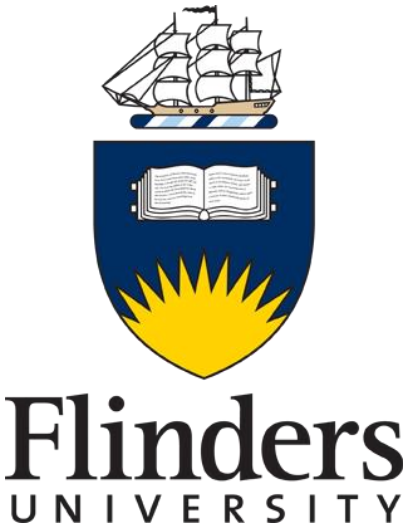
# PROBING THE INTERACTIONS BETWEEN SALIVARY PROTEINS AND WINE TANNINS USING SURFACE ANALYTICAL TOOLS

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## Abstract

Taste and mouth-feel are very complex phenomena, influenced by a wide range of processes. Astringency plays a crucial part in evaluating the flavour profile of wine, especially red wine. Astringency is described as the drying, roughing and puckering of the epithelium felt in the mouth as a result of interactions between polyphenolic compounds from wine (tannins) and salivary proteins. Tannins are phenolic polymers found in plant–derived food and beverages. In wine, tannins are extracted from grape skin and seeds. Tannins from grapes constitute one of the most important quality parameters of wines, since they contribute to the organoleptic characteristics such as bitterness and astringency. Astringency has been studied largely during the last decades, since it represents an important quality attribute of red wine. One of the major mechanism behind astringency is the loss of lubrication as a result of binding between tannins and salivary proteins. Mucins are large (0.5-20MDa), highly glycosylated salivary protein, which are important components for oral lubrication and wear protection. Studies of the binding mechanisms between polyphenols and salivary mucins are rare. This is surprising given the pre-eminent role of mucins in creating the viscoelasticity of saliva. In order to fill the void, we used Human Mucin as our protein of interest along with Bovine Submaxillary Mucin and Human Whole Saliva.

There are two main drivers behind the binding of wine tannin with salivary proteins and mucin: hydrogen bonding and the hydrophobic effect. These drivers of binding are tested in our research by varying pH and ethanol concentration of model wine. The probing of the binding between salivary proteins and tannins are investigated by surface analytical techniques and rheology.

Surface Plasmon Resonance (SPR) spectroscopy is a surface sensitive technique used to monitor the kinetics of binding in real time and the results from SPR highlighted the effect of ethanol and pH of wine on binding between mucin and tannins. SPR results showed the

influenced of hydrogen and hydrophobic interaction on astringency. Further, Quartz Crystal Microbalance with Dissipation (QCM-D) experiments of Human Whole Saliva (HWS) and its interaction with tannins in model wine highlighted the change in viscoelasticity/ rigidity of HWS film as a result of the interaction. Using Atomic Force Microscopy (AFM) we were able to visualize the topography of Human whole saliva and the impact of tannins to the salivary network. Rheological studies on HWS and Mucin protein revealed the change in viscosity when they bind to tannins. The results from these experiments validate our hypothesis that

"*The primary function of MG1, mucin protein is oral lubrication. When the Human whole saliva reacts with wine polyphenols, the mucin protein forms insoluble complexes, which lead to the decrease in oral lubrication. Thus an increased astringency will be perceived by the wine drinker".*

Polysaccharides in wine originate from grape berry fruit cellulose and cell wall of yeast. These macromolecular polysaccharides not only affect the sensory properties by altering the mouthfeel of the wine but also plays a vital role in stability and viscosity of wine during wine production and storage. Polysaccharides have the ability to affect the interaction between tannins and salivary protein. This ability of polysaccharides to prevent aggregation and flocculation of tannins and protein complexes can modify astringent response in wine. Two approaches have been proposed in the literature to understand the capacity of polysaccharides in inhibiting protein-tannin interactions. We are able to identify that the polysaccharides form soluble complexes with tannin and this prevents the interaction between tannin and protein to form insoluble ternary complexes which elicit astringency. This mechanism was identified through SPR, QCM-D and AFM studies on HWS, tannins and polysaccharides.

## Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university, and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Shyamsundar Muthuramalingam

## Acknowledgements

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Personally, I felt very lucky to have my wonderful wife, Amritha who was with me through thick and thin for the whole period. Her confidence in me gave the courage to complete this milestone. To my dad, you are my inspiration, and I will make you proud.

# Glossary of abbreviations

| Abbreviation | Definition |
|---|---|
| AFM | Atomic force microscopy |
| AGP | Arabinogalctan-proteins |
| BSM | Bovine submaxillary mucin |
| GST | Grape seed tannin |
| HM | Human mucin |
| HRPs | Histidine rich proteins |
| HWS | Human whole saliva |
| LSCM | Laser scannin confocal microscope |
| MPs | Mannoproteins |
| NMR | Nuclear magnetic resonance |
| PP | Parallel-plate |
| PRPs | Proline-rich proteins |
| QCM-D | Quartz crystal microbalance with dissipation |
| RG II | Rhamnogalacturonan |
| RU | Reflectivity unit |
| ST | Skin tannin |
| SPR | Surface plasmon resonance |
| WP | Wine polysaccharide |
| WT | Wine tannins |

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“In vino veritas.”

# Objective and scope of the thesis

This thesis is using biophysical techniques to describe the astringency sensation, which is one of the main taste factors influencing the consumer preference during wine selection. The research methods used in this project are novel in the field of oenology. The unique combination of surface analytical tools such as Surface Plasmon Resonance spectroscopy (SPR), Quartz Crystal Microbalance with Dissipation (QCM-D) and microscopy techniques like Atomic Force Microscopy (AFM) and Laser Scanning Confocal Microscopy (LSCM) can give access to clear understanding of astringency mechanism. The field of sensory and flavour perception is vast and complicated, the results from this thesis underline the importance of using novel techniques to unravel complex sensory perceptions like astringency which is common in oenology.

The introductory chapter (section one) gives an overview of the astringency sensation and also provides general information on the polyphenols of wine and salivary proteins. The role of polysaccharides in red wine astringency is also discussed. A short introduction into food rheology and the importance of rheological studies on Human whole saliva is reported.

Literature review and previous works on this field are discussed with relation to surface analytical tools and other biophysical techniques used to study the interaction between salivary proteins and wine polyphenol. Importance is given to the analytical studies conducted to understand the astringency sensation in red wine. Imaging techniques used by various researchers are also discussed to highlight the importance of imaging the Human salivary film and the mucin protein. Section one also emphasises the knowledge gap that exists in this field and put forward the research hypothesis which will bridge the gap.

Section two describes the materials involved in this research and the various methodologies applied. Materials ranging from different wine tannins, salivary proteins and model wine solution preparation are discussed. Since sample preparation and handling impacts the outcome

of this research work, the sample preparation and surface modification strategies employed are explained in detail. The data acquisition method for both the SPR and QCM-D is discussed comprehensively.

The results from all the techniques are compiled, interpreted and discussed in detail in the results and discussion section of this thesis. In this section, the main drivers of binding of the salivary protein with wine tannins are discussed. The effect of ethanol and pH of the model wine solution on the binding between salivary protein Human Mucin (HM), Human whole Saliva (HWS) and Bovine Submaxillary Mucin (BSM) is discussed by results from SPR and QCM-D. The results from SPR and QCM-D are interpreted in such a way that it will be straightforward to understand even for the novice in these techniques.

The results from AFM imaging helps in visualizing the protein-taannin complex topography. The results from rhelogical study and LSCM provides a holistic approach to understanding the astringency sensation. Moreover, the binding nature of different types of tannins and the impact of temperature on the binding is also discussed. The role of wine polysaccharides in astrigency and the mechanism behind the polysaccharides impact on the interaction between protein-tannin complex is presented in the form of SPR, QCM-D and AFM results. The results from this study benefit not only the researchers but also to wine maker in greater extents as it helps in better understanding the role of polysaccharides in perceiving the astringency sensation.

# Section One

## Introduction

"*Wines are known by their taste, brightness, colour, aroma-The requisite criterion of truly good wines are, that they possess strength, beauty, fragrance, coolness and briskness.*"
*-P.P Carnell, "a treatise on family wine making," London,1814, p.40*

# 1 GENERAL INTRODUCTION

Wine and winemaking contribute significantly to the Australian economy. A recent report by the Australian grape and wine authority indicates that there are about 2,900 Australian wineries, and the total annual wine production has been estimated at 1.2 billion litres with a gross value of 5.9 billion AUD (Economics 2015). Research and development into the wine-making processes, wine quality, and improvement of crop management and modification of the sensory perception greatly improves the economic benefits received from wine by Australia. The Australian Wine Research Institute (AWRI) based in Adelaide, South Australia is the Australian grape and wine industry's own research organisation. It supports a sustainable and successful grape and wine industry through world-class research, practical solutions and knowledge transfer. The role of science and technology to improve quality and quantity of wine making is one of the strategic importance to AWRI and major Australian Universities.

The role of sensory properties of food in general and of wine, in particular, is of significant importance to the food industry, and there is significant research into understanding the taste perception in order to improve the quality of wine (Gawel 1998; Cheynier 2006; Bajec and Pickering 2008). Taste and mouthfeel are major determinants of consumer preference and acceptance, not only in wine but also for other food and beverages (Guinard JX 1996). Mouthfeel properties of wine, unlike taste is experienced through the sense of touch and the related signals transmitted to brain by the trigeminal nerve. The tests performed to analyse mouth feel properties of wine involves "mouthfeel wheel" which has hierarchically arranged terminologies like 'adhesive, 'aggressive' and 'powder' associated with complex characteristics of red wines (Gawel 1998).

Wine evaluation for sensory properties is primarily carried out by using people as the measuring instruments. Earlier wine assessments were performed directely by winemakers and wholesalers. Since this practice possessed biases and eccentricities, trained testers are now used

to carry out wine evaluation. The common terms, which are used in sensory evaluation to describe the wine qualities are aroma, bouquet, taste, flavour and body. The majority of wine evaluations are carried out using tests like pair test, duo-trio test and triangle test in wine assessment. Although wine-tasting panels undergo extensive sensory training with series of tasting involving a wide variety of wines, the process is unlikely to eliminate genetic based idiosyncrasies (Lawless 1984). New technologies can help in understanding the taste and mouth feel properties of wine in detail. One such technology is Surface Plasmon Resonance spectroscopy (SPR), which was primarily used for real-time detection and monitoring of biomolecular interactions. SPR experiments provide quantitative information on reaction specificity, concentration of molecules, kinetics and affinity of the reacting molecules. This information is essential for the progress of a full understanding of the molecular recognition processes involved as well as for areas such as the design of receptor-targeted therapeutics (Tanious, Nguyen et al. 2008). In wine evaluation, the two terms of great importance are 'body' and 'astringency'. The body of the wine is dependent on the alcohol content, sugar content and other dry extract compounds in wine. The body of the wine is the 'fullness' or 'roundness' touch of the wine in mouth. Astringency is primarily described as a feeling; a sensation felt all around inside the mouth and developed over time. It is a sensation of dryness perceived in the mouth after being in contact with an astringent agent. A comprehensive explanation of astringency sensation is presented in chapter 1.2. In order to understand the astringency sensation in detail, we used SPR technology for the first time in oenology. As SPR has the potential to monitor the binding of wine tannins with salivary proteins in real time and also the kinetics of the reaction, unravelling the astringency mechanism will be much easier than ever before.

Moreover, by the use of complementary techniques such as Quartz Crystal Microbalance with Dissipation (QCM-D), Atomic Force Microscopy (AFM) and rheology, the overall binding nature of wine tannins and salivary proteins was studied under the perspective of food scientists

and nanotechnologists. This holistic approach in probing the astringency mechanism and the potential role played by Mucin, a salivary protein involved in astringency will be of great help not only to winemaker and researchers but also to the consumer.

## 1.1 THE COMPLEXITY OF TASTE

Taste plays a significant role not only as a sensory stimulus but also a defence mechanism employed to detect toxins and recognise high nutrient foods (Bajec and Pickering 2008). Taste is an intricate sensation with a diverse range of varieties but not limited to sweet, sour, salty bitter and savoury (He, Shi et al. 2006). Taste is perceived by a specialized neuroepithelial cells called taste receptor cells (TRCs). These TRCs are organised as a cluster into onion shaped end organs as taste buds. TRCs detect various soluble chemicals that encounter our tongue. The mechanisms by which the taste receptor cells interact with various chemicals range from employing $Na^+$, $K^+$ and $Ca^+$ ions channels to elicit taste stimuli and the participation of specific membrane receptor to mediate the taste transduction mechanisms (Kinnamon and Margolskee 1996). Each basic taste perceived by the tongue employs one or more types of mechanisms to detect the sensation. Primarily, the chemical constituent present in the food give raise to the different taste perception, for example, sour taste is brought out by the acids (Roper 1983). The hydrogen ions act as primary functional part that interacts with the specific proton receptor. Salt perception is brought out by the interaction of $Na^+$ and $Cl^-$ ions with the cation ion channels like ENaC as reported by Roper (Bigiani and Roper 1994). Tastes like sweetness, bitterness and umami employs G-protein coupled receptors and glutamate receptors (Naim, Seifert et al. 1994; Chaudhari, Yang et al. 1996).

The complex nature of taste helps in the sense of smell by identifying the aromas and also it serves as tactile stimuli which arise as a result of the loss of lubrication in mouth apart from recognising mechanical and trigeminal stimuli (Engelen 2012). The advancement in food manufacturing technology outdated the old values of consumer behaviour, who anticipated and

accepted an unbalanced flavour, as long as the product delivered health benefits. The modern day consumer prefers not only high nutrient value foods but also has an emphasis on taste and flavour perception (Lesschaeve and Noble 2005). Hence it is important for researchers and food technologists to understand the taste mechanism in full and redefine the flavour profile at various levels. Flavour perception is a multisensory experience which not only involves taste and smell but also includes tactile cues (mouthfeel), visual cues (food colour) and auditory contribution (crispness and crunchiness) (Spence 2013). A detailed analysis of the taste mechanism would involve understanding the molecular basis of taste with all its receptor and taste cells combined, which is beyond the scope of this thesis. Moreover, the temperature of the meal, fragrance and the texture of the food also play an important part of the taste experience. Astringency is an important sensation, which will be the topic of discussion in this thesis as it has both commercial value in the wine industry and the complex mechanism in perceiving this sensation.

## 1.2 ASTRINGENCY

The ATSM (American Society for Testing and Materials) defined astringency as "the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins" (ASTM 1989). Earliest discussion on astringency was found in Moncrief's Chemical senses book, which describes astringency as a "contracting or drying taste"(Moncrieff 1946). In general, astringent compounds have the ability to bind with and cross-links proteins. Encyclopaedia Britannica (1957) defines astringents as "a group of agents which tend to shrink the mucous membranes and raw surfaces and dry up secretions." In medicine, astringent agents are used in treatment of haemorrhage and inhibiting secretions at mucosal surfaces (Joslyn and Goldstein 1964). Astringents are also used in different fields like leather processing, cosmetics and beverages for its shrinking effects on tissues. Astringents are

classified into salts of metallic cations (e.g. aluminium and zinc), dehydrating agents (e.g. ethanol and glycerine), mineral acids and various phenolic compounds (e.g. tannins)

The word astringency is derived from Latin “ad stringere” which in term refers “to bind”. Although astringency can be defined simply as “to bind”, the mechanism of perceiving astringent sensation is more complex. Researchers are suggesting two different mechanisms of perceived astringency and this emphasise the complexity of astringency (Schiffman, Suggs et al. 1992; Simon, Hall et al. 1992; Baxter, Lilley et al. 1997; Charlton, Baxter et al. 2002). The first mechanism being, treating astringency as a taste like sour, salty and bitter, which arises as a result of activation of signal transduction pathway by astringent compounds (Spielman 1990). The second mechanism involves treating astringency as a tactile phenomenon, which occurs by binding and precipitation of salivary proteins by polyphenols (Kallithraka, Bakker et al. 1998). There have been studies trying to differentiate whether astringency is a tactile sensation as a result of loss of lubrication in the mouth due to the binding polyphenols to salivary proteins. Astringency as a tactile sensation is better understood by the working of innervation and transduction of tactile, gustatory and oral receptors in the mouth. In the oral region, there are three important cranial nerves for transducing sensory information namely trigeminal, facial and glossopharyngeal (Engelen 2012). The trigeminal nerve which is in orofacial region is primarily involved in tactile sensation by the activation of the chorda tympani nerve during the interaction of salivary proteins with phenolic compounds (Biedenbach and Chan 1971). On the other hand, astringency is a quality of taste like others involving signal transductions through taste receptors (Spielman 1990; Breslin, Gilmore et al. 1993; Kallithraka, Bakker et al. 1998).

A taste test in the wine industry covers major tastes like sweetness, bitterness, and mouthfeel. Astringency also plays a crucial part in evaluating the flavour profile of the wine, especially red wine. The preference of consumer on astringent taste ranges widely from highly astringent to none, a control over the astringency taste according to the needs of consumer will give

winemakers an added advantage in their profitability. Therefore, the scientific community in the last decade experimented mainly in unravelling the astringency and transfer the knowledge about controlling astringent taste in wine to the wine industry (Lee and Lawless 1991; Obreque-Slier, Peña-Neira et al. 2010; Rinaldi, Gambuti et al. 2012). Lesschaevae and Noble detailed factors like viscosity of the wine, level of ethanol, ionic strength, degree of polymerization of tannin subunits and the polymer size of tannins contribute to the astringency of red wine (Lesschaeve and Noble 2005). Luck et al. suggested that the interaction of astringent agents like tannins with salivary proteins and glycosaminoglycans, a type of mucopolysaccharides seems to be responsible for the loss of saliva lubricity (Luck, Liao et al. 1994). In Baxter et al.'s study on astringency, they concluded that the interaction process of tannin and proteins resulting in precipitation of protein-tannin complexes was alleged to be the main mechanism in reduced lubrication in oral cavity (Baxter, Lilley et al. 1997).

Among the techniques employed to control astringency, the use of polysaccharides like rhamnogalactans and mannoproteins showed scientific evidence of modulating astringency by reducing the astringent sensation in model wine solutions (Vidal, Francis et al. 2004). One of the main goals of this thesis is directed towards understanding the molecular basis of understanding the role of polysaccharides in inhibiting/mediating astringency.

## 1.3 *VITIS VINIFERA*

The scientific name for the common grapevine is *Vitis vinifera*. The Vitis genus has physical attributes of elongated flower clusters, fruits that adheres to the fruit stalk and shed petals at maturity and a shredding bark. According to archaeological evidences from turkey and Georgia, the domestication of *Vitis vinifera* occured around the early sixth millennium B.C (Cavalieri, McGovern et al. 2003)

Although the plant is of economic importance and cultivated throughout the world by wine producing nations, this plant is native to western Asia and Europe between 30° N and 50° N

(Keller 2015). Grapevines are predominantly planted for wine production, FAO estimates that almost seventy percent of grapevines was used to make wine, 27% was consumed as fresh fruit, 2% was used to produce raisins and the remaining was processed to grape juice and vinegar (Anderson and Nelgen 2015).

The grapefruit from this plant is used by winemakers with or without skin to produce red and white wine respectively. Grapefruit is a berry, which contains more than 25% sugar in the form of glucose and fructose. It also contains mallic acid, tartaric acid and vitamin C (Parry, Su et al. 2006). The pigment anthocyanin is responsible for the red colouration of grape skin. A detailed explanation of the different tannins and the other phenolic compounds present in grape will be introduced in the following sections.

## 1.4 POLYPHENOLS IN WINE

Understanding the structure and role of polyphenols is crucial in oenology to produce better quality wines, as these molecules are involved primarily in taste, texture, and fullness of the wine (Cheynier, Dueñas-Paton et al. 2006). Phenols are basic building blocks of polyphenols, a number of phenols joined in groups and forms different polyphenols. Phenols are benzene rings ($C_6H_6$) with substituted hydroxyl group in the place of one of the hydrogens ($C_6H_5OH$). This substitution can occur in one or several locations in the benzene ring. This combination along with the different grouping of phenol blocks gives rise to complexity in structure of naturally occurring polyphenols. To better understand the polyphenols in wine, the grapefruit structure should be considered.

### 1.4.1 Structure of grapefruit

Fruit formation occurs after fertilization when the ovary ripens resulting in pericarp. Primarily, viticultural practices, genetic factors and climatic changes influence the fruit set of grapevines (Jackson and Lombard 1993). The endocarp, mesocarp, and exocarp collectively make up the pericarp. A single grape is a fleshy fruit is called a berry, which has a fleshy pericarp and seeds

(Keller 2015). The grapefruit has three major components, the skin, pulp and the seed. Each component contains different polyphenols, and each polyphenol has a unique structure and function. The skin of the grapefruit has a clear outer layer, made of epidermal cells and the inner layer contains more than half a dozen layer of hypodermal cells (Esau 1948). The pulp portion comprises of external and internal mesocarp, which is connected by the vascular tissues. The seed portion of grapefruit has a double layer seed coat with inner testa cells and outer cuticle. The parancheymal layer in the seed holds the seed phenolics and responsible for the seed tannins (Hellman 2003). Polyphenols are classified into flavonoids and non-flavonoids according to their structure and hydrocarbon backbone arrangements. The location and composition of flavonoids and non-flavonoids on seed, skin, pulp and stem of grapefruit are shown in *figure 1*. Compared to flavanoids, the non-flavanoids are present in lower concentrations except hydroxycinnamic acid, which is present in higher concentration in white wine (Jackson 2008). Non-flavanoid compounds that are present in conjugated form of glycosides and esters are benzoic acid and cinnamic acid, which are often called as acid phenols. The grape seed contains 65% of total phenols; the skin harbours 30% and the rest 4-5% present in the juice of the grape fruit (Singleton 1982).

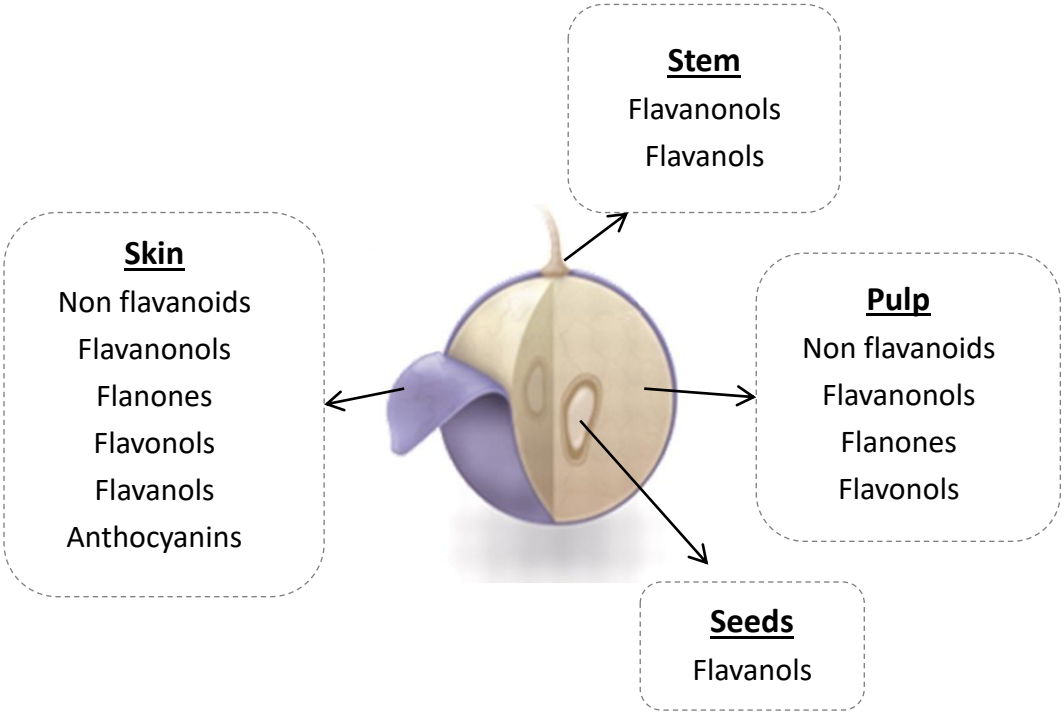



***Figure 1:*** *Structure of grapefruit with the presence of polyphenols [Picture adapted from "Managing wine quality"(Reynolds 2010)]*

### 1.4.2 Flavanoids

Flavanoids are present in three different forms in grape namely, flavan-3-ol, flavonols, and anthocyanins. They are classified based on the number and localisation of hydroxyl and methoxyl groups in the benzene ring. The flavonoids are more complex comprising a C15 skeleton with an aromatic and benzodihydropyran ring bearing another aromatic ring in position 2. Flavanoids are usually present in the seed, skin, and stem of grape. Flavonoids exist in three forms: free or bonded with other flavonoids, non-flavonoids or a combination of both. Flavanoids contribute majorly to wine colour by the presence of pigment anthocyanin and also to the astringency due to their tannin content. Additionally, flavonoids provide a first-line of defence against microbial pathogens and also have anti-oxidant properties by quenching the effects of reactive oxygen radicals (Jackson 2008). Moreover, flavonoids also play a major role in pollen fertility and protect the grapefruit from UV-induced and nucleic acid damages (Cheynier 2006). Flavanonols and flavones are also present in wine in lower concentrations.

### 1.4.3 Flavan-3-ols

Flavan-3-ols largely contribute to the bitterness in wine and also in parts to the astringency taste (Andersen 2006). In grapes, flavan-3-ols exists as monomers, oligomers and polymers, the generic structure of flavan-3-ols is shown in *figure* 2. Flavan-3-ols are found mostly in seeds and skins of grape fruit, although trace amount of monomers and dimers were reported to be present in the pulp of the teinturier grape varieties (Cheynier 2006).

Flavan-3-ols can also take four isomeric forms namely (-)-epicatechin and (+)-catechin apart from (-)-epicatechin-3-O-gallate. Flavan-3-ols also forms monomers of the grape proanthocyanidins or procyanidins. These procyanidins are known to be involved in astringency by binding to salivary proteins.

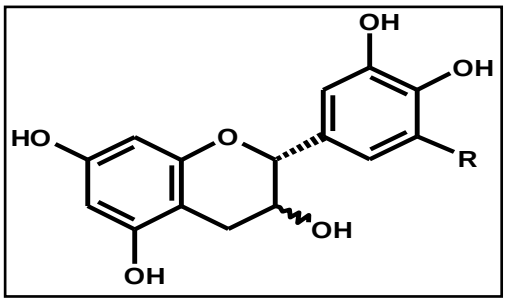



***Figure 2:*** *Chemical structure of Flavan-3-ols (R-OH=Gallocatechin, R-H=Catechin))*

### 1.4.4 Flavonols

Grape skin is rich in flavonols and protects the fruit against UV-A and UV-B lights by acting as a sunscreen in both the red and white grapes (Mabry, Markham et al. 1970). The falvonol skeleton is 3-hydroxyflavone and the molecules are very different from other flavonoids as they have a double bond between position two and three and a ketone group at position four of the carbon ring. These flavonols are a major group of flavonoids and are found in the form of 3 glycosides of quercetin, isorhamnetin, kaempferol and myrcetin as shown in *figure 3*. In nature, quercetin and kaempferol are available in about 280-350 different glyosidic combinations.

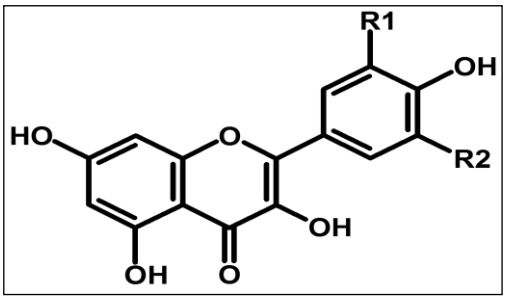
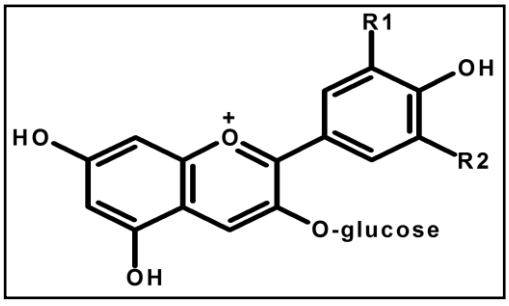



***Figure 3:*** *Structure of Flavonols (Substitution on R1, R2 = H, OH)*

### 1.4.5 Anthocyanins

Anthocyanins, which are usually present in the hypodermal cells of the grape skin are primarily involved in pigmentation. In plants, the colour of the fruit depends on various parameters such as the specific anthocyanin, its glycosidic form, concentration in the tissue cells, interaction with metals and pH (Margalit 2012).

Common grapevine has 3-monoglucosides of anthocyanidins and is based on cyanidin, peonidin, delphinidin, petunidin, pelargonidin and malvidin. In most of the fruits, there is one particular anthocyanin, which dominates its colour, (for example cyanidin dominates in apple, fig and peach), whereas, the *Vitis vinifera* grapes contain all the above-mentioned six anthocyanidins. Anthocyanins are relatively unstable in young red wine because of their susceptibility to oxidation and degradation. During the wine ageing process, the anthocyanin monomers polymerises with other flavonoids to produce red coloured polymers. These polymers are proved to be more stable towards oxidative decoloration (Jurd 1967). The structure of anthocyanin and the possible substitutions are shown in the *figure* below.




| Anthocyanin | $R_1$ | $R_2$ |
|---|---|---|
| Cyanidin | OH | H |
| Delphinidin | OH | OH |
| Peonidin | $OCH_3$ | H |
| Petunidin | $OCH_3$ | OH |
| Malvidin | $OCH_3$ | $OCH_3$ |

***Figure 4:*** *Structure of anthocyanin (left) and the substitutions (right)*

## 1.5 TANNINS

Tannins are phenolic polymers, usually found in apples, plant-derived foods and beverages like tea and wine (Soares, Vitorino et al. 2011). The primary role of tannins during fruit development is to serve as anti-feedents. Tannins, because of their astringent sensation serve as a defence mechanism in protecting the seeds and fruits against animal consumption.

In general, tannins are usually present in wine. The skin, seed, and pulp of grape all hold tannins, apart from this, during the winemaking process, the tannins from oak barrels are released into the wine. In red wine, tannins form the major part of the phenolic group. During the ageing process, the flavonoid units like catechin and epicatechin polymerise to form dimers, trimers and up to decamers. Hence, during ageing of wine and fermentation, tannins undergo several changes. These changes significantly affect the mouthfeel properties of wine (Obreque-Slier, Peña-Neira et al. 2010). On structural basis tannins are classified in four categories as shown in *figure* 5a. In customary way, tannins are broadly classified into two categories; hydrolysable and condensed tannins. Tannins, which can be fractionated hydrolytically into their component by treatment with hot water or with enzymes tannases are classified as hydrolysable tannins. Hydrolysable tannins are gallotannins and ellagatannins, the former has galloly units bound to diverse polyol catechin, or tritepenoid units. The latter has at least two galloyl units which are C-C coupled with each other, and does not contain a glycosidically linked catechin unit. Haslam defined hydrolysable tannins as esters of gallic acid and its derivatives (Roberts 1990). The complex tannins have catechin units, which are bound glycosidically to a gallotannin or an ellagitannin unit.

Condensed tannins are mainly found in grape plants and are present in abundant quantities in red wine, whereas hydrolysable tannins are usually found in oak barrels and are released during winemaking process or from fermentation.

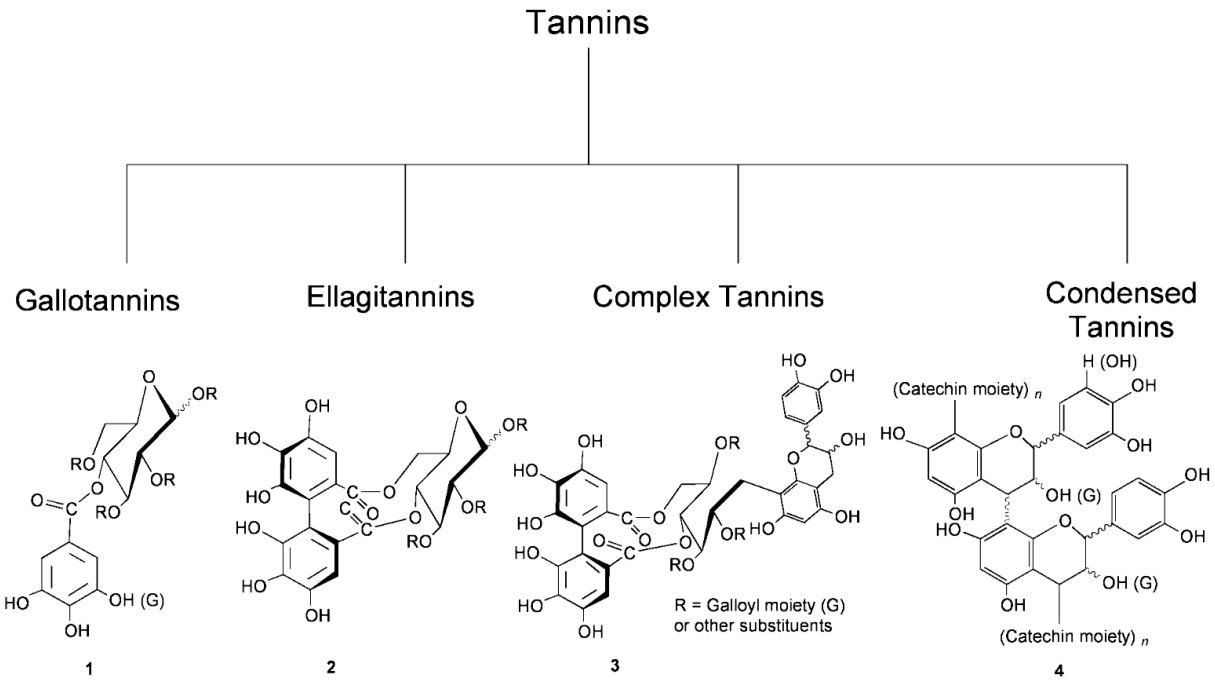



***Figure 5a:*** *Classification of tannins by structural characteristics (Khanbabaee and van Ree 2001). Figure adapted from Karamali Khanbabaee and Teunis van Ree*

### 1.5.1 Condensed tannins

Tannins, which are non-hydrolysable and have oligomeric and polymeric proanthocyanidins were classified as condensed tannins. Condensed tannins are also called as proanthocyanidins.

As shown in *figure 5b,* the monomers of flavan-3-ols catechin and epicatechin polymerise to form the condensed tannins of various chain length (oligomers or polymers) with different subunits (Schofield, Mbugua et al. 2001). Condensed tannins have the ability to bind to salivary proteins and reduce the oral lubrication of mouth which results in astringent sensation (Jones and Mangan 1977). The degree of polymerization of condensed tannins impacts their specific reaction with salivary proteins. Studies by Lu and Bennick using SDS-PAGE and Western blot experiments showed that salivary protein like basic PRPs bind to condensed tannins more effectively than acidic and glycosylated PRPs (Lu and Bennick 1998).

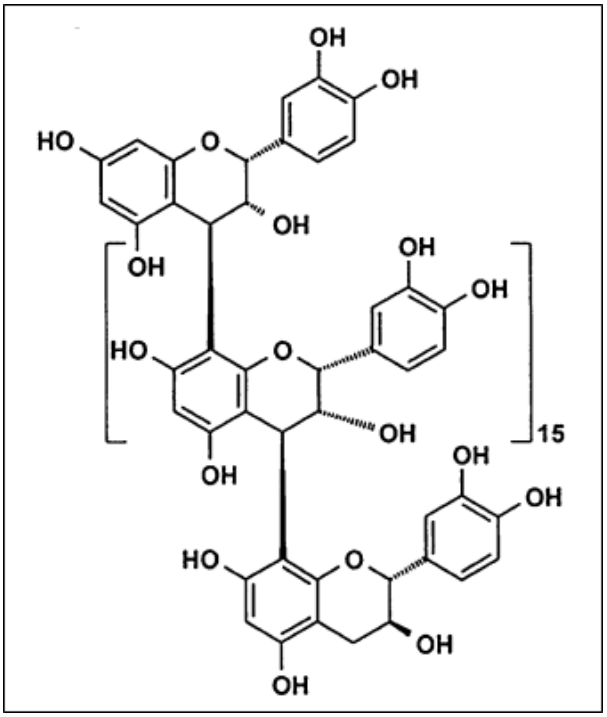



***Figure 5b:*** *An example of condensed tannin structure formed by polymerization of 15-epicatechin monomers with epicatechin at each end*

## 1.6 HUMAN WHOLE SALIVA (HWS)

Human saliva is secreted mainly by three glands in mouth, namely parotid, sublingual and submandibular. Each salivary gland performs a unique function, and the composition of secretion varies between glands (Mandel 1987). Human saliva contains mostly water, apart from enzymes (amylases and carbonic anhydrases), proteins and inorganic substances. Saliva is the first physical contact that a molecule encounters when entering the mouth. Interactions happen before any taste is perceived.

Though the primary function of saliva is to aid digestion, it also possesses antimicrobial and lubricating properties. In this thesis, Human whole saliva (HWS) served as a thin film for the absorption studies using surface analytical tools. Saliva acts as an initial reaction medium, both for astringency and the perception of any taste in the mouth. Proteins present in saliva are

usually referred here as salivary proteins. In the following sections, a brief discussion on the various human salivary proteins will be presented.

### 1.6.1 Human Salivary Proteins

Human salivary proteins are involved in taste perception, lubrication, digestion, enamel formation and microbial protections (Van Nieuw Amerongen, Bolscher et al. 2004). Human whole saliva is mainly composed of glycoproteins like Mucin as shown in *figure* 6, which helps in lubrication. Most often, two highly diverse families of salivary proteins, histadine-rich proteins (HRPs) and proline-rich proteins (PRPs), have been used as single molecular targets for the study of polyphenols causing astringency (Mehansho, Butler et al. 1987; Lu and Bennick 1998). The larger size of mucin protein masks the effects of other protein in taste perception studies, so researchers usually remove mucin and use other proteins like histidine-rich protein and proline-rich proteins (Bennick 2002). The advantage of using Human whole saliva as a reaction medium in this research is that the entire spectrum of proteins in saliva is taken into account, without neglecting mucins.

HRPs have the ability to bind both hydrolysable and condensed tannins, and they are present in very low percentage (2.6%) in HWS (Oppenheim, Xu et al. 1988). HRPs also carry out antimicrobial functions (Lamkin and Oppenheim 1993). Isolation and structural determination of 12 different HRPs (HRP 1-12) have been accomplished for HWS in the last decade, and HRPs 1, 3 and five are found to be the majority one (Oppenheim, Yang et al. 1986).

PPRs oblige diverse functions in oral health and taste perception. Three different types of PRPs are secreted by parotid salivary glands each with a unique amino acid sequence (Steinert and Marekov 1995). PRPs prevent the adsorption tannins in alimentary canals and act as a defense mechanism by interacting with tannins. The role of acid PRPs is to bind to calcium and help in the formation of protein film in the mouth to prevent the continuous deposition of calcium phosphate (Bennick, Chau et al. 1983). The role of basic PRPs involves the binding of polyphenol and thus in taste perception (Luck, Liao et al. 1994). Glycosylated PRPs perform

the function of reducing the mouth friction and increasing oral lubrication apart from its antibacterial activities.

