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Mei Wang

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Mei Wang. Salivary properties involved in astringency sensitivity in human differ as a function of age. Biochemistry, Molecular Biology. Université Bourgogne Franche-Comté, 2022. English. NNT : 2022UBFCK045 . tel-04140631

HAL Id: tel-04140631

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**THESE DE DOCTORAT DE L'ETABLISSEMENT UNIVERSITE BOURGOGNE
FRANCHE-COMTE
PREPAREE A L'UNIVERSITE DE BOURGOGNE**

Ecole doctorale n°554
Environnement – Santé

Doctorat de Biochimie et biologie moléculaire

Par

Mei Wang

Les propriétés de la salive impliquées dans la sensibilité à l'astringence chez
l'homme différent en fonction de l'âge

Thèse présentée et soutenue à Dijon, le 20 Décembre 2022

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Titre : Les propriétés de la salive impliquées dans la sensibilité à l'astringence chez l'homme diffèrent en fonction de l'âge

Mots clés : astringence, protéines salivaires, vieillissement, flavour

Résumé : L'astringence est une caractéristique sensorielle importante des aliments et des boissons contenant des polyphénols. Cependant, la perception de l'astringence chez les personnes âgées est peu documentée. L'hypothèse actuelle sur le mécanisme de l'astringence implique l'agrégation de la pellicule mucoale par les tanins et un rôle protecteur par les protéines salivaires, en particulier les protéines riches en proline (PRP). Le but de ce travail était d'évaluer la sensibilité à l'astringence en fonction de l'âge et de la salive (débit et composition). Cinquante-quatre panélistes (30 âgées et 24 jeunes) ont participé à cette étude. L'astringence a été évaluée par la procédure 2-Alternative Forced Choice. Pour les PRP salivaires, une méthodologie de western blot a été mise au point pour évaluer spécifiquement les PRP glycosylées (gPRP) et basiques (bPRP). Les résultats ont montré que le seuil d'astringence était significativement plus élevé dans le groupe des personnes âgées que dans le groupe des jeunes.

Une corrélation négative entre le flux salivaire et la valeur seuil a été observée uniquement dans le groupe des jeunes. En ce qui concerne les quantités de PRP, les résultats ont montré qu'il n'y avait pas de différence entre les jeunes et les personnes âgées concernant les niveaux de g- et bPRP. Cependant, une corrélation négative entre les niveaux de bPRP et le seuil d'astringence a été observée dans le groupe des personnes âgées alors qu'une corrélation positive a été observée avec le niveau de gPRP dans le groupe des jeunes. Il s'agit de la première étude décrivant une différence dans la perception de l'astringence orale entre les sujets jeunes et âgés. Les quantités de PRP salivaires semblent expliquer cette différence mais seulement à l'échelle de chaque groupe. Ces résultats suggèrent que des mécanismes différents interviennent dans la sensibilité à l'astringence en fonction de l'âge. Des analyses complémentaires prenant en compte d'autres protéines salivaires liant les tanins devraient être réalisées à l'avenir afin de décrypter ces mécanismes au sein deux populations.

Title : Salivary properties involved in astringency sensitivity in human differ as a function of age

Keywords : astringency, salivary proteins, ageing, flavour

Abstract: Astringency is an important sensory characteristic of foods and beverages containing polyphenols. However, astringency perception in elderly people is not documented. Current hypothesis on astringency mechanism involved aggregation of the mucosal pellicle by tannins and its protective role by salivary proteins in particular proline rich-proteins (PRPs). The aim of the present work was to evaluate sensitivity to

A negative correlation between salivary flow and threshold value was observed in the young group only. When it comes to PRP amounts, the results showed that there was no difference between young and elderlies regarding g- and bPRP levels. However, a negative correlation between bPRPs levels and astringency threshold was observed in elderly group while a positive correlation was observed for gPRPs level in the young group.

astringency in function of age and saliva (flow and composition). Fifty-four panelists including (30 elderlies and 24 young) participated in this study. Astringency was evaluated by 2-Alternative Forced Choice procedure. For salivary PRPs, a western blot methodology was set up to evaluate specifically glycosylated (gPRPs) and basic PRPs (bPRPs). Results showed that the astringency threshold was significantly higher in the elderlies compared to the young group.

