatant, revealed the proteins mainly reactive with polyphenols by comparison with the control saliva. Evidence of the qualitative and quantitative changes in salivary protein profile after tasting tannin solutions and wines was also made by HPLC [143]. Interactions and precipitation of low molecular weight salivary proteins with procyanidins confirmed the involvement of different families of salivary proteins in the development of astringency [144]. The use of salivary proteins involves the collection of human saliva from different healthy volunteers according to a specific protocol, and it must take into account the salivary flow to limit the effect of individual differences in astringency perception due to subjects' saliva characteristics [145].

6.3 Nephelometry: pros and contras

Nephelometry is a method that allows a direct estimation of the amount of protein/tannin complexes by measuring the scattered light in the solution that results from the gradual formation of a cloudy precipitate corresponding to the soluble aggregate. Chapon [146] proposed this technique by studying the interactions between beer polyphenols and proteins involved in the colloidal instability of beer. Similarly, the haze formed between salivary proteins and polyphenols represents the first step in the development of astringency and can be measured with a turbidimeter [147, 148]. A continuous flow method was also used to study the interactions between grape extracts and wine with BSA at different concentrations [149]. Globular proteins and PRPs were used to measure a relative tannin specific activity of procyanidin oligomers from grape seeds [30], and PRPs showed the strongest affinity. Human salivary proteins have been considered as the most suitable model proteins. For this reason, in turbidity measurement, whole human saliva [148] and mucin, a high molecular weight salivary protein [150], were used as model proteins for astringency assessment. Based on polyphenol/mucin reactivity, a micro-plate assay was also developed [151]. Tannic acid [150], grape seed extracts [151], wine extracts [63], tannin fractions added to model solutions [152] were analysed by nephelometry. The turbidity of the solution, formed by the tannin-protein aggregates, linearly correlated with astringency. However, no direct analysis of wines was carried out. Lastly, instead, wine samples were analysed trough nanotechnology such as localised surface plasmon resonance (LSPR) combined with surface imprinted polymers, as a measure of the interactions of polyphenol with salivary protein and then astringency [153].

6.4 Sensory analysis: pros and contras

The sensory analysis represents the human response to wine tasting. A sensory panel can provide information about the sensory properties of a product, but significant training is required before the panel becomes a reliable sensory instrument. Astringency is a difficult sensory attribute to evaluate, owing to particular

characteristics of the sensation. Generally, it is evaluated by tasting but can suffer from individual subjectivity. The feeling can take over 15 seconds to develop fully and is known to build in intensity and become increasingly difficult to clear from the mouth over repeated exposures [19, 154]. Carry-over effects can occur. When wines or tannic solutions are evaluated by a well-trained panel using established sensory methodologies, the panel leader can expect to obtain reliable information about the intensity in the perceived astringency of the samples. Screening, selection, training, and panel maintenance are exercises that help the panel attain proficiency before sample evaluation. Classical methodologies widely applied are descriptive and rating sensory analyses. The first helps to distinguish between samples by a qualitative description of their sensory properties [75] and the second permits to scale samples according to the intensity of the perception. However, time-intensity (TI) is a temporal methodology widely used. This method consists of recording one by one the intensity evolution of given attributes [155]. However, TI showed some limitations because it is time-consuming due to the evaluation of only a few attributes at the same time [156]. Furthermore, carry-over effects can overcome when assessing the temporal perception of an attribute [157]. To overcome these drawbacks, Pineau et al. [156] developed a new method called temporal dominance of sensations (TDS), which consists of identifying and rating sensations perceived as dominant until the perception ends. Before the development of this method, a similar experimental approach was successfully used to describe the temporality of sensations in wines [158]. Astringency, a dynamic sensation, takes many seconds to develop after the basic tastes, and the duration depends on the wine. Notwithstanding, TDS can be difficult when panellists had select the dominant attribute and score its intensity, but proper training can overcome this problem [159].