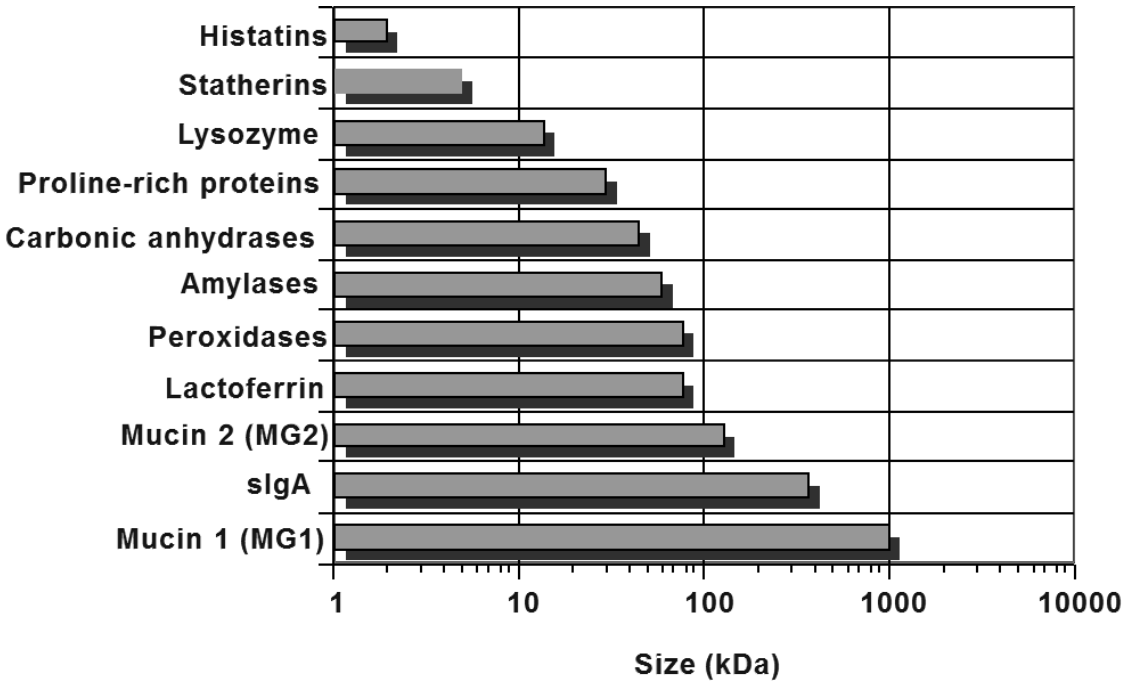



***Figure 6:*** *Major salivary components and their size [Picture adapted from(van Nieuw Amerongen, Bolscher et al. 2004)]*

#### *1.6.1.1 Salivary Mucins*

Mucins are proteins of interest in this research because of their oral lubrication properties. These glycosylated proteins are classified as MG1 and MG2 by their molecular weight. MG1 has higher molecular weight in the range of 200 to 2500 kDa, and MG2 has lower molecular weight of between 150 to 200 kDa. The viscoelasticity of salivary fluid is because of the MG1, and hence MG1 is responsible for oral lubrication (Bansil and Turner 2006). Mucin absorbed to human tongue give rise to molecular boundary lubricating layer and renders the surface with low friction coefficient. Nowadays, Mucins MG1 and MG2 are referred as MUC5B and MUC7 respectively. Studies on Mucins by Inoue et al. using viscometer and Neva meter revealed that MUC5B contributes to the viscosity and MUC7 to spinnbarkeit (i.e. stringy and stretchy quality) of human saliva (Inoue, Ono et al. 2008)

The polymeric structure of Mucins is formed by protein monomers with over 5000 amino acid residues. Although the high molecular weight of mucin and the high degree of glycosylation makes it hard to characterize mucin using light scattering techniques and NMR, the larger size helps in imaging mucin through transmission electron microscopy (TEM) and AFM (McMaster, Berry et al. 1999; Round, Berry et al. 2002). TEM Studies on Mucin reveals the linear conformation with few loops and hairpin turns, Higher concentrations of Mucin 5B at around pH value 7 form viscous gels by the formation of disulphide bond between the thiol groups of their cysteine residues (Round, Berry et al. 2002). Mucin have the ability to bound to various surfaces in the oral epithelia by forming hydrogen bonds, hydrophobic and electrostatic interactions because of its glycosylated and unglycosylated regions in its linear structure (Di Cola, Yakubov et al. 2008).

These high molecular weight mucin proteins present an impressive structure of "bottle-brush" configuration as shown in *figure 7* with linear polypeptides, and the mucin monomers are linked by disulphide bonds. Researchers suggests that this bottle brush configuration of mucins may be involved in the formation of thin lubricative layer between the oral epithelia and unattached mucins in the lubrication boundary (Yakubov, Papagiannopoulos et al. 2007)

Mucin proteins have high carbohydrate content, and the size of mucin proteins ranges from 0.5-20MDa, depending on the type of salivary gland they were secreted from (Coles, Chang et al. 2010). Mucin proteins are negatively charged because of the sialic acid residues. Atomic force microscopy analysis have shown structures with 100 to 1500nm in lengths(Round, Berry et al. 2002). Earlier studies on Mucin isolation and staining was conducted by Mayer in 1896 by using sequence of tannic acid-ferric chloride (Mayer 1896). Pizzolatto and Lillie in 1973 used a similar technique to understand the mechanism of interaction between tannic acid and mucins. Their experiment involved isolation of tannin-mucin bound complexes in a fixed tissue using chelating and bleaching agents. They identified that mechanism of tannin-mucin complex involves van den Waal's forces, adsorption and attachments to the hydroxyl, amino and

carboxyl radicals. This study highlighted the complexity of tannin-mucin interaction and the need for further understanding of this mechanism by employing Mucin a s model protein (Pizzolato and Lillie 1973). Jones and Mangan in 1977 used bovine salivary mucoprotein and condensed tannin to prove the effectiveness of PEG as marker for rumen volume determination when feeding tannin containing legumes to cattle (Jones and Mangan 1977). In their experiments, they concluded that at temperature below 25° and concentration of 2000mol/mol of protein around the pH of 4.0 and 7.0 there was formation of insoluble complex of mucin with tannin. They also observed that at temperature of 39° there was an absence of insoluble protein-tannin complex and they attribute this to the weak hydrogen bonding between mucin-tannin complexes. The instability of tannin-mucin complexes at temperature of 39° suggest that the reaction site of tannin is primarily with the polysaccharide sidechain on the mucin protein. This was due the fact that Strumeyer and Malin in their study using yeast invertase enzyme highlighted the importance of carbohydrate side chain in the mucin glycolprotein (Strumeyer and Malin 1970). The results from their study concluded that the carbohydrates protect the polypeptide backbone of Mucin and thereby preventing access to the tannin molecule and hence the tannin interacts weakly with the carbohydrates and forms reversible complex.

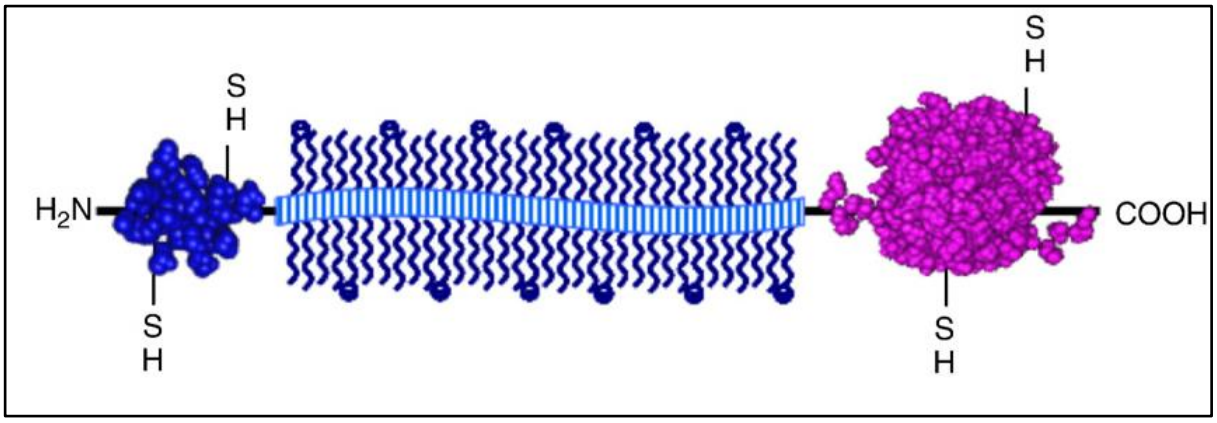



***Figure 7:*** *Structure of Mucin Protein with bottle brush configuration [Picture adapted from:(Coles, Chang et al. 2010)]*

## 1.7 COMPLEXITY OF ASTRINGENCY MECHANISM

Astringency is not only a complex sensation; it is a commercially important taste quality, which impacts the food and beverage industry. Joslyn and Goldstein article on "astringency of fruit and fruit products in relation to phenolic content" in 1964 opened the floodgates on different models to predict astringency mechanism and reviewed variety of analytical methods available for tannin and astringency assay (Joslyn and Goldstein 1964). Analytical techniques to determine the tannin content in coffee and wine developed in the later part of 19$^{th}$ century contributed significantly in understanding the role of tannin in fruit ripening. Lowenthal in 1877 used potassium permanganate to titrate against wine with indigo carmine as regulator was adopted as standard procedure to determine the concentration of tannins in wine till 1912 (Loewenthal 1877). Lowenthal and Proctor redox titration method had its disadvantages and was further improved by Folin and Danies calorimetric procedure to quantify tannin contents in fruits. Even until 1954, in the paper published by Bate and Smith astringency was defined by correlating with quantity of active tannin present in the fruit. (Bate-Smith 1954). The importance of different classes of tannins, the classification phenolic compounds and the impact of degree of polymerization of phenolic compounds was not realised until the isolation leucoantocyanins by Swain in 1962 (Swain 1962). The earlier measurements involved mixture of varying molecular size of tannins and often involve more than one species of tannins. These leucoanthocyanins are precursors to catechins and involved in binding with protein via hydrogen. Previously researchers were unware of the impact of the polymerisation and the localization of tannins in different parts of the fruits. Moreover, recent research supports that even small differences in the configuration of these phenolic compounds contributes greatly to the flavour profile, for example, epicatechin which is a chiral isomer of catechin is more astringent than the latter (Thorngate and Noble 1995). Moreover, these condensed tannins have the mean degree of polymerisation (mDp) from 3 to 80 with varying gallolyated units Souquet et al, used HPLC to purify these tannins phenolic acids and employed LC-Mass spectrometric

analysis to distinguish between the terminal units containing falvan-3-ols and the extension units containing derivatives of toluene – α-thiols (Souquet, Cheynier et al. 1996).

The studies based on isolation of phenolic compounds and use of analytical techniques to correlate their interaction to astringency with a redox agent or formation of various coloured products using electrophilic reagents or precipitation from solution using various salts were primitive stages of understanding the complexity of astringency. In order to decipher the astringency mechanism and the perception of astringency with respect to sensory studies, salivary protein models or single protein targets paved way for further advancements.

One of the important class of salivary protein which was used in model studies more often are PRPs, this protein is primarily secreted by paratoid and submandibular salivary glands is present in high volume on the whole human saliva (Muenzer, Bildstein et al. 1979; Mehansho, Hagerman et al. 1983). PRPs have a strong binding affinity with wine polyphenols due to their open and flexible structure that promotes hydrogen bonding (Asquith 1985; Luck, Liao et al. 1994). Hagerman and Butler employed competitive binding assay to understand the molecular level interaction between the proanthocyanidins with globular PRPs (Hagerman and Butler 1981). The assay was similar to the antigen-antibody interactions and used tannins as a binding agent and labelled the PRPs with Iodine-125 which acts as a labelled ligand. The competitive assay included unlabelled proteins and polymers as competing ligands for the tannins. The research concluded that the interaction of PRPs to tannins was highly specific compared to the other proteins and precipitation of PRP was observed closer to its isoelectric point. This study demystified the non-specificity of protein-tannin interactions and revealed that protein size, conformation and charge played an important role in the precipitation of protein. The study identified that hydrogen bonding between the hydroxyl group of tannin and the carbonyl group of the protein was a major force of interaction. Their study also highlighted the affinity of polyphenols for salivary protein PRP which has extended and random configuration in solution

with high proportion of proline residues in their sequences. As mentioned in section 1.6.1, PRPs are classified as basic PRPs, acidic and glycosylated, Lu and Bennick used binding assays to differentiate the effect of deglycosylation on the PRP and showed that carbohydrates in glycosylated PRPs inhibit the binding of PRPs to condensed tannins (Lu and Bennick 1998). In the same study, they showed that trypsic digestion of acidic PRPs showed a significant increase in binding to tannins as a result of inhibition of proline-poor N-terminal and the proline-rich C-terminal regions interactions in native acidic PRPs. An important study by Charlton et al. using DLS and NMR highlighted the importance self-association of Epigallocatechin gallate (EGCG) by stacking the polyphenolic rings to one above the another and binding of this to multiple sites of PRP (Charlton, Baxter et al. 2002).

Astringency was not only debated as a tactile sensation or taste but also debated based on the function of astringency. Researchers argued that the primary function of astringency was to act as a protective defence mechanism to inhibit the harmful effects of high tannin concentration or as a warning or alarm mechanism to cue to animals to avoid substances that are high in tannins. Glendinning in 1992 devised experiment to prove that astringency was a defence mechanism using PRP and tannic acid and studied the ingestive responses in mice (Glendinning 1992). The study was based on the hypothesis that high PRP levels helps mice to survive in tannin rich nutrients environments by forming insoluble complexes and thereby decreasing the toxic effects of tannins. High level secretion of PRP was induced in mice by injecting chronic isoproterenol and behavioural testing was conducted using control and tannic acid solutions. The whole mouth saliva was compared to tannin binding capacity and the relative PRP concentrations. The results based on the drinking pattern of the rodent between control and tannic acid experiments concluded that salivary protein PRP influence the ingestive response for tannic acid by diminishing the free concentration of tannic acid and protecting the averse taste quality of it. Alternate to Glendinnings approach Mehansho et al. fed rats with high tannin sorghum diet and documented the changes in saliva secretion by monitoring the gene expression. The results from

their experiments involving amino acid analyses and cell free translation of mRNAs concluded that the secretion of PRPs increased twelve folds since the introduction of high tannin diet and the PRPS affinities toward tannins also saw a ten-fold increase (Mehansho, Hagerman et al. 1983). Kim et al. research article in 2004 considered the inhibitory effects of tannin on iron absorption in rodents. Their hypothesis was that tea inhibits iron absorption on animal unaccustomed to tannin-rich foods that habituated animal of tea consumption. They devised experiments with control, short term tea diet and long-term tea diet. They measured the iron-absorption ($^{59}FeCl_3$) using gamma ray whole body counting radioactivity measurements. Biochemical analyses revealed that rats employed adaptive mechanism for overcoming the inhibitory effects of tea-tannins on iron absorption by increasing the PRP in saliva secretion (Kim, House et al. 2004). Prinz and Lucas hypothesised an entirely opposite view for astringency, they stated that the primary function of astringency is to warm or alarm to detect tannins in diet (Prinz and Lucas 2000). As mentioned earlier, tannins have some deterrent effect in nutrient absorption, it is necessary to detect them and warn against them. Results from Glendinning, Mehansho and Kim concluded that PRPs react with the tannins and remove them before they inhibit digestion down the small intestine and gut. Prinz and Lucas built a Boothroyd friction tester to prove that PRPs involved in astringency by reducing the lubrication qualities of saliva on interaction with tannic acid. The friction tester involved measuring the friction between two surfaces of steel and rubber and the viscosity of saliva was measured between the two surfaces with addition of tannic acid or water. The interaction between tannic acid and PRPs in saliva resulted in increased higher coefficient of friction and decreased viscosity of saliva which underlies that astringency is a tactile phenomenon (Prinz and Lucas 2000). This results in decrease bolus formation and signal the harmful effects of tannins by eliciting astringency. Astringency mechanism has been critically debated as to whether it is a tactile sensation or a taste sensation (Gawel 1998). Researchers suggest as the precipitation of proteins by polyphenols is mediated by mechanoreceptors, astringency should be called a tactile sensation

(Simon, Hall et al. 1992; Breslin, Gilmore et al. 1993). Other investigators argue that since the tannins bind with proteins in the epithelium of the oral surface and stimulate the taste receptors, astringency should be a taste sensation (Robichaud and Noble 1990; Siebert, Troukhanova et al. 1996). The above-cited contradictions in understanding astringency mechanism highlight the importance of using analytical techniques to examine astringency sensation in modern times.

## 1.8 STUDIES ON ASTRINGENCY IN RED WINE

In red wine, astringency is a fundamental quality, which gives character and body to the wine. The tactile sensation of astringency arises as a result of binding between polyphenol, and salivary protein depends on factors like acidity and ionic strength of the medium (Gawel 1998; Fontoin, Saucier et al. 2008). The nature of binding, whether it is hydrogen bonding or hydrophobic interactions, was also heavily argued in the scientific community (Kallithraka, Bakker et al. 1998; Charlton, Baxter et al. 2002; Bajec and Pickering 2008). For example, the interaction between human salivary protein fragment $IB7_{14}$ and B3 red wine tannin c (catechin-4α, 8-catechin) was studied using $^{1}H$ magic angle spinning NMR, circular dichroism, electrospray ionization mass spectrometry, and molecular modelling by Simon et al. The findings from the experiments revealed that the principal driving forces toward interaction between the tannin and salivary protein fragment were governed by hydrogen bonding between the carbonyl functions of proline residues in protein and both the phenol and catechol -OH groups of the wine tannin (Simon, Barathieu et al. 2003). Baxter et al. used NMR to study the interaction between series of polyphenols with Proline-rich proteins (PRPs), and they concluded that the polyphenol rings stake against the prolines by hydrophobic interactions. Moreover, their results highlighted the self-association of polyphenol rings which is dependent on the number of aromatic rings in the polyphenol along with their size and hydrophobicity (Baxter, Lilley et al. 1997). In their research, the nature of the binding site for the association of polyphenol favours with the β-sheet conformation of PRPs with proline ring facing outwards.

J**ö**bstl et al. used combination of biophysical techniques like circular dichroism, Small-Angle X-ray Scattering, Dynamic Laser Light Scattering, Viscometry and diffusion NMR to study the interaction between basic PRPs and epigallocatechin gallate (EGCG) polyphenol. Instead of isolating PRPs, they used β-casein as their model protein because of its similarities with basic PRPs. Their results presented a three-stage model for the development of protein-polyphenol complexes. In the first stage, the protein compacts itself from the extended coil conformation on binding to the polyphenols, which results in decreased protein size. On the second stage, when the polyphenol concentration is increased, it causes crosslinking among the different protein molecules and results in insoluble protein-polyphenol complexes. With the addition of more polyphenols, it results in forming larger aggregates of protein-polyphenols, which will eventually precipitate out of the solution. This three-stage model was presented as proof of concept for the development of astringency sensation in the mouth (Jobstl, O'Connell et al. 2004).

Researchers also used turbidity measurements and sensory evaluation methods to determine the interaction between polyphenols and tannins (Monteleone, Condelli et al. 2004; Carvalho, Póvoas et al. 2006; Mercurio and Smith 2008). The reactivity of salivary proteins with physiologically active concentration of polyphenol samples was studied by Carvalho et al. using nephlometry technique (Carvalho, Póvoas et al. 2006). In Llaudy et al.'s study of turbidity measurements, ovalbumin was used as a precipitating agent to bind excess proteins and tannins via electrostatic interactions and hydrogen bonding (Llaudy, Canals et al. 2004). Their results concluded that a greater change in turbidity was found as equal amounts of ovalbumin were added to the tannic acid solutions. This result was then correlated with higher levels of astringency as determined by sensory evaluations in red wines (Llaudy, Canals et al. 2004; Burin, Falcão et al. 2010).

By correlating sensory based astringency on blind wine tasting and the compositional analysis of red wines using multiple linear regression, Cliff et al. were able to model the astringency sensation solely based on the phenolic contents (Cliff, Brau et al. 2002). Astringency response with relation to change in time (carry over effect) also plays an important role in perceiving the sensation. Traditional sensory measurements for astringency involves measuring the stimulus at immediate impact of tannins at threshold concentrations and these methods failed to address the time-course of astringency to reach maximum intensity. Singleton et al. in their experiment involving white grapes sensory qualities measured the astringency, bitterness over different periods (over two hours) of tasting sessions (Singleton, Sieberhagen et al. 1975). They observed that astringency increased over time and the same wine sample tasted with increasing time period showed increase in perceived astringency. Guinard et al hinged on their hypothesis and further advanced the sensory effects of repeated ingestion of wine with varying tannin-content over a 5 and 30-seconds and 20-40 seconds time interval between measurements (Guinard, Pangborn et al. 1986). They performed this experiment with a panel of 10 judges over nine sessions on different days. A computerised time intensity measurement was used and model with tannic acid and water was used as standard and controls. Two different time intensity measurements were conducted, one involving the astringency measurement when the intensity decayed to zero and the other between ingestions irrespective of the intensity decay. Their results from the study interpreted that astringency over time changes with repeated ingestion of wine and there was increase in total duration of perceived astringency between 5-30 seconds measurements. This was because various layers of protein and tannin binding occurs and form a sandwich architecture. On first wine sip the tannins interacts with salivary proteins and on further ingestion tannins combine with salivary proteins and membrane protein and building a layer of protein-tannin complexes. This reduces the oral lubrication and the time interval to reach normal oral lubrication increase as there are multiple layers. The fixed time point experiments (measurement at 20 & 40 seconds) suggested that maximum astringency intensity

at 20 seconds was higher than the intensity at 40 seconds. This was due to greater accumulation of tannins at shorter time and the astringency decay over longer time in a single sip. Noble and Lesschaeve contributed greatly in recent times to underline the time-dependent nature of astringency in wine and tea consumption. The number of sips and the time interval between each sip affected perceived astringency, as with the number of increase in sips there is an increase in astringency maximum intensity and plateauing after third sip (Lesschaeve and Noble 2005).Researchers also used other techniques like DLS, ellipsometry and nephlometry to study the polyphenol-protein complexes (Al-Hashimi and Levine 1989; Glantz, Natiella et al. 1989; Hahn Berg, Rutland et al. 2003; Hannig, Döbert et al. 2004; Cárdenas, Elofsson et al. 2007).

### 1.8.1 Surface characterisation studies in literature

Kaneda et al. used a lipid coated Quartz Crystal Microbalance (QCM) - sensor chip to mimic the oral surface (tongue in particular). In their, experiment they studied the relationship between the adsorption to or desorption of beer from a lipid membrane and related it with sensory evaluation. They concluded that the modification of electrostatic and hydrophobic interactions of the inorganic and organic beer components with tongue and throat surfaces evaluates the astringency sensation (Kaneda, Takashio et al. 2001). In Yao et al. study QCM was used to monitor the frequency changes detected during the experiment and then they were empirically modelled to reflect the change in mass of adsorption of polyphenols. The morphological and conformational changes of the salivary film by the papain hydrolysis and the effect of pH, ionic strength and temperature of papain solutions was studied using Atomic force microscopy (AFM) experiments by Yao et, al. (Yao, Xiao et al. 2012). Salivary proteins like PRPs and HRPs are used in isolation and as single molecular protein targets for the binding of tannins from model wine like solutions. Results from that experiments concluded that the influence of structural changes in condensed tannins had a greater impact on astringency (de Freitas and Mateus 2001).

### 1.8.2 Taste dilution analysis and other techniques

Taste dilution analysis (TDA) test are standard in the wine industry, not only to test the astringency but also to other tastes like bitterness, sweetness and supplementary qualities of wine. TDA was one of the earliest forms of analysing flavour and aroma in food and beverage through serial dilution of the beverage or food. This technique was later perfected by Frank et al. (Frank, Ottinger et al. 2001). The sensory TDA usually includes a descriptive analysis of a sample with the reference compound (Frank, Ottinger et al. 2001). Lopez et al. experiments on taste dilution analysis of five different wines used sensory panels to assess the intensity of the basic tastes and in-mouth sensations of serially diluted wine concentrates (Lopez, Mateo-Vivaracho et al. 2007). The results from sensory analysis were then compared with fractions of wine concentrates submitted to HPLC-mass spectrometry analysis to screen for known tastants of the same wines. This study played an important role in optimizing the TDA experiments and served as a model experiment in analysing bitterness and astringency sensations by taste dilution chromatograms. Apart from TDA, previously researchers used several assays like gelatin precipitation methods to estimate tannin compositions in wine. In order to do that, they measured the turbidity or precipitation by precipitating the BSA protein or gelatin when added to the tannins and correlated the percentage value of precipitation to the astringency intensity (Bate-Smith 1954).

### 1.8.3 Importance of analytical techniques in this research

The role of SPR in this research is to monitor the binding between HWS, Human Mucin and BSM with polyphenols in wine and also to investigate the major driving factors of the interaction process. Complementary techniques like QCM-D and AFM was used to support the claims made from SPR experiments. The advantage of these surface analytical techniques is to study the interaction with the Human saliva as a whole unlike using single protein targets and without any major sample preparation technique which affects the nature of HWS.

## 1.9 ROLE OF POLYSACCHARIDES IN WINE

Wine polysaccharides like cellulose and pectic polysaccharides are derived from cell wall material of grape berry fruit (Doco, Williams et al. 2003). The mesocarp portion of grape berry fruit harbours these cell wall materials. Polysaccharides are carbohydrates with a degree of polymerization higher than 20. During fermentation, along with grapes, the cell wall of microorganisms containing polysaccharides gets disrupted and blends with the wine matrix (Vidal, Williams et al. 2003). The concentration of polysaccharides in wine from both the cell wall of grapes and microorganism fermentation ranges from 0.2 to $2gL^{-1}$ (Reynolds 2010). The concentration of polysaccharides in wine depends on several factors like variety of the grape used, grape maturity stage, vinification method and the solubilisation of the macromolecular components in grape (McManus, Davis et al. 1985).

### 1.9.1 Origin of polysaccharides

Arabinogalactan-proteins (AGP), rhamnogalacturonan (RG-II) and pectic polysaccharides which originate from grape cell wall material and mannoproteins (MPs) like mannans from yeast fermentation which are released after autolysis. These are the primary source of polysaccharides in wine (Brillouet, Bosso et al. 1990). MPs are polysaccharides with 90% mannose and phosphoric acids (Vidal, Williams et al. 2003). RG-II is an acidic polysaccharide with 12 different glycosidic residues on a galacturonic acid main chain as shown in *figure* 8 below (Carvalho, Mateus et al. 2006). AGPs and RG-II constitute more than 50% and MPs represent 35% of the total polysaccharide concentration in wine (Ozawa, Lilley et al. 1987). Polysaccharides rich in arabinose and galactose (PRAGs) and rhamnogalacturonan I (RGI) are also present in small quantities (Pellerin, Vidal et al. 1995).

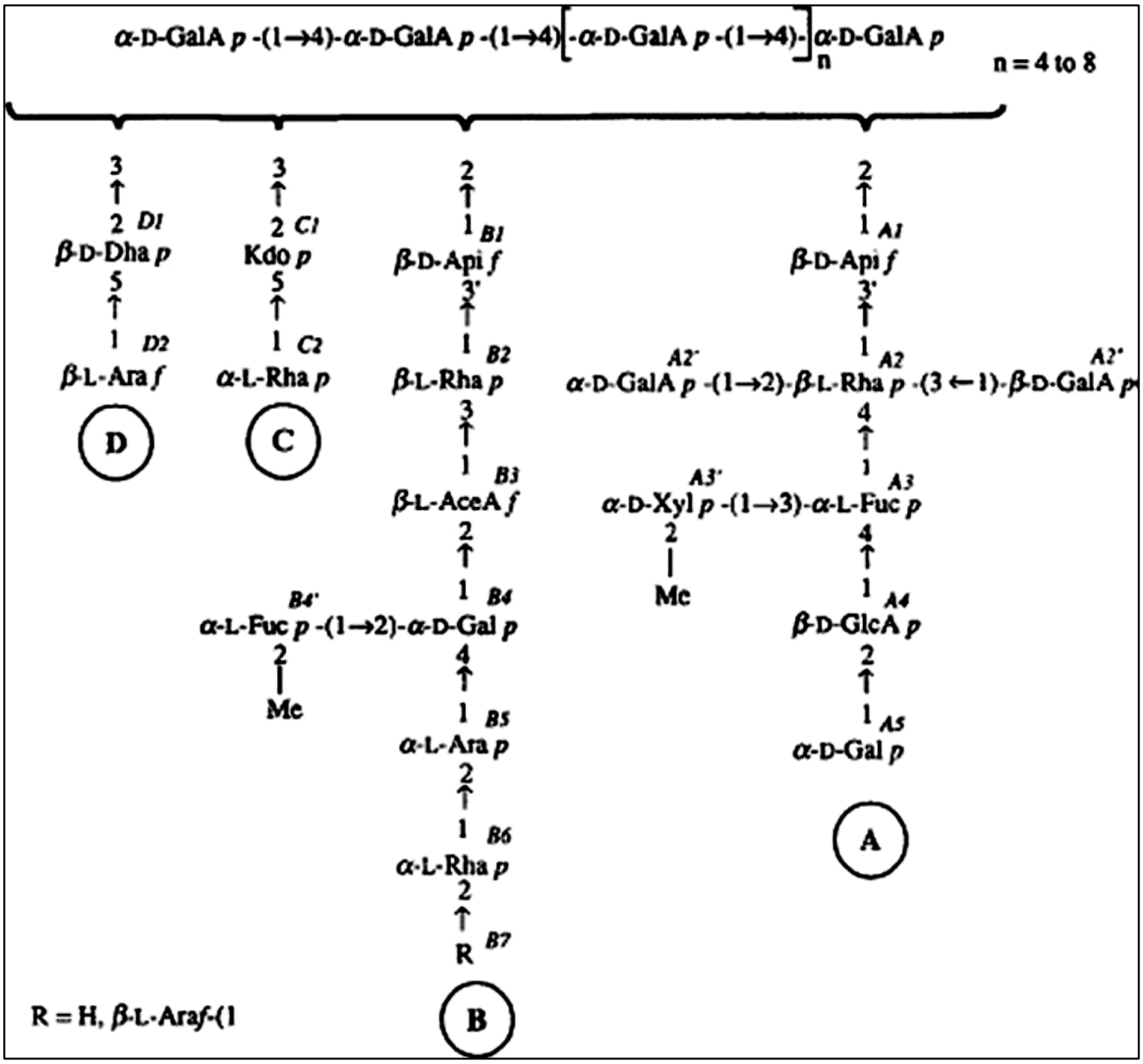



***Figure 8****: RG-II polysaccharide hypothetical structure with oligo glycosidic side chains (A-D) predicted by Pellerin et al. [Picture adapted from (Pellerin, Doco et al. 1996)]*

### 1.9.2 Functions of polysaccharides in wine

The macromolecular polysaccharides not only affects the sensory properties by altering the mouthfeel of the wine but plays a vital role in stability and viscosity of wine during wine production and storage (Escot, Feuillat et al. 2001). Polysaccharides are primarily responsible for the mellowness of the wine (Semichon 1927). Polysaccharides also inhibit the interaction between salivary proteins and wine tannins by forming soluble protein–tannin-polysaccharide complexes (Taira, Ono et al. 1997). When the grapefruit ripens, the water soluble pectin fragments increases as a result of a softening of the cellular structure; these pectin fragments

are able to prevent the tannins from forming complexes with salivary protein which can result in a modified astringency response (Hayashi, Ujihara et al. 2005). Thus, polysaccharides in wines are considered as protective colloids which have the ability to prevent aggregation and flocculation of tannins and protein complexes (Carvalho, Mateus et al. 2006). Polysaccharides also play a vital role in the prevention of haze formation in white wines (Escot, Feuillat et al. 2001; Jones, Gawel et al. 2008).

### 1.9.3 Analytical techniques used to characterise polysaccharides in wine

Chromatographic techniques like anion-exchange chromatography and size exclusion chromatography are prominent methods used to identify polysaccharides present in wine (Vidal, Williams et al. 2003). Complex polysaccharides like RG II was purified using size exclusion chromatography and by the use of ion-exchange chromatography the neutral and acidic fractions of the polysaccharides were separated. The molecular weight of the RG II was determined by high-performance size exclusion chromatography and low-angle laser-light-scattering techniques (Doco and Brillouet 1993). Though Fourier transform infrared spectroscopy (FTIR) was commonly used to analyse levels of tartaric acid, alcohol, glucose and fructose in wine, Coimbra et al. used FTIR to predict the type of polysaccharide in wine dry extracts and quantified the monosaccharide constituents in the sample. Using a calibration model, the researchers predicted the type of wine making process (like maceration) involved in producing a particular wine (Coimbra, Gonçalves et al. 2002; Boulet, Williams et al. 2007).

### 1.9.4 Biophysical techniques used to study polysaccharide interaction with wine

Poncet-Legrand et al. used DLS to study the effects of various types of polysaccharides added to the model wine solution containing grape seed tannins. In their research, they showed that when there are no polysaccharides present in the model wine solution (pH 3.4 buffers containing 2 $gL^{-1}$ tartaric acid, 12% ethanol and grape seed tannins), the particle size of the tannins was increased as they aggregated together. When mannoproteins polysaccharides (concentration of 0.1g $L^{-1}$) were introduced the aggregation of tannins were relatively slow and eventually stopped

as monitored by DLS. Polysaccharides like RG II monomers had no impact on the tanning aggregation. On the other hand, the addition of RG II dimers increased the aggregation of tannins (Poncet-Legrand, Doco et al. 2007).

de Freitas et al. used nephlometry to study the influence of polysaccharides on solubilisation of protein-tannin complexes. In their research, they used BSA as a model protein to complex with grape seed procyanidin polyphenol. They noted that the addition of polysaccharides like xanthan, gum arabic, arabinogalactan and polygalacturonic acid restricts the formation of insoluble BSA-procyanidin aggregates. Their results were plotted in nephelometric turbidity units, and it showed a reduced aggregation size percentage with increased carbohydrate addition to the protein-tannin solution (de Freitas, Carvalho et al. 2003).

The advantage of using nephlometry to study the interaction between tannins, salivary proteins and polysaccharides in forming the aggregates is the measurement at macromolecular level with importance to the physical and chemical driving forces. However, in NMR studies the interactions are considered over molecular level (Ozawa, Lilley et al. 1987).

The interaction between polysaccharides, salivary proteins and tannins which result in the formation of soluble or insoluble complexes were investigated by other techniques like fluorescence quenching, DLS, isothermal titration calorimetry and mass spectrometry (Simon, Barathieu et al. 2003; Frazier, Papadopoulou et al. 2006; Poncet-Legrand, Edelmann et al. 2006; Soares, Gonçalves et al. 2009).

### 1.9.5 Mechanisms of Interaction of polysaccharides with tannins and proteins

The interaction between salivary protein and wine tannins are affected by factors like molecular weight of the tannins (Hagerman and Butler 1981), temperature of the wine (Kawamoto and Nakatsubo 1997), ionic strength (Hagerman, Rice et al. 1998), structure of polyphenols binding to the protein (Hagerman and Butler 1978), ethanol concentration (Noble 1990) and more importantly the type of polysaccharides in wine (Carvalho, Mateus et al. 2006).

Two approaches have been proposed in the literature to understand the capacity of polysaccharides in inhibiting protein-tannin interactions and hence astringent response in wine. Researchers like Haslam, Carvalho and Luck (Haslam 1974; Luck, Liao et al. 1994) suggested the following mechanisms (Refer *Figure 9* )

1. The polysaccharides inhibit the association by forming soluble ternary complexes with protein-tannin complexes and increase their solubility in aqueous medium
2. Polysaccharides compete with tannins and in some cases, encapsulate tannins and thereby interfere with their ability to bind to salivary proteins

Soares et al. used DLS measurements to study the aggregate sizes of the gum arabic (polysaccharide) and β-cyclodextrin (as a tannin model) and their binding to α-amylase and concluded that the polysaccharides followed the second type of mechanism to bind to tannins (Soares, Gonçalves et al. 2009). Carvalho et al. found that increasing the ionic character of polysaccharides enhances their effectiveness in inhibiting protein-tannin aggregation and thereby it supports the first mechanism (Carvalho, Mateus et al. 2006). Polysaccharides in wine are classified as neutral or acidic based on their net charge. Studies on polysaccharides inhibition of tannin-protein aggregation showed that acidic polysaccharides have greater impact on astringency reduction (Taira, Ono et al. 1997; Riou, Vernhet et al. 2002; Vidal, Francis et al. 2003).

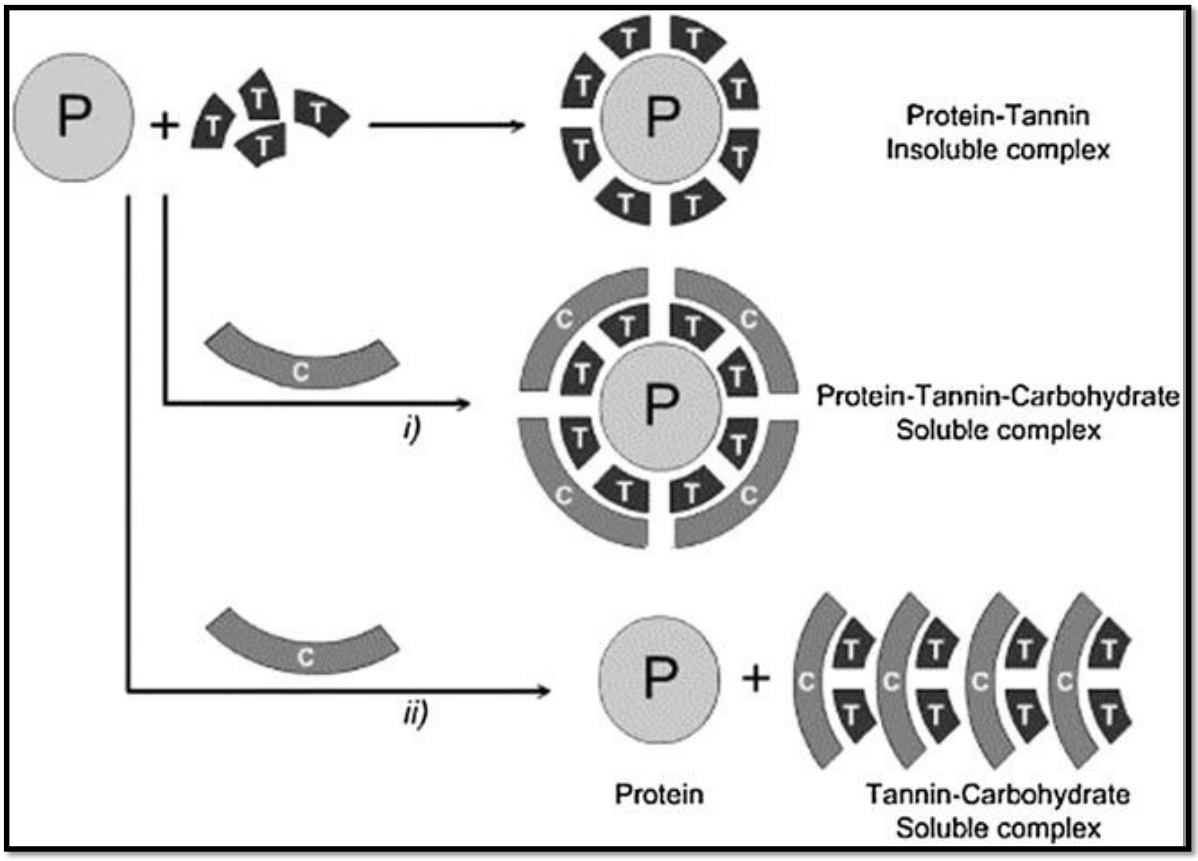



***Figure 9**: Proposed mechanism of interaction of polysaccharides with tannins and salivary proteins (i) Formation of soluble ternary complexes with tannins and proteins (ii) competitive binding of polysaccharides with tannins. Picture adapted from [(Mateus, Carvalho et al. 2004)]*

Polysaccharides with neutral and anionic charges have been shown to disrupt the binding between tannins and polyphenols (McManus, Davis et al. 1985; Ozawa, Lilley et al. 1987). Studies on ionic polysaccharides like pectin, gum arabic and polygalcturonic acid demonstrated the inhibition of polyphenol binding with salivary proteins (Luck, Liao et al. 1994; de Freitas and Mateus 2001). Riou et al. showed that when RG-II polysaccharides are present in wine, they promote self-aggregation of proanthocyanidins, whereas MPs and AGP inhibit the self-aggregation of proanthocyanidins (Riou, Vernhet et al. 2002). The interaction between polysaccharides and tannins are also affected by the ionic strength and ethanol concentration of the wine (Poncet-Legrand, Doco et al. 2007).