This is the first study describing a difference in oral astringency perception between young and elderly subjects related to respective salivary properties. Salivary PRP amounts should explain this difference but only at the group scale. These results suggest that different mechanisms occur in astringency sensitivity as a function of age. Further analysis considering other salivary tannin binding proteins should be performed in the future in order to decipher these mechanisms in both populations.

Acknowledgement

First of all, I would like to extend my deep gratitude to my supervisors, Dr. Gilles Feron and Dr. Francis Canon, for their patient guidance, insightful teaching, continuous encouragement and support through the whole process of my thesis. Without their guidance and especially the efforts they have made to revise the manuscript, it would be a really difficult task for me to complete my thesis. Meanwhile, I really appreciate my supervisor, Dr. Gilles Feron for his help and consideration of my life in France. This is a great relief to me as an overseas student.

I would like to thank Helene Brignot and Chantal Septier, who gave me a lot of guidance on the experiment, which enabled the experiment to be completed smoothly. In addition, I would like to thank other colleagues, subjects and all the people involved in the experiment for their contribution to the thesis.

My sincere gratitude also goes to Prof. Jianshe Chen, Dr. Martine Morzel, Dr. Vanderberghe-Descamp and Dr. Loïc Briand for their acceptance to serve on my thesis and time to attend my thesis defense. Meanwhile, I would like to express my special thanks to Prof. Chen. Without him, I would not be where I am today.

My sincere gratitude also goes to Dr. Claire Sulmont-Rosse and Dr. Sylvain Guyot for their insightful comments and encouragement on my thesis committee during the first three years of my doctoral career.

I would also like to express thanks to my friends, who are always there for me when I need them to accompany with me, even though we are miles apart. They are willing to spend their time on listening to me, chatting with me and sharing with me.

Last but not least, I am deeply indebted to my parents, who gave me immense support, encouragement, care and comfort over the past four years, especially during the special pandemic time, they worried about me a lot but without a word of complaint. At the same time, they gave me great courage and strength. As a Chinese poet wrote, “Such kindness of warm sun, can’t be repaid by grass”. I will do my best to repay my parents in the years ahead. I also owe my deeply gratitude to my grandparents, who give me their loving considerations, encouragement and great confidence in me all through these years. I would also like to extend my great gratitude to my motherland, China scholarship council for sponsoring my PhD study in France, I will put my heart and soul into serving my country after I go back to China. I love my parents, my grandparents and my motherland.

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Communications

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Wang, M., Septier, C., Brignot, H., Martin, C., Canon, F., & Feron, G. (2022). Astringency Sensitivity to Tannic Acid: Effect of Ageing and Saliva. *Molecules*, 27(5). doi:10.3390/molecules27051617

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Wang M., Septier, C., Brignot, H., Martin, C., Canon, F., & Feron, G. Role of saliva in perception of astringency in human in function of age. 4th Asian Sensory and Consumer Research Symposium, December 5-7, 2021, Online.

Wang M., Septier, C., Brignot, H., Martin, C., Canon, F., & Feron, G. Role of saliva in perception of astringency in human in function of age. Forum des Jeunes Chercheurs UBFC, June 16-17, 2022, Dijon, France.

Wang M., Septier, C., Brignot, H., Martin, C., Canon, F., & Feron, G. Salivary properties involved in astringency sensitivity IN human differ as a function of age. 10th European

Conference on Sensory and Consumer Research. September 13-16, 2022, Turku, Finland.