It is also essential to discuss and familiarise with the terms associated with astringency. A vocabulary of 33 terms has been proposed by a combined panel of experienced tasters and winemakers to describe the mouthfeel characteristics of red wines [160]. The check-all-that-apply (CATA) question that consists of a list of subqualities from which the panellists have to select all the options they consider appropriate to that wine has been utilised for the characterisation of the astringency subqualities of Tannat wine [161]. Recently, a sensory method that combines CATA approach and training in astringency subqualities with touch-standards resulted very useful for investigating the astringency characteristics of red wines [24, 25, 162]. In any case, intense training is necessary to distinguish astringency from other tastes, especially bitterness, and to reveal the different qualitative attributes. Fatigue and loss of *stimuli* memory may occur, particularly with panellists who are unfamiliar with astringency, and when too many samples are presented. Training is also expensive and time-consuming. However, it is necessary to investigate the astringency subqualities of red wines. Sensory analysis is of fundamental importance, but in some cases, it is not possible to perform, so the replacement with an analytical instrument able to measure astringency could help in research as well as in the winery.

6.5 Correlation between sensory and analytical analysis

Because astringency is one of the main attributes for wine quality, winemakers are interested in an analytical and objective method to evaluate it. No method can substitute entirely sensory analysis, but a method that results in a reproducible index has to correlate quite well with it. A statistically significant correlation between the sensorial and analytical methods is necessary.

The gelatin index has represented the almost widely analytical method for estimating astringency in red wine [136]. Besides, it furnished only approximate results [137]. Successively, a positive correlation ($R2 = 0.56$) between the gelatin index and

time-intensity data was obtained only at a low concentration of polyphenols utilising 29 wines judged by 10 panellists [163]. A method that used the ovalbumin in alternatively to gelatin as a precipitation agent was proposed to determine astringency [137]. Ten wines were tested by 10 expert enologists evaluating the astringency on a scale from 1 to 100. The method resulted in more reproducible than the gelatin index and was positively correlated (R2 = 0.77) with sensory analysis. This method was also used to assess the astringency of Greek wines, and a good correlation was found (R2 = 0.93) [164]. Another predictive model for astringency estimation was based on phenolic compounds and colour analysis of 34 wines by 12 judges on a 9-point intensity scale [165]. Multiple regression generated three possible models to predict astringency from analytical data, the most simple depended on total phenolics and co-pigmented anthocyanins, besides the predicted astringency plotted versus observed astringency resulted in low but acceptable correlation from a sensory perspective.

Monteleone et al. [150] proposed a predictive model by measuring the polyphenol-mucin reactivity in which the capability of polyphenolic extracts to induce astringency was estimated on their ability to develop turbidity in the *in vitro* assay. They found a linear relation between astringency perceived by 30 trained judges and the mucin index for tannic acid model solutions (R2 = 0.993) grape seed extracts (R2 = 0.996), and phenolic extracts (R2 = 0.95) [63].

In a study by Kennedy et al. [166], 40 red wines were evaluated by a panel consisting of three winemakers and two enologists for the astringency intensity scored from zero to 10. The aim was to correlate astringency and tannin concentration measured by different analytical methods: absorption at 280 nm, phloroglucinolysis, gel chromatography, and BSA protein precipitation. The analytical method having the strongest correlations with perceived astringency was the protein precipitation one (R2 = 0.82). Protein precipitation represents the method the most similar to the physiological response to astringent *stimuli* and can be used as an *in vitro* tool for understanding how tannin can modulate astringency perception. Generally, it was assumed that the most suitable proteins for evaluating astringency are the salivary PRPs. However, other proteins in whole human saliva were preferentially precipitated by increasing tannin solutions [142]. Successively, the percentage decrease of two salivary proteins after the precipitation with tannins, measured by electrophoresis, represented an indicator of the reactivity of tannin. The saliva precipitation index (SPI) was well correlated with the sensory evaluation of the astringency of 57 red wines (R2 = 0.97) made by 18 trained assessors [167].