## 1.10 IMPORTANCE OF RHEOLOGICAL STUDIES

Rheology was initially termed and used in the late 1920's by E.C Bingham and M. Reiner. The term rheology is defined as "the science of deformation and flow"(Bingham and Sarver 1920). In general, when forces are applied to solid, liquid or gases (Bodies), they deform and rheology deals with the study of the deformation of bodies and their effects. Ideal solids on the application of force deform elastically and on the removal of force will be back to its original shape, whereas ideal liquid flows on the application of force and will not come back to its original form like solids as the energy is dissipated within, similar for gases as well. The materials in the real world are neither ideal solids nor ideal fluids.

Rheological studies are used in many food manufacturing and packaging industries, polymers and paint industries and most of the materials transportation units. Rheology allows understanding of the structural and flow properties of material, which enables to design user-friendly and intelligent materials. Rheological studies predict the behaviour of material in complex process operations like extrusion, polymer film blowing and spraying in industries using developed models.

To understand the application of rheology in this thesis, some basic concepts and terms are introduced in the following sections.

### 1.10.1 Fundamental Parameters in rheology

The basic parameters in rheology are defined by applying force between two parallel plates with liquid between them and the displacement of the top plate with a stationary bottom plate. The displacement of liquid under laminar flow forms the core premises of this definition *(Table 1).*

***Table 1:*** *Definition of Fundamental Parameters in Rheology*

| **Terms** | **Symbols** | **Definition** | **Units** |
|---|---|---|---|
| **Shear Stress** | σ | Force applied per unit area | Pascals |
| **Strain** | ɣ | Deformation per unit length | Dimensionless |
| **Shear Rate** | D | The relation between velocity of flow and thickness between layers | $s^{-1}$ |
| **Viscosity**<br><br>1. **Dynamic viscosity**<br>2. **Kinematic viscosity** | η<br><br>η<br><br>ν | Measure of resistance to fluid flow<br><br>Ratio of shear stress to shear rate<br><br>Ratio of dynamic viscosity to density of material | Pascalsecond (Pas)<br><br>Pas or Poise<br><br>$mm^2/s$ or Centistokes |
| **For Purely Elastic Solids (Based on Hooke's law) (Todhunter 1886)**<br>1. **Shear Modulus**<br><br>**For Newtonian liquids (Newton, Motte et al. 1850)** | σ= G ɣ<br><br>G<br><br>σ= ηD | The stress is directly proportional to strain for relatively small stresses<br><br>Viscosity is the ratio of shear stress to shear rate | <br><br>Pa<br><br>Pas |

### 1.10.2 Flow curves

Flow curves are a visual representation of materials in relation to the shear stress and shear rate. In flow curves as shown in *figure* 10, the shear stress is on the Y-axis and shear rate on the X -axis. Whereas, viscous curves have viscosity on the Y-axis and shear rate on X-axis. Based on the nature of the viscous curves or flow curves, liquids are either Newtonian liquids or non-Newtonian liquids

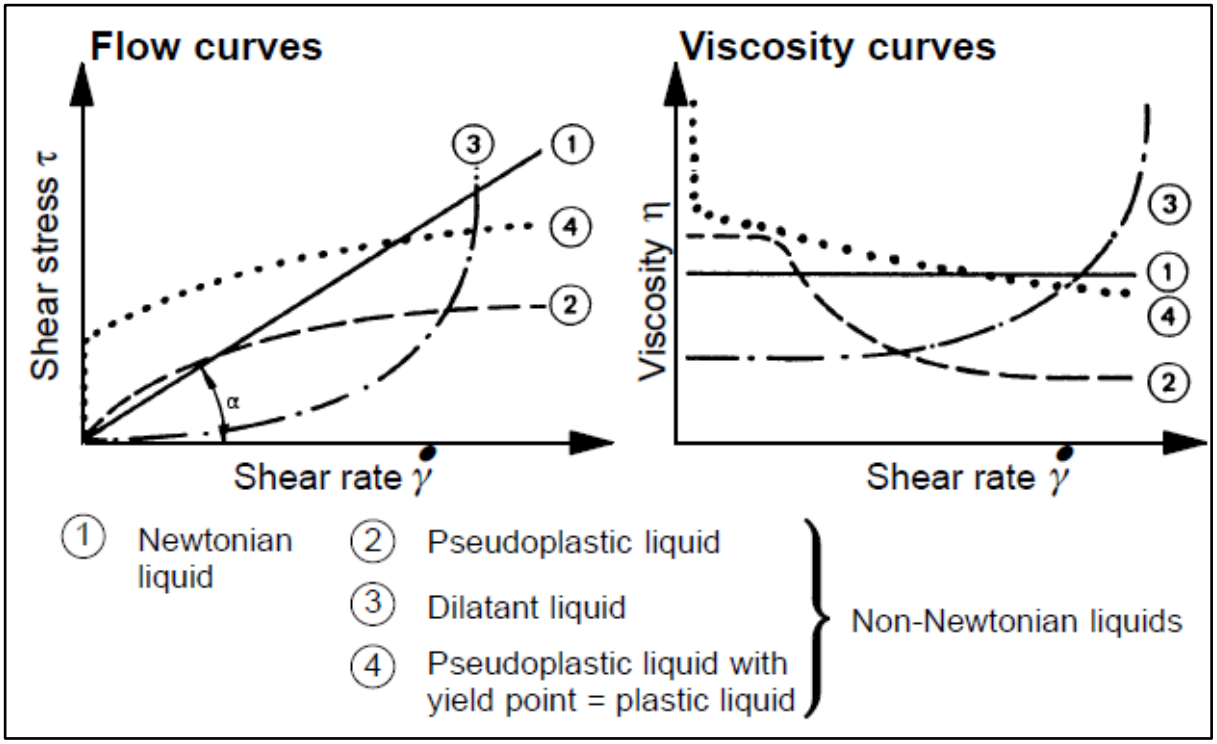



***Figure 10**: Flow behaviour of different fluids based on flow and viscosity curves [Picture adapted from (Schramm 1994)]*

Understanding the basics of flow curve and viscous curves helps in understanding the structural and flow behaviour of the material.

The measured viscosity of the material depends on the temperature, shear rate, time, pressure, pH value, magnetic and electric field strength of the material apart from the main physical and chemical nature of the material (Barnes, Hutton et al. 1989).

### 1.10.3 Importance of food rheology

Food rheology is a crucial part in understanding the deformation of food substance during the mastication of food in mouth. Rheological studies on bolus and saliva are increasing rapidly not only because of its importance in designing novel food products with desired texture and taste but also to fulfil the consumer preferences to certain food products (Stokes 2012). The rheology of food in the oral cavity is a dynamic process, undergoing different stages with various applied shear stress and physical transformations (Stokes, Boehm et al. 2013). Nowadays rheological research in food encompasses various techniques ranging from Universal texture analyser to the

tribological studies (i.e. study of friction and lubrication between surfaces in relative motion) to depict the complete picture of the oral rheology of food.

In order to understand the complex rheology of food in the oral cavity, it is important to understand the rheological properties of Human whole saliva (HWS) and also the role of salivary proteins in perceiving astringency. Astringency in red wine, the topic of discussion in this thesis is better understood by conducting rheological studies on saliva, salivary protein and with tannins.

### 1.10.4 Rheology of Human whole saliva

Although HWS has approximately 99 % water, the rheology of HWS is not like an ideal liquid (Gibbins and Carpenter 2013). Saliva in the oral cavity acts as fluid and blends with food and also form thin film protective coatings over the oral cavity or salivary pellicles. The salivary gland, saliva flow rate, shear rate and saliva collection time are the determinants of the viscoelastic properties of HWS (Levine, Aguirre et al. 1987; Stanley and Taylor 1993). Reports suggest that the viscosity of saliva depends on the shear rate and hence saliva is classified as non-Newtonian fluid (i.e. with increasing shear rate the viscosity decreases in viscosity curve) (Van der Reijden, Veerman et al. 1992).

The viscosity of saliva also depends on the method of saliva collection for rheological studies. For example, the viscosities of stimulated and unstimulated saliva are quite different; even in the stimulation methods itself, the viscosity of saliva differs between acid stimulation and mechanical stimulation (Stokes and Davies 2006). Not only the collection method, the handling and storage of the saliva sample for rheological experiments also have an enormous effect on the viscosity of the saliva (Schipper, Silletti et al. 2007). Researchers usually centrifuge at 1000 x g and add protease inhibitors to prevent degradation. In some rheological studies on human saliva, experiments are performed by removing high molecular weight mucin protein to reduce the viscous nature of saliva (Vitorino, Lobo et al. 2004; Hu, Xie et al. 2005).

The salivary film has various levels of organisation regarding its structural geometry; it forms pellicles, continuous network scaffolds, weak gel phase and a fluid electrolyte phase (Schwarz 1987). The complex structure of saliva prompts researchers to treat saliva as a soft tissue rather than fluid (Glantz, Natiella et al. 1989). The rheological study on HWS hence relies on a large number of factors affecting the outcome of the results. Though saliva has a range of function from digestion to taste perception, the scope of the rheological studies performed in this research is pertinent to oral lubrication and astringency perception through salivary protein and tannin binding.

### 1.10.5 Oral Lubrication and Salivary Mucins

Oral lubrication occurs when the salivary film is confined between two surfaces. The surface hardness of the oral cavity ranges from hard teeth (enamel and dentine) to soft (tongue) and viscous mucosal layer. Lubrication by saliva between the surfaces not only protects the oral cavity but also increases the taste perception (Waterman, Blom et al. 1988). Balmer and Hirsch in 1978, estimated that the shear rate inside mouth ranges from 160 to $60s^{-1}$ during digestion of food (Balmer and Hirsch 1978).The lubricating properties of saliva help to prevent abrasions and reduce friction during food digestion. In oral lubrication, there are primarily three stages like boundary lubrication, mixed lubrication and hydrodynamic lubrication. The viscoelasticity, contact pressure, surface energy and surface roughness defines the fluid behaviour in these regimes (Coles, Chang et al. 2010). These three regimes are represented by a Stribeck curve, in which friction coefficient is plotted as a function of fluid viscosity and shear velocity divided by the load with corresponding lubrication film thickness.

Lubricating properties of saliva in rheological studies primarily depend on saliva viscosity and the physical structure or the surface characteristics of Mucin adsorption (Van der Reijden, Veerman et al. 1993). Researchers used techniques like tribolometer, wear testing and AFM to study the lubricating properties of HWS (Hahn Berg, Rutland et al. 2003; Turssi, Faraoni et al.

2006). Though saliva is a complex mixture of water, proteins, enzymes, sugars and lipids and inorganic molecules, the lubricating properties of saliva are primarily due to the presence of glycosylated mucin proteins, statherin and PRPs (Tabak 1995; Proctor and Carpenter 1998; Rantonen and Meurman 1998).

### 1.10.6 Lubricating properties of Mucins

The structure of salivary mucins and their types are already discussed in the salivary proteins section of this thesis. In this section, the lubricating nature of mucin proteins will be presented. Though there are four different genetic types of Mucins (MUC1-4, MUC5B, MUC5AC and MUC6-8), MUC5B and MUC7 are the only two types of mucins secreted by a salivary gland in the oral cavity (Inoue, Ono et al. 2008). The high molecular weight MUC5B was previously called MG1 and the low molecular weight MUC7 as MG2 (Wickstrom, Davies et al. 1998). The molecular elasticity properties of salivary mucin MUC5B helps in binding to the food during the mastication stage in digestion (Haward, Odell et al. 2011). The interaction of mucin with food compounds involves their ability to form complexes via hydrophobicity/hydrophilicity, hydrogen-bonding and electrostatic interactions (Bansil and Turner 2006). Researchers showed that when the high molecular weight mucins are removed, the viscosity of saliva is similar to that of water (Veerman, Valentijn-Benz et al. 1989). On the basis of mucin behaviour under rheological studies, mucins are characterised as gel MUC5B, sol MUC5B and MUC7. Among the three fractions the gel MUC5B, which has supramolecular aggregates of mucins in a filamentous network and acts as a weak gel. The fluidity of saliva is attributed to this weaker gel forming MUC5B because of its low structural rigidity.

The glycosylation of mucin protein defines its physiochemical properties. For example, the glycosylation in MUC5B is heterogeneous (i.e. heavily glycosylated in some regions and less in other regions) and this influences the viscoelastic behaviour of saliva. These glycosylated regions of mucin interact with water and extend their random coil conformation, which provides an essential barrier to prevent the oral cavity from abrasion (Macakova, Yakubov et al. 2010).

The structural conformation changes of mucin depend on the ionic strength and pH (Lee, Müller et al. 2005). The change in pH and ionic strength can alter the structural conformation change from random coil to rod-like structure and then to weak gel phase (Kočevar-Nared, Kristl et al. 1997).

### 1.10.7 Rheological approach to astringency

Oral lubrication and the astringent sensation are closely related to each other. In order to demonstrate the efficacy of boundary lubrication properties of HWS Bongaerts et al. used PDMS surfaces coated with saliva that mimics the oral surfaces. The results are drawn at the conclusion of the boundary lubricating regimes of HWS and compared it with its bulk viscoelastic properties. Moreover, their results highlighted the importance of hydration for the efficient lubricating properties of HWS, which can impact astringency. (Bongaerts, Rossetti et al. 2007). Experiments by Rossetti et al. on the surface shear elasticity of HWS with epicatechin and epigallolate catechin revealed that the interfacial shear elasticity of saliva reduced significantly on reaction with the above-mentioned polyphenol. As a result of interaction between the polyphenol and salivary protein aggregates, there is a decrease in oral lubrication, which was one of the proposed mechanism behind astringency sensation (Rossetti, Yakubov et al. 2008).

### 1.10.8 Importance of rheological studies in this research

Rheological measurements in this research will reveal the viscosity of HWS at constant shear rate. The change in viscosity of HWS as a result of interaction with tannins and the effect of tannins on mucins viscosity will also be addressed. The shear thinning nature of HWS and the influence of tannin on the binding strength of tannin-protein interaction will be elucidated in detail.

## 1.11 INTRODUCTION TO SALIVARY FILM IMAGING

The science of interpretive uses and application of microscopes is known as microscopy (Rochow 2012). Microscopy plays a major role in the visualisation of micro and nano-structures which are invisible to the naked eye (Dean 2011). The major function of any used microscopic technique is to increase the resolution. There are many different types of microscopes each of which has its distinct function and application. Microscopy employs different principles to obtain or characterise a particular sample ranging from diffraction, reflection, refraction and electromagnetic radiation. In scanning probe microscopy, the sample topography or characterization involves the interaction of a scanning probe with the surface of the object of interest. Choosing the perfect microscopy technique involves knowledge about the physical and chemical characters of the sample and the information researchers are interested in attaining from the sample(Bard, Fan et al. 2001). Sample preparation, behaviour and handling of samples during imaging process play a vital role in the outcome of the microscopy results.

The salivary film topography and thickness will be visualised, and the results are discussed in chapter three of this thesis. In this section, attention is paid to AFM technique for imaging of salivary proteins and their interaction with wine tannins and polysaccharides

### 1.11.1 Salivary film imaging in literature

The complex structure of salivary films has been visualised or imaged by various techniques around the world by researchers over the past few decades (Schipper, Silletti et al. 2007). Visualisation of the salivary film reveals the network properties, globular proteins and macromolecular organisation in its structure (Glantz, Natiella et al. 1989). Imaging techniques like transmission electron microscope (TEM), scanning electron microscope (SEM), AFM and LSCM have been used to characterise salivary film, micelles and salivary proteins (Glantz, Natiella et al. 1989; Rykke, Smistad et al. 1995; Rykke, Young et al. 1997; Sheehan and Thornton 2000; Van Nieuw Amerongen, Bolscher et al. 2004).

Initially, during the late 1980's Glantz et, al used TEM imaging to visualise thin (90-100nm) section of the whole saliva and suggested the complex micro-architectural model of the salivary film with dense phase containing globular structures (Glantz, Natiella et al. 1989). Researchers visualised the weak gel porous nature of the salivary film and also characterised the salivary micelles (Cárdenas, Elofsson et al. 2007). Enzymatic action like hydrolysis on the HWS and the induced conformational changes in the protein structure or the morphological changes in HWS were studied by AFM (Yao, Xiao et al. 2012). More recently the structure of mucin protein showing individual fibres with a broad distribution of contour lengths has been identified using AFM (Bansil and Turner 2006).

Although a lot of microscopy studies were performed on the imaging of salivary film on in-vitro and adsorbed surfaces, very few have used microscopy to investigate the interaction between tannins and salivary proteins in its native state.

## 1.12 KNOWLEDGE GAP IN ASTRINGENCY RESEARCH

The analytical techniques and TDA tests in characterising astringency sensation used numerous ways like single protein target binding, isolated aroma compound taste test and model wine like solutions. One of the major drawback in those studies being the removal of large size mucin protein from the saliva film to better study topography and the impact of other proteins on binding to the tannins. Notably, researchers focussed mainly on the role of PRPs to bind to polyphenols and the factors that drove the binding (Haslam 1974; Hagerman and Butler 1981). The overall research aimed at understanding the mechanism of astringency as perceived by human mouth and the factors involved in the binding process of tannin-salivary protein complexes. A better understanding of the Human saliva rheology and imagining of the salivary film will lead to an enhanced understanding of the influence of saliva on mouth feel, sensory perception and oral processing of wine. More importantly the dilemma in understanding the

polysaccharide interaction with protein-tannin complexes and proposing a valid mechanism is still debated among researchers. These knowledge gaps will be bridged in this research.

## 1.13 BRIDGING THE KNOWLEDGE GAP BY SURFACE ANALYTICAL TOOLS

Surface analytical tools used in this research are surface plasmon resonance (SPR) spectroscopy and quartz crystal micro-balance with dissipation (QCM-D). SPR is a non-destructive technique so that we can study the HWS close to reality, and we can obtain information on the kinetics of their interactions in real-time (Homola, Yee et al. 1999). QCM-D is a surface sensitive technique with nanogram level sensitivity (Höök, Vörös et al. 2002) thus enabling the measurement of the adsorbed mass on to the HWS film. QCM-D also requires no molecular tags making it one of the very few label-free techniques available to monitor surface interactions (Dixon 2008).

## 1.14 RESEARCH HYPOTHESIS FOR ASTRINGENCY

The hypothesis of this research is,

"*The primary function of MG1, mucin protein is oral lubrication. When the Human whole saliva reacts with wine polyphenols, the mucin protein forms insoluble complexes, which lead to the decrease in oral lubrication. Thus an increased astringency sensation will be perceived by the wine drinker".*

The nature of the interaction (i.e. Hydrogen bonding or Hydrophobic interaction) between the protein and tannin was analysed by the changing the ionic strength and ethanol concentration of the wine. Additionally, factors like the temperature of the wine, types of tannins present in the wine and predominantly the changes in viscoelasticity of the HWS film during the binding process were tested, and the results are discussed in section five of this thesis. The theoretical aspects of primary analytical techniques SPR and QCM-D will be discussed below.

# Section Two

## Materials and Methods

# 2 MATERIALS AND METHODOLOGY

## 2.1 MATERIALS USED

### 2.1.1 Human Whole Saliva (HWS)

Human whole saliva was collected by an unstimulated method from a single volunteer throughout the course of the experiments. To avoid variations in protein profile and abnormalities, salivary samples were collected at least one hour before the ingestion of any food or beverages and the collection timing was set standard throughout the course of the research. The volunteer selected was a healthy male and non-smoker. Moreover, as Human whole saliva contains components of different sources, the handling and storage are crucial. The salivary sample collected were centrifuged for 10 minutes at 10000 x g using a microcentrifuge (Thermo Fisher, Australia) to remove cellular debris and bacteria and stored at 4°C for future measurements.

### 2.1.2 Human Mucin Isolation and Bovine Submaxillary Mucin (BSM)

Human mucin (HM) purified from the whole saliva of the volunteer was identified as MG1, and the preparation of this mucin was done according to Wickström and co-workers (Wickström, Davies et al. 1998). Isolated Mucins were stored at 4°C. BSM was purchased from Sigma-Aldrich Co. (USA) (Lot No: 039K7003V) and stored at -20°C.

### 2.1.3 Tannin Extraction

The tannins used in this research are Grape Seed Tannin (GST), Skin Tannin (ST), and Wine Tannin (WT). GST is rich in catechin and epicatechin whereas, ST has procyanidins and prodelphidins as their main ingredients.

The tannins are extracted and provided by Dr Paul Smith from Australian Wine Research Institute (AWRI) at the University of Adelaide, Urrabrae. The following protocol was used by the researchers at AWRI to extract tannins:

The seeds were removed from preveraison Tannat grapes, scraped clean to remove pulp, and extracted for 24 hours with 70% acetone/water. Using a rotary evaporator at 30°C, acetone was removed under vacuum. Ethyl acetate was used to extract the excess water further by liquid-liquid separation method. After removing the ethyl acetate fraction, residual ethyl acetate in the water extract was removed under vacuum. The tannin was isolated from the extract using toyopearl media (Sigma-Aldrich, Castle Hill, NSW, and Australia). The sample was loaded onto a column washed with $H_2O$/0.05% trifluoroacetic acid (TFA) and then by fifty percentages of MeOH/$H_2O$/0.05%TFA, finally eluting the tannin with 2:1 acetone/$H_2O$/0.05% TFA. Before freeze-drying the tannin samples for storage, the excess acetone was removed under vacuum. Skin and seed tannins from commercially available grape and preveraison were also extracted using 2:1 acetone: $H_2O$ that contains 0.01%v/v of TFA. Final extract was purified according to Bindon et al. in Toyopearl chromatography media (Bindon, Smith et al. 2010).

### 2.1.4 Grape seed tannins (GST)

Tannins extracted from the method mentioned above had a molar mass of 2702g/mol, calculated by the Mercurio and Smith method (Mercurio and Smith 2008). GST 10 µM and GST 5 µM concentrations were prepared by dissolving the GST stock solution in model wine and used for all the experiments.

### 2.1.5 Wine Tannin (WT)

Young wine tannins were extracted from Leconfield Cabernet Sauvignon (Coonawarra, Australia) commercial wine as mentioned in the literature (Jeffery, Mercurio et al. 2008). The molar mass of WT was found to be 3549g/mol by following the method of Mercurio and Smith (Mercurio and Smith 2008). Wine tannins are primarily used in the study of the effect of wine temperature on the binding of salivary proteins, by dissolving WTs in the model wine solution.

### 2.1.6 Model wine solution

The practice of using model wine solution or wine like solution in astringency studies is for its simplicity. The natural wine has numerous compounds and factors to be considered which will

influence the experimental process. In model wine solution, deionized water, ethanol (Sigma-Aldrich, Castle Hill, NSW, and Australia) and Mallic acid (4g/L) (Sigma-Aldrich, Castle Hill, NSW, and Australia) are used. Zero percentage (v/v) and 14.4% (v/v) of ethanol were used for the study of the influence of ethanol on binding, and the pH was varied to 3.0, 4.0 and 7.0 by using NaOH (Sigma-Aldrich, Castle Hill, NSW, Australia) for the ionic strength influence studies. 2g/L of tannin concentration was used in model wine solution as it is mid-tannin concentration on red-wines.

## 2.2 MATERIALS AND METHODS FOR POLYSACCHARIDE EXPERIMENTS

The polysaccharides for the experiments were generously provided by Dr Paul Smith and his team from the Australian Wine Research Institute (AWRI), Adelaide.

### 2.2.1 Polysaccharide composition

Instead of usually occurring polysaccharide, our composition of wine polysaccharide had 50% of Mannoprotein, 14.1% polysaccharides rich in arabinose and galactose, which are known as PrAGs and 19.1% Rhamnogalacturonan II.

### 2.2.2 Purification of polysaccharides

The proteins and phenolics in the wine were removed by passing it through a FPX66 column twice and then concentrated to 30-fold under reduced pressure. Three volumes of cold ethanol were added to the concentrated wine to precipitate the polysaccharides. After centrifugation at 4000g for 15 mins at 4°C, pellets were collected and dissolved in water. The dissolved polysaccharides were dialysed (7kDa MWCO) against water and finally freeze-dried and stored at 4°C.

### 2.2.3 Preparation of polysaccharide solutions

Different concentration of polysaccharide solutions was prepared by diluting the stock polysaccharide solution in model wine at a temperature of 25° C. Concentrations ranging from

0.05mg/ml to 0.2mg/ml polysaccharide solutions were filtered through a 0.2µm cellulose ester membrane (Millipore) and added to grape seed tannin (GST) extractions.

## 2.3 SPR MATERIALS AND METHODOLOGY

### 2.3.1 SPR glass slide

A LaSFN9 glass slide with a refractive index of 1.85 at 633nm from Hellma Optik, Jena was used for film formation and adsorption studies on SPR spectroscopy. The glass slides (LaSFN9) were cut at a dimension of 50 x 26mm to fit the flow cell and cleaned with Hellmanex®II solution (1:50; Hellmanex®II: 18.2MΩ Milli-Q Water) in a sonicator for 15 minutes. The cleaned glass slides were repeatedly rinsed for five times with Milli-Q water and then eventually dried with a stream of pure Nitrogen gas. Gold was deposited using a low sputter current onto the glass surface using a Quorumtech K757X Sputter coater (ProSciTech Pty Ltd, Australia) and a PST VS57x03-Au gold target was used (ProSciTech Pty Ltd, Australia).

### 2.3.2 SPR Spectroscopy Instrumentation

A customised SPR setup with Kretschmann geometry was used for all the SPR experiments (Kretschmann and Raether 1968). A He/Ne laser from Uniphase, San Jose, CA and wavelength of 632.8nm was used as a monochromatic light source. The laser beam was aligned parallel to the optical bench and the optical axis of the laser was defined by adjusting optical bench and the iris diaphragm. The laser beam was setup in such a way that it was reflecting back on itself at sample angle theta (Θ) 0° and 180° degree.

Once the laser beam is aligned with the goniometer axis, the next step involved sample preparation and assembly of prism and sample. An index matching fluid of n=1.7 was used to match the refractive index of the LaSFN9 glass substrates. A very small amount of the liquid is dripped carefully onto the non-evaporated sample side and the prism base is positioned free of bubbles onto the liquid film. The prism and sample was positioned for measurements in solution as shown in the following figure:

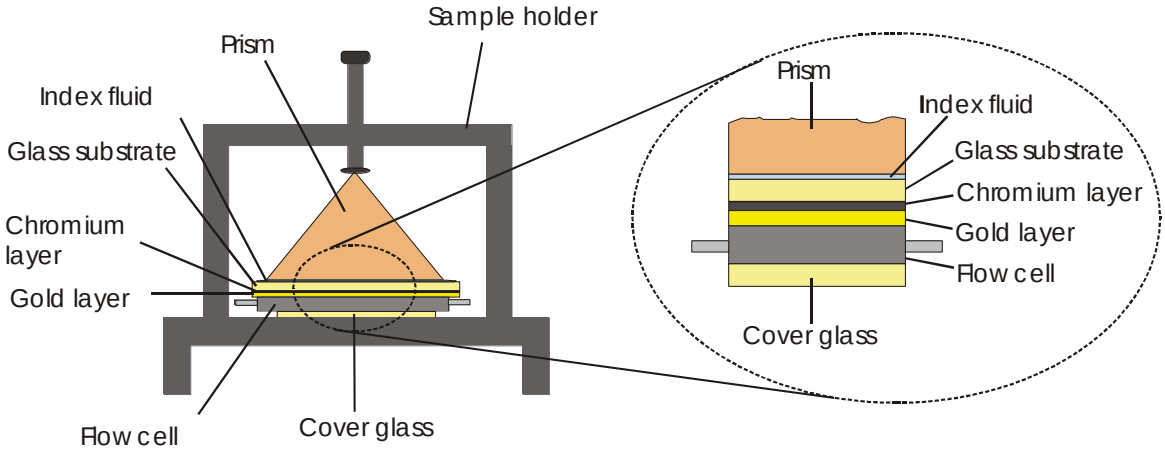



***Figure 11a:*** *Schematic showing the assembly of prism and sample for measurements in solution*

The He/Ne laser was directed through the glass prism which was optically matched with the sample glass slide, and the refracted light is collected by a customised photodiode detector as shown in *figure 11b*.

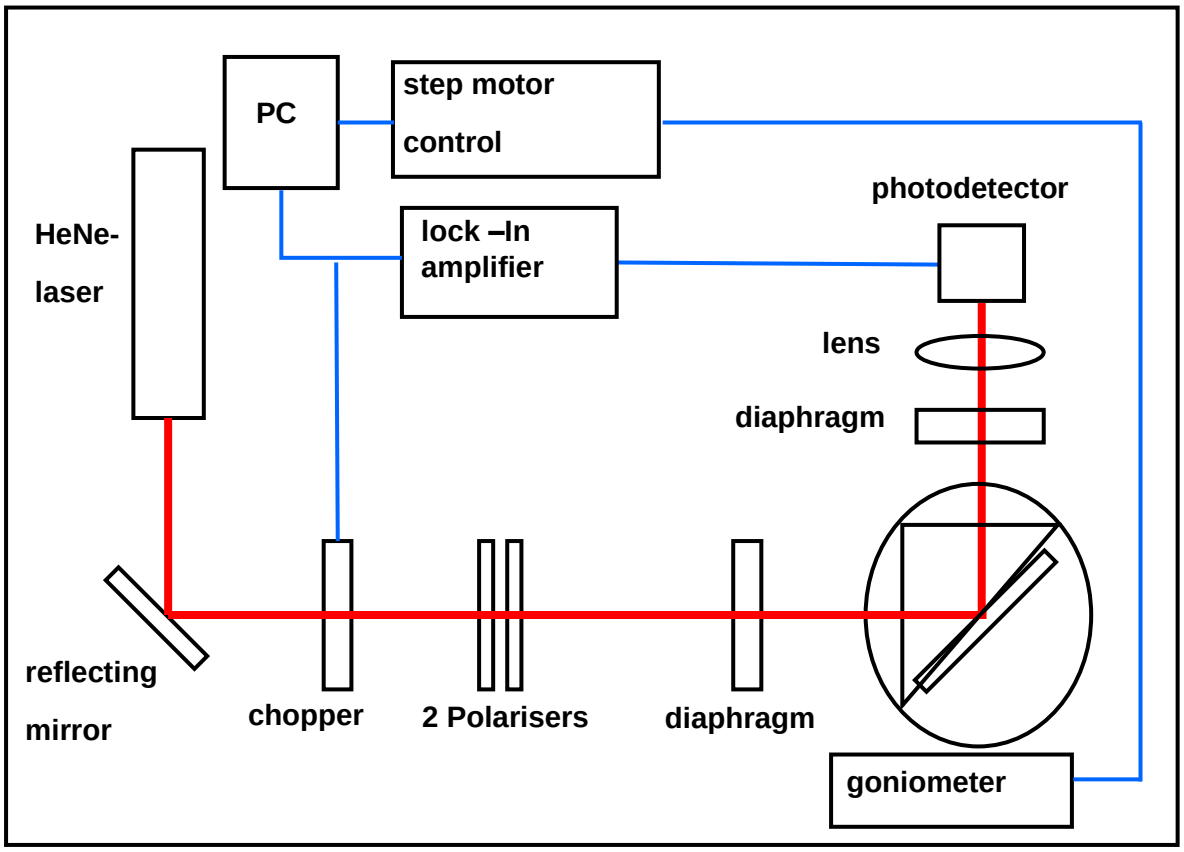



***Figure 11b****:* Schematic of SPR instrumentation set-up

### 2.3.3 Detection Principle

The detection principle relies on an electron charge density wave phenomenon that arises at the surface of a metallic film when the light is reflected at the film under specific conditions.

In total internal reflection (i.e. when the angle of incidence is greater than the critical angle), the light incident on the reflecting interface leaks an electric field intensity into the lower refractive index medium this is called an evanescent wave field. This evanescent wave field amplitude decreases exponentially decaying over a distance of ~1/4 wavelength beyond the surface (approx.300nm). When the surface of the higher refractive index (i.e. LasFN9 glass) is coated with gold, then the reflection is not total and then there exists SPR angle (i.e. angle at which the intensity of reflected light reaches a minimum or dip) which gives rise to plasmons. These plasmons resonate because of the wave length of the surface plasmons matches with the wave vector of the incident light. The resonance conditions are very sensitive to change in refractive index of the solution. This change in solute refractive index changes the positon of the SPR angle dip. The change in SPR angle is noted with the addition of salivary film and by performing a SPR scan over a specified angle area. Typically, these SPR scans consist of recording the reflected intensity from the adsorbed layer as a function of the scattering angle in degree (i.e. Reflectivity units or RU) and this is shown in *figure* 12a below.

A characteristic curve features a minimum in reflectivity, where the position of the minimum depends on the material adsorbed on the gold interface. Changes in the adsorbed layer can thus be followed by monitoring changes in the reflectivity signal. The angular dependence can be described theoretically using a layer model, where each layer is described by the optical thickness (i.e. the combination of layer thickness and refractive index). However, very accurate fitting of soft multilayered systems is difficult, since it requires knowledge of either the exact thickness of a layer or its refractive index. However, for typical protein layer, the change in the resonance angle can be converted into a layer thickness. To quantify the amount of tannins bound to the different protein films (HWS, HM, and BSM) the shift in the minimum angle is

expressed as RU (reflectivity units) with 1 RU = 1pg/mm$^2$ of mass bound to the sensor surface (Skoog 2007)

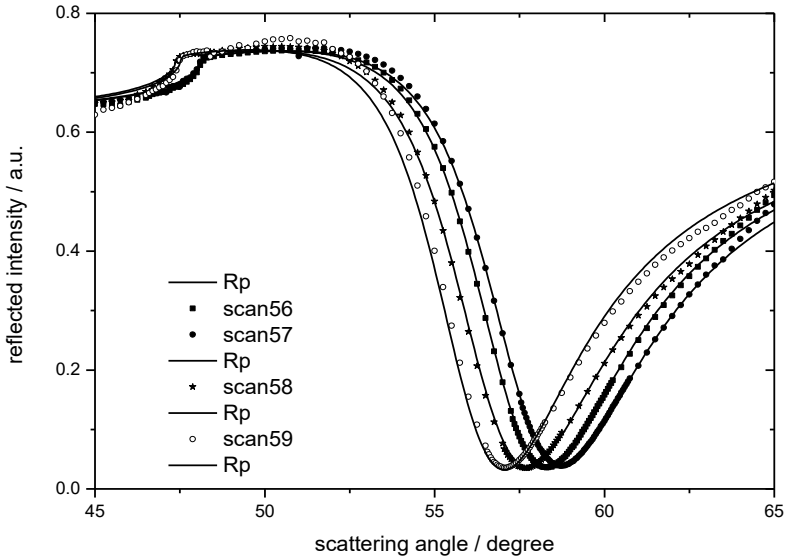



***Figure 12a:*** *Typical scanning curves of SPR sensorgram fitted with curves corresponding to the layer thickness of the analyte*

#### *2.3.3.1 Data collection for SPR*

A standard measurement consisted of measuring a baseline reflectivity using an SPR chip coated with a thin (~49 nm) gold layer, immersed in saline solution. Next, a layer of protein film was adsorbed onto the gold film by adding the respective protein (HWS, HM, and BSM) into the flow cell. Unbound proteins were removed by washing with saline solution. The protein film was then exposed to wine solution and the binding of GST to the film was monitored. Finally, the unbound tannins were eluted with model wine solution. A cleaning procedure was used to regenerate the SPR chip to perform further measurements. The entire protocol involved in SPR binding measurement is depicted in the *figure* 12b below.

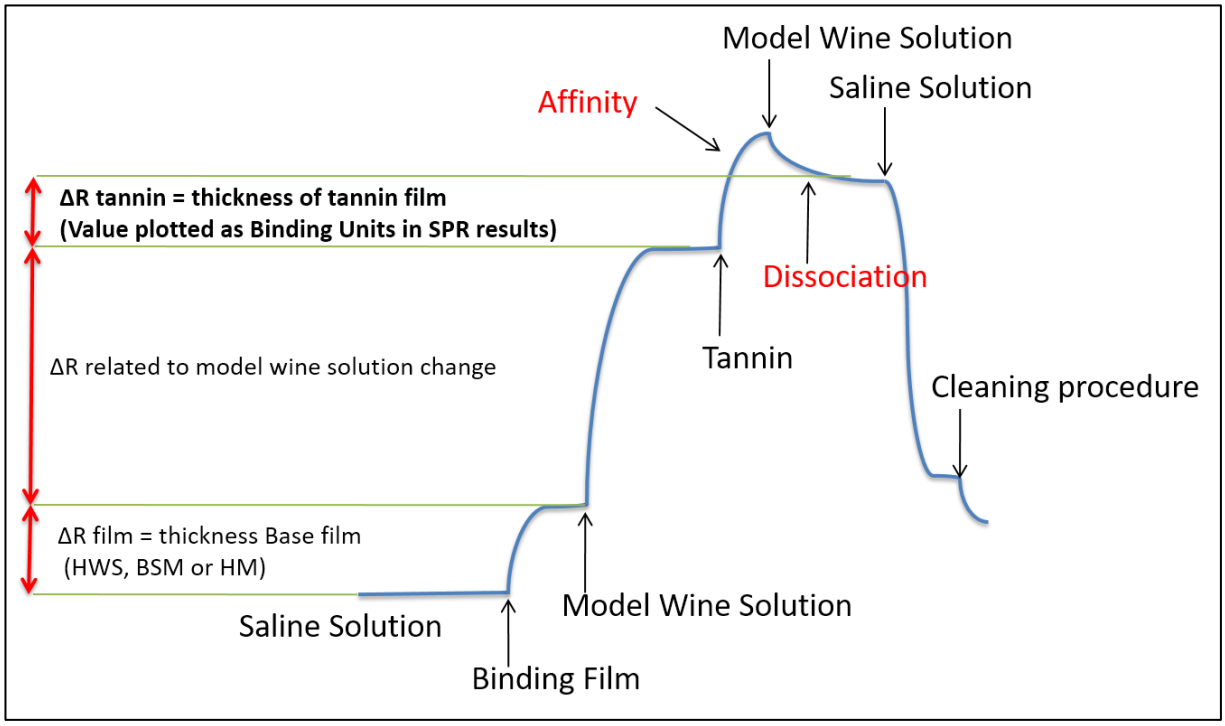



***Figure 12b:*** *Schematic depicting the steps involved in SPR experiment and the thickness of tannin film which is expresses as RU is used in results and discussion section*

## 2.4 QCM-D Experiments Materials and Methodology

QCM-D works on the principle of measuring the frequency change of oscillating quartz crystal when a particular voltage is applied which corresponds to the mass of the substance absorbed to the crystal.