GENERAL INTRODUCTION

General introduction

With the aging of society, the proportion of elderly people worldwide is growing rapidly. It has been reported that by the middle of this century, the global population aged 60 and over is projected to expand by more than three times, to reach nearly 2.1 billion (Assad-Bustillos, Tournier, Septier, Della Valle, & Feron, 2019).

Ageing is often accompanied by deterioration in oral health (e.g. reduced saliva secretion), which could alter flavour perception, leading to changes in food choices, eating habits and dietary intake. These alterations of feeding behaviour could be one factor compromising health status and increase dietary-related diseases e.g. increase of oxidative stress in older people (Vandenberghe-Descamps, Laboure, Septier, Feron, & Sulmont-Rosse, 2018).

Polyphenolic compounds are known to be effective scavengers of reactive oxygen species and may also function indirectly as antioxidants (Koleckar et al., 2008). Therefore, the supplementation of food with polyphenols could be a strategy to maintain a good general health status, especially in the elderly. However, polyphenols can be at the origin of the astringency sensation.

Astringency is an important sensory attribute of food and beverage that containing polyphenols, especially tannins. It is defined 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” by the American Society for Testing of Materials (ASTM). It is usually accepted that the interactions of salivary proteins, especially proline-rich proteins (PRPs), with tannins lead to their aggregation and/or precipitation, causing a loss of the saliva lubrication in oral cavity. It may also involve the adsorption of tannins on the oral mucosa, either by mechanoreceptors after the increase of the friction forces at the surface of the epithelial cells or by the detection of the aggregation of the mucosal pellicle (Ployon et al. 2018), which may involve the transmembrane mucin MUC1 as recently proposed by Canon et al. (2021) (Canon et al., 2021; M. Wang et al., 2022). In those two hypotheses, PRPs play a protective role and prevent astringency by binding tannins before their interaction with the mucosal pellicle. Astringency is perceived as a quality parameter and desired in balanced levels in some foodstuffs like red wine. However, it is usually regarded as an unpleasant oral sensation when perceived with high intensity, which limits the use and promotion of polyphenols at moderate levels in food despite their health benefits. These benefits on health are particularly beneficial to the elderly people. However, the astringency perception in elderly people is not documented, although the influence of ageing on the perception of other taste modalities has been widely documented. The modifications of saliva properties, including salivary flow rate and salivary composition, are considered to be key factors impacting food perception.

Therefore, in the context of an ageing population around the world, the objective of the present work is to investigate the role of salivary properties (salivary flow rate and salivary protein composition) in astringency sensitivity, while considering its evolution as a function

of age. A particular focus is performed on the role of PRPs.

This work is divided into six chapters. The first chapter presents a literature review on the saliva and its role in each taste modalities perception, and the effects of aging on salivary properties. It also presents the effects of aging on taste and olfaction perception and the causes of age-related taste and olfactory impairments. Last but not least, it presents hypothesis on astringency mechanisms, tannins properties, proline-rich protein (PRPs) properties, the interaction between tannins and PRPs and the impacts of tannins and PRPs properties on astringency perception.

The second chapter presents the methods and materials that have been used in the present work. It includes i) the sensory methodology (2-alternative forced choice) that was set up in preliminary experiment and was used in formal sensory experiment, ii) the methodologies to evaluate the PRPs levels that were attempted to be developed in preliminary biology experiment and iii) western-blot analysis that was set up in formal biology experiment that to explore the amounts of PRPs in the saliva of subjects. In addition, dot-blot was adopted to perform the comparison of MUC5B pixel volume ratio in the young and elderly group.

The third chapter presents the work that has been conducted on sensory experiment, for which fifty-four panelists including 30 elderly people (70-87 years) and 24 young people (24-35 years) were recruited. Astringency sensitivity was evaluated by 2-alternative forced choice method, salivary flow rate was determined by collecting saliva from subjects before and after test sessions. Furthermore, salivary protein amounts were also measured by Bradford protein assay. Bovine serum albumin (BSA) was used as the standard for calibration.