7. The saliva precipitation index (SPI)

The SPI represents a useful tool to assess the physiological response to astringents, measuring the astringency of red wine indirectly. This index evaluated the precipitation of salivary proteins occurring during the tasting of an astringent stimulus. The SPI, analysing the salivary protein pattern by SDS-PAGE electrophoresis, has been improved considering the in-mouth temperature (37°C) for the binding reaction, the choice of resting saliva, and the ratio saliva:wine. The excess of saliva with respect to wine (2:1) in a static environment permits to measure the binding capacity of tannins better [167]. Successively, to reduce the time and solvents, the chip electrophoresis replaced the SDS-PAGE, providing similar results [168]. In the last years, the SPI has been used for different technological practices proving useful information for winemakers and enologists to manage the style and quality of red wines.

7.1 Applications of SPI in winemaking

7.1.1 Enological practices

In winemaking, the clarification process is fundamental to stabilise and clarify the wine by adding exogenous proteins into wine [169]. Proteins used for fining interact with wine tannins by a mechanism similar to that occurring during the tasting. The interaction protein-tannin, binding, and precipitation determine a decrease in polyphenolic compounds responsible for the sensation of astringency [170]. The SPI was used to evaluate the efficacy of the fining of different proteins at different concentrations in Aglianico [171], and Sangiovese wines [172]. In Aglianico, the gelatin (animal protein) and patatin (plant protein) showed similar efficacy in diminishing wine polyphenols reactive towards salivary proteins, and then astringency, whilst in Sangiovese it depended on the polyphenolic content of the wine. The information provided by SPI was useful to understand that each wine, with peculiar polyphenolic composition, should be treated maintaining the ratio anthocyanins and tannins such as to assure a modulation of astringency and at the same time a correct evolution of the colour during ageing.

A common practice is the utilisation of enological tannins as a substitute for oak barrels to improve colour stability and taste and is authorised by the International Organisation of the Vine and Wine (OIV) for musts and wines clarification [173]. Commercial preparations of tannins of different origins showed different abilities in precipitating salivary proteins: condensed tannins resulted in higher SPI and astringency than hydrolysable tannins. The addition of tannins in wines modify the astringency or not depending on the wine phenolic content. The SPI was useful to understand the effect of tannins addition on wine astringency in order not to compromise overall wine quality [83]. Similarly, after a moderate oxidation (21 mg/L of oxygen equivalent), the addition of 2 g/L of enological tannins did not result in an increase in the reactivity of wine tannins towards salivary proteins after 30 days of treatment. This effect was also shown in the oxidation process in the presence of acetaldehyde [174]. The SPI seems to be sensitive to reaction-products such as polymers of flavanols and anthocyanins formed directly or via a molecular bridge (e.g., acetaldehyde) [31, 175], and new-formed proanthocyanidins [93, 176]. This may explain why during the oxidation of red wines, the SPI followed a different trend from BSA reactive tannins [174, 177, 178].

7.1.2 Ageing

The decrease of astringency with time has been shown to depend on the reduced concentration of tannins due to precipitation [31, 68], but the trend is not strictly related to the age of wine [179]. The astringency of red wine decreases during ageing because of the changes in the structure of tannins due to cleavage reactions generating low molecular weight species [31], polymerisation without the participation of anthocyanins and subsequent precipitation [95], direct or indirect condensation with anthocyanins [180], and the formation of flavan-3-ol sulfonates by SO2 [181]. Wine becomes soft and mellow for the decline of tannin mean degree of polymerisation [182], velvet and mouth-coating for the formation of the polymeric pigments [24], or satin for lower content of flavans and astringent tannins (measured by SPI), and higher formation of polymers [183] after ageing. Studies on Sangiovese wine revealed that the astringency profile changed from an unripe, dry astringency towards rich, full-body, and mouth-coating sensations after about 2 years of ageing [184]. However, pucker sensations can appear if the oxidation is excessive [24, 25]. Astringency subqualities have been able to discriminate wines of different