The change in the mass and the frequency changes are related by the Sauerbery relationship (Sauerbrey 1959)

$\Delta m = -C \cdot \Delta f$ (Sauerbrey relation)

Where;

$\Delta m$= Change in mass per unit area ($g/cm^2$)

$\Delta f$= Change in frequency, in Hz

C= Sensitivity factor for the quartz crystal

Conventional QCM has been used to analyse mass changes on rigid surfaces, most effectively in air or vacuum. The advantage of used QCM-D is not only, its nanogram level of sensitivity in detecting mass change but also its potential in monitoring the viscoelastic properties of the film or surface when analytes are adsorbed. The dissipation values are calculated based on the principle that when the voltage applied to the oscillating crystals is disconnected, the oscillation amplitude of the crystal decays exponentially. The time taken for the oscillating crystal to come to stationary will depend on the rigidity of the film.

QCM-D experiments were performed on the Q-sense$^{TM}$ instrument from Q-sense, Vastra Frolunda, Sweden. The QCM-D experiments were conducted under the guidance of Prof Nico Voelcket at the Ian Wark Institute, University of South Australia, Mawson Lakes. Adelaide.

### 2.4.1 QCM-D Sensor

A Q-Sense $^{TM}$ instrument mode E4 with an ISMATEC high precision pump (Q-Sense, Vastra Frolunda, Sweden) was used for QCM-D experiments. The pump flow rate was set at 0.15ml $min^{-1}$. QCM sensors have a circular piece of quartz crystal sandwiched between two metal electrodes. The quartz crystal was cut at 35° 25' to the Z axis of the crystal. This cut was popularly known as “AT cut”. Quartz sensor employed in the experiments were standard 50 nm gold coated AT cut quartz sensor crystals (Q-sense, QSX 301- Standard Gold). The sensors in QCM-D were connected in parallel as shown below for validation and reproducibility.

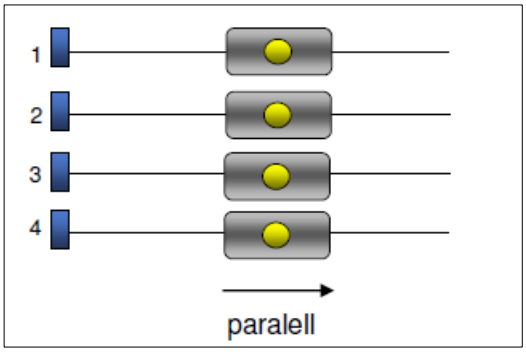



***Figure 13a:*** *QCM-D sensors connected in parallel.*

### 2.4.2 Quartz sensor cleaning

The quartz sensors with gold coat were cleaned by treating them in piranha solution of 98% $H_2SO_4$ and 30% $H_2O$ for five minutes then subsequently placed in UV/ozone chamber for ten minutes. The crystals were then rinsed thoroughly with pure ethanol and Milli-Q water (18.6 MΩ) and finally dried under a stream of Nitrogen gas.

### 2.4.3 Cleaning and binding of saliva and GST

The binding of a salivary film to the quartz crystal surface on the Q-sense $^{TM}$ instrument (model E4 from Q-sense, Vastra Frolunda, Sweden) with a fundamental frequency of 5 MHz was carried out by pumping Human whole saliva (HWS) to quartz crystal surface by the pump. Unbound saliva was washed with saline solution and then the model wine buffer was introduced. Finally model wine solution with GST was introduced and the frequency change and dissipation value changes are noted.

### 2.4.4 Data acquisition for QCM

Human whole saliva of 1mg/ml in saline solution was injected onto the cleaned QCM sensor surface for incubation at 25°C for 30 mins, and the changes of frequency for steady state condition was noted. A rinsing step was carried out with the saline solution to remove the unbound saliva and the shift in frequency was noted at this point. Baseline was recorded by zeroing the changes in frequency value and each measurement were repeated in triplicate and mean and SD values were calculated.

### 2.4.5 QCM-D Methodology

Before the start of the QCM-D experiment, saline solution was pumped and a baseline was recorded. Then the salivary film was formed on the sensor surface by injecting saliva directly through the pump. Unbound saliva was eluted by washing with saline solution. The model wine solution was introduced into the tubing and then washed with saline solution. A baseline was recorded for the model wine solution as well. Finally, the model wine solution containing dissolved GST was pumped for the adsorption measurements through the flow cell and the

remaining unbound tannins were eluted. The layer arrangements in QCM-sensor for this research is shown in the *figure* below.


***Figure 13 b:*** *Schematic depicting the various layers involved in QCM-Experiment*

In the measurement section, the change in resonance frequency of crystals are noted with the mass change associated with the binding. The dissipation value of the oscillation crystal and the energy of the piezoelectric sensor was noted to reflect the viscoelasticity value of the monitored film.

The frequency and dissipation values are plotted in excel as obtained from the QCM-D is shown in *figure* 13c below. In the *figure*, the varied colours represent the odd overtones (3, 5, 7, 9 and 11) which were plotted apart from the fundamental resonance frequency of the Quartz crystals. Each step in the whole process is depicted by arrow head with the corresponding name and the time point of introduction (Seconds). The rise and drop in the values reflect the mass adsorbed and the changes in viscoelasticity induced by the model wine solution and GST on the HWS film.

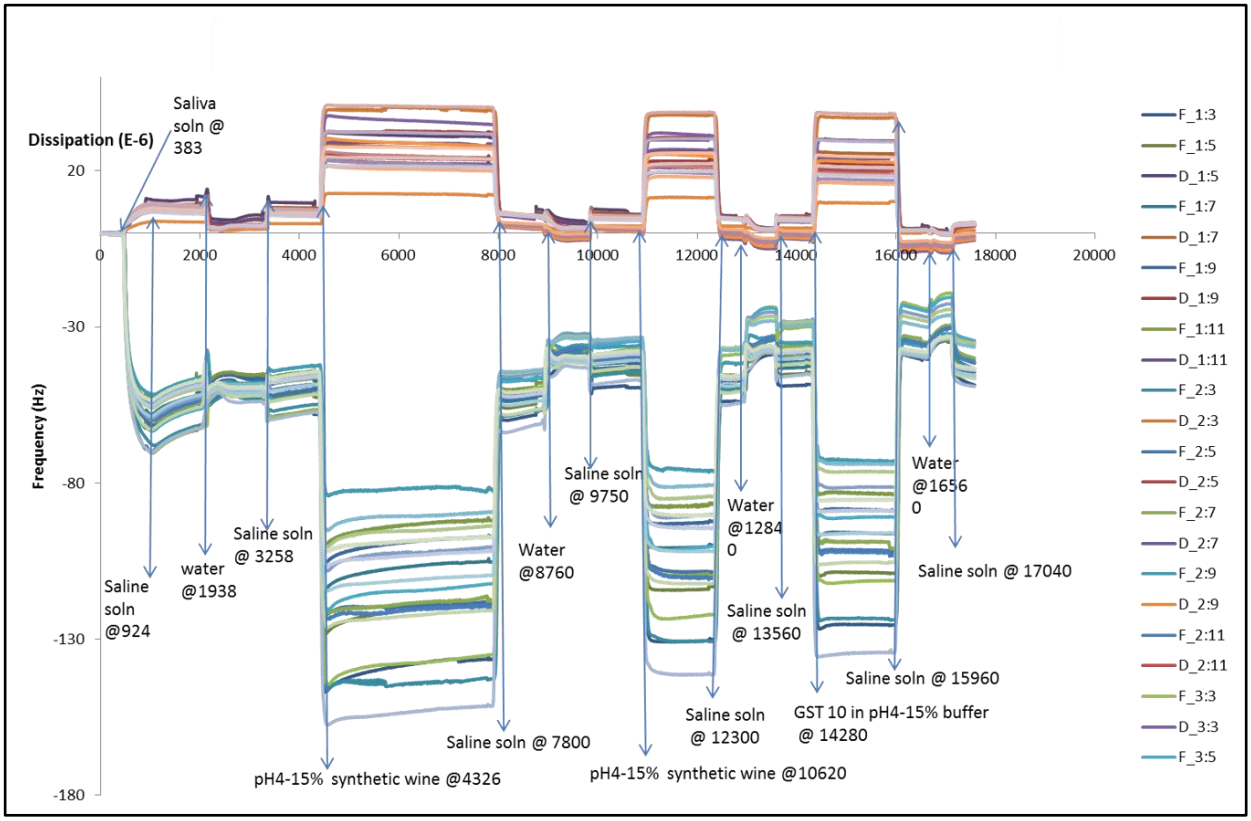



***Figure 13c:*** *A raw graph is showing the step by step measurements from the QCM-D instrument.*

## 2.5 ATOMIC FORCE MICROSCOPY (AFM)

AFM experiments were performed at Mawson Institute in University of South Australia, Mawson Lakes, and Adelaide under the guidance of Prof. Nico Voelcker.

AFM imaging was carried out on NanoWizard III BioAFM (JPK Instruments, Berlin). The images were taken on the salivary film and tannins and scanning of the samples were performed in at least three different areas of size 5 x 5 $\mu m^2$ and 10 x 10 $\mu m^2$. The imaging was conducted in both air and liquid environment at 22°C by soft contact and contact mode respectively. To remove the offset and tilt of each image first–order flattening was applied for the height images. The soft contact mode was based on fixing the surface load to the repulsive electrostatic barrier so that the tip was not in direct contact with the salivary film and imaging of the film was possible without alterations. The root-mean-square (RMS) roughness based on a mean line (Rq)

and average roughness (Ra) were determined using the JPKSPM data processing software. A FORT (AppNano) tip with spring constant in the range of 1.2-6.4N/m was used.

### 2.5.1 Use of soft-contact mode in AFM experiments

AFM has been used historically to image the topography of a surface via the scanning probe which is in contact with the substrate. This traditional technique has proven to be an excellent one for hard surfaces. However, when the surface is soft, delicate and viscous in nature such as the adsorbed layer of Human whole salivary film, then the contact imaging technique can prove to be destructive. Moreover, the viscous nature of HWS film makes the probe sticking to the HWS surface creating a drag and makes the imaging process more difficult. In an aqueous system like HWS, the electrical-double-layer repulsive forces present can be utilised to obtain more satisfactory image contrast. This method of imaging in the repulsive electrostatic regime has been proved to be the key to image HWS film at solid/liquid interfaces, as it permits imaging at non-zero probe-sample separations and, in turn, more delicate imaging.

### 2.5.2 Protocol

AFM imaging of salivary film and all the experiments of tannin and polysaccharide additions was performed on the template stripped gold coated SPR sensor surface (Microscopic specimen glass from Hellma Optik, Jena, refractive index n=1.6). To maintain consistency throughout different techniques (SPR, QCM-D and AFM) and to have a uniform surface for adsorbing the salivary film, this simple surface was chosen.

### 2.5.3 Measurement in fluid

The imaging on the fluid environment of the sample was performed on a liquid cell. This allows the imagining of the salivary films adsorbed proteinaceous layers without the major perturbations of globular proteins and salivary micelles as the interaction with the probe was set at the repulsive electrostatic regime.

## 2.6 MATERIALS AND METHODS FOR LSCM

The LSCM (Laser Scanning Confocal Microscope) experiments were performed at Max-Planck Institute for Polymer research at Mainz, Germany under the guidance of Prof. Thomas A. Vilgis.

### 2.6.1 Sample preparation

Human whole saliva 1ml was centrifuged at low speed (1000g) and the incubated with GST 100µl along with Rhodamine B (10µl) in a 6-well flat bottom cell culture plates (Corning Co-star, Sigma-Aldrich, Germany) for 5 minutes at room temperature. Rhodamine B (Merck, Darmstadt, Germany) for fluorescence with an excitation wavelength of 553 nm was used to stain the salivary proteins.

### 2.6.2 LSCM Instrumentation

LSCM experiments were performed on Olympus FluoView FV1000 confocal microscope (Olympus Optical co. Hamburg, Germany). The set-up uses multi-line Ar laser and diode laser. Two types of scanning units, filter-based and spectral detection, were also provided along with conventional illumination modules which are designed for long-duration time-lapse experiments. The information flow from confocal microscope to the control unit is depicted in *figure* 14.

The advantage of using confocal microscope compared to conventional microscopy is the ability to collect serial optical sections from thick specimens and the elimination of image degrading of out-of-focus information. Although higher magnification such as those obtained by TEM, could not be reached in LSCM, it has the advantage of studying samples without drying or freezing (Schipper, Silletti et al. 2007). This is crucial with respect to HWS and Human Mucin as drying and freezing could change the conformation of protein, which eventually affect its binding properties. Moreover, most modern confocal microscopes are easier to operate, and the image processing software gives information about 3-D visualisation of the specimen along

with multicolour imaging and targeting specific region of the sample (Claxton, Fellers et al. 2006).

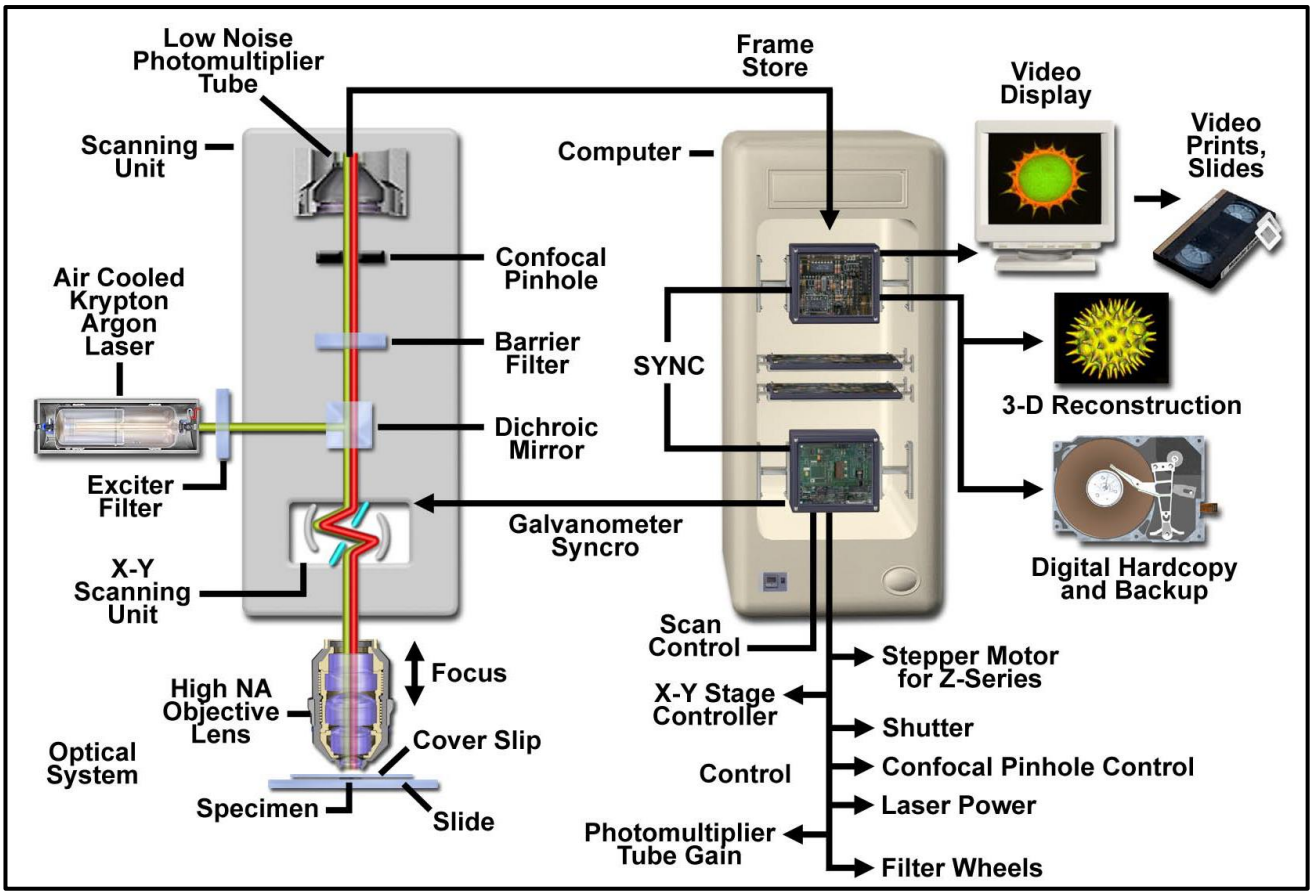



***Figure 14**: Information flow schematic of laser scanning confocal microscope, [Picture adapted from (Claxton, Fellers et al. 2006)]*

## 2.7 MATERIALS AND METHODOLOGY FOR RHEOLOGICAL STUDIES

### 2.7.1 Rheometry

The measuring method and the devices used to measure rheological properties like viscosity, shear modulus and elasticity is called as rheometry. One of the important considerations in rheological studies is the type of instrument or the geometry used to conduct the measurements (Barnes, Hutton et al. 1989). The outcome of the results from rheological studies is primarily dependent on the type of geometry used. The most commonly used geometries in food rheological studies are cone and plate, parallel plate and concentric cylinders.

### 2.7.2 Rheometer

Rheological studies were carried out at Max-Planck Institute for Polymer Research in Mainz, Germany under the guidance of Prof. Dr Thomas A. Vilgis in the food science and statistical physics of soft matter group. Rheological experiments were performed on the Bohlin Gemini Advanced rheometer (Malvern Instruments Ltd, UK).

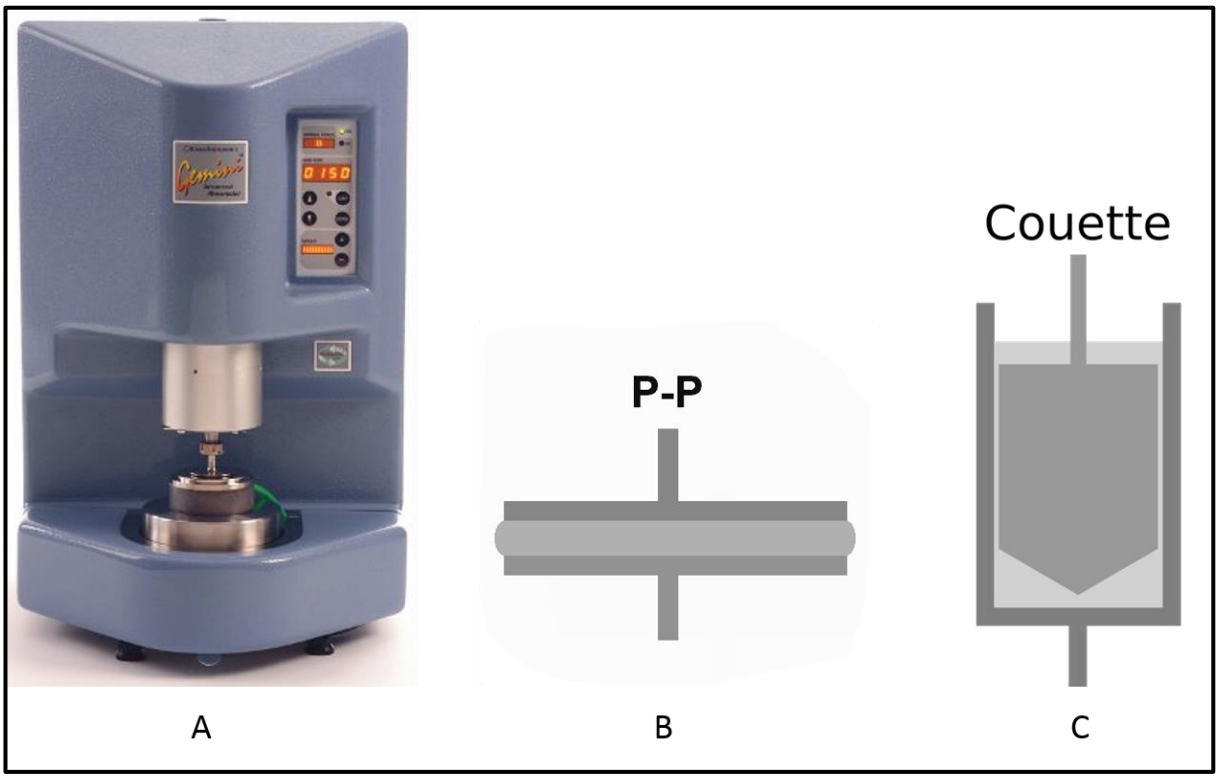



***Figure 15:*** *Rheometry used A) Bohlin Gemini advanced rheometer with B) Parallel-plate geometry and C) Couette geometry*

The geometries used are a parallel plate (PP DIN40), and Couette geometry (refer *figure* 15). The geometries of the parallel plate are referred by the diameter of the upper plate. In the rheological performed here, used a 40mm diameter which is referred as PP40. The diameter of the lower plate is usually larger or same size as the upper plate.

### 2.7.3 Methodology

Rheological measurements are initially carried out on the alginate polymer beads to calibrate the instrument and set up the control parameters. Both parallel plate and Couette geometries were used to test the difference in viscosity measurement by two geometries. Viscosity measurement of a constant shear rate (60 $s^{-1}$) was used over 200 seconds for the test. The temperature was set at 21°C. Calibration tests showed very negligible differences in the viscosity values for varying alginate polymer beads concentrations between two geometries. In a parallel plate, the distance between two plates was set to be 1000 µm and the volume was adjusted to cover the two plates. Oscillation test, shear rate ramp measurements and frequency measurements were carried out in the alginate concentration to get a better understanding of the operations of the rheometer and the calibrating the two different geometries and optimising the parameters of the measurements.

# Section Three

## Results and Discussion

# 3 RESULTS AND DISCUSSION

The result and discussion section highlights the results obtained from SPR, QCM-D, AFM, LSCM and rheological experiments. Mostly, the factors influencing the binding of tannins with salivary proteins (HWS, HM and BSM) is presented in the first half. The results from all the above mentioned techniques are analysed not only in their own but also with complement to each other technique. The effect of temperature of the wine and the type of tannin present in the wine also determine the binding properties of tannin and salivary protein. This is also discussed in the subsequent section. Finally, the role of polysaccharide in the interaction between tannins and salivary protein is investigated and the mechanism of binding is elucidated.

## 3.1 MAJOR DRIVERS OF BINDING OF TANNIN AND SALIVARY PROTEINS

The primary surface analytical techniques used in this research are SPR and QCM-D. In order to observe the effect of ethanol content and the pH levels of wine, series of experiments were performed in SPR and QCM-D. The major part of this research is dedicated to the study of the interaction between polyphenols in wine (i.e. tannins) and salivary proteins as an ensemble and mucin separately. Layers of Human Whole Saliva (HWS), Bovine Submaxillary Mucin (BSM) and Human Mucin (HM) protein were adsorbed on gold surfaces and the subsequent binding of tannins to these layers were studied using SPR and QCM-D techniques.

The factors that affects this mechanism are pH, ethanol levels, concentration of tannins and proteins and temperature of the wine.

### 3.1.1 The effect of ethanol (hydrophobicity) on the binding of tannins

The effect of ethanol and pH on the binding between tannins and salivary proteins has been investigated by SPR and their results are shown in the *Figure 16.* The experimental methodology involved forming HWS film on the gold coated SPR chip and then grape seed tannins were dissolved in the model wine solution with varying ethanol content and pH. In a

similar fashion BSM and HM was used as a base film or absorbing layer that will interact with the GST.

SPR measurements have been performed at 14.4% and 0% ethanol concentration in model wine buffer with pH 3, 4 and 7. The wide variation in ethanol content level was chosen to highlight the role of ethanol (hydrophobic interaction) in the interaction between salivary protein and tannins. The layer thickness of the GST layer has been measured by SPR for three different films namely HWS (Blue), BSM (Orange) and HM (Grey) and the results are shown in *figure* 16.

The binding thickness of GST to HWS, BSM and HM were expressed in RU (Reflectivity Units) in the ordinate. Along the X-axis pH values (3, 4 and 7) and their corresponding ethanol (0% and 14.4%) concentration is expressed for each film. The initial observation shows that the binding thickness for the GST to the three different pH of the model wine (pH 3, 4 & 7) shows an increased RU for the wine solution without ethanol (0%) than compared to the binding value of the GST film with ethanol (14.4%) in the model wine solution.

The results discussed below for the *figure* 16 address two specific issues. They are

- The presence of ethanol (14.4%) in model wine solution gives rise to the self-association of tannins through hydrophobic interaction
- The effect of the ethanol on the ability of mucins to bind to grape seed tannins

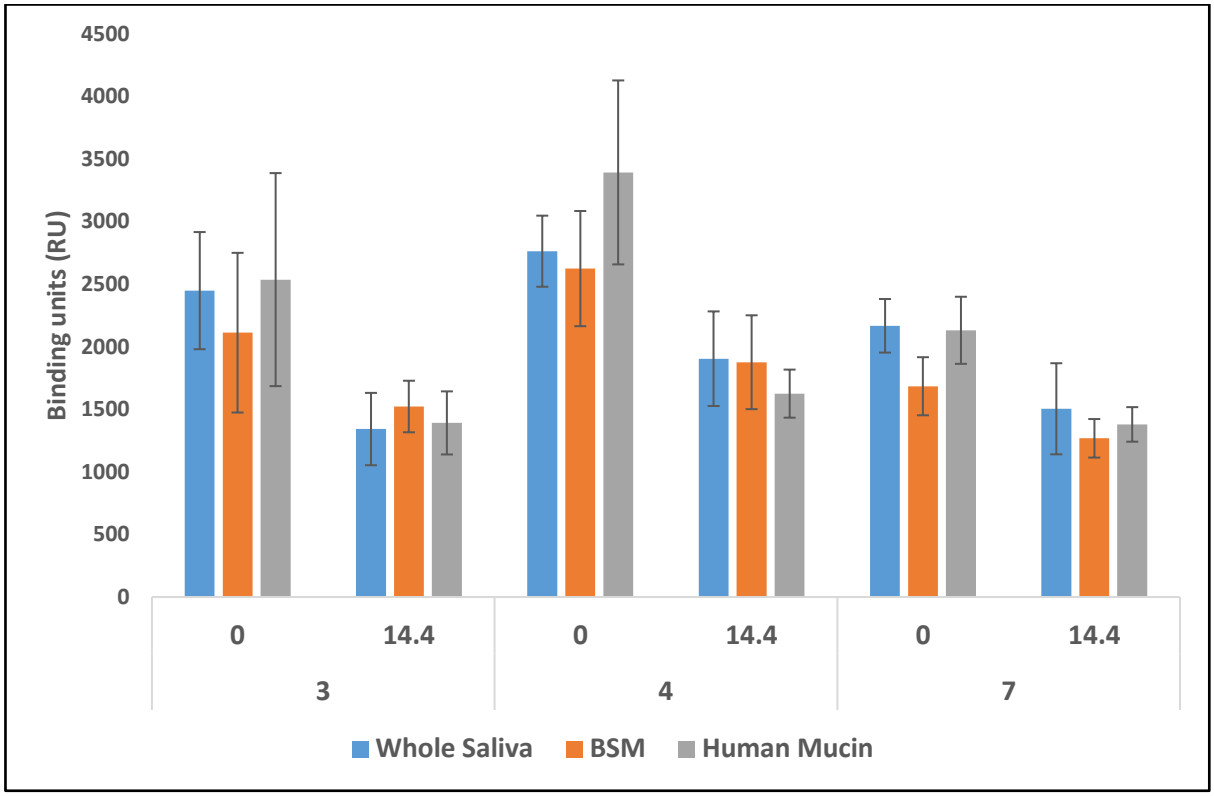



***Figure 16:*** *Variations in film thickness of GST bound to three different films on the SPR sensor chip. The effect of ethanol concentration of the model wine on the binding of GST to HWS, BSM and HM*

#### *3.1.1.1 Hydrophobicity of Ethanol*

The reason attributed to the decrease in binding of tannins to the salivary protein in the presence of ethanol (14.4%) in the model wine (Figure 16) is the self-association of tannin by hydrophobic bonds. This limits the formation of protein-tannin aggregates, which contributes to the lower protein-tannin precipitation. Hence, the perceived astringency also decreases in the presence of ethanol.

The expressed layer thickness values (RU) reflect the binding thickness of the GST to the particular film. There exists a distant correlation between the RU values and the astringency sensation. When there is ethanol in the solution it correlates to lower astringency as compared to the one with no ethanol in model wine. Moreover, proteins like Mucin, PRPs and HRPs that are present in the HWS film undergoes a conformational change because of the hydrophobic nature of ethanol, which results in modified binding sites in the proteins. On the other hand,

when there is no ethanol in model wine solution (0%), then there are less conformational changes in the protein structure as induced by ethanol. This allows the tannins to bind to protein at multiple sites which enables them to form protein-tannin complexes.

#### *3.1.1.2 Effect of Ethanol on Human Mucin Binding*

The binding to HM is highest at pH four at ethanol level 0%, when compared to BSM and HWS. This supports the research hypothesis that mucins are primarily involved in binding of polyphenols in wine apart from PRPs. The results were further supported by the Mackova et. al studies on the influence of ionic strength on the salivary film(Macakova, Yakubov et al. 2010). The authors concluded in that the mucin proteins are hydrated in their glycosylated outer layer and protrude into the bulk fluid. These glycosylated mucins extend the random coil conformation by interacting favourably with water. The protrusion of elongated mucin in the hydrated layer presents more binding sites for the protein to form protein-tannin complexes as shown in *figure 17.* These protein-tannin complexes crosslink with the other complexes and ultimately precipates out resulting in astringency. Thus the binding of mucins and tannins bring outs the perceived astringency. The presence of ethanol, on the other hand, reduces the ability of Mucin to bind to tannins or even to other proteins (*Figure 17 (b))* and the electrostatic forces collapses and entangle the protein modification. This results in protein folding with fewer binding sites for the tannins and thus less binding to the tannins (Gawel 1998).

After the binding of mucins to tannins and when the concentration of tannins is increased, the protein-tannin complexes precipitates out of the solution as a result of coalescing colloidal proteins. This may result in increase of the perceived astringency.

The main aim of the study is to recognise hydrophobic interaction as one of the major driving factor in the binding of tannin and proteins in model wine. Moreover, the effect of ethanol on the ability of mucin to bind with the tannins. In order to achieve that model wine without ethanol was used.

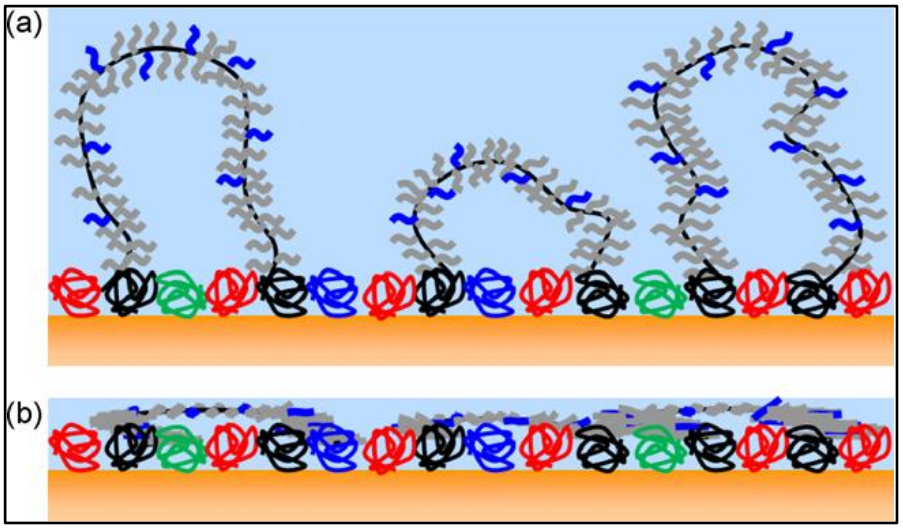



***Figure 17:*** *The proposed structure of Mucin the salivary film by Mackova et, al. (a) Extended conformation in the absence of ethanol (b) Electrostatic collapsed structure in the presence of ethanol.[Picture reproduced from(Macakova, Yakubov et al. 2010)]*

### 3.1.2 Effect of pH (Hydrogen Bonding) on the binding of tannin

Further to the alcohol content, the pH of wine has a significant influence on the perceived astringency. Whereas, alcohol controls mainly the hydrophobic character of biomolecules, the pH of the wine influences the charge of protein, depending on the isoelectric point of the biomolecule (Oh, Hoff et al. 1980). It also influences the capability of the protein to form hydrogen-bond networks with itself and with other molecules. The hydrogen bonding can be established between the hydroxyl groups of phenolic compounds and carbonyl and amide groups of proteins. The pH of the solution has a pronounced effect on the protein-tannin interactions.

The pH of the wine plays a major role in the binding of tannins with salivary proteins. Most of the wine pH varies between 3 to 6 which can bring about substantial changes in astringent taste of the wine(Perez-Maldonado, Norton et al. 1995). The change in the pH of the model wine in this study also had a significant influence on the binding of GST to the films (*Figure 16*). In

particular, around pH 4, the Human Mucin showed the highest binding for the GST at 0% ethanol concentration than any of the other experimental conditions.

The carbonyl functions on mucin protein which has the peptide linkage, act as hydrogen acceptor from the hydrogen donating phenolic –OH groups in the tannins (Baxter, Lilley et al. 1997; Wickström, Davies et al. 1998). This results in the formation hydrogen bond and complexations between the protein and tannins. Researchers have also shown that the hydrogen bond formation of hydrophobic domains of mucins with phenolic hydroxyls resulted in creating soluble tannin-protein complexes (Jones and Mangan 1977).

The pH of the solution had an effect on the binding of tannins to the proteins in HWS film, both with and without ethanol. Literatures suggest that the highest binding for salivary protein could be observed at pH 5 with lower values at pH 3 and pH 7. The isoelectric point of Mucin lies between 2 and 3 (Bansil and Turner 2006). Hence the binding was highest for mucin at pH 4 than pH 7.The trend was similar for the whole saliva and BSM.

The structure and conformational change of the salivary proteins depends on the pH of the wine solution (Payne, Bowyer et al. 2009). Moreover, in acidic environment the number of tannins in phenolate form is much higher (Guinard, Pangborn et al. 1986) and thus the effect of pH can be found in the both the salivary film and  in tannins.

The pH dependence of the tanning binding correlates with the changes in the perceived astringency of wine. Proteins with acidic pI have higher affinities to complex with tannins at lower pH, whereas basic proteins aggregate preferentially at higher pH. In literature, it has been shown that the acidic environment can promote the precipitation of salivary protein leading to the loss of lubrication (Fontoin, Saucier et al. 2008).

In the later part of this thesis, the rheological properties and the viscoelasticity of the salivary film experimentation results will discuss in detail about the molecular process relationship between astringency and pH.

#### *3.1.2.1 Discussion*

The results discussed in this section emphasis the factors that drive the binding of tannins in wine to the salivary proteins and also helps to understand the mechanism behind astringency sensation.

The interaction between tannins and salivary protein are mediated by hydrogen bonding and hydrophobic interaction. The results were also corroborated with literatures proposed mechanism. The mechanism behind astringency sensation involves the following three steps as per literature and shown in *figure* 17 (Gawel 1998; Fontoin, Saucier et al. 2008; McRae and Kennedy 2011);

- The formation of protein-tannin complexes via hydrophobic interaction and hydrogen bonding between tannins and proteins
- The cross-linking between protein-tannin complexes as a result of self-association of bound tannins with protein aggregates
- The precipitation of protein-tannin complexes as a result of colloidal particles by coalescing protein aggregates.

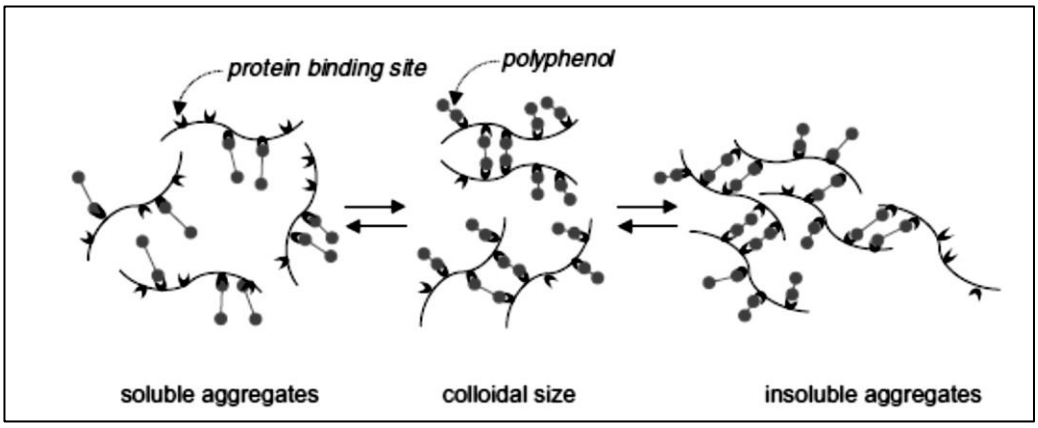



***Figure 17c:*** *Schematic showing the stages involved in protein-tannin binding and precipitation of polyphenols by proteins (Monteleone, Condelli et al. 2004)*

The above mentioned mechanism of interaction between tannins and salivary proteins is explained by addressing the major drivers of the binding process using SPR.

### 3.1.3 QCM-D data analysis and interpretation

The results from QCM-D experiments are of vital importance, as it reveals essential information about the changes in viscoelastic properties of the adsorbed film and the mass change during the binding of tannins to salivary protein.

The *figure 18* below shows the transformed/analysed QCM-D experimental data from the raw data received from QCM instrument.

In the y-axis, the positive values (1st quadrant) indicate the dissipation measurement and the negative part of y-axis (4th quadrant) indicate the frequency (Hz) value of the oscillating crystal.

**Steps involved in QCM-D Experiment**

- Formation of Salivary (HWS) on the QCM sensor surface
- Introduction of Model wine with specific pH (3&4) and ethanol percentage (0% &14.4%); this is termed as buffer run
- Introduction of GST dissolved in model wine solution

Each step in the experiment and their corresponding dissipation and frequency values were translated into the four different coloured columns (representing four sensors on the bar graphs along with the step name before them. These four columns in each step correspond to the four sensors that are connected in parallel in QCM-D instrument, and the whole experiment was repeated thrice for statistical analysis in four similar sensors and the error bars were plotted using Standard deviation values.

To estimate the viscoelastic properties of the adsorbed film, literature suggested the use of Voigt-based viscoelastic models (Richter, Mukhopadhyay et al. 2003).