The fourth chapter presents the work on biology experiment. Western-blot method was developed to explore the amounts of PRPs in saliva of subjects. In addition, dot-blot method was developed in the supplementary experiment to quantitatively compare the MUC5B amount in the young and elderly group with the aim to identify other factors explaining the difference of astringency threshold between young and elderly populations.

The fifth chapter is a general discussion, conclusion, and perspectives for the further studies.

The final chapter is a substantial abstract of this thesis in French.

CHAPTER 1: STATE OF THE ART

CHAPTER 1: State of the art

1. Saliva

1.1 Basic concept of saliva secretion

Saliva is a physiological fluid that plays a significant role in oral cavity and food oral processing. The hypotonic fluid shows a pH of 6.0-7.0 at rest, during stimulation it increases to 7.4 (Spirk et al., 2019). It is secreted by three pairs of major salivary glands (parotid, submandibular and sublingual) and several minor salivary glands (buccal, labial, lingual, and palate) (Figure 1) (Feron, 2018; Xu et al, 2019). Salivary glands consist of two main cell types: one is the acini, which produce the saliva, and the second are ductal cells, conveying the saliva to the mouth. Acinar cells can be categorized into mucous and serous acinar cells. Mucous cells secrete viscous mucin in the vacuoles, while serous cells secrete water and enzymes. Most of the acinar cells in parotid glands are serous, while those of the sublingual and minor glands are mucous. In the submandibular gland, 10% of acinar cells are mucous, and 90% are serous (Toan & Ahn, 2021). The other is the ductal cells, which modify and convey the saliva to the mouth (Carpenter, 2012). Saliva secretion is deemed to be a two-stage process, with initial secretion of an aqueous plasma-like primary fluid by the acinar cells and its subsequent modification during passage through the water impermeable ductal cell system (Dodds, 2005).

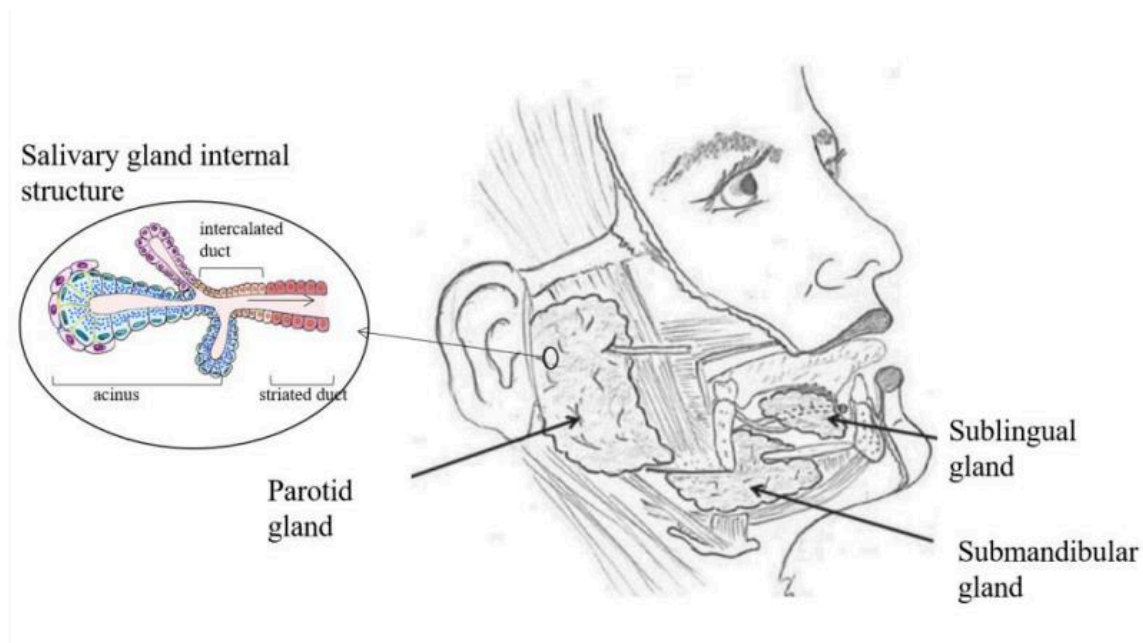


Figure 1. Human salivary glands (F. Xu, Laguna, & Sarkar, 2019).