denominations with a chemical age of 3–5 years, more than other wine parameters [25]. Red wine benefits of a moderate oxygenation during ageing favouring changes in tannin structures that, affecting their reactivity towards proteins, can modulate wine astringency. The SPI was utilised to objectively evaluate changes in astringency as a function of oxygen uptake before and after bottling [185]. Although conflicting results were reported for astringency after micro-oxygenation of wines, a significant variation of wine reactivity towards salivary proteins and, then, in wine astringency was observed after 42 months of ageing in bottle only in low pH wines. Moreover, oxygen permeating towards closures determined changes in wine phenolics detectable only using SPI. It was significantly lower when the bottles were sealed with closures at high oxygen transfer rate (OTR). Such differences were not perceived by sensory analysis, demonstrating that SPI can be more sensitive in revealing slight differences in the reactivity of tannins. Lastly, the effect of ageing on the precipitation of salivary proteins is a function of ageing time, wine pH and phenolic composition, and oxygen level in red wine. The decisive role of pH on wine astringency has been confirmed in a recent work of Forino et al. [92], in which the SPI was used to measure wines with different pH levels (3.7–3.2) obtained by adding strong acids or bases, which made the wine unsafe to taste. The binding and precipitation of wine tannins with saliva proteins was favoured at low pH values, and this effect was dominant with respect to the tannins content. Previously, the tartaric acid addition in wine, modifying the pH, resulted in high SPI [186], due to the increase of tannins in the phenolate form, and therefore to an increase of hydrogen bonding with salivary proteins. It is also likely that at low pH increases the accessibility of the binding sites leading to enhanced Van der Waal interactions and hydrogen bonding between proteins and polyphenols [187]. However, other parameters, such as ethanol, fructose, and mannoproteins have been shown to influence astringency and SPI [186]. The effect of mannoproteins on the inhibition of salivary protein precipitation was also showed in Aglianico and Sangiovese wines after 12 months of ageing. The sensory analysis confirmed a reduction in wine astringency. Some mannoproteins interact with tannins forming higher molecular weight structures that prevent the binding with salivary proteins, and thus are not able to elicit astringency [94]. Mannoproteins can also act as steric stabilisers limiting the binding with tannins [112]. Wine polysaccharides inhibit tannin-salivary proteins interaction by a mechanism that involves the formation of protein-tannin complex firstly, probably ruled by hydrophobic interactions and stabilised by hydrogen bonds, and then the polysaccharides can act by a ternary mechanism through the encapsulation of this complex, increasing its solubility. However, the efficiency depends on the polarity of both salivary proteins and tannins [188]. Beyond the molecular mechanism, mannoproteins can highly influence the qualitative sensory perception of astringency, conferring positive subqualities of astringency to red wines [162].

8. Conclusions

Astringency is still a complex phenomenon, and despite the many efforts from researchers, it is not fully understood. However, the different *in vitro* assessments have been shown to be useful in evaluating the wine astringency. They could replace the sensory evaluation when there is no possibility of tasting wines: for low sample availability, when tasting is not permitted (as in the pandemic period due to Covid-19) or unsafe, or when too many samples must be tasted. An analytical method for astringency may be potentially useful not only in research purposes but also in the optimisation of the winemaking process and may help wine producers to improve wine quality.

Conflict of interest

The authors declare no conflict of interest.

Nomenclature

BSA	bovine serum albumin
CATA	check-all-that-apply
CD	circular dichroism
CMC	critical micelle concentration
DLS	dynamic light scattering
FT-MIR	Fourier transform mid-infrared
OIV	International Organisation of the Vine and Wine
ITC	isothermal titration microcalorimetry
LSPR	localised surface plasmon resonance
M1	high molecular-weight mucin
M2	low molecular-weight mucin
mDP	mean degree of polymerisation
MRs	mechanoreceptors
MCP	methylcellulose precipitable-tannin
NP-HPLC	normal-phase HPLC
NMR	nuclear magnetic resonance
OTR	oxygen transfer rate
PRPs	proline-rich proteins
SPI	saliva precipitation index
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPE	solid-phase extraction
TDS	temporal dominance of sensations
TI	time-intensity

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