The successful fitting of the Voigt model required the values of the thickness, density, viscosity, elasticity and overtone number. The calculation of $\Delta D_n$ involves the following equation:

$$\Delta D_n = \frac{1}{d_Q \rho_Q}\left[\eta_l \rho_l \frac{d_p\, \mu_p \omega_n^2}{\mu_p^2 + \omega_n^2 \eta_p^2}\right]$$

In this equations $d$, $\rho$, and $\omega_n$ stand for thickness, density, and $2\pi f_n$ (i.e. angular frequency of oscillation) respectively. The subscripts $Q$, $p$, and $l$ stand for quartz crystal, protein film, and wine medium respectively. The unknown components are the effective viscoelastic properties of the film, which arises as a result of interaction between tannins in model wine and proteins in HWS, like the shear elastic modulus, $\mu$, and the shear viscosity $\eta$.

Though, the overtone number, thickness and density of the adsorbed film are known, the viscosity and elasticity were unknown, so it was hard to calculate these parameters as in each step involves not only salivary film and the tannin but also it had wine buffer. This process was much more complicated than anticipated. As an alternative, the Sauerbery relation was used, and the dissipation values were proportional to the viscoelasticity/stiffness of the film (i.e. hard rigid films have lower dissipation values, and the soft, viscous film has higher values of dissipation) and the change in frequency is directly proportional to mass change.

Upon addition of model wine solution to the pre-adsorbed salivary film, the contents and pH of the model wine solution cause a significant change in frequency. At the same time the dissipation value decreased, indicating that the ethanol (14.4%) in the model wine solution causes shrinkage of the film *(see also: figure 17(b*)), resulting in a more rigid film (*figure 18*). In the final step, the GST dissolved in the model wine solution was added to the pre-adsorbed salivary film. A significant frequency change indicates the adsorption of tannins from the model wine solution to the pre-adsorbed salivary film. Moreover, the dissipation value decreased in half from the original dissipation value of the HWS film. Which resonates with our hypothesis that the addition of tannins will make the salivary film more rigid and stiff. The viscosity of the film was lowered and has a grainy morphology of HWS (refer results and disscusion from AFM imaging section 3.2). As result of rigid film, there is a decrease in oral lubrication, which triggers roughing or puckering of human palate otherwise called as astringent sensation.

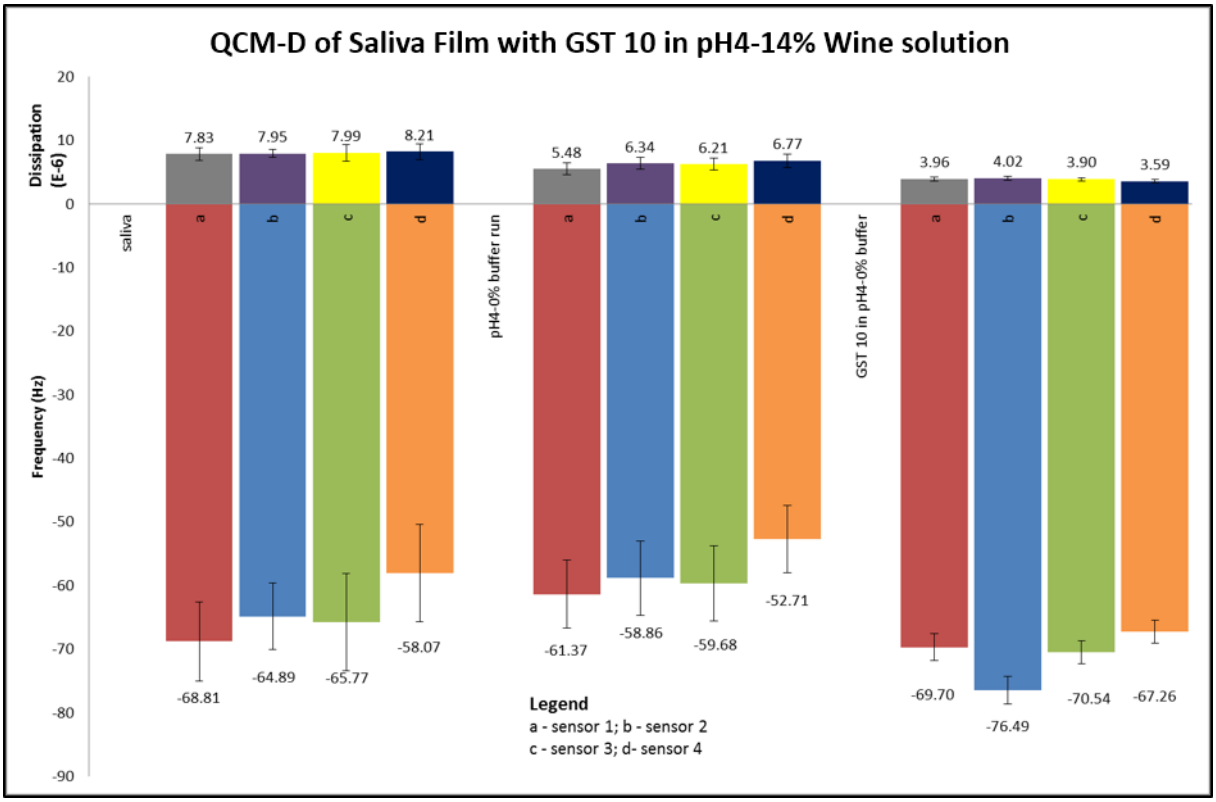



***Figure 18:*** *QCM-D graph showing the frequency change and the dissipation value for the saliva film adsorbed onto the gold-coated quartz crystal and their interaction with model wine buffer and GST*

### 3.1.4 QCM-D result for different model wine buffer on salivary film

The *figure 19* and *table 2* below shows the QCM-D results of collective data for the different model wine buffers (i.e. two ethanol concentrations 0%and 14.4% at pH 3 and pH 4). Four different experimental conditions (Model wine of pH 3-0%, pH 3-14%, pH4-0% & pH4-15%) and their results (like *figure* 19) were combined and presented as a single figure for simplicity and better understanding. The data of frequency change for all the four sensors were averaged and presented in one for each step (For example saliva-green colour bar graph). Even though the effect of pH is not pronounced, a significant change in dissipation value is observed for the film impacted by model wine that has ethanol. Moreover, in the absence of ethanol the HWS film is more viscous and the salivary proteins are elongated which binds more tannins. This is evident from the difference in frequency change across the experiments (i. e. Δf for pH 3-0% Ethanol is -12 between HWS film before adding tannins and after the addition, whereas Δf for

pH 3-14% value is -4). This result is supported by studies on other system literature. For example, Macakova et al. in their experiment using pre-absorbed salivary film on quartz crystal showed that the absorbed film of saliva is a heterogeneous structure (Macakova, Yakubov et al. 2010) due to the trapped water in between and the irregular topography of the film. This hydrated layer helps in the binding of salivary protein more efficiently to the wine tannins.

The initial salivary film is highly viscoelastic and as the addition of model wine buffer alone shows changes in dissipation value which correspond to the changes in viscoelasticity of HWS film. The presence of ethanol in the model wine buffer significantly contributes to the shrinking of salivary film and making it more rigid as shown in the decreasing dissipation values in each graph (Refer to *Table 2* as well). The similar trend was observed in all the experimental conditions.

The QCM-D results complement the results from SPR regarding the effect of the hydrogen bonding and hydrophobic interaction of GST in the model wine buffer.

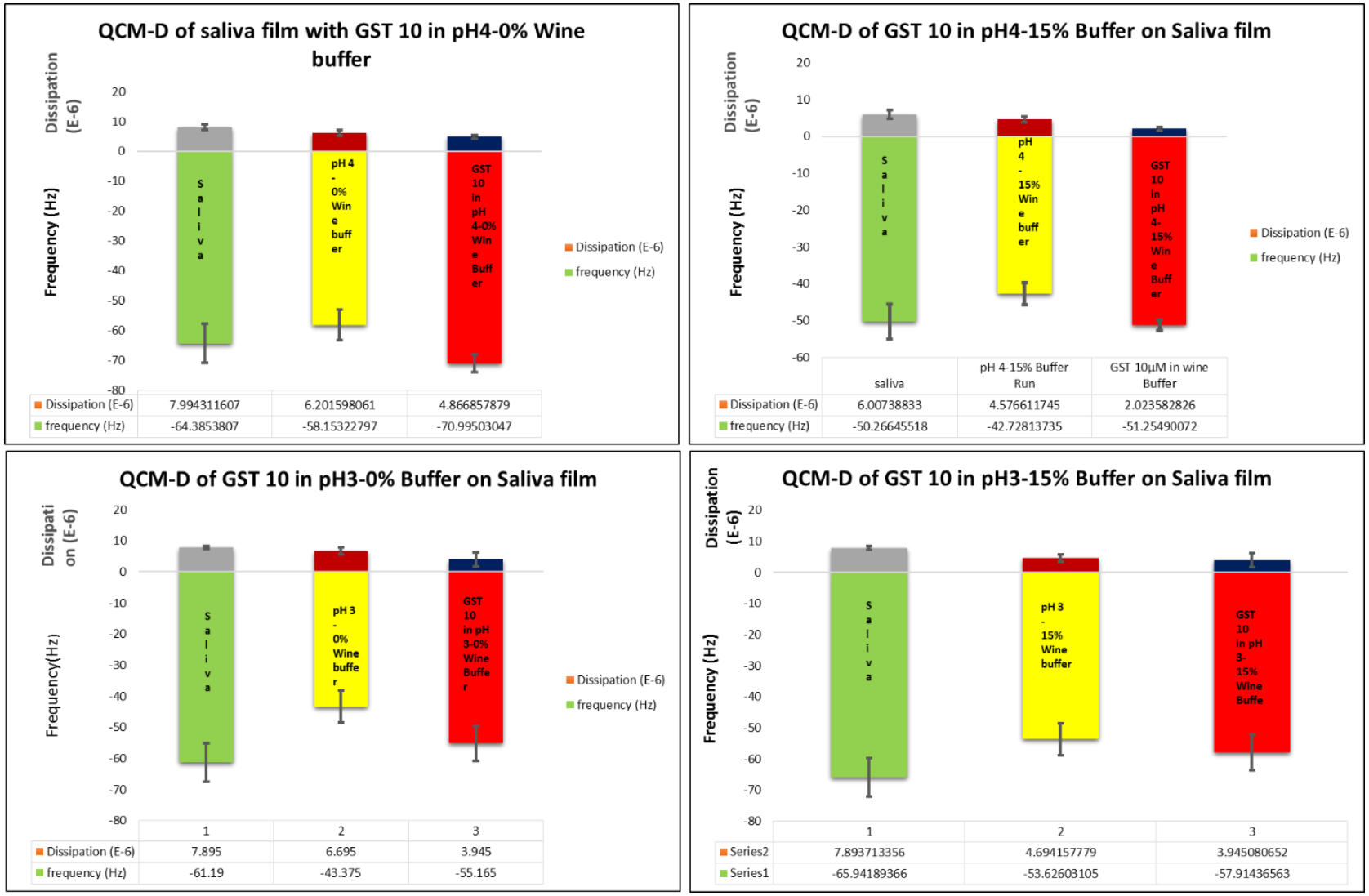



***Figure 19:*** *QCM-D results of model wine buffer with different pH and alcohol concentration of 0 & 15% interacting with salivary film*

***Table 2:*** *The frequency and dissipation values of four different model wine buffer on saliva film, influence of wine buffer and the addition of GST*

| **Model Wine Solution** | | **Frequency (Hertz)** | | | **Dissipation** | | |
|---|---|---|---|---|---|---|---|
| pH | Ethanol concentration | Saliva | Buffer | GST | Saliva | Buffer | GST |
| 3.0 | 0% | -61.2 | -43.4 | -55.2 | 7.9 | 6.7 | 3.9 |
| 3.0 | 15% | -65.9 | -53.6 | -57.9 | 7.9 | 4.7 | 3.9 |
| 4.0 | 0% | -64.4 | -58.2 | -70.9 | 7.9 | 6.2 | 4.9 |
| 4.0 | 15% | -50.3 | -42.6 | -51.3 | 6.0 | 4.6 | 2.0 |

### 3.1.5 Conclusion for the role of ethanol and pH on wine astringency

In conclusion, the two main drivers of binding of tannins to salivary protein are hydrophobic interaction and hydrogen bonding. We have also shown the interaction of salivary protein mucin with GST in both environments of the presence and absence of ethanol and the conformational changes induced in the mucin structure as a result of hydrophobicity. QCM-D studies gave an insight into the viscoelasticity of physiological salivary film and the transformation of the soft/viscous film to rigid/hard/compressed film after the addition of GST to the film. This decodes the mechanism behind astringency that the rigidity of the film is one of the major factor and serves as a proof of concept.

## 3.2 RESULTS AND DISCUSSION FOR AFM IMAGING

In *figure* 20, the AFM image of the salivary film (a) adsorbed on the glass coated with template-stripped gold is shown. Template stripping process using gold was used because of their ability to provide ultra-smooth homogenous flat surfaces (Vogel, Zieleniecki et al. 2012). This enabled us to perform efficient surface roughness measurements and depth profiling in AFM experiments.

The salivary film was formed by placing HWS on the slide for 3h and then rinsing extensively with Milli-Q water to elute the unbound saliva. The slide was then dried with a stream of nitrogen gas and placed in a desiccator. Imaging was performed at the room temperature.

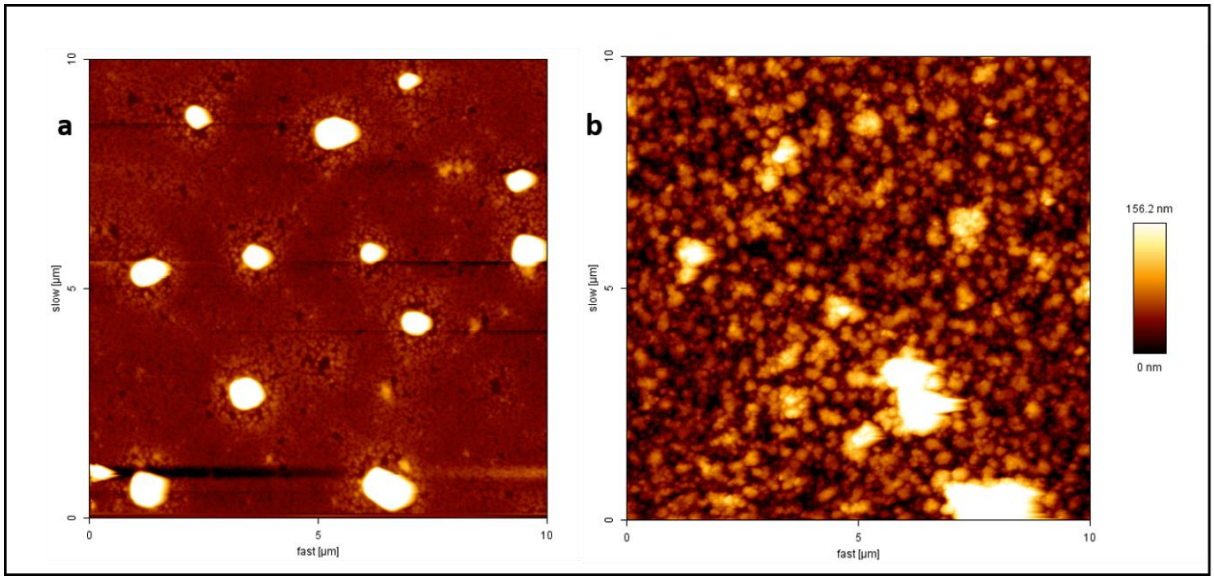



***Figure 20*: *AFM image of salivary film (HWS) marked as (**a**), and tannin added salivary film as (**b**) in air with soft contact mode on area of 10x10 µm$^2$***

The AFM image of the salivary film (*Figure* 20(a)) shows that the film with two different sublayers which has larger aggregates that protrude from the very thin dense inner layer of the film (the white dots in *figure* 20a indicates the larger aggregates). The size and shape of these adsorbed aggregates vary in their height and width and it is in good agreement with earlier studies by various researchers (Hannig, Döbert et al. 2004; Cárdenas, Arnebrant et al. 2007).

Moreover, the type of substrate in which these aggregates are adsorbed determines the length and height of the aggregates (Cárdenas, Elofsson et al. 2007). Meckel reported that the aggregates in HWS film are mostly proteins of salivary origin with a high amount of acidic amino acids (Meckel 1965). Similar studies by other researchers on the characterization of the salivary film concluded that it has proteins like alpha-amylase, albumin, cysteine-containing phosphoprotein and salivary mucins (Levine, Aguirre et al. 1987; Jensen, Lamkin et al. 1992; Yao, Grogan et al. 2001)

### 3.2.1 Hypothesis Validation

In *Figure* 20 (b) grape seed tannin (GST) of concentration 5µM along with model wine buffer was added to the salivary film and scanned in the area of 10 x10 $\mu m^2$. The AFM image of *figure* 20 (b) in comparison with *Figure* 20(a) shows the granular morphology of the HWS added with grape seed tannins. This supports the hypothesis that

"*The grape seed tannins (GST) on interaction with salivary proteins forms insoluble complexes and these grain-like structures causes a reduction in lubrication in the mouth which serves as the basis of astringency mechanism.*"

Surface roughness parameter is quantified by the vertical spacing of a real surface from its ideal form. If these spacing are large, the surface is rough; if they are small the surface is smooth.

The surface measurements values are calculated for Figure 20(a) and (b), the rougher surface has higher Ra and Rq value and smoother surface has low Ra & Rq values.

The role of tannins in precipitating the salivary proteins and inducing the conformational changes in the protein structure is self-explanatory from the *figure* 20 (b).

The observed film was more granular, and the surface roughness measurements varied vastly when compared to the surface roughness of HWS film alone (Refer *Table* below).

| Interaction | Rq (RMS) | (Ra) |
|---|---|---|
| Salivary Film (HWS) | 4.85 nm | 2.98 nm |
| HWS with Grape seed tannins | 21.18 nm | 17.41 nm |

*Ra = Average roughness

^Rq = Root mean square based on mean line of 2.5um

### 3.2.2 Depth Profiling using AFM

Depth profiling is the measure of the deepest valley across the surface profile analysed from the baseline. Depth profiling technique was used not only to determine the thickness of the HWS film; it also showed the topographical change that occurred to the HWS films when tannins were added to it. The HWS film was rehydrated with 0.1M NaCl buffer, and a hard contact force was applied to open the film by scratching. The inner network structure of the HWS film was revealed on opening, and the continuous imaging and depth profiling estimated the thickness to be approximately 60 nm for the HWS film (*Figure* 21). The globular protein structures are visible along with salivary micelles( the white circles on Fig. 21), the results from the imaging of HWS film and the film thickness are in coherence with published results by Cardenas et.al (Cárdenas, Arnebrant et al. 2007). When the thickness of the absorbed HWS film increases drastically along with the surface roughness value, then the film has coarser structure and that affects the morphology of the film.

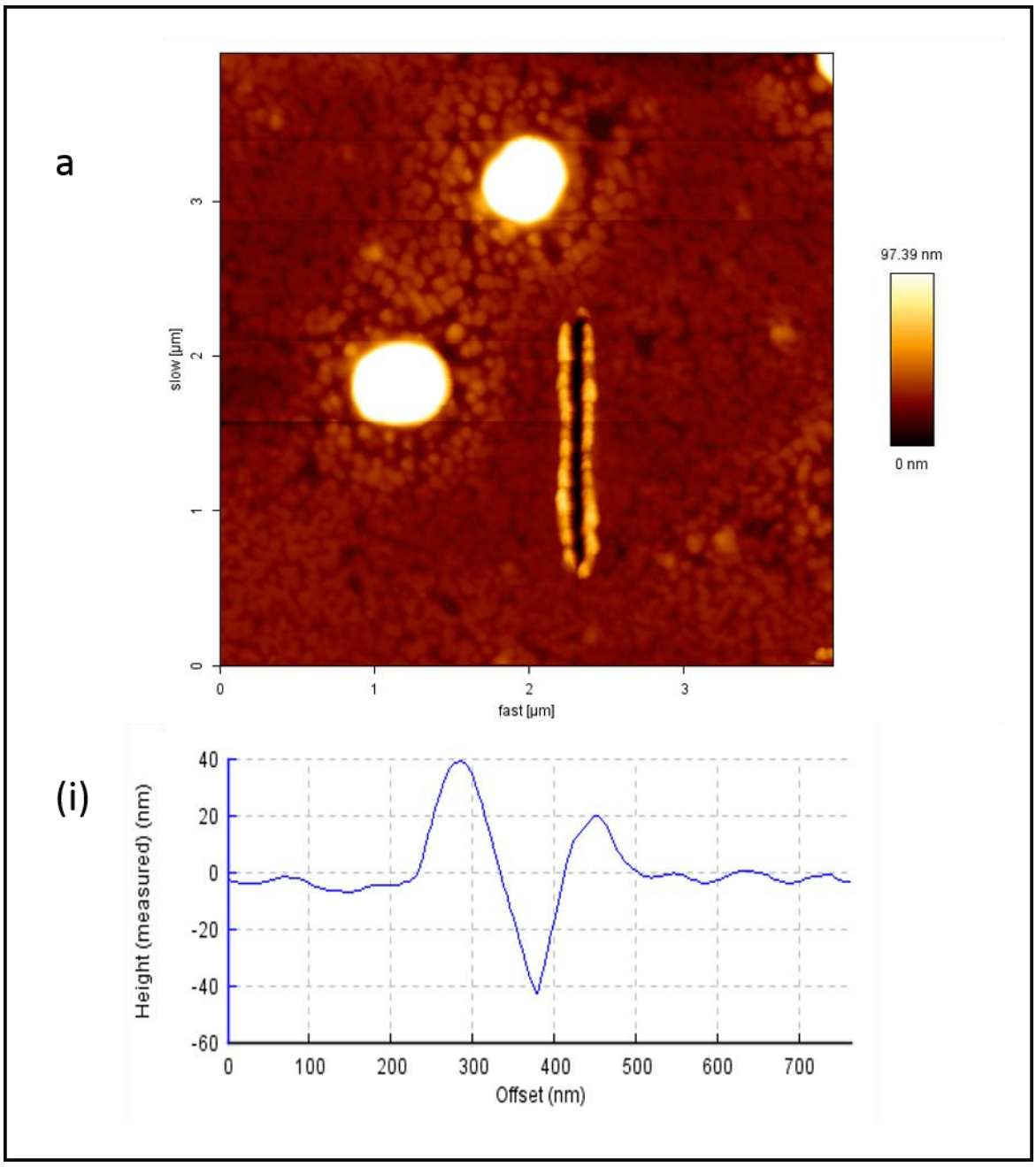



***Figure 21*****:** *Thickness measurement of the salivary film by a scratch method in contact mode AFM imaging. Area 5 x 5 µm$^2$. The thickness of the film approx ≈ 60nm (a) Salivary film with scratch open (i) Depth profile analysis of the scratch with the salivary film to calculate the thickness of the bound salivary film.*

*Figure* 22 (b) shows the AFM image of HWS film added with tannins in it. The scratch open of the film on applying hard contact reveals the thickness of the film and also the film has a granular morphology when compared to HWS film in the surface roughness measurement (*figure* 21 (a)). The insoluble protein-tannin complexes give the grainy nature of the film and

as a result, the film thickness and surface roughness are increased when compared to HWS film (*Figure* 21 (b)) and thickness of the film was calculated to be approximately 150 nm (*figure* 22 (ii)) by depth profile analysis.

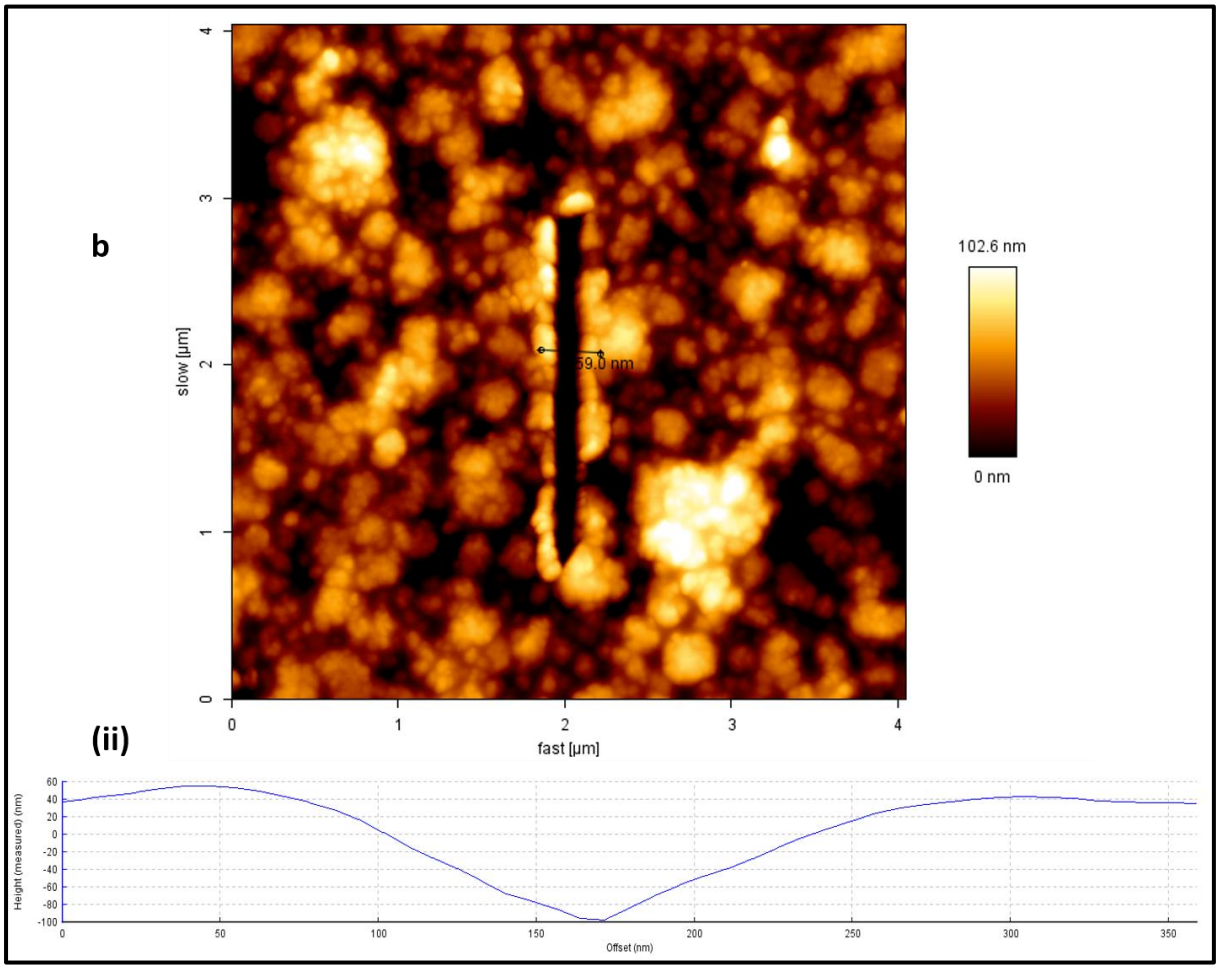



***Figure 22:*** *Depth profile analysis of the HWS film with added tannins. The scratch open reveal the thickness of the film (Approx. ≈ 150nm)*

### 3.2.3 Conclusion for AFM

AFM studies revealed the change in morphology of HWS film before and after the addition of tannins. The surface roughness and depth profiling measurements provides a strong evidence of formation of granular protein-tannin complexes, which are the causal factors in reduced oral lubrication.

### 3.2.4 Imaging of salivary proteins by LSCM

By using LSCM, initially the human whole saliva was imaged, the proteins were selectively stained red, and the results are clearly visible in *figure* 23. On the left, hand side the red dots represent the diverse family of salivary proteins as imaged. To the right, there is a large globular structure lying amongst the salivary proteins (*Figure* 23, right) and they are called as salivary micelles. These micelles consist of individual particles or cluster of particles with different sizes and shapes (Rantonen and Meurman 1998). The size of the micelles as determined by other researchers and found to be between 40 to 400 nm. Researchers used amino acid analysis on salivary micelles and proved that the amino acid composition was different from that of whole saliva (Soares, Lin et al. 2004).

The salivary proteins are α-amylase, mucins, lactoferrin, PRPs, HRPS and cystatins, were also identified by previous researchers by using various proteomics approaches (Glantz, Natiella et al. 1989; Wickström, Davies et al. 1998).

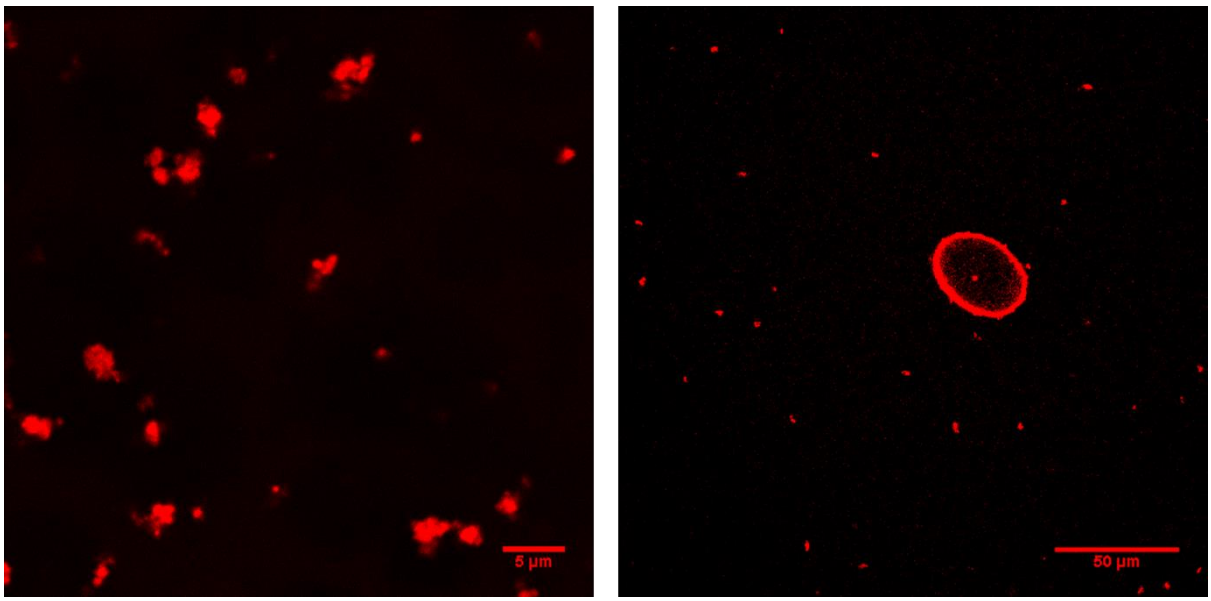



***Figure 12:*** *LSCM image of HWS showing the salivary proteins stained red on the left and salivary micelle on the right*

### 3.2.5 Human Mucin Imaging

Human mucin isolated from the HWS was imaged using LSCM, and the structure of the mucin is shown in *figure* 24. The Human mucin is stained red with rhodamine B, and the long chain conformation is visible from the image. Mucin protein is primarily involved in oral lubrication and the main protein of interest in this research with respect to its role in binding to tanning and hence astringency perception. Mucin proteins entanglements are primarily responsible for the gel properties of Human whole saliva (Wickstrom, Davies et al. 1998) . Mucins composed of disulphide linked subunits (Cárdenas, Elofsson et al. 2007). The mucins are high molecular weight (2-50MDa) and have a complex structure with domains involving hydrophobic/hydrophilic, hydrogen bonding and electrostatic interactions (Bansil and Turner 2006). This larger size of the mucin is very useful for imaging and researchers used AFM and TEM to image the mucin in its random coil structure of length 200-600 nm (Van Klinken, Dekker et al. 1995). As it is evident by now that the conformation of the mucin protein depends on the pH and ionic strength (Bansil and Turner 2006).

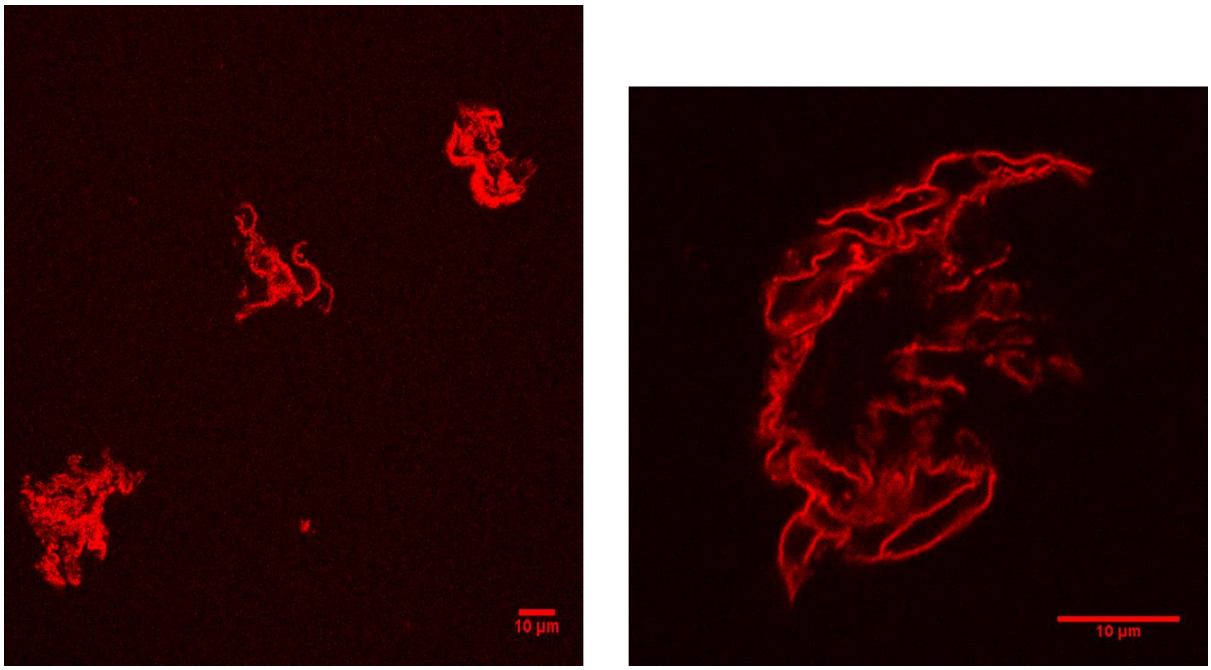



***Figure 24:*** *CLSM image of isolated Human mucin from HWS (left), individual Mucin protein structure (right)*

### 3.2.6 Imaging of tannins with Human Mucins

In order to study the impact of tannins when bound to salivary protein mucin, the Human Mucin is stained red in the GST extract. *Figure* 25 shows the conformational change induced by tannins to the mucins when compared to the *figure* 24. Due to time constraint, only one fluorescence probe (Rhodamine B), if multiple probes were used to visualise the bound tannins and the 3-D image of the Mucin-tannin complex will elucidate the interaction visually.

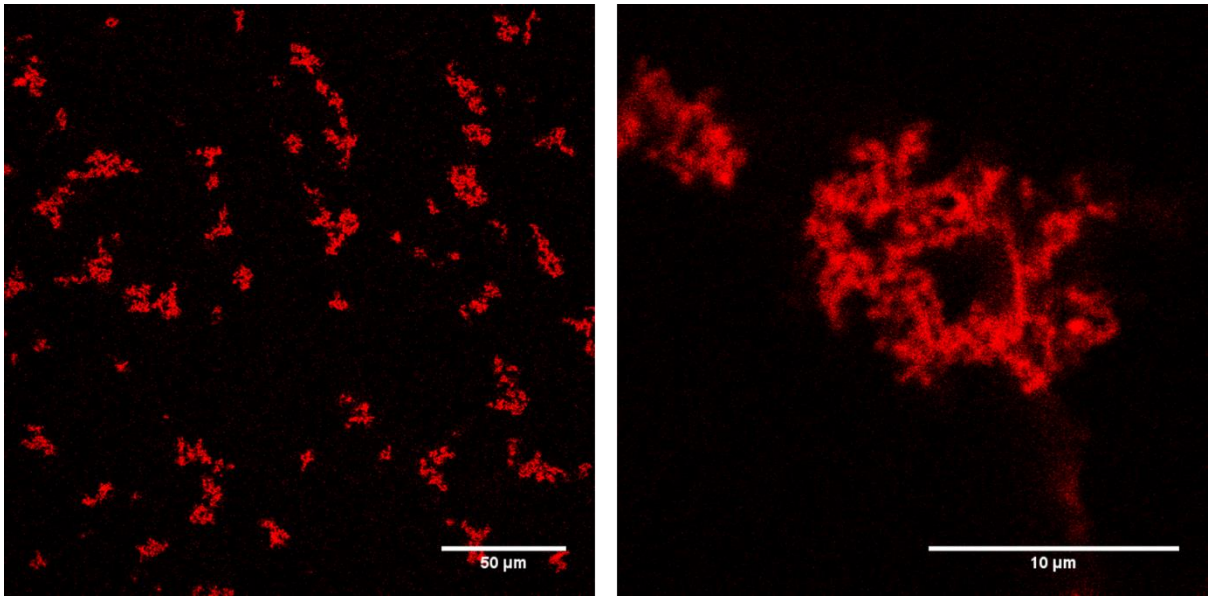



***Figure 25:*** *CLSM image showing the interaction between Human Mucin and GST (left), the conformational change induced by tannins to the mucins (right)*

### 3.2.7 Conclusion for LSCM

The primary objective of the research is to visualise the proteins in HWS by LSCM and to visualise mucin which is isolated from the HWS. The interaction between mucin and wine tannins were also characterised by LSCM with the help of the fluorophore. The microscopy technique used here is a complementary system to support the results from SPR, QCM-D and AFM. Hence advanced imaging with multiple fluorophores was not used as it is beyond the scope of the primary objective of this thesis.

## 3.3 RESULTS AND DISCUSSION FOR RHEOLOGY EXPERIMENTS

The outcomes from the rheology experiments are primarily affected by the type of rheometer chosen and the saliva collection method to perform the experiments. Hence, prudent steps were taken in selecting the rheometer and the saliva collection method. Below is the description of saliva collection technique.

Human whole saliva was collected by unstimulated method from a healthy non-smoking volunteer. The time of sample collection was set to be standard to avoid any interference and saliva was collected before 2 hrs of ingestion of food or beverages. Mouth was rinsed with water at least 3-5 minutes prior to sample collection. The saliva was collected by passive drooling procedure and protease inhibitor was added. Researchers suggests that the viscosity of saliva decreases with time and when stored at room temperature, so it is important that rheological measurements are taken immediately (Stokes and Davies 2007). The measurements were performed within one day of sample collection.

### 3.3.1 Viscosity measurement at constant shear rate

Viscosity measurement at constant shear rate was performed to understand the difference in viscosity values by two different methods (i.e. parallel plate and Couette method). Though a Universal value for shear rate in mouth for non-Newtonian fluid is still debated in the scientific community (Wood 1968; Shama and Sherman 1973; Stanley and Taylor 1993), a constant shear rate of 60 $s^{-1}$ was used in the viscosity measurement experiments in this research. The temperature was kept constant at 22°C.