1.2 Saliva properties

Oral health and physiological processes present in the oral cavity can be affected by the rheological properties of saliva. Lubrication is essential to prevent abrasion in the mouth and form cohesive food bolus during oral processing (Gibbins & Carpenter, 2013). Whole saliva can be classified as a non-Newtonian fluid exhibiting a shear thinning behavior, which is due to the presence of large glycoproteins such as mucins (Mosca & Chen, 2017). The saliva produced by the different salivary glands shows different rheological properties. Submandibular and sublingual salivas contain much more mucins that result in a shear thinning viscosity. Parotid saliva does not contain mucins. It is considered as a Newtonian fluid, as its viscosity and shear are independent and slightly higher than the viscosity of water (Vanderreijden, Veerman, & Amerongen, 1993). It was shown that stimulated saliva has a higher friction coefficient than unstimulated saliva, probably due to the increase of the parotid gland secretion which have a higher protein content and a lower viscosity of parotid saliva (Prinz, de Wijk, & Huntjens, 2007). The properties of saliva can be affected by intrinsic factors and extrinsic factors. Intrinsic factors refer to individual characteristics such as age, gender, health status, emotional stress that lead to a large inter-individual variability in the characteristics of saliva (Mosca & Chen, 2017). Extrinsic factors refer to the type of stimulation and salivary collection time. It has been reported that salivary flow, composition and rheology, buffer capacity, amylase activity, mucin and total protein concentration can be affected by the type of stimulation (Engelen et al., 2007). It has been also shown that saliva samples collected in the morning and afternoon differed in terms of protein, lipocalin and uric acid concentration, lysozyme activity, and amylolysis (Neyraud, Palicki, Schwartz, Nicklaus, & Feron, 2012). Therefore, these factors should be taken into account when researchers perform saliva investigation and data analysis.

1.2.1 Salivary flow

Salivary flow depends on the physiological status. There are stimulated saliva, which is secreted mainly by the parotid gland and unstimulated saliva, which is secreted mainly by the submandibular and sublingual glands (Mosca & Chen, 2017). Unstimulated flow (approximate flow rate 0.1-0.5 mL/min) (Boehm, Yakubov, Stokes, & Baier, 2020) is the result of low-level autonomic stimulation by the higher centers, including the orbitofrontal cortex and amygdala of the brain, via the salivary centers within solitary tract nuclei in the brainstem to act on salivary glands to produce saliva (Carpenter, 2012). In the unstimulated state, the contribution to the whole mouth saliva volume is approximately 60% from the submandibular glands, 25% from the parotid glands, 7%-8% from the sublingual glands and 8% from the minor salivary glands (Pedersen, Sorensen, Proctor, & Carpenter, 2018). Stimulated saliva (approximate flow rate 1-5 mL/min) (Boehm et al., 2020) is a constant process in conscious humans that is upregulated by a reflex mostly stimulated by taste and chewing (Carpenter, 2012). With stimulation, the parotid gland increases its contribution to the whole mouth saliva volume by 10% (Dawes, 2008). Generally, taste and chewing are considered as factors that stimulate saliva secretion. The mean flow rates during chewing varied between 3.15 and 4.94 mL/min (Dawes, 2008). Citric acid is often used as taste stimulant, because it generates by far the largest salivary flows, often at a tenth of the concentration of other stimulants, such as sweet, salty, bitter, and umami (Hodson & Linden,