Initial viscosity measurements at a constant shear rate (60 $s^{-1}$) were performed in parallel-plate (PP) method and Couette method. In the parallel plate geometry one of the major disadvantage being the variation in shear rates ranges from zero in the middle to a maximum value at the outside rim (Stokes 2012). As a result, the viscosity of saliva varies across the radius of the

plate when compared to the Couette method (Refer *figure* 26). This was evident when the shear rate was set constant at 60 $s^{-1}$. The Couette method also possess difficulty like filling the high viscous saliva sample in the Couette system and it requires larger sample volume (around 5 ml for each measurements). This makes it difficult to perform multiple measurements in single day. Moreover, Couette geometry usually have a large mass and large inertia and could cause trouble when performing high frequency measurements

Unstimulated Human whole saliva collected was immediately centrifuged at a low speed of 100 x g without affecting its elasticity behaviour. To avoid the risk of shear-induced effects, a small waiting time of 10s was applied. During the waiting period, there are chances of film formation on the surface of the film and to avoid that small amount of oil was added to the salivary sample. Viscosity measurements were performed with a fixed shear rate on undiluted HWS (▲) along with HWS mixed with diluted GST 10 µM (◊), GST 50µM (▼) and GST 100 µM (●) (*Figure* 26). The concentration of tannins in the measurement of viscosity of protein-tannin mixture is vital. As tannins are multidentate ligands (i.e. ability to bind more than one protein at a time or bind to multiple sites in one protein) the ratio of protein to tannins and the concentration of tannins and proteins determines formation of protein-tannin aggregates, which will precipitate and result in astringency. Moreover, the amount of HWS used was restricted because of the P-P geometry in viscometer. Tannins are usually present in with the normal wine concentration (2g/L) and was diluted in the molar concentrations of GST 10 µM, GST 50 µM and GST 100 µM to make it proportional to the amount of saliva (1350µL). The viscosity difference between Couette method (■) and parallel-plate method (▲) on unstimulated whole saliva was approximately 2 mPas. The viscosity of HWS under constant shear rate of 80 $s^{-1}$ was 8 mPas in studies by Haward et al (Haward, Odell et al. 2011). The difference in viscosity measurement, correlated value of viscosity from literature and the disadvantages associated with Couette method made us to choose PP geometry as a standard geometry for the rest of the

experiments. The viscosity values and standard deviation bars reported for HWS and grape seed tannins were obtained averaging over the three-complete set of experiments.

The viscosity measurements for undiluted HWS saliva showed an increase in viscosity when compared to the saliva mixed with different tannin concentrations. This is common and expected as the HWS is viscous in natural state than the HWS interacted with tannins. The decrease in viscosity with increasing molar concentration of tannins supports the central dogma behind astringency.

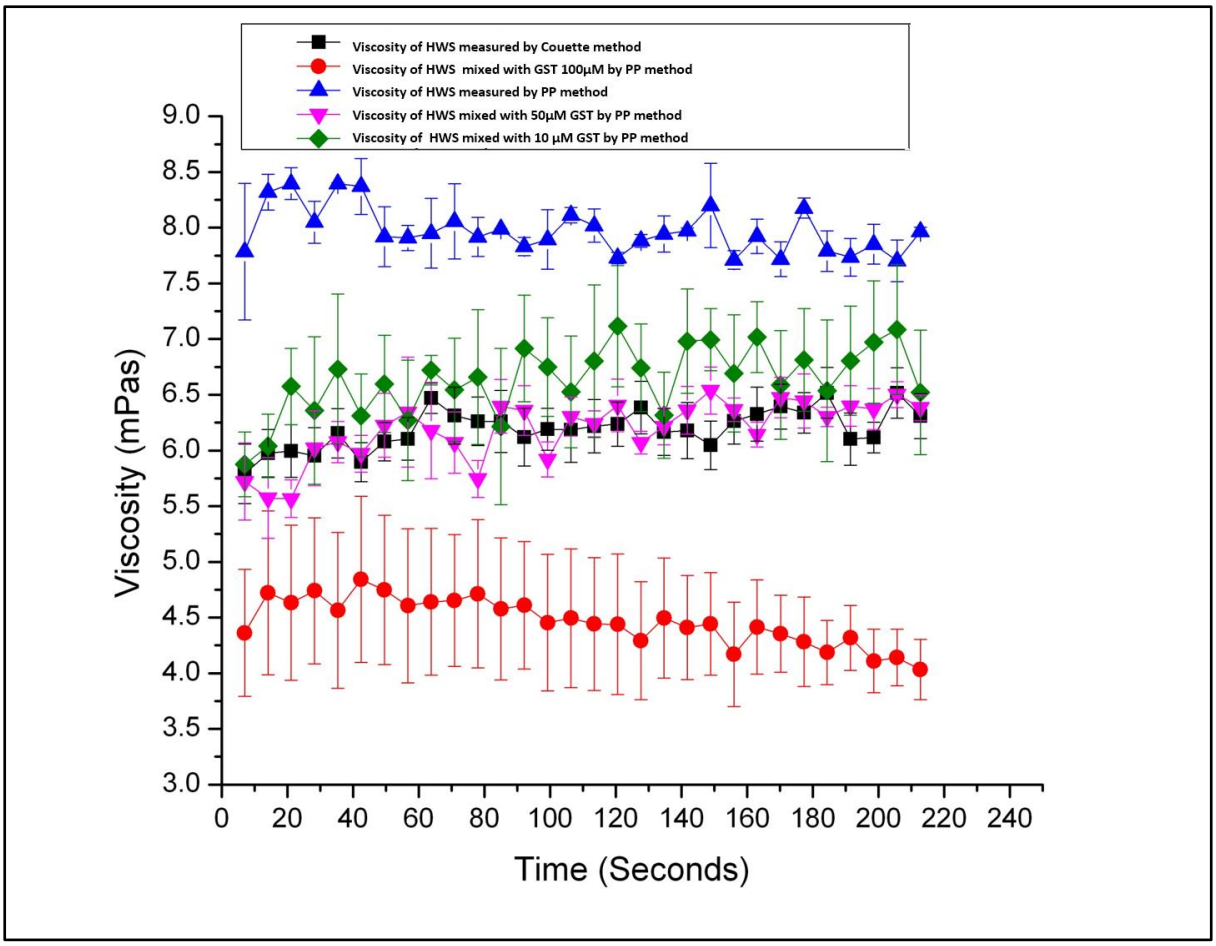



***Figure 26:*** *Viscosity measurement of undiluted Human whole saliva, undiluted GST, GST10µM, GST100 µM by PP method and coquette method at constant shear rate of* $60s^{-1}$.

The reason behind the decreased viscosity value when the threshold concentration of tannins (GST 100 µM (●) (*Figure* 26)) are added to HWS, is due to the fact that the tannins and salivary proteins react to form filamentous thread like structures which can be seen in naked eye. This is due to the aggregation of tannins with salivary proteins, and they form insoluble ternary complexes which precipitates at higher concentration. These visible filamentous structures are precipitated out from the HWS and gives a decreases viscosity values as they affect the lubrication of saliva.

Lowering the concentration by dilution of tannin (GST of 10µM and 100 µM) with the wine buffer from the GST stock solution resulted in decreased viscosity values.

### 3.3.2 Viscosity measurement of Human Mucin and tannins at constant shear rate

As mentioned in the literature section of this thesis, the lubricating properties of Human whole saliva is primarily attributed to Human Mucin (MG1). The aim of this experiment is to highlight the role played by Mucin in oral lubrication and the impact caused by tannins on binding with the Mucin

The results from the viscosity measurement of Human Mucin isolated from Human whole saliva and human mucin mixed with grape seed tannins experiment results are shown in *figure 27.* About 1350µl of Human Mucin isolated from HWS was placed between two parallel plates and the gap was set to be 1000µm. A constant shear rate of 60 $s^{-1}$ was applied between the two plates, and the readings were plotted for 210 seconds (*figure* 27 black line). The experiment was replicated thrice. The same experimental procedure was repeated another three-time with the tannins (GST 100µM) mixed with Human Mucin and the results were plotted (*Figure* 27 red line).

The addition of the tannins to the mucins results in the reduction of viscosity almost by half. This validates our hypothesis that

*"The Mucin is one of the primary proteins involved in oral lubrication, and the decrease in viscosity will lead to reduced oral lubrication and thus increased astringency sensation."*

The reason attributed in association with literature and from the results for the reduced viscosity value for Mucins that are bound to the tannins is due to the fact that Mucins form hydrogen bonds by their peptide carbonyl to the –OH group of the tannins (Wu, Csako et al. 1994). When the Mucin protein-tannin complexes aggregate it reduces the viscosity of the model wine solution and this can be correlated with oral lubrication. Salivary mucins are highly solvated in solution, and exist as expanded linear random coils. These mucins that occur naturally in higher concentration can aggregate to form viscous gels by molecular entanglement and hydrophobic interactions. When the viscous Mucins bind to the tannins then there is a conformational change in mucin structure and becomes weaker gel.

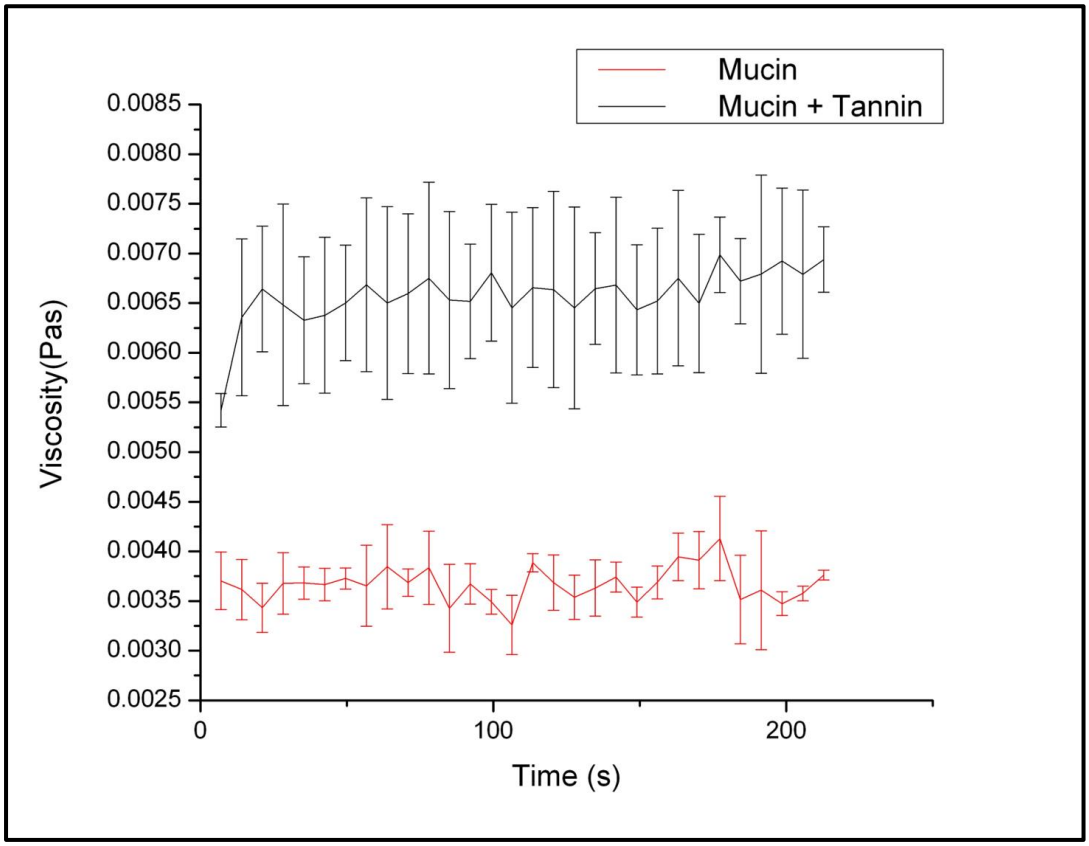



***Figure 27:*** *Viscosity measurement of Human Mucin and Human Mucin with added tannins at constant shear rate of 60 $s^{-1}$ by PP method*

Coefficient of friction is the ratio of force of friction between two bodies and the force pressing them together. From literature, it is evident that the Mucin proteins have very low coefficient of friction, but when they form hydrogen bonding with the tannins the coefficient of friction value increases and thus the loss in lubrication (Coles, Chang et al. 2010). Although, Mucins are known to facilitate lubrication in boundary, mixed and hydrodynamic regimes of the Stribeck curve. In order to understand the molecular mechanism of lubrication of Mucin proteins and to determine the regime (Boundary, mixed or hydrodynamic lubrications) where the lubrication loss occurs techniques like Mini traction machine and soft-tribology are helpful (Bongaerts, Rossetti et al. 2007). These tribological studies will help us to better understand the salivary mucin oral lubrication and it is one of the main future directions of this project.

### 3.3.3 Shear rate ramp measurements

The efficient lubricating properties of saliva depends on its viscosity and more importantly the modification associated with changes in shear rate. Shear rate ramp measurements are commonly used to detect the flow behaviour of fluid of interest. In *figure* 28, the viscosity of saliva shows a minimum increase when the shear rate is decreased below the lower shear-limit threshold rate (around 0.001 $s^{-1}$) and this rise didn't continue indefinitely, instead the viscosity attains a plateau known as First Newtonian Plateau.

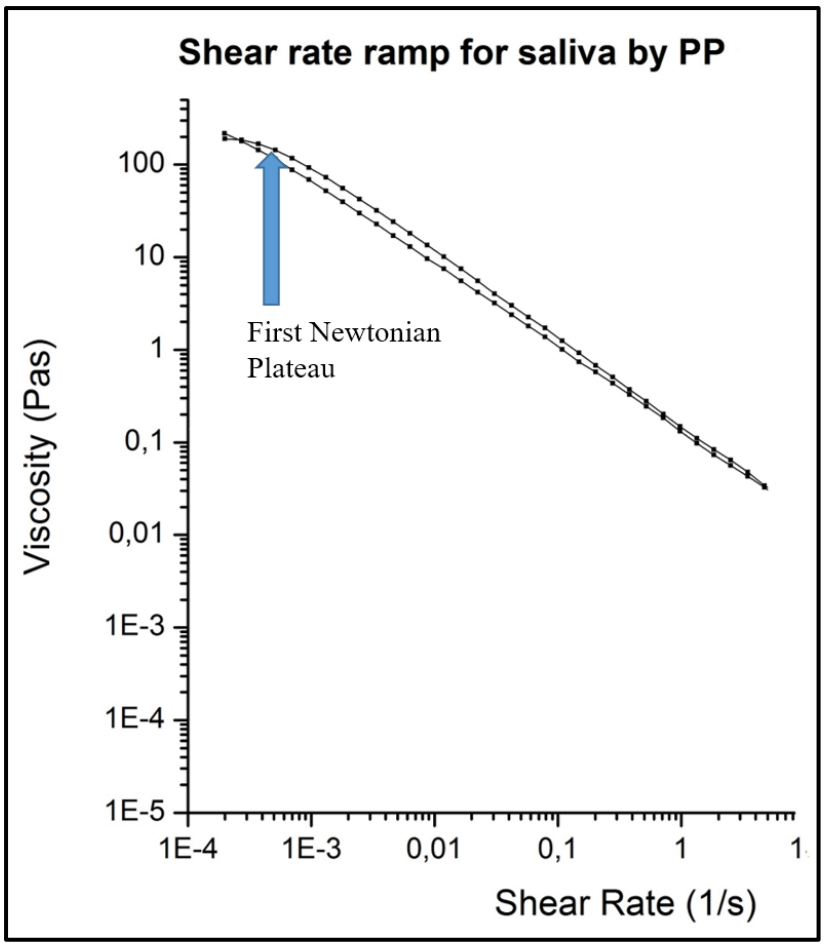



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***Figure 28:*** *Shear rate ramp viscosity measurements for HWS*

HWS is a non-Newtonian fluid with shear thinning nature and the shear rate ramp also shows a minor decrease in viscosity for saliva with tannins and wine buffer. The viscosity of model wine buffer (black line in *figure* 29) is the lowest as it has no lubricating component. These results are evident from the shear rate ramp measurements as shown in the f*igure* 29, and the shear thinning nature of saliva mixed with tannins (GST100 µM) and saliva with wine buffer are also shown. The shear rate ramp measurements are carried out in controlled rate (CR) mode with a gap of 1000 µm between the two plates. In shear rate ramp measurements, weaker interactions are studied at low shear rate and stronger interactions are at the higher shear rate. The vast range of shear rate applied showed the shear thinning nature of HWS but it concealed the noticeable effect of viscosity as a result of binding between tannins and salivary proteins.

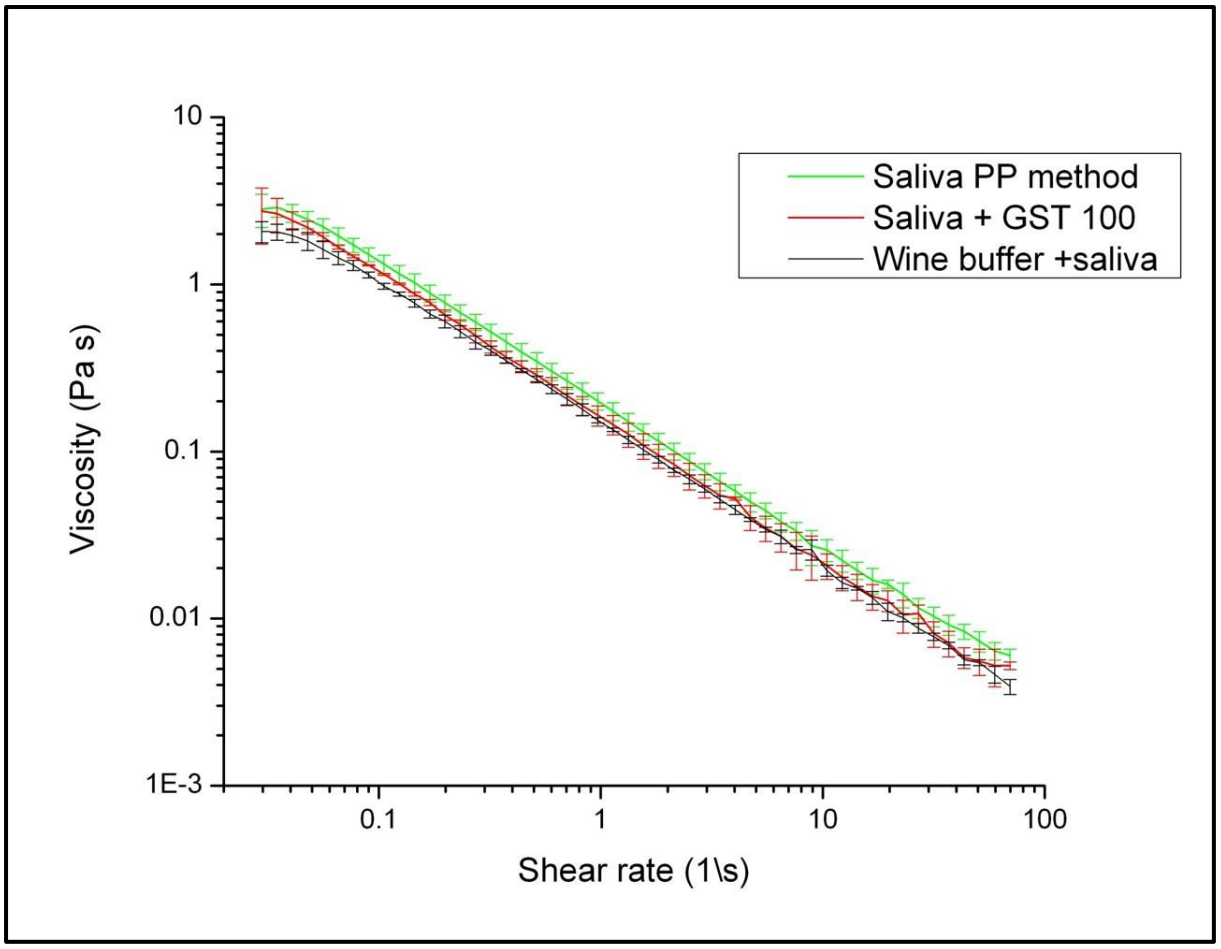



***Figure 29:*** *Shear rate ramp measurements against viscosity for undiluted saliva, saliva mixed with tannins and saliva with wine buffer by parallel plate method.*

### 3.3.4 Frequency sweeps measurements

Oscillatory frequency sweeps allow us to probe and identify the nature of the layer structuring present in the HWS film. The sample is exposed to small-deformation oscillations covering a range of frequencies to assess the structural response to deformations of longer or shorter timescales. In this experiments, the applied frequency range lies between 0.01 Hz-10 Hz. The two main parameters in this experiment is:

**G' the in-phase, elastic or storage modulus**

**G" the out-of-phase, viscous or loss modulus**

The frequency sweep measurements also help in determining the movement of viscous component (G") and elastic component (G') with respect to the speed at which saliva is moved. The oscillation of undiluted saliva sample over a range of frequencies will provide the viscoelastic characteristics over a range of timescales as high frequencies relate to short times, and low frequencies relate to the long time scale. In this experiment, the larger slope value of G' indicates the low strength of the HWS film. This reflects the weaker gel characteristic of salivary film under high frequency sweeps.

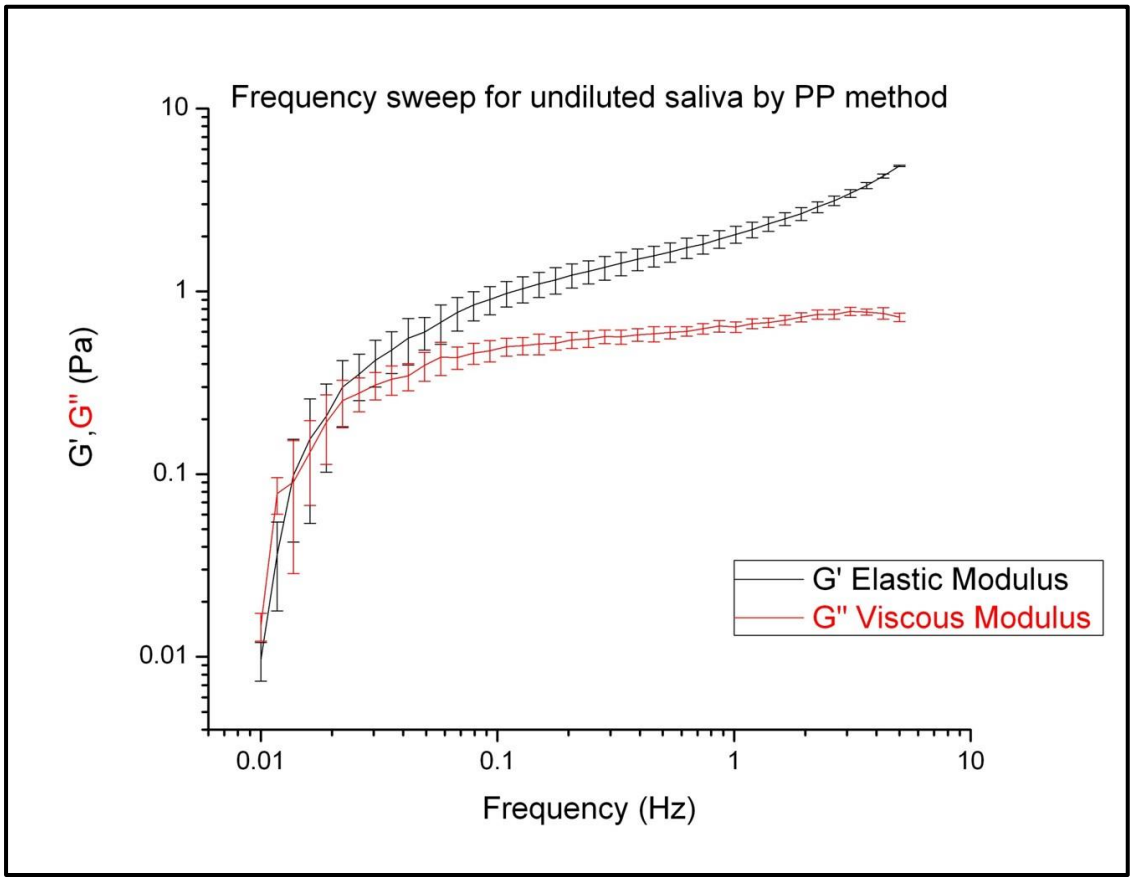



***Figure 30:*** *Frequency sweeps measurement on Human whole saliva by PP method.*

### 3.3.5 Amplitude sweeps measurements or strain sweep measurement

In oscillation strain sweeps measurement, the sample is subjected to small amplitude oscillatory (i.e. clockwise then counter clockwise) strain.

Amplitude sweep measurements are performed by oscillating at a fixed frequency (1Hz was fixed) and slowly increasing the applied amplitude (strain) (from 0.001 to 100). The measured values of the viscoelasticity will remain constant until a point, when the applied stress becomes too high and then the induced strain will start to cause the HWS film to break or 'rupture' (some flow on the deformation will be obtained). In order to characterize the visco-elastic HWS, measurement of strain amplitude dependence on the storage and loss moduli is critical.

The measured value of elasticity (G') will be falling while the measured viscous component (G") will start to increase(see *figure* 31 below).

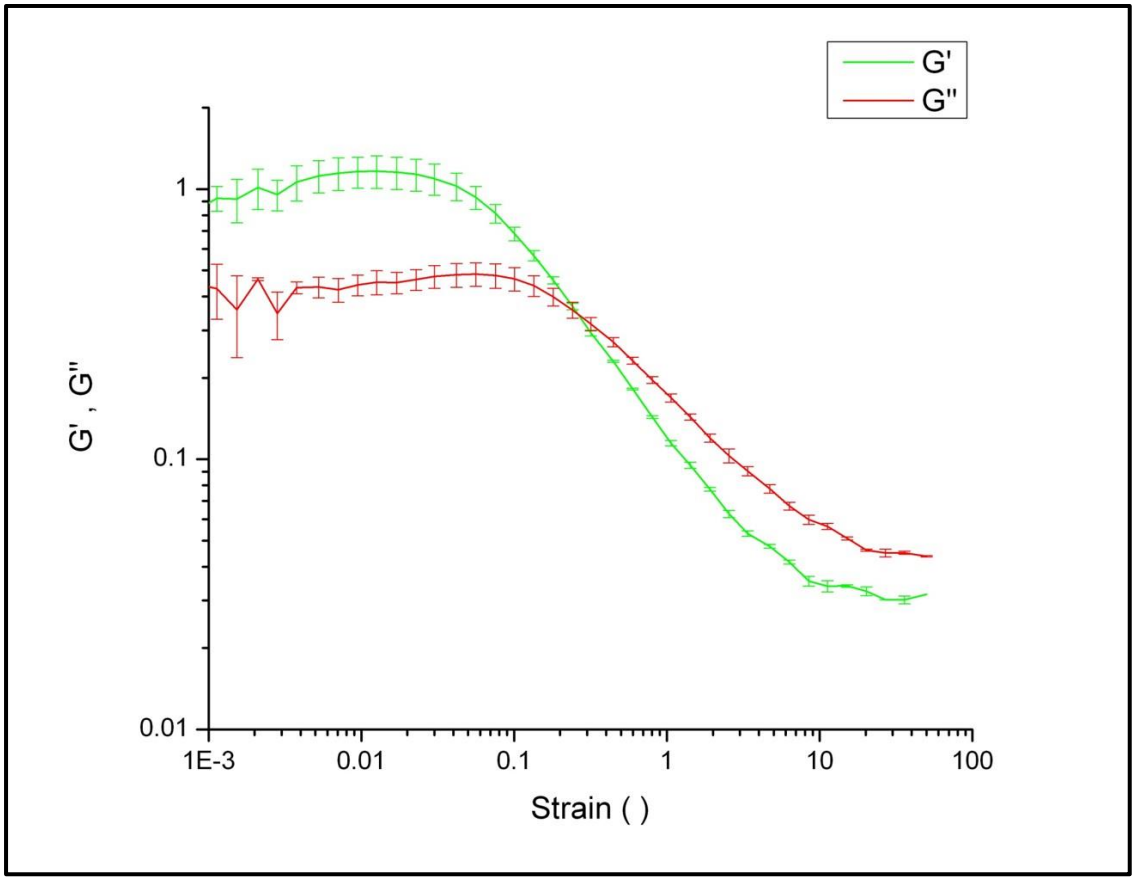



***Figure 31:*** *Amplitude sweeps measurements on Human whole saliva by PP method*

The rheological properties of viscoelastic HWS is independent of strain up to a critical strain level. The critical strain for HWS is 0.1 as shown in *figure* 31. Beyond this strain level, the HWS behaviour is non-linear and the storage modules decreases. The salivary film has solid-like properties and their networks is highly structured as long as the G'>G" and on increasing the strain level above critical strain the film ruptures and the highly ordered structure breaks down and becomes progressively more fluid-like, the moduli decline and G" exceeds G' eventually. The strength of the cohesive forces in the HWS film is reflected by tan $\delta = (G''/G')$.

The results from the amplitude measurements on both the Human whole saliva and the HWS added with tannins are shown in *figure* 32. The critical strain of HWS 0.1 whereas, the critical strain for HWS with added tannins is 0.5. This shows that the strain required to break the hydrophobic and hydrogen bonding between the tannins and salivary proteins are higher than to rupture the HWS without tannins in it. The difference in the strain required to break the HWS with added tannins film gives an insight to the binding strength between the tannins and salivary proteins.

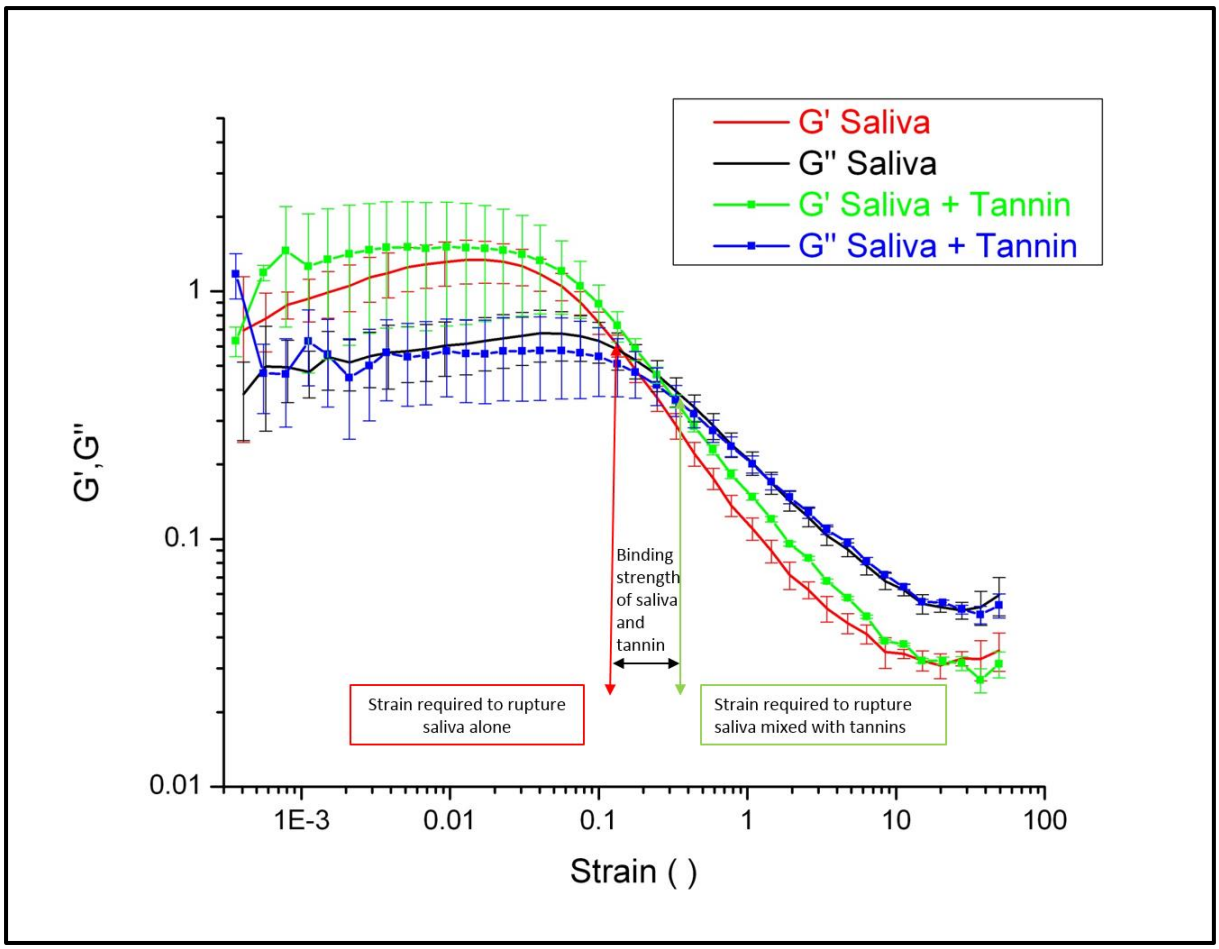



***Figure 3213:*** *Amplitude sweep measurements on human whole saliva and the saliva added with tannins. Inset is showing the binding strength of the tannin and saliva with respect to the strain.*

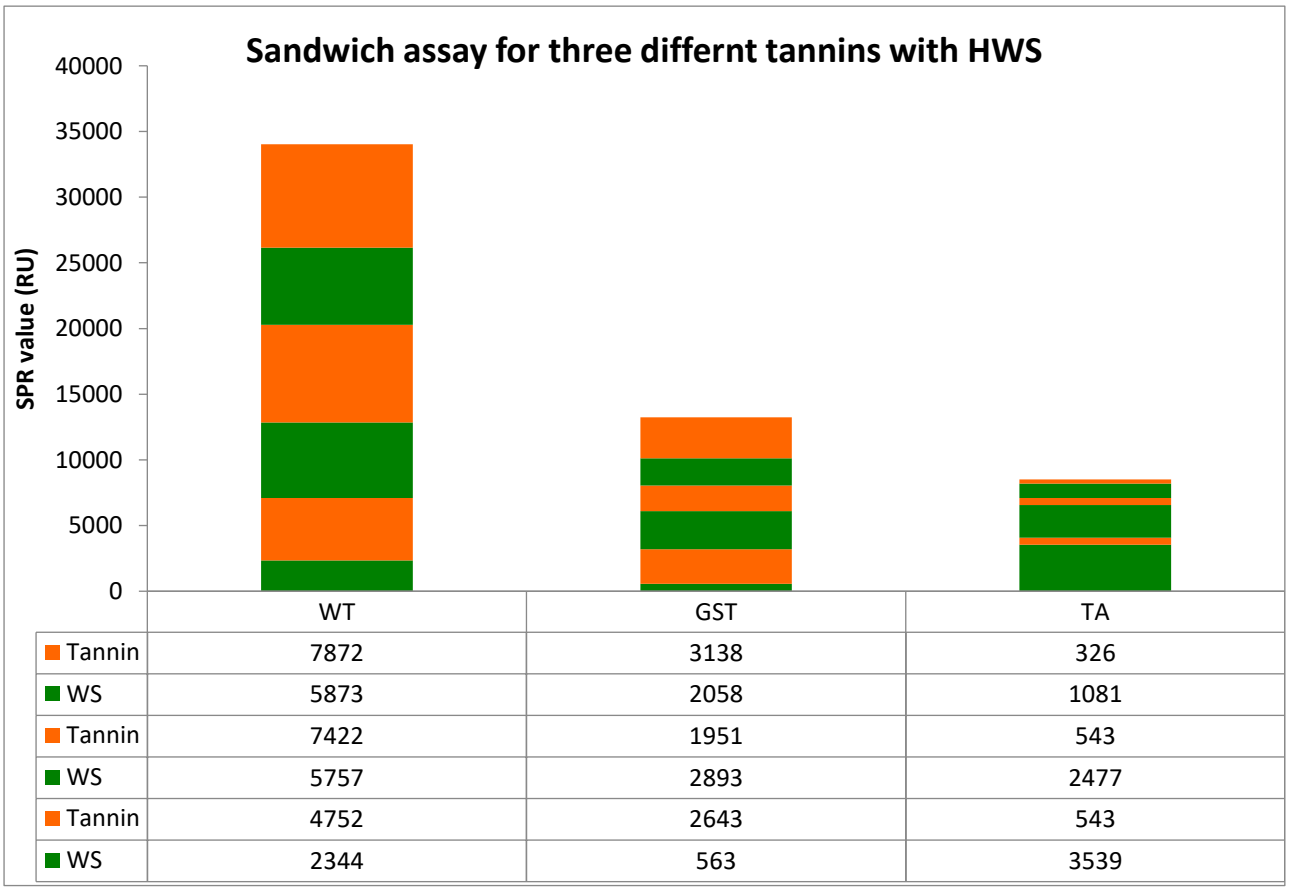
## 3.4 CONCLUSION FOR RHEOLOGY EXPERIMENTS

The preliminary rheological experiments on HWS and HM highlighted the viscosity of saliva and Human Mucin. Moreover, on interaction with tannins, the viscosity of HM decreases by half. The frequency and strain sweeps measurements on HWS depicted the viscoelastic nature of HWS. Results from strain sweep measurements showed the critical strain value for HWS and the increase in critical strain value as a result of binding with tannins.

## 3.5 BINDING OF DIFFERENT TYPES OF TANNINS TO SALIVARY FILM

The structure and origin of the three different tannins (i.e. Grape Seed Tannin, Tannic Acid and Wine Tannins) vary significantly and hence their binding to the salivary proteins also show inconsistencies. As it is already evident that the binding of these tannins contributes to the taste sensation like astringency and bitterness (Monteleone, Condelli et al. 2004; Soares, Mateus et al. 2007). The graph below *(Figure 33)* shows layer thickness of different tannins bound to the Human whole salivary film.

In this experiment, we have used sandwich-like assay (layer by layer assembly of HWS film and tannins) to deduce the binding of tannins to HWS. Initially, a layer of HWS was formed on the gold slide and then the interaction of different types of tannins were monitored by SPR.




| | WT | GST | TA |
|---|---|---|---|
| Tannin | 7872 | 3138 | 326 |
| WS | 5873 | 2058 | 1081 |
| Tannin | 7422 | 1951 | 543 |
| WS | 5757 | 2893 | 2477 |
| Tannin | 4752 | 2643 | 543 |
| WS | 2344 | 563 | 3539 |

***Figure 33:*** *Graph illustrating the SPR value for different types of tannins bound to human whole saliva*

This multilayer assay experiment was performed to visualize the interaction between GST, WT and TA with the proteinaceous salivary film. The results from SPR shows that the whole saliva interactions with various tannins takes place only in the surface, creating a sandwich assembly with constant wide-layers. This is evident from the binding thickness of each layer of HWS film and the subsequent tannin film.

The molecular mass and the size of the tannins plays a major role in this interaction process. On analysing the kinetics of the reaction of different tannins with the HWS, the rate constant values were calculated by plotting the real time kinetic data. The average time for the wine tannins to bind to HWS film takes 9.3 mins whereas the average time for tannic acid took only 4.62 mins as shown in *Figure* 34. The reason being the smaller size and low molecular weight for the tannic acid, it took less time to reach and bind to proteins on the surface.

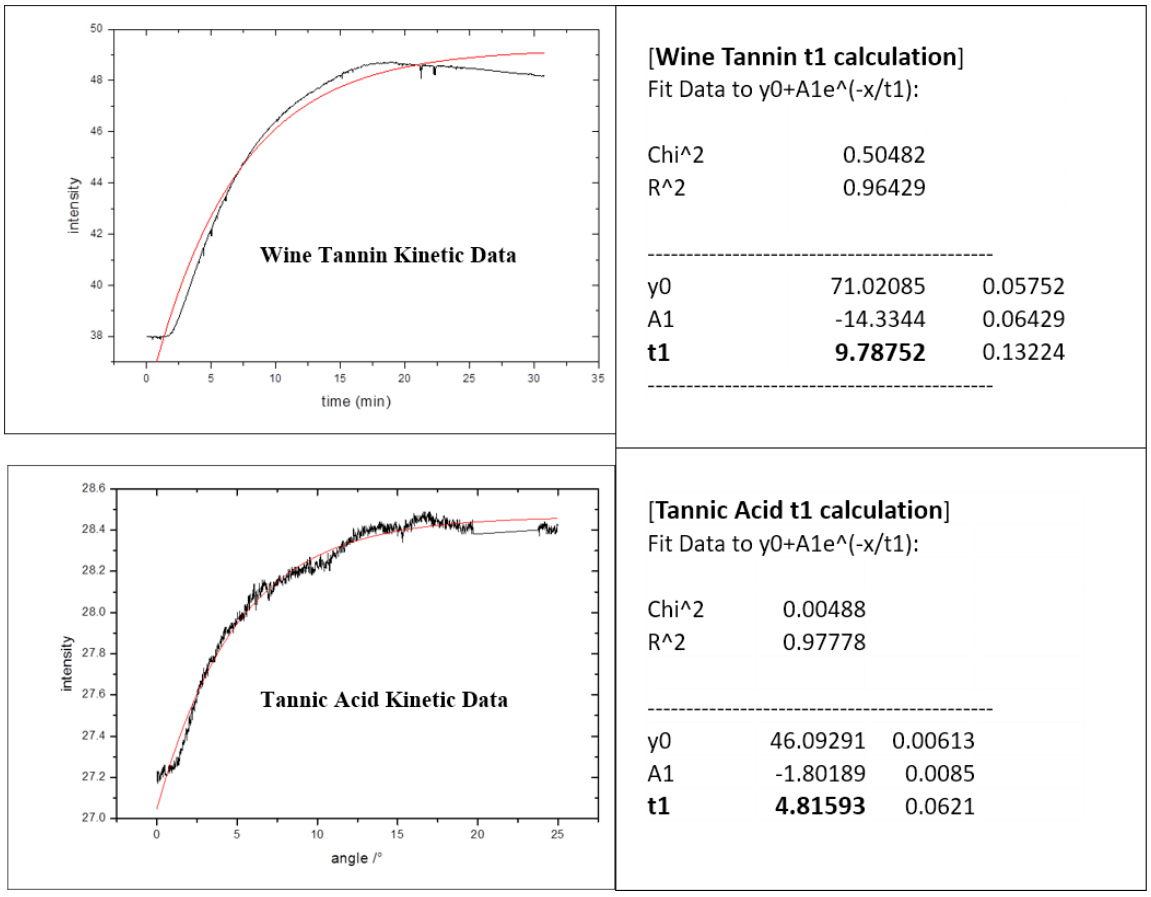



***Figure 34:*** *The average time calculation for tannic acid and wine tannin from affinity kinetics data*

The role of four different types of tannins with varied molar concentration and results of their binding property with the HWS are shown in figure 35.

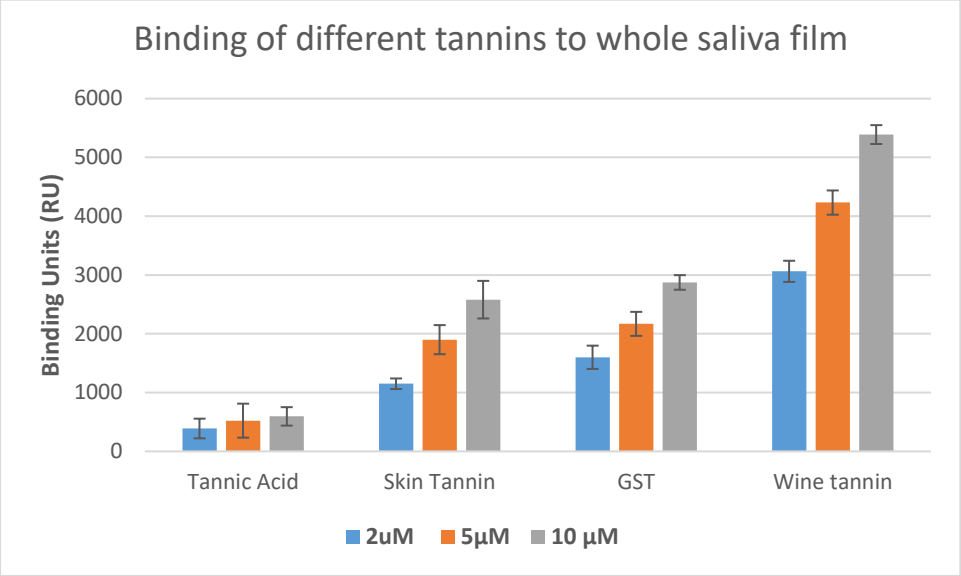



***Figure 35:*** *Binding of different types of tannins found in wine with varying concentrations to the Human Whole Saliva*

The molar mass of tannins used in our experiments are

Tannic acid – 1702 g/mol

Skin Tannin – 2271 g/mol

Grape Seed Tannin- 2702 g/mol

Young Wine Tannin (Leconfield 2007) – 3549 g/mol

Regarding the molar mass of the tannins, the trend is WT > GST > ST > TA and the order of affinity of different tannins to HWS are TA < ST < GST <WT. Wine tannins are atypical in their interaction as their molecular weight depends on the maturity of the wine as shown in *figure* 36 below. In Oenology, winemakers are experienced of the verity that the wine astringency decreases during aging/maturation "softening" than the intensity of younger wine. In our experiments, we used wine tannins from young wines.

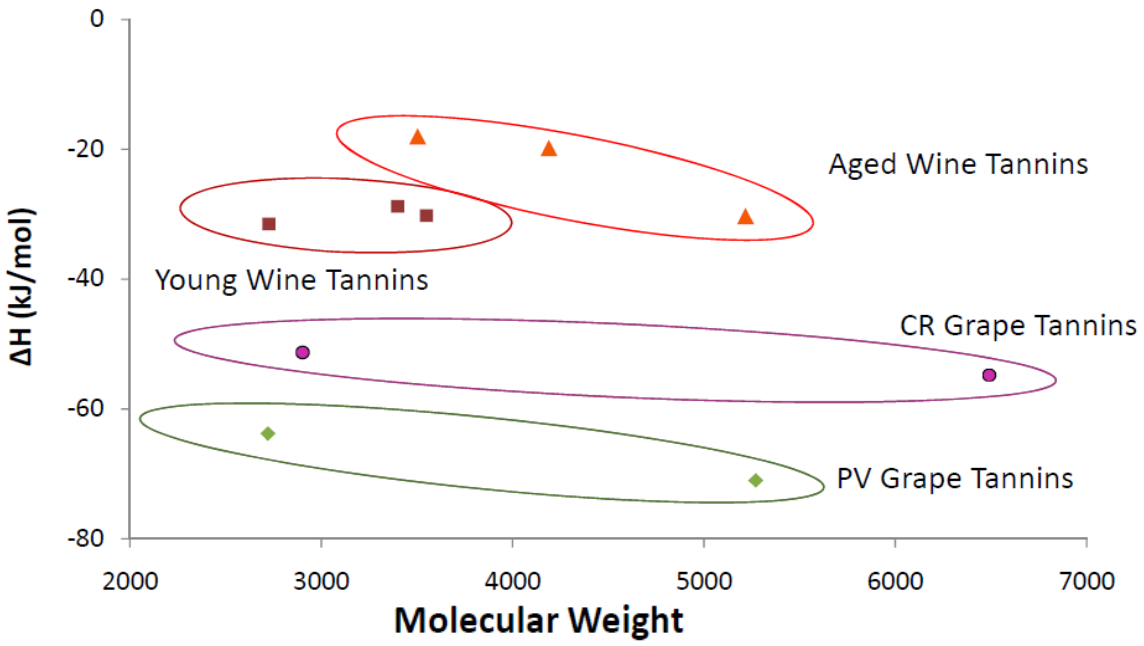



***Figure 36:*** *Molecular weight of aged tannins and their corresponding enthalpy change as measured in ITC by McRae et, al [Picture adapted from(McRae, Falconer et al. 2010)].*

## 3.6 EFFECT OF TEMPERATURE ON BINDING OF WINE TANNINS TO SALIVARY FILM

It is a normal practise to serve red wine at room temperature and white wines chilled. The serving temperature of wine affects sensory and mouth feel properties of the wine. The reason behind serving white wines colder is thought to be to suppress the inherent sweetness and enhance the acidity of the wine. On the other hand, red wines are served at room temperature in order to increase its aroma and decrease the inherent bitterness and astringency of the red wine (Jackson 2009). The temperature of the wine not only affect the taste properties but also the aromatics in a wine's fragrance. Hence, temperature change can either increase or decrease the detection of particular aromatics.

The effect of temperature on wine sensory properties are usually studied by serving wines with different temperature to a trained sensory panel. The panel scored the wines on aroma,

sweetness, acidity, bitterness and astringency. The scores were then analysed using ANOVA and the results were compared alongside chemical analysis of different components on the wine.

The temperature also has a significant effect on the astringency of wine. To probe this effect, the binding of tannins to a film of the whole saliva has been observed. Wine Tannins were dissolved in model wine solution (pH 4 and 14.4% ethanol) and added to a pre-adsorbed salivary film. At 22˚C, binding of 6225 ± 150 RU was observed, while at 4˚C a change of 4482± 170 RU has been observed. The higher binding at higher temperatures correlates with an increase in astringency. The greater binding thickness/layer thickness at higher temperature than the chilled wine tannin is associated with activation of wine tannins as the temperature increases and the volatility of aromatic components is also promoted, increasing the perception of the wine's fragrance and mouth-feel (Green and Frankmann 1988).

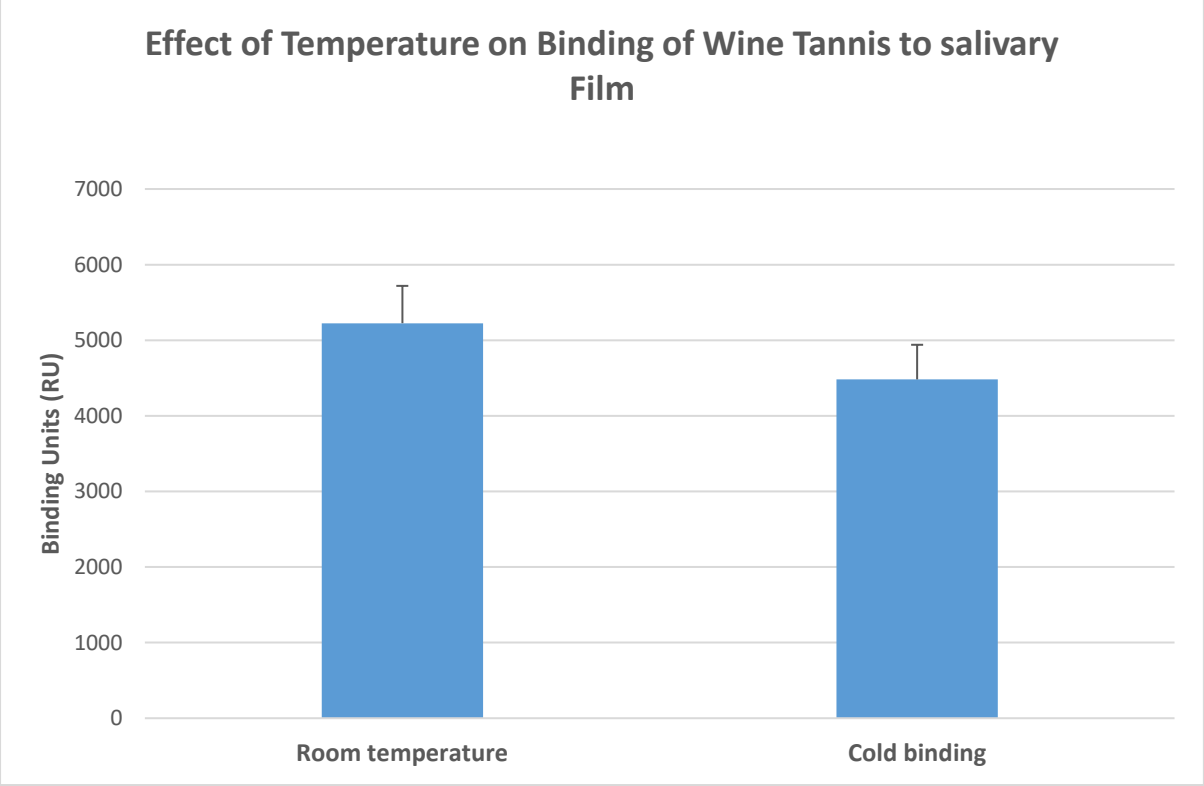



***Figure 14:*** *Binding of Wine Tannin (WT) to Human whole saliva as measured by SPR with temperature 4˚C and 22˚C.*

## 3.7 PROBING THE ROLE OF POLYSACCHARIDE IN ASTRINGENCY

SPR experiments on the polysaccharide interactions with salivary proteins and tannins benefits researchers and wine-makers to understand the role played by polysaccharides by binding with protein-tannin complexes and thereby reducing the perceived astringency. Researchers proposed two different binding mechanisms for polysaccharide interaction with the salivary proteins and wine tannins and they are:

1. The polysaccharides inhibit the association by forming soluble ternary complexes with protein-tannin complexes and increase their solubility in aqueous medium
2. Polysaccharides compete with tannins and in some cases encapsulate tannins and thereby interfere with their ability to bind to salivary proteins

The aim of this research is to investigate the mechanism of interaction of polysaccharides with the tannins and salivary proteins, by using SPR, QCM-D and AFM.

#### *3.7.1.1 SPR Reaction steps*

In order to understand the mechanism of binding of polysaccharides, HWS film was adsorbed on the gold coated SPR chip as mentioned in the section 2.3.3.1 of this thesis. Wine polysaccharides of varying concentrations (from 50µg/ml to 200µg/ml) was added to the GST 5µM (13.6µg/ml) solution in a vial. The mixed tannin-polysaccharide mixture was then allowed to interact with the HWS film. The binding value or the layer thickness this interaction was plotted in the *figure* 38a.

Results from SPR experiments shows the competitive binding of polysaccharides and tannins with salivary proteins. The initial binding reaction of GST with HWS is labelled as **1** in the bar chart below. The subsequent addition of polysaccharide to the tannin mixture, and the measured binding value of the GST with polysaccharides decreases till the maximum threshold of polysaccharide (200µg/ml) concentration is reached (**4**). It is important to note that, under the

experimental conditions described, control experiments practically did not induce any polyphenol/carbohydrate precipitation in the absence of protein.

1. Interaction of GST 5µM (13.6µg/ml) with HWS film – **Negative Control**
2. Interaction of GST 5µM and wine polysaccharides (WP 50µg/ml) with HWS
3. Interaction of GST 5µM and wine polysaccharides (WP 100µg/ml) with HWS
4. Interaction of GST 5µM and wine polysaccharides (WP 200µg/ml) with HWS
5. Interaction wine polysaccharides (WP 200µg/ml) with HWS film – **Positive Control**

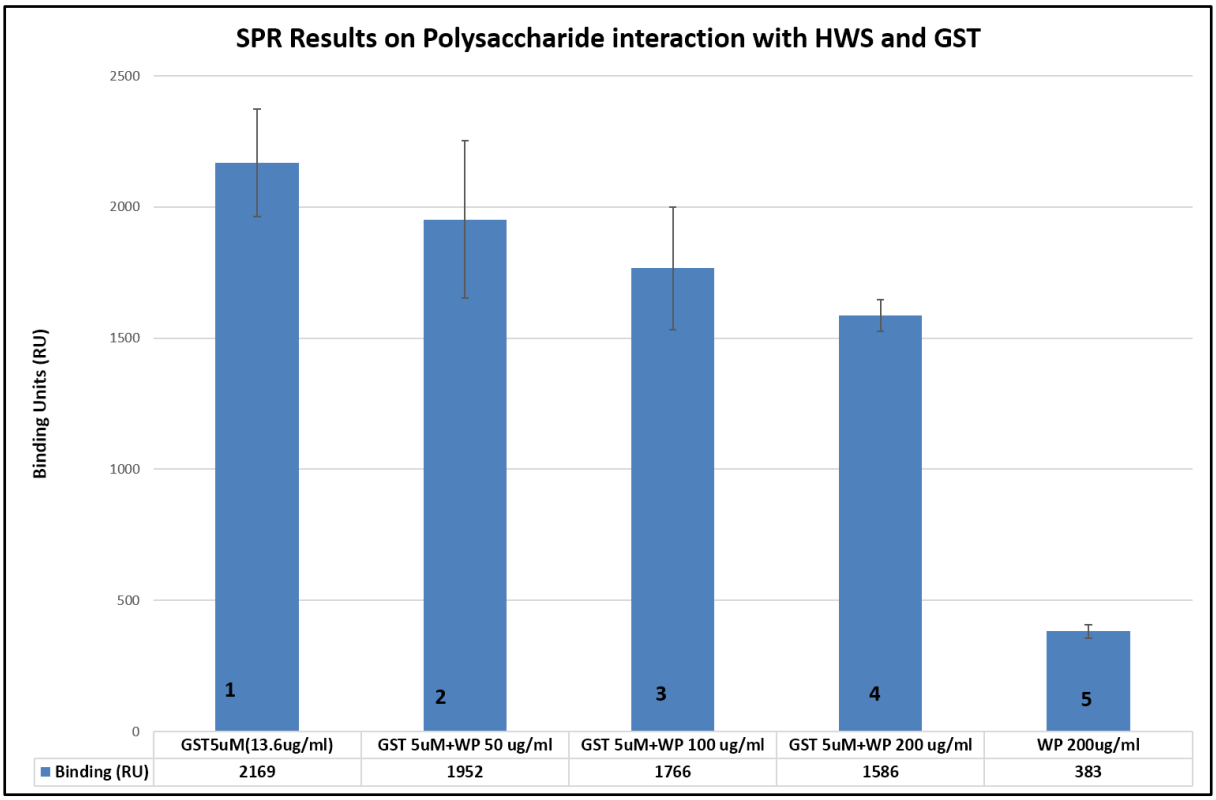



***Figure 38a:*** *SPR results from the interaction of HWS film with GST and polysaccharides. The numbered bars indicating the binding thickness values of the named reaction (i, e.* **1***-binding of GST 5µM with HWS film)*

The GST without the addition of polysaccharides (**1**) serves as negative control, it shows the binding thickness of GST with salivary as in normal condition. The higher value of layer thickness shows the formation of largest network of protein-tannin insoluble complexes. These

complexes then precipitate out the tannins, which serves as a basic mechanism of astringency sensation. The positive control in this study is the wine polysaccharide with the threshold/maximum concentration without tannins interacting with the HWS film (**5**). It is evident from the bar chart that on increasing the polysaccharide concentration, the binding thickness of the GST with HWS decreases and this can be correlated to astringency sensation. The larger binding values of GST has strong astringent sensation as more protein-tannin insoluble complexes were formed, whereas lower binding indicates less protein-tannin insoluble aggregates. It is evident from the SPR results that the interaction between polysaccharides and wine tannins and salivary proteins follows mechanism two (i.e. the formation of soluble tannin-polysaccharide complexes and reduces astringency) as shown in the figure below

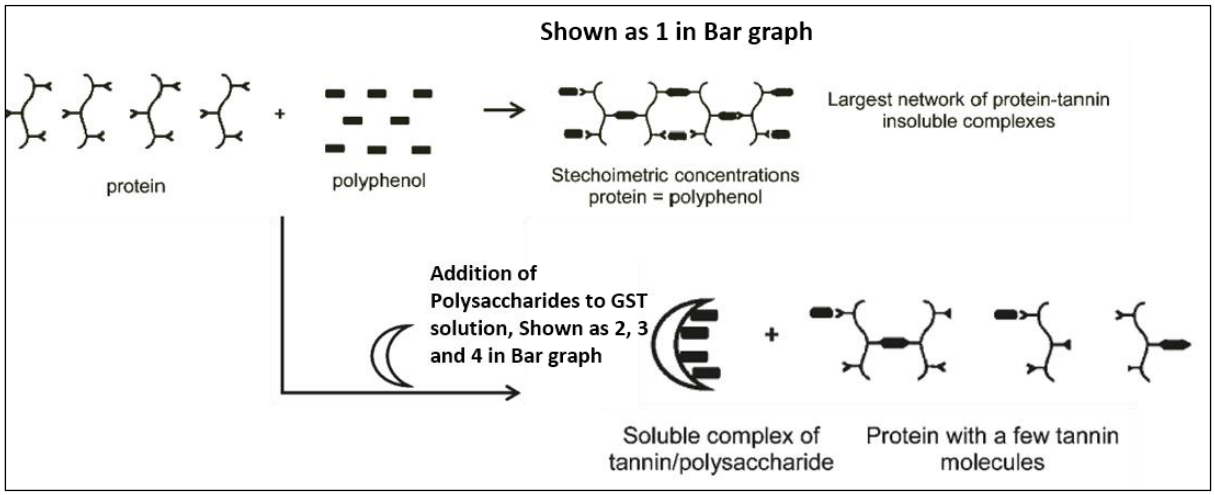



***Figure 39b:*** *Possible Mechanism of polysaccharides interaction with tannins by directly competing with salivary protein for the binding of tannins and thereby forming soluble complexes of tannin-polysaccharides.*

The molecular basis of this process is explained by the ability of water soluble pectin-fragments (i.e., RG II) to form hydrogen bonding between the oxygen atom of the polysaccharide and the phenolic hydroxyl group and also via hydrophobic interactions helps to encapsulate polyphenols and prevent their interaction with proteins (de Freitas, Carvalho et al. 2003). This

molecular association between the polysaccharides and polyphenols will compete with protein aggregation to polyphenol and hinders the formation of protein-tannin insoluble complexes. Many studies have shown that the presence of polysaccharides may reduce astringency, but only when combined with increased viscosity (Lyman and Green 1990, Smith et al. 1996).
In order to prove the concept, results from QCM-D will provide insights into the viscoelasticity of the soluble tannin-polysaccharide complexes and AFM imaging technique allowed us to image this polysaccharide association with protein and tannins.

### 3.7.2 Methodology and Results of QCM-D Experiments

The primary objective of the QCM-D experiments was to understand the role of polysaccharides interactions with tannins and proteins and the changes in visco-elastic properties caused by the polysaccharides. The concentration of ethanol and ionic strength of the solution have also been shown to impact tannin-polysaccharide interactions as well as tannin-protein interactions. In this experiment, we used GST 5µM and wine polysaccharides (WP 200µg/ml) in model wine buffer of pH 4 and ethanol 14.4%.
In QCM-D analysis, the initial baseline measurement with saline solution was noted, and the corresponding frequency changes are zeroed. The dissipation and frequency values from four sensors, which are connected in parallel are noted. HWS film was absorbed on the gold coated quartz sensor surface, and the frequency values along with dissipation are measured and plotted under the name HWS Film *(Figure* 40). Subsequently, unbound saliva was washed away with model wine buffer (pH 4 & ethanol 14.4%) and the final thickness or the frequency change is noted under HWS film after buffer elution. Now tannins (GST) along with model wine buffer was introduced and allowed to interact with HWS film and after buffer elution, the frequency and dissipation values are plotted under GST on HWS. The final step involves the introduction of wine polysaccharides into the system, and the readings are entered as GST with WP on HWS on *figure* 40. The dissipation and frequency values from four sensors, which are connected in

parallel are noted. The experiment was repeated thrice and the standard deviation was calculated and the error bars were included with the values.

The frequency and dissipation value correlates to the mass change ($\Delta m$) and viscoelastic nature of the adsorbed film respectively. At the end of the second step in the QCM-D experimental methodology, the approximate mass change and HWS film viscoelasticity was recorded by measuring $\Delta f$ and dissipation value. The higher dissipation value corresponds to more viscous or softer films and the larger dissipation value corresponds to rigid or harder film.

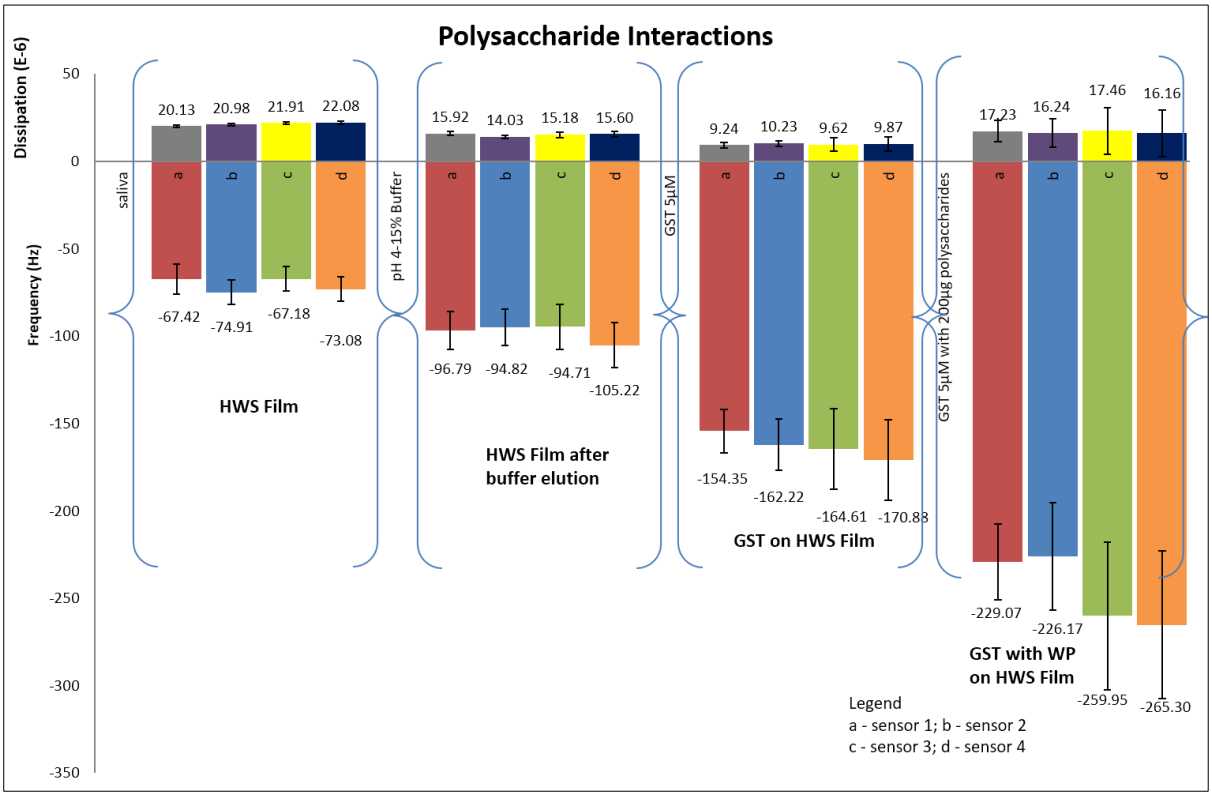



***Figure 40:*** *QCM graph showing the frequency change and the dissipation value for the saliva film adsorbed onto the gold-coated quartz crystal and their interaction with model wine buffer and GST and wine polysaccharides*

#### *3.7.2.1 Discussion*

The formation of salivary film results in change in frequency, this correlates to mass change ($\Delta m$) and the dissipation value shows that the saliva's structural properties, which is similar to that of a softer film. The elution of unbound saliva by model wine solution changes the structure of salivary by making it less soft HWS film (Note the decrease in dissipation value in *figure* 40. The subsequent addition of GST not only makes the salivary film rigid, but there is a large change in frequency value. The introduction of wine polysaccharides (WP 200µg/ml) to the HWS film along with GST results in increased value of dissipation, this suggests that there exists a competitive binding between polysaccharides and salivary proteins towards polyphenol.

The formation of soluble tannin-polysaccharide complexes in the system enables salivary protein with few bound tannin molecules. The results from polysaccharide addition corresponds to the increase in viscoelasticity of adsorbed salivary film. This shows that polysaccharides also moderate astringency perception through their ability to increase the viscosity of solutions by synergistically enhancing the viscoelasticity of human whole saliva. The results from the QCM-D experiments are further corroborated with studies by de Freitas et al. and Ozawa et al. In their study, they showed that the polysaccharides that are able to develop gel-like structures such as xanthan gum or hydrophobic pockets such as cyclodextrin are able to trap the tannins and by this way they inhibit the protein-tannin associations (Ozawa, Lilley et al. 1987; de Freitas, Carvalho et al. 2003)

### 3.7.3 AFM imaging of HWS with polysaccharides and tannins

To establish the role of the polysaccharides in inhibiting the binding of tannins with salivary proteins, AFM imaging of wine tannins with polysaccharides (*Figure* 41(a)) and salivary proteins with tannins and polysaccharides (*Figure* 41(b))were carried out. The objective of the experiment is to understand the interaction between wine tannin and polysaccharide and to prove that the polysaccharides like RG-II involved in co-aggregation with the tannins, and this interaction resulted in enhanced particle size of the polysaccharides as seen in *figure* 41(a). This is to prove that the mechanism (2) is followed by polysaccharides when inhibiting tannins from binding to salivary proteins. This competitive binding between tannins and polysaccharides leaves the salivary proteins unbound which can result in modified astringency response (Mateus, Carvalho et al. 2004). The factors that affects the binding between polysaccharides and tannins are the degree of polymerisation (mDp) of the tannins, ionic character of the polysaccharides and the type of the tannins (Scollary, Pásti et al. 2012). Surface roughness measurements also reveal the impact of polysaccharides on the structural morphology of the HWS film

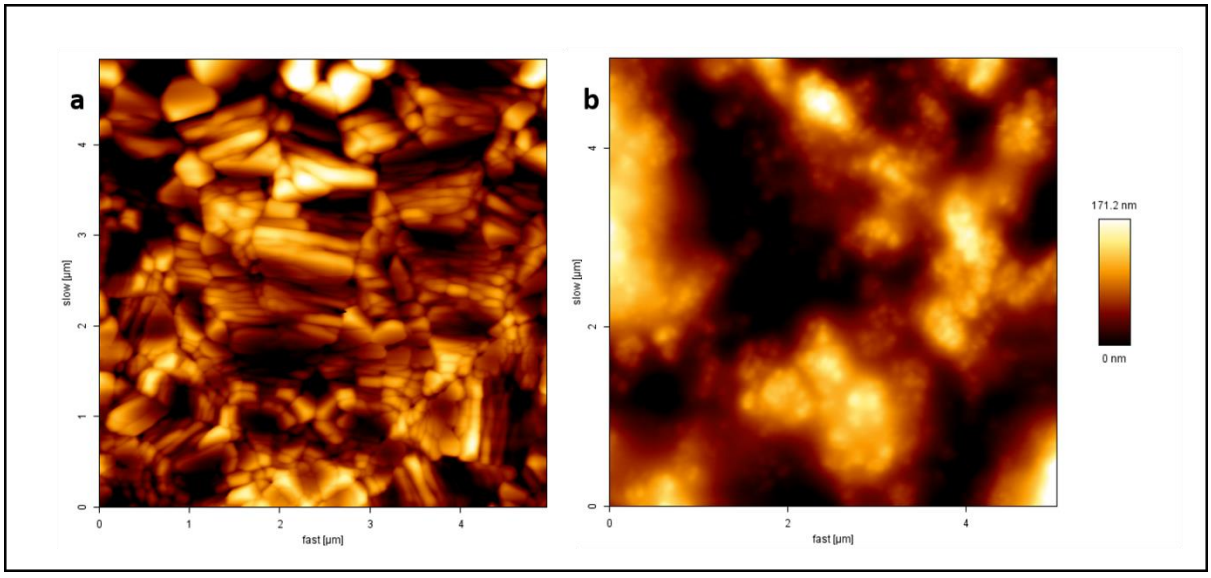



***Figure 41*: *AFM image of wine tannin with wine polysaccharides marked as (**a**) and salivary film with polysaccharides along with tannins in the ternary complex as (**b**). Area 5 x 5 µm$^2$***

### 3.7.4 Surface Roughness Measurements in AFM

The surface measurement experiments are primarily used in the AFM imaging to show the change in morphology of the adsorbed HWS film as a result of binding with tannins and polysaccharides. The higher value of average roughness indicates the rigid/granular film. The average roughness (Ra) and the root mean square roughness (Rq) values were obtained from the AFM imaging on HWS film (*figure* 20a), HWS film with tannins (*figure* 20b) and HWS with tannin and polysaccharides (*Figure* 41 (a) and(b)). The Ra values are the arithmetic mean of the height of the surface profile. The values are shown in Table 3 below. The roughness measurements of HWS film serves as a control or basis for other two readings. On addition of tannins (GST 5µM) to the salivary film, the tannins and salivary proteins form an insoluble complex as seen in *figure* 20(b) in section 3.2 which results in granular morphology and as an outcome there is a marked increase in surface roughness (Table 3). On the other hand, the HWS film which has the added tannins and wine polysaccharides forms soluble ternary complexes as shown in *figure* 41 (b) and as a result in the roughness of the film decreased dramatically, which proves the point that the addition polysaccharides in wines in reduces the astringency.

***Table 3:*** *The Ra and Rq values of salivary film along with tannins and also polysaccharides showing their effect on the interaction mechanisms*

| Interaction | Rq (RMS) | (Ra) |
|---|---|---|
| Salivary Film (HWS) | 4.85 nm | 2.98 nm |
| HWS with Grape seed tannins | 21.18 nm | 17.41 nm |
| HWS with grape seed tannins and wine polysaccharides | 6.80 nm | 5.52 nm |

*Ra = Average roughness

^Rq = Root mean square based on mean line of 2.5um

## 3.8 CONCLUSION FOR SPR, QCM-D AND AFM EXPERIMENTS ON POLYSACCHARIDES

The primary role of polysaccharides in inhibiting the binding of tannins with salivary proteins was well established with surface analytical tools like SPR and QCM-D. Results from SPR studies highlights the competing interaction between polysaccharides and wine tannins to the adsorbed protein film. The varying layer thickness and the positive and negative wine polysaccharide controls represents the binding thickness value of polysaccharides to protein-tannin complex. Moreover, the mechanism by which the polysaccharides inhibits the binding was also shown with SPR and QCM-D. The mechanism of interaction of polysaccharides with tannins was identified as, “the wine polysaccharides compete with tannins and in some cases, encapsulate tannins and thereby interfere with their ability to bind to salivary proteins”

QCM-D results revealed the nature of the film that is bound to polysaccharide. The addition or the presence of polysaccharides makes the salivary film viscous/soft compared to the salivary film which has tannin present. This increase in viscoelasticity of the film plays an important role in astringency as the parameter is directly proportional. The results from AFM experiments like surface roughness measurements showed the changes in the surface topography and the film thickness on the presence and absence of polysaccharides.

# 4 OVERALL DISCUSSION AND FUTURE DIRECTIONS

## 4.1 ROLE OF ETHANOL AND PH OF THE WINE

Results from the SPR and QCM-D reveals that the polyphenol association with proteins is a mainly a surface phenomenon. As wine polyphenols are multidentate ligands, this allows them to bind simultaneously (via different phenolic groups) at more than one point to the salivary protein surface effectively. Moreover, the concentration of ethanol and pH of the wine plays an important role in binding properties of the mucin. The hydrophobicity of ethanol plays a vital role in inducing the conformation changes in Mucin, which in turn impacts the binding with tannins. The pH can affect both the protein film and the tannins. Researchers suggested that a lower pH of wine (around 3) can lead to an increase in associated phenol groups, which allows for more hydrogen bonds formation (Guinard, Pangborn et al. 1986). This pH dependence of the tanning binding correlates with the changes in the perceived astringency of wine.

In future, various pH and ethanol concentrations will need to be studied to understand the extent of impact by hydrophobic and hydrogen-bonding interactions. Currently, we are working on high-throughput experimentation with the use of custom-made SPR chips, which allows the study of sixteen different combination of pH and ethanol concentrations found in wine. This opens up a whole new paradigm shift in the study of interaction between salivary proteins and wine tannins. Diffent types tannins, and their interactions with varied salivary proteins like PRPs and HRPs can be studied in a single sensor chip with SPR imager instruments.

## 4.2 IMAGING AND RHEOLOGICAL STUDIES ON HWS, GST AND HM

Results from AFM imaging of HWS film and HWS with GST featured the topography of the film and highlighted the changes induced by GST on interaction with HWS film. The depth profiling technique in AFM was greatly helpful as we were able to scratch open the HWS film and reveal the thickness of the adsorbed HWS film. Moreover, the change in thickness of the HWS film interacted with GST mimics the tannin build-up in oral surface during wine consumption. This result supports the hypothesis that the perceived astringency increases over time. LSCM imaging of Human Mucin and other salivary protein with Rhodamine dye gave a glimpse into the realm of salivary proteins and salivary micelles. The conformation change induced by tannins on Human Mucin was visualized in LSCM imaging. Further advancements include using different fluorophores to tag the salivary protein and tannins and image the interaction using LSCM. This will present the 3-D view of the altered protein structure on binding with tannins.

Rheological studies on HWS film highlighted the shear thinning nature of saliva and its non-Newtonian behaviour. Preliminary results from frequency sweep and strain sweep measurements showed the changes in storage and loss moduli of HWS film bound to tannins. The decrease in viscosity of Human Mucin on binding to tannins complements the results from SPR and adds strength to our hypothesis that the mucins are involved in oral lubrication and when they interact with tannins the lubrication decrease. Though researchers like Professor.Stokes and his group at University of Queensland has done a lot of interfacial rheology in saliva viscoelasticity and tribological studies on oral processing, our aim is to further advance the research in the direction of tribology to get a complete picture of red wine astringency.

## 4.3 ROLE OF POLYSACCHARIDES IN ASTRINGENCY

The main highlight from the polysaccharide interaction with wine tannin and salivary protein study was the identification of mechanism behind the interaction. The combination of SPR, QCM-D and AFM results depicted a clear picture of binding of polysaccharides with wine tannins to form soluble complex as opposed to the formation of protein-tannin-polysaccharide soluble ternary complexes. Researchers debated between these two mechanisms for the past decade and now we are able to highlight one of the proposed mechanism. The surface roughness measurement result from AFM provides an insight into the tannin-polysaccharide topography and the granular morphology of salivary film as a result of interaction with GST. These results strengthen our hypothesis that astringency arises as a result of decrease in oral lubrication in the mouth.

Rheological studies on the polysaccharides-tannin complexes were planned but due to time constrain, it was not performed. One of the future directions of this project would involve the rheological studies of tannin-protein-polysaccharide complexes, this will complement the results from our research. Apart from RG II and MPs, polysaccharides like Xanthan, Gum Arabic and RG I need to be investigated for their potential role in reducing astringency or other flavour perception. In conclusion, the project highlighted the main drivers of binding involved in salivary protein and tannin interaction and the role of polysaccharide in this interaction.