2006). The salivary flow rate triggered by 5% of citric acid is 7.07 ± 2.16 mL/min (S. Watanabe & Dawes, 1988b), which is a protective mechanism as acidic pH may alter the teeth. Therefore, authors concluded that sour stimulation is a much more effective salivary stimulus than mechanical stimulation from chewing in producing the flow of saliva (S. Watanabe & Dawes, 1988a). During sleep, the flow rate is negligible (approximately 0-0.1 mL/min) (S. Watanabe & Dawes, 1988b). It is the reason why our teeth are particularly susceptible to attack at this time by oral microorganisms. It has been proposed that the total volume of saliva secreted each day is about 600 mL (S. Watanabe & Dawes, 1988a). When unstimulated salivary flow rate is less than 0.1 mL/min or chewing-stimulated whole mouth saliva flow rates are below 0.7 mL/min, it is regarded as hyposalivation (Pedersen et al., 2018). In addition, the salivary flow rates of minor glands are less than 1 μ L/min (Carpenter, 2012).

1.2.2 Salivary components

Saliva mainly consists of 99% of water, almost 3000 proteins and peptides, electrolytes and small molecules (Canon, Neiers, & Guichard, 2018; Dawes, 2008).

More than 95% of salivary proteins belong to the major salivary protein families, which include acidic, basic and glycosylated PRPs, amylase, mucins (MUC5B and MUC7), cystatins, histatins and statherin (Helmerhorst & Oppenheim, 2007).

Basic PRPs and glycosylated PRPs are secreted exclusively by parotid glands, whereas acidic can be secreted by submandibular and parotid glands. These PRPs contain high levels of proline (25-42%), glycine (16-22%) and glutamine (15-28%), which are 70% of all amino acids. They constitute about 70% of the total content of parotid saliva proteins (Y. Lu & Bennick, 1998; Sarni-Manchado, Canals-Bosch, Mazerolles, & Cheynier, 2008). PRPs have been considered as a “first line of defense” against the detrimental effects of polyphenols in the diet (Sarni-Manchado et al., 2008).

Amylase is the single most abundant protein in parotid saliva. It is generally considered to be associated with the initial digestion of starch-containing foods. However, the contact time of salivary amylase and food starch is generally short, and the acidic pH of gastric fluid mostly stops the amylolytic activity after swallowing (Laguna, Fiszman, & Tarrega, 2021).

Mucins are high-molecular-weight glycoproteins of submandibular/sublingual saliva with an elongated structure, it is the main contributor to the viscoelastic behavior of saliva (Michael W.J.Dodds, 2005). In addition, mucins are also secreted by minor glands, which are important in maintaining a mucin-rich layer adjacent to the mucosa.

Cystatins are protease inhibitors in submandibular/sublingual saliva (Carpenter, 2012).

Histatins are characterized by a high content of histidine and are small molecules (3-5 kDa) found in both parotid and submandibular/sublingual saliva. It has been reported that histatins have also a high affinity for tannins (Bennick, 2002; Shimada, 2006), it also has potent

anti-candida effects (Michael W.J.Dodds, 2005).

Statherin has important roles in binding calcium to form enamel pellicle on the teeth surface. Statherin also has a function of lubricating the teeth, which is crucial to preventing the teeth from chipping and wearing during chewing (Carpenter, 2012).

The main electrolytes in whole saliva include sodium, potassium, calcium, magnesium, chloride, bicarbonate, phosphate, thiocyanate and fluoride. Of the salivary electrolytes, calcium, phosphate, bicarbonate and fluoride are of particular importance for oral health (Dawes, 2008). The net effect of saliva is supersaturated with respect to hydroxyapatite, which is the main mineral of teeth (Dawes, 2008). Thus, tooth mineral will not dissolve in saliva or plaque fluid. Bicarbonate in saliva is the main buffering component against acid, but it is effective at high salivary flow rates because its concentration increases markedly with the flow rate. The fluoride concentration in saliva is low, about 1 micromole per liter, but this helps keep the saliva somewhat more supersaturated with respect to fluorapatite than to hydroxyapatite (Pan & Darvell, 2007).