## 4.4 OUTLOOK

The importance of salivary protein mucin on astringency mechanism which was ignored during past years is highlighted in this research. Two major factors i.e., hydrogen bonding and hydrophobic effect which drives the binding of mucin with wine tannins were addressed and proved with specific experimental studies using SPR as a primary surface analytical tool. The binding of different types of tannin like GST, ST and WT to human saliva depends on the degree of polymerisation and size of the tannin molecules were also studied using the affinity kinetics data from SPR. The complex astringency mechanism which involves various textural properties and sub terms like grainy, velvety and puckering were hard to address using traditional techniques like taste dilution assay, nephelometry and time intensity methods. This complex nature which arose because of different phenolic compounds interactions with various salivary protein was addressed because of using Human saliva as whole in its native form. The use of SPR imaging in the protein-tannin interaction facilitated greatly in using the saliva without denaturing the proteins. This use of surface analytical tools to probe this interaction opens new avenues in the field of oenology. Though, a complete understanding of flavour profile of wine requires traditional taste assessment by qualified assessors, novel techniques employed in this research will further the understanding of basic tastes like bitterness, sweetness, sourness and astringency. In terms of further advancing this research, in white wines, haze formation (protein instability) has been studied in detail but advancement in textural and mouthfeel properties like sourness, bitterness and astringency were lacking. This research bias will soon be addressed in our following studies on white wine.

The role of polysaccharides was primarily to provide stability and viscosity of wine during production and storage and played a significant role in sensorial properties. The SPR and QCM-D results not only highlighted the interaction of polysaccharides with protein-tannin complexes but also deciphered the primary mechanism behind the interaction. The results are crucial to

winemakers to control the flavour profile and strength of wine by altering polysaccharide quantity during wine production. The polysaccharide addition varies differently with respect to the composition and quantity, irrespective of it this research proved that all polysaccharides tend to decrease the astringency by some degree. Further research is needed to understand the impact of wine matrix which involves polyphenolic compounds, proteins, organic acids and carbohydrates. Wine aroma profile in head space and wine too greatly influenced by the addition of polysaccharides to wine and researchers are currently employing combination of gas chromatography-olfactometry and mass spectrometry to further the understanding of polysaccharide interaction with volatile compounds.

Rheology and imaging studies on Human whole saliva and salivary mucin proved the complexity and the difficulties in handling saliva as a research material. The variability and instability of saliva sample was surmounted by minimising storage time and restricting sample size to the minimum. Rheological studies highlighted the role of mucin as a primary lubricant and the interaction of tannin with mucin affected the viscosity of saliva in that way contributing to the astringency. Initial saliva rheological studies in this thesis draw attention to the fact that rheological properties of whole saliva differ prior to contacting wine tannins as it possesses gel phase and solid phase in its network. The non-Newtonian nature of saliva was proved using shear rate ramp measurements and this contributed to the lubricating properties of saliva. Advancement in tribology and oral textural studies by employing mini traction machines helps to further understand the lubricating properties of mucin in saliva. The experiments involve PDMS ball and disk which serves biomimetic surfaces for studying the role of lubricating regimes and the influence of food components.

# REFERENCES


Al-Hashimi, I. and M. Levine (1989). "Characterization of in vivo salivary-derived enamel pellicle." Archives of oral biology **34**(4): 289-295.

Andersen, O. M., Markham, K.R., Ed. (2006). In Flavonoids, Chemistry, Biochemistry and Applications. Flavans and proanthocyanidins. FL, USA, Taylor and Francis CRC Press.

Anderson, K. and S. Nelgen (2015). Global wine markets, 1961 to 2009: a statistical compendium, University of Adelaide Press.

Asquith, T. (1985). "Induction of proline-rich protein biosynthesis in salivary glands by tannins." FASEB J **44**: 1097.

ASTM (1989). "Standard definitions of terms relating to sensory evaluation

of materials and products." Annual book of ASTM standards.

Bajec, M. R. and G. J. Pickering (2008). "Astringency: Mechanisms and Perception." Critical Reviews in Food Science and Nutrition **48**(9): 858-875.

Balmer, R. and S. Hirsch (1978). The non-Newtonian behaviour of human saliva. AIChE symposium series on biorheology.

Bansil, R. and B. S. Turner (2006). "Mucin structure, aggregation, physiological functions and biomedical applications." Current Opinion in Colloid & Interface Science **11**(2–3): 164-170.

Bard, A. J., F.-R. F. Fan, et al. (2001). "Introduction and principles." Scanning Electrochemical Microscopy **2**.

Barnes, H. A., J. F. Hutton, et al. (1989). An introduction to rheology, Elsevier.

Bate-Smith, E. (1954). "Astringency in foods." Food **23**(124).

Baxter, N. J., T. H. Lilley, et al. (1997). "Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation." Biochemistry **36**(18): 5566-5577.

Baxter, N. J., T. H. Lilley, et al. (1997). "Multiple Interactions between Polyphenols and a Salivary Proline-Rich Protein Repeat Result in Complexation and Precipitation†." Biochemistry **36**(18): 5566-5577.

Bennick, A. (2002). "Interaction of plant polyphenols with salivary proteins." Critical Reviews in Oral Biology & Medicine **13**(2): 184-196.

Bennick, A., G. Chau, et al. (1983). "The role of human salivary acidic proline-rich proteins in the formation of acquired dental pellicle in vivo and their fate after adsorption to the human enamel surface." Archives of oral biology **28**(1): 19-27.

Biedenbach, M. A. and K. Y. Chan (1971). "Tongue mechanoreceptors: comparison of afferent fibers in the lingual nerve and chorda tympani." Brain Research **35**(2): 584-588.

Bigiani, A. and S. D. Roper (1994). "Reduction of electrical coupling between Necturus taste receptor cells, a possible role in acid taste." Neuroscience Letters **176**(2): 212-216.

Bindon, K. A., P. A. Smith, et al. (2010). "Interaction between Grape-Derived Proanthocyanidins and Cell Wall Material. 1. Effect on Proanthocyanidin Composition and Molecular Mass." Journal of Agricultural and Food Chemistry **58**(4): 2520-2528.

Bingham, E. C. and L. A. Sarver (1920). "FLUIDITIES AND SPECIFIC VOLUMES OF MIXTURES OF BENZYL BENZOATE AND BENZENE. XXIX." Journal of the American Chemical Society **42**(10): 2011-2022.

Bongaerts, J., D. Rossetti, et al. (2007). "The lubricating properties of human whole saliva." Tribology Letters **27**(3): 277-287.

Boulet, J. C., P. Williams, et al. (2007). "A Fourier transform infrared spectroscopy study of wine polysaccharides." Carbohydrate Polymers **69**(1): 79-85.

Breslin, P. A. S., M. M. Gilmore, et al. (1993). "Psychophysical evidence that oral astringency is a tactile sensation." Chemical Senses **18**(4): 405-417.

Brillouet, J.-M., C. Bosso, et al. (1990). "Isolation, purification, and characterization of an arabinogalactan from a red wine." American journal of enology and viticulture **41**(1): 29-36.

Burin, V. M., L. D. Falcão, et al. (2010). "Colour, phenolic content and antioxidant activity of grape juice." Ciência e Tecnologia de Alimentos **30**: 1027-1032.
Cárdenas, M., T. Arnebrant, et al. (2007). "Human Saliva Forms a Complex Film Structure on Alumina Surfaces." Biomacromolecules **8**(1): 65-69.
Cárdenas, M., U. Elofsson, et al. (2007). "Salivary Mucin MUC5B Could Be an Important Component of in Vitro Pellicles of Human Saliva: An in Situ Ellipsometry and Atomic Force Microscopy Study." Biomacromolecules **8**(4): 1149-1156.
Carvalho, E., N. Mateus, et al. (2006). "Influence of Wine Pectic Polysaccharides on the Interactions between Condensed Tannins and Salivary Proteins." Journal of Agricultural and Food Chemistry **54**(23): 8936-8944.
Carvalho, E., M. J. Póvoas, et al. (2006). "Application of flow nephelometry to the analysis of the influence of carbohydrates on protein-tannin interactions." Journal of the science of food and agriculture **86**(6): 891-896.
Cavalieri, D., P. E. McGovern, et al. (2003). "Evidence for S. cerevisiae Fermentation in Ancient Wine." Journal of Molecular Evolution **57**(1): S226-S232.
Charlton, A. J., N. J. Baxter, et al. (2002). "Polyphenol/peptide binding and precipitation." Journal of Agricultural and Food Chemistry **50**(6): 1593-1601.
Charlton, A. J., N. J. Baxter, et al. (2002). "Polyphenol/Peptide Binding and Precipitation." Journal of Agricultural and Food Chemistry **50**(6): 1593-1601.
Chaudhari, N., H. Yang, et al. (1996). "The Taste of Monosodium Glutamate: Membrane Receptors in Taste Buds." The Journal of Neuroscience **16**(12): 3817-3826.
Cheynier, V. (2006). In Flavonoids - Chemistry, biochemistry

and applications;. Flavonoids in wine. O. M. Andersen, Markham, K. R.,. FL, USA, CRC Taylor & Francis.
Cheynier, V., M. Dueñas-Paton, et al. (2006). "Structure and properties of wine pigments and tannins." American Journal of Enology and Viticulture **57**(3): 298-305.
Claxton, N. S., T. J. Fellers, et al. (2006). "Laser scanning confocal microscopy." Department of Optical Microscopy and Digital Imaging, Florida State University, Tallahassee, http://www. olympusconfocal. com/theory/LSCMIntro. pdf.
Cliff, M. A., N. Brau, et al. (2002). "Development of predictive models for astringency from anthocyanin, phenolic and color analyses of British Columbia red wines." Journal international des sciences de la vigne et du vin **36**(1): 21-30.
Coimbra, M. A., F. Gonçalves, et al. (2002). "Fourier transform infrared spectroscopy and chemometric analysis of white wine polysaccharide extracts." Journal of agricultural and food chemistry **50**(12): 3405-3411.
Coles, J. M., D. P. Chang, et al. (2010). "Molecular mechanisms of aqueous boundary lubrication by mucinous glycoproteins." Current Opinion in Colloid & Interface Science **15**(6): 406-416.
de Freitas, V., E. Carvalho, et al. (2003). "Study of carbohydrate influence on protein–tannin aggregation by nephelometry." Food Chemistry **81**(4): 503-509.
de Freitas, V. and N. Mateus (2001). "Structural Features of Procyanidin Interactions with Salivary Proteins." Journal of Agricultural and Food Chemistry **49**(2): 940-945.
Dean, Z. (2011). "Microscopy Techniques Using Fluorescence Imaging."
Di Cola, E., G. E. Yakubov, et al. (2008). "Double-Globular Structure of Porcine Stomach Mucin: A Small-Angle X-ray Scattering Study." Biomacromolecules **9**(11): 3216-3222.
Dixon, M. C. (2008). "Quartz crystal microbalance with dissipation monitoring: enabling real-time characterization of biological materials and their interactions." Journal of biomolecular techniques: JBT **19**(3): 151.
Doco, T. and J.-M. Brillouet (1993). "Isolation and characterisation of a rhamnogalacturonan II from red wine." Carbohydrate Research **243**(2): 333-343.
Doco, T., P. Williams, et al. (2003). "Polysaccharides from grape berry cell walls. Part II. Structural characterization of the xyloglucan polysaccharides." Carbohydrate polymers **53**(3): 253-261.
Economics, G. (2015). "Economic Contribution of the Australian Wine Sector."

Engelen, L. (2012). Oral Receptors. Food Oral Processing, Wiley-Blackwell**:** 15-43.
Esau, K. (1948). "Phloem structure in the grapevine and its seasonal changes." Hilgardia **18**(5): 217-296.
Escot, S., M. Feuillat, et al. (2001). "Release of polysaccharides by yeasts and the influence of released polysaccharides on colour stability and wine astringency." Australian Journal of Grape and Wine Research **7**(3): 153-159.
Fontoin, H., C. Saucier, et al. (2008). "Effect of pH, ethanol and acidity on astringency and bitterness of grape seed tannin oligomers in model wine solution." Food Quality and Preference **19**(3): 286-291.
Frank, O., H. Ottinger, et al. (2001). "Characterization of an intense bitter-tasting 1 H, 4 H-quinolizinium-7-olate by application of the taste dilution analysis, a novel bioassay for the screening and identification of taste-active compounds in foods." Journal of agricultural and food chemistry **49**(1): 231-238.
Frazier, R. A., A. Papadopoulou, et al. (2006). "Isothermal titration calorimetry study of epicatechin binding to serum albumin." Journal of Pharmaceutical and Biomedical Analysis **41**(5): 1602-1605.
Gawel, R. (1998). "Red wine astringency: a review." Australian Journal of Grape and Wine Research **4**(2): 74-95.
Gibbins, H. and G. Carpenter (2013). "Alternative mechanisms of astringency–what is the role of saliva?" Journal of Texture Studies **44**(5): 364-375.
Glantz, P.-O., J. R. Natiella, et al. (1989). "Structural studies of human saliva." Acta Odontologica **47**(1): 17-24.
Glendinning, J. I. (1992). "Effect of salivary proline-rich proteins on ingestive responses to tannic acid in mice." Chemical Senses **17**(1): 1-12.
Green, B. G. and S. P. Frankmann (1988). "The effect of cooling on the perception of carbohydrate and intensive sweeteners." Physiology & behavior **43**(4): 515-519.
Guinard, J.-X., R. M. Pangborn, et al. (1986). "Preliminary studies on acidity-astringency interactions in model solutions and wines." Journal of the science of food and agriculture **37**(8): 811-817.
Guinard, J.-X., R. M. Pangborn, et al. (1986). "The Time-Course of Astringency in Wine upon Repeated Ingestion." American Journal of Enology and Viticulture **37**(3): 184-189.
Guinard JX, M. R. (1996). "The sensory perception of texture and mouthfeel." Trends Food Sci. Technol. **7**: 213-219.
Hagerman, A. E. and L. G. Butler (1978). "Protein precipitation method for the quantitative determination of tannins." Journal of Agricultural and Food Chemistry **26**(4): 809-812.
Hagerman, A. E. and L. G. Butler (1981). "The specificity of proanthocyanidin-protein interactions." Journal of Biological Chemistry **256**(9): 4494-4497.
Hagerman, A. E., M. E. Rice, et al. (1998). "Mechanisms of Protein Precipitation for Two Tannins, Pentagalloyl Glucose and Epicatechin16 (4→8) Catechin (Procyanidin)." Journal of Agricultural and Food Chemistry **46**(7): 2590-2595.
Hahn Berg, I. C., M. W. Rutland, et al. (2003). "Lubricating properties of the initial salivary pellicle—an AFM study." Biofouling **19**(6): 365-369.
Hannig, M., A. Döbert, et al. (2004). "Initial salivary pellicle formation on solid substrates studied by AFM." Journal of nanoscience and nanotechnology **4**(5): 532-538.
Haslam, E. (1974). "Polyphenol–protein interactions." Biochemical Journal **139**(1): 285.
Haward, S. J., J. A. Odell, et al. (2011). "Extensional rheology of human saliva." Rheologica acta **50**(11-12): 869-879.
Hayashi, N., T. Ujihara, et al. (2005). "Reduction of Catechin Astringency by the Complexation of Gallate-Type Catechins with Pectin." Bioscience, Biotechnology, and Biochemistry **69**(7): 1306-1310.
He, Q., B. Shi, et al. (2006). Food Chem. **95**: 250.

Hellman, E. W. (2003). "Grapevine structure and function." Oregon Viticulture. Hellman, EW (Ed.). Oregon State University Press, Corvallis: 5-19.
Homola, J., S. S. Yee, et al. (1999). "Surface plasmon resonance sensors: review." Sensors and Actuators B: Chemical **54**(1): 3-15.
Höök, F., J. Vörös, et al. (2002). "A comparative study of protein adsorption on titanium oxide surfaces using in situ ellipsometry, optical waveguide lightmode spectroscopy, and quartz crystal microbalance/dissipation." Colloids and Surfaces B: Biointerfaces **24**(2): 155-170.
Hu, S., Y. Xie, et al. (2005). "Large‐scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two‐dimensional gel electrophoresis‐mass spectrometry." Proteomics **5**(6): 1714-1728.
Inoue, H., K. Ono, et al. (2008). "Rheological properties of human saliva and salivary mucins." Journal of Oral Biosciences **50**(2): 134-141.
Jackson, D. and P. Lombard (1993). "Environmental and management practices affecting grape composition and wine quality-a review." American Journal of Enology and Viticulture **44**(4): 409-430.
Jackson, R. (2008). "Grapevine structure and function." Wine Science—Principles and Aplications: 50-105.
Jackson, R. S. (2009). Wine tasting: a professional handbook, Academic Press.
Jeffery, D. W., M. D. Mercurio, et al. (2008). "Rapid Isolation of Red Wine Polymeric Polyphenols by Solid-Phase Extraction." Journal of Agricultural and Food Chemistry **56**(8): 2571-2580.
Jensen, J., M. Lamkin, et al. (1992). "Adsorption of human salivary proteins to hydroxyapatite: a comparison between whole saliva and glandular salivary secretions." Journal of dental research **71**(9): 1569-1576.
Jobstl, E., J. O'Connell, et al. (2004). "Molecular model for astringency produced by polyphenol/protein interactions." Biomacromolecules **5**(3): 942-949.
Jones, P. R., R. Gawel, et al. (2008). "The influence of interactions between major white wine components on the aroma, flavour and texture of model white wine." Food Quality and Preference **19**(6): 596-607.
Jones, W. T. and J. L. Mangan (1977). "Complexes of the condensed tannins of sainfoin (Onobrychis viciifolia scop.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH." Journal of the Science of Food and Agriculture **28**(2): 126-136.
Joslyn, M. A. and J. L. Goldstein (1964). "Astringency of Fruits and Fruit Products in Relation to Phenolic Content." Advances in Food Research **13**: 179-217.
Jurd, L. (1967). "Anthocyanidins and related compounds—XI: Catechin-flavylium salt condensation reactions." Tetrahedron **23**(3): 1057-1064.
Kallithraka, S., J. Bakker, et al. (1998). "EVIDENCE THAT SALIVARY PROTEINS ARE INVOLVED IN ASTRINGENCY." Journal of Sensory Studies **13**(1): 29-43.
Kaneda, H., M. Takashio, et al. (2001). "Adsorption to or desorption of beer components from a lipid membrane related to sensory evaluation." Journal of bioscience and bioengineering **92**(3): 221-226.
Kawamoto, H. and F. Nakatsubo (1997). "Effects of environmental factors on two-stage tannin-protein co-precipitation." Phytochemistry **46**(3): 479-483.
Keller, M. (2015). Chapter 1 - Botany and Anatomy. The Science of Grapevines (Second Edition). San Diego, Academic Press**:** 1-57.
Keller, M. (2015). Chapter 6 - Developmental Physiology. The Science of Grapevines (Second Edition). San Diego, Academic Press**:** 193-265.
Khanbabaee, K. and T. van Ree (2001). "Tannins: classification and definition." Natural product reports **18**(6): 641-649.
Kim, H.-S., W. A. House, et al. (2004). "Habitual tea consumption protects against the inhibitory effects of tea on iron absorption in rats." Nutrition research **24**(6): 383-393.

Kinnamon, S. C. and R. F. Margolskee (1996). "Mechanisms of taste transduction." Current Opinion in Neurobiology **6**(4): 506-513.
Kočevar-Nared, J., J. Kristl, et al. (1997). "Comparative rheological investigation of crude gastric mucin and natural gastric mucus." Biomaterials **18**(9): 677-681.
Kretschmann, E. and H. Raether (1968). "Notizen: Radiative Decay of Non Radiative Surface Plasmons Excited by Light." Zeitschrift für Naturforschung A **23**(12): 2135-2136.
Lamkin, M. S. and F. G. Oppenheim (1993). "Structural features of salivary function." Critical Reviews in Oral Biology & Medicine **4**(3): 251-259.
Lawless, H. T. (1984). "Flavor description of white wine by "expert" and nonexpert wine consumers." Journal of Food Science **49**(1): 120-123.
Lee, C. B. and H. T. Lawless (1991). "Time-course of astringent sensations." Chemical Senses **16**(3): 225-238.
Lee, S., M. Müller, et al. (2005). "Porcine gastric mucin (PGM) at the water/poly (dimethylsiloxane)(PDMS) interface: influence of pH and ionic strength on its conformation, adsorption, and aqueous lubrication properties." Langmuir **21**(18): 8344-8353.
Lesschaeve, I. and A. C. Noble (2005). "Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences." The American Journal of Clinical Nutrition **81**(1): 330S-335S.
Levine, M., A. Aguirre, et al. (1987). "Artificial salivas: present and future." Journal of dental research **66**(2 suppl): 693-698.
Llaudy, M. C., R. Canals, et al. (2004). "New Method for Evaluating Astringency in Red Wine." Journal of Agricultural and Food Chemistry **52**(4): 742-746.
Loewenthal, J., Z (1877). anal. Chem **16**.
Lopez, R., L. Mateo-Vivaracho, et al. (2007). "Optimization and validation of a taste dilution analysis to characterize wine taste." J Food Sci **72**(6): S345-351.
Lu, Y. and A. Bennick (1998). Arch. Oral Biol. **43**: 717.
Lu, Y. and A. Bennick (1998). "Interaction of tannin with human salivary proline-rich proteins." Archives of Oral Biology **43**(9): 717-728.
Luck, G., H. Liao, et al. (1994). "Polyphenols, astringency and proline-rich proteins." Phytochemistry **37**(2): 357-371.
Mabry, T. J., K. R. Markham, et al. (1970). The ultraviolet spectra of flavones and flavonols, Springer.
Macakova, L., G. E. Yakubov, et al. (2010). "Influence of ionic strength changes on the structure of pre-adsorbed salivary films. A response of a natural multi-component layer." Colloids and Surfaces B: Biointerfaces **77**(1): 31-39.
Mandel, I. D. (1987). "The functions of saliva." Journal of dental research **66**(2 suppl): 623-627.
Margalit, Y. (2012). Concepts in wine chemistry, Board and Bench Publishing.
Mateus, N., E. Carvalho, et al. (2004). "Influence of the tannin structure on the disruption effect of carbohydrates on protein–tannin aggregates." Analytica Chimica Acta **513**(1): 135-140.
Mayer, P. (1896). "Mitt. zool." Stn. Neapel **12**: 303.
McManus, J. P., K. G. Davis, et al. (1985). "Polyphenol interactions. Part 1. Introduction; some observations on the reversible complexation of polyphenols with proteins and polysaccharides." Journal of the Chemical Society, Perkin Transactions 2(9): 1429-1438.
McMaster, T. J., M. Berry, et al. (1999). "Atomic Force Microscopy of the Submolecular Architecture of Hydrated Ocular Mucins." Biophysical Journal **77**(1): 533-541.
McRae, J. M., R. J. Falconer, et al. (2010). "Thermodynamics of Grape and Wine Tannin Interaction with Polyproline: Implications for Red Wine Astringency." Journal of Agricultural and Food Chemistry **58**(23): 12510-12518.
McRae, J. M. and J. A. Kennedy (2011). "Wine and Grape Tannin Interactions with Salivary Proteins and Their Impact on Astringency: A Review of Current Research." Molecules **16**(3): 2348-2364.
Meckel, A. (1965). "The formation and properties of organic films on teeth." Archives of oral biology **10**(4): 585-IN516.

Mehansho, H., L. G. Butler, et al. (1987). Annu. Rev. Nutr. **7**: 423.
Mehansho, H., A. Hagerman, et al. (1983). "Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels." Proceedings of the National Academy of Sciences **80**(13): 3948-3952.
Mercurio, M. D. and P. A. Smith (2008). "Tannin Quantification in Red Grapes and Wine: Comparison of Polysaccharide- and Protein-Based Tannin Precipitation Techniques and Their Ability to Model Wine Astringency." Journal of Agricultural and Food Chemistry **56**(14): 5528-5537.
Moncrieff (1946). The Chemical Senses. New York, John Wiley and Sons.
Monteleone, E., N. Condelli, et al. (2004). "Prediction of perceived astringency induced by phenolic compounds." Food Quality and Preference **15**(7–8): 761-769.
Muenzer, J., C. Bildstein, et al. (1979). "Purification of proline-rich proteins from parotid glands of isoproterenol-treated rats." Journal of Biological Chemistry **254**(13): 5623-5628.
Naim, M., R. Seifert, et al. (1994). "Some taste substances are direct activators of G-proteins." Biochemical Journal **297**(3): 451-454.
Newton, I., A. Motte, et al. (1850). Newton's principia: The mathematical principles of natural philosophy, Geo. P. Putnam.
Noble, A. (1990). Bitterness and astringency in wine. Bitterness in Foods and Beverages. I. R. Rousseff. Amsterdam, Elsevier**:** 145–158.
Obreque-Slier, E. a., A. l. Peña-Neira, et al. (2010). "Comparative Study of the Phenolic Composition of Seeds and Skins from Carménère and Cabernet Sauvignon Grape Varieties (Vitis vinifera L.) during Ripening." Journal of Agricultural and Food Chemistry **58**(6): 3591-3599.
Oh, H. I., J. E. Hoff, et al. (1980). "Hydrophobic interaction in tannin-protein complexes." Journal of Agricultural and Food Chemistry **28**(2): 394-398.
Oppenheim, F., T. Xu, et al. (1988). "Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans." Journal of Biological Chemistry **263**(16): 7472-7477.
Oppenheim, F., Y.-C. Yang, et al. (1986). "The primary structure and functional characterization of the neutral histidine-rich polypeptide from human parotid secretion." Journal of Biological Chemistry **261**(3): 1177-1182.
Ozawa, T., T. H. Lilley, et al. (1987). "Polyphenol interactions: astringency and the loss of astringency in ripening fruit." Phytochemistry **26**(11): 2937-2942.
Parry, J., L. Su, et al. (2006). "Chemical compositions, antioxidant capacities, and antiproliferative activities of selected fruit seed flours." Journal of agricultural and food chemistry **54**(11): 3773-3778.
Payne, C., P. K. Bowyer, et al. (2009). "Interaction of astringent grape seed procyanidins with oral epithelial cells." Food Chemistry **115**(2): 551-557.
Pellerin, P., T. Doco, et al. (1996). "Structural characterization of red wine rhamnogalacturonan II." Carbohydrate research **290**(2): 183-197.
Pellerin, P., S. Vidal, et al. (1995). "Characterization of five type II arabinogalactan-protein fractions from red wine of increasing uronic acid content." Carbohydrate research **277**(1): 135-143.
Perez‐Maldonado, R. A., B. W. Norton, et al. (1995). "Factors affecting in vitro formation of tannin‐protein complexes." Journal of the Science of Food and Agriculture **69**(3): 291-298.
Pizzolato, P. and R. D. Lillie (1973). "MAYER'S TANNIC ACID-FERRIC CHLORIDE STAIN FOR MUCINS." Journal of Histochemistry & Cytochemistry **21**(1): 56-64.
Poncet-Legrand, C., T. Doco, et al. (2007). "Inhibition of grape seed tannin aggregation by wine mannoproteins: Effect of polysaccharide molecular weight." American Journal of Enology and Viticulture **58**(1): 87-91.
Poncet-Legrand, C., A. Edelmann, et al. (2006). "Poly(l-proline) interactions with flavan-3-ols units: Influence of the molecular structure and the polyphenol/protein ratio." Food Hydrocolloids **20**(5): 687-697.

Prinz, J. F. and P. W. Lucas (2000). "Saliva tannin interactions." Journal of Oral Rehabilitation **27**(11): 991-994.
Proctor, G. B. and G. H. Carpenter (1998). "The function of salivary proteins and the regulation of their secretion by salivary glands." Biomedical Reviews **9**: 3-15.
Rantonen, P. J. and J. H. Meurman (1998). "Viscosity of whole saliva." Acta odontologica Scandinavica **56**(4): 210-214.
Reynolds, A. G. (2010). Managing Wine Quality: Viticulture and Wine Quality, Elsevier.
Richter, R., A. Mukhopadhyay, et al. (2003). "Pathways of lipid vesicle deposition on solid surfaces: a combined QCM-D and AFM study." Biophysical journal **85**(5): 3035-3047.
Rinaldi, A., A. Gambuti, et al. (2012). "Precipitation of Salivary Proteins After the Interaction with Wine: The Effect of Ethanol, pH, Fructose, and Mannoproteins." Journal of Food Science **77**(4): C485-C490.
Riou, V., A. Vernhet, et al. (2002). "Aggregation of grape seed tannins in model wine—effect of wine polysaccharides." Food Hydrocolloids **16**(1): 17-23.
Roberts, M. (1990). "Edwin Haslam. Plant Polyphenols—Vegetable Tannins Revisited Cambridge University Press, Cambridge, 1989. £37.50." Phytotherapy Research **4**(4): ii-ii.
Robichaud, J. L. and A. C. Noble (1990). "Astringency and bitterness of selected phenolics in wine." Journal of the Science of Food and Agriculture **53**(3): 343-353.
Rochow, E. (2012). An introduction to microscopy by means of light, electrons, x-rays, or ultrasound, Springer Science & Business Media.
Roper, S. (1983). "Regenerative impulses in taste cells." Science. **220**(4603): 1311-1312.
Rossetti, D., G. E. Yakubov, et al. (2008). "Interaction of human whole saliva and astringent dietary compounds investigated by interfacial shear rheology." Food Hydrocolloids **22**(6): 1068-1078.
Round, A., M. Berry, et al. (2002). "Heterogeneity and persistence length in human ocular mucins." Biophysical Journal **83**(3): 1661-1670.
Round, A. N., M. Berry, et al. (2002). "Heterogeneity and persistence length in human ocular mucins." Biophysical Journal **83**(3): 1661-1670.
Rykke, M., G. Smistad, et al. (1995). "Micelle-like structures in human saliva." Colloids and Surfaces B: Biointerfaces **4**(1): 33-44.
Rykke, M., A. Young, et al. (1997). "Transmission electron microscopy of human saliva." Colloids and Surfaces B: Biointerfaces **9**(5): 257-267.
Sauerbrey, G. (1959). "Verwendung von Schwingquarzen zur Wägung dünner Schichten und zur Mikrowägung." Zeitschrift für physik **155**(2): 206-222.
Schiffman, S. S., M. S. Suggs, et al. (1992). "Chorda tympani and lingual nerve responses to astringent compounds in rodents." Physiology & behavior **51**(1): 51-63.
Schipper, R. G., E. Silletti, et al. (2007). "Saliva as research material: biochemical, physicochemical and practical aspects." Archives of oral biology **52**(12): 1114-1135.
Schofield, P., D. Mbugua, et al. (2001). "Analysis of condensed tannins: a review." Animal Feed Science and Technology **91**(1): 21-40.
Schramm, G. (1994). A practical approach to rheology and rheometry, Haake Karlsruhe.
Schwarz, W. (1987). "The rheology of saliva." Journal of dental research **66**(2 suppl): 660-666.
Scollary, G. R., G. Pásti, et al. (2012). "Astringency response of red wines: potential role of molecular assembly." Trends in Food Science & Technology **27**(1): 25-36.
Semichon, L. (1927). "Les pectines des raisins et le moelleux des vins." Chimie et Industrie **17**: 25-32.
Shama, F. and P. Sherman (1973). "Variation in stimuli associated with oral evaluation of the viscosities of glucose solutions." Journal of Texture Studies **4**(2): 254-262.
Sheehan, J. K. and D. J. Thornton (2000). Heterogeneity and size distribution of gel-forming mucins. Glycoprotein Methods and Protocols, Springer**:** 87-96.
Siebert, K. J., N. V. Troukhanova, et al. (1996). "Nature of Polyphenol-Protein Interactions." Journal of Agricultural and Food Chemistry **44**(1): 80-85.

Simon, C., K. Barathieu, et al. (2003). "Three-Dimensional Structure and Dynamics of Wine Tannin−Saliva Protein Complexes. A Multitechnique Approach†." Biochemistry **42**(35): 10385-10395.

Simon, S., W. Hall, et al. (1992). "Astringent-tasting compounds alter ion transport across isolated canine lingual epithelia." Pharmacology Biochemistry and Behavior **43**(1): 271-283.

Singleton, V. (1982). Grape and wine phenolics: background and prospects. Grape and Wine Centennial Symposium, Proceedings of the University of California, Davis.

Singleton, V., H. Sieberhagen, et al. (1975). "Composition and sensory qualities of wines prepared from white grapes by fermentation with and without grape solids." American Journal of Enology and Viticulture **26**(2): 62-69.

Skoog, D. A. H., F. J.; Crouch, S. R. (2007). Principles of Instrumental Analysis. Belmont,CA, Thomson Higher Education.

Soares, R. V., T. Lin, et al. (2004). "Salivary micelles: identification of complexes containing MG2, sIgA, lactoferrin, amylase, glycosylated proline-rich protein and lysozyme." Archives of Oral Biology **49**(5): 337-343.

Soares, S., N. Mateus, et al. (2007). "Interaction of Different Polyphenols with Bovine Serum Albumin (BSA) and Human Salivary α-Amylase (HSA) by Fluorescence Quenching." Journal of Agricultural and Food Chemistry **55**(16): 6726-6735.

Soares, S., R. Vitorino, et al. (2011). "Reactivity of Human Salivary Proteins Families Toward Food Polyphenols." Journal of Agricultural and Food Chemistry **59**(10): 5535-5547.

Soares, S. I., R. M. Gonçalves, et al. (2009). "Mechanistic approach by which polysaccharides inhibit α-amylase/procyanidin aggregation." Journal of agricultural and food chemistry **57**(10): 4352-4358.

Souquet, J.-M., V. Cheynier, et al. (1996). "Polymeric proanthocyanidins from grape skins." Phytochemistry **43**(2): 509-512.

Spence, C. (2013). "Multisensory flavour perception." Current Biology **23**(9): R365-R369.

Spielman, A. I. (1990). "Interaction of Saliva and Taste." Journal of Dental Research **69**(3): 838-843.

Stanley, N. and L. Taylor (1993). "Rheological basis of oral characteristics of fluid and semi-solid foods: a review." Acta psychologica **84**(1): 79-92.

Steinert, P. M. and L. N. Marekov (1995). "The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked components of the human epidermal cornified cell envelope." Journal of Biological Chemistry **270**(30): 17702-17711.

Stokes, J. R. (2012). 'Oral' Rheology. Food Oral Processing: Fundamentals of Eating and Sensory Perception. J. C. Lina Engelen, Wiley-Blackwell.

Stokes, J. R. (2012). "'Oral'Rheology." Food oral processing: Fundamentals of eating and sensory perception: 225-263.

Stokes, J. R., M. W. Boehm, et al. (2013). "Oral processing, texture and mouthfeel: From rheology to tribology and beyond." Current Opinion in Colloid & Interface Science **18**(4): 349-359.

Stokes, J. R. and G. A. Davies (2006). "Viscoelasticity of human whole saliva collected after acid and mechanical stimulation." Biorheology **44**(3): 141-160.

Stokes, J. R. and G. A. Davies (2007). "Viscoelasticity of human whole saliva collected after acid and mechanical stimulation." Biorheology **44**(3): 141-160.

Strumeyer, D. H. and M. J. Malin (1970). "Resistance of extracellular yeast invertase and other glycoproteins to denaturation by tannins." Biochem J **118**(5): 899-900.

Swain, T. a. B.-S., E.C, Ed. (1962). Flavonoid compounds. In: 'Comparative Biochemistry'.

Tabak, L. A. (1995). "In defense of the oral cavity: structure, biosynthesis, and function of salivary mucins." Annual review of physiology **57**(1): 547-564.

Taira, S., M. Ono, et al. (1997). "Reduction of persimmon astringency by complex formation between pectin and tannins." Postharvest Biology and Technology **12**(3): 265-271.

Tanious, F. A., B. Nguyen, et al. (2008). Biosensor ‐ Surface Plasmon Resonance Methods for Quantitative Analysis of Biomolecular Interactions. Methods in Cell Biology, Academic Press. **Volume 84:** 53-77.

Thorngate, J. H. and A. C. Noble (1995). "Sensory evaluation of bitterness and astringency of 3R(−)-epicatechin and 3S(+)-catechin." Journal of the Science of Food and Agriculture **67**(4): 531-535.

Todhunter, I. (1886). A History of the Theory of Elasticity and of the Strength of Materials: Galilei to Saint-Venant, 1639-1850.-v. 2. pt. 1-2. Saint-Venant to Lord Kelvin, University Press.

Turssi, C. P., J. J. Faraoni, et al. (2006). "Analysis of potential lubricants for in vitro wear testing." Dental Materials **22**(1): 77-83.

Van der Reijden, W., E. Veerman, et al. (1992). "Shear rate dependent viscoelastic behavior of human glandular salivas." Biorheology **30**(2): 141-152.

Van der Reijden, W., E. Veerman, et al. (1993). "Rheological properties of commercially available polysaccharides with potential use in saliva substitutes." Biorheology **31**(6): 631-642.

Van Klinken, B. J., J. Dekker, et al. (1995). "Mucin gene structure and expression: protection vs. adhesion." American Journal of Physiology - Gastrointestinal and Liver Physiology **269**(5): G613-G627.

Van Nieuw Amerongen, A., J. Bolscher, et al. (2004). "Salivary proteins: protective and diagnostic value in cariology?" Caries research **38**(3): 247-253.

van Nieuw Amerongen, A., J. G. M. Bolscher, et al. (2004). "Salivary Proteins: Protective and Diagnostic Value in Cariology?" Caries Research **38**(3): 247-253.

Veerman, E., M. Valentijn-Benz, et al. (1989). "Viscosity of human salivary mucins: effect of pH and ionic strength and role of sialic acid." Journal de biologie buccale **17**(4): 297-306.

Vidal, S., L. Francis, et al. (2003). "The mouth-feel properties of grape and apple proanthocyanidins in a wine-like medium." Journal of the science of food and agriculture **83**(6): 564-573.

Vidal, S., L. Francis, et al. (2004). "The mouth-feel properties of polysaccharides and anthocyanins in a wine like medium." Food Chemistry **85**(4): 519-525.

Vidal, S., P. Williams, et al. (2003). "The polysaccharides of red wine: total fractionation and characterization." Carbohydrate Polymers **54**(4): 439-447.

Vitorino, R., M. J. C. Lobo, et al. (2004). "Identification of human whole saliva protein components using proteomics." Proteomics **4**(4): 1109-1115.

Vogel, N., J. Zieleniecki, et al. (2012). "As flat as it gets: ultrasmooth surfaces from template-stripping procedures." Nanoscale **4**(13): 3820-3832.

Waterman, H., C. Blom, et al. (1988). "Rheological properties of human saliva." Archives of oral biology **33**(8): 589-596.

Wickstrom, C., J. Davies, et al. (1998). "MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage." Biochem. J **334**: 685-693.

Wickström, C., J. R. Davies, et al. (1998). "MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage." Biochem. J. **334**(3): 685-693.

Wood, F. (1968). "Psychophysical Studies on the Consistency of Liquid Foods." Rheology and Texture of Food Stuffs, SCI Monograph(27): 40-49.

Wu, A. M., G. Csako, et al. (1994). "Structure, biosynthesis, and function of salivary mucins." Molecular and cellular biochemistry **137**(1): 39-55.

Yakubov, G. E., A. Papagiannopoulos, et al. (2007). "Molecular Structure and Rheological Properties of Short-Side-Chain Heavily Glycosylated Porcine Stomach Mucin." Biomacromolecules **8**(11): 3467-3477.

Yao, J.-W., Y. Xiao, et al. (2012). "Effect of various pH values, ionic strength, and temperature on papain hydrolysis of salivary film." European Journal of Oral Sciences **120**(2): 140-146.

Yao, Y., J. Grogan, et al. (2001). "Compositional analysis of human acquired enamel pellicle by mass spectrometry." Archives of Oral Biology **46**(4): 293-303.