Table 1. Salivary main components and their main secretion sources and roles.

	Main Salivary Components	Main Secretion Sources	Functions
Organic	Basic PRPs	Parotid glands	Anti-viral and a high affinity for tannins
	Acidic PRPs	Submandibular and parotid glands	Bind to the surface of teeth and involve in the formation of the dental pellicle
	Glycosylated PRPs	Parotid glands	Provide oral lubrication, and prevent bacterial agglutination
	Amylase	Parotid glands	Initial digestion of starch-containing foods
	Mucins	Submandibular/sublingual glands	Main contributor to the lubrication and viscoelastic behavior of saliva
	Cystatins	Submandibular/sublingual glands	Cysteine proteases inhibitors
	Histatins	Parotid and submandibular/sublingual glands	Antifungal and a high affinity for binding tannins
	Statherin	Submandibular/sublingual glands	Binding calcium to form enamel pellicle on the

			teeth surface
Inorganic	Calcium	Submandibular/sublingual and parotid glands	Maintenance of teeth mineral integrity
	Phosphate	Submandibular/sublingual and parotid glands	Main mineral of teeth
	Bicarbonate	Submandibular/sublingual and parotid glands	Main buffer against acid
	Fluoride	Submandibular/sublingual and parotid glands	Keep the saliva more supersaturated with respect to fluorapatite than to hydroxyapatite

1.3 Saliva functions

Saliva serves multiple functions that are maintaining oral health and also during food consumption. One of the most important functions is to contribute to the lubrication and protection of the surfaces of the oral cavity. Saliva is also helping to the formation of pellicle, which is a protein layer that covers the surface of tooth enamel and other exposed buccal surfaces (Laguna et al., 2021). The buffering capacity of specific components (e.g. bicarbonate) can maintain salivary pH around 7.0, therefore, protecting the teeth and oropharyngeal mucosa from dietary acids or acids produced from the oral bacterial fermentation of carbohydrates (Pedersen et al., 2018). Moreover, saliva is supersaturated with hydroxyapatite, which prevents tooth mineral from dissolving in saliva or plaque fluid, unless the saliva or plaque is acidified (Dawes, 2008). Last but not least, saliva contains substances such as lysozyme, histatins and mucins that have antibacterial, antiviral and/or antifungal properties which modulate the oral microbiota in different ways (Dawes, 2008).

During oral food consumption, saliva plays three major roles. At first, during mastication process, food is broken down into smaller particles to form a bolus moisturized and lubricated by saliva, which can be safely swallowed. During this process, salivary mucins play a key role. They bind masticated food into a coherent and slippery bolus that can easily slide through the oesophagus. Saliva enzymes also initiate the digestion of carbohydrates at the beginning of the process of food digestion, such as alpha-amylase (Munoz-Gonzalez et al., 2018; Vandenberghe-Descamps et al., 2016). Secondly, saliva plays an essential role in oral perception of food flavour (taste, texture, aroma etc.). The sensation of taste is activated during the initial phase of food ingestion, which is important for the differentiation of essential nutrients from harmful and potentially toxic substances. Saliva acts as a solvent of tasting compounds as food particles need to be in fluid in order to reach the taste receptor cells located in the taste buds within the lingual papillae (fungiform, foliate and vallate papillae). In addition, it has been shown that saliva composition can influence taste sensitivity because the upper surface of receptor cells are bathed by saliva (Pedersen et al., 2018).

Furthermore, texture perception is also related to saliva composition. Finally, during in-mouth food breakdown, saliva can affect aroma release depending on aroma compounds affinity with saliva, or salivary enzymes produce new aroma compounds (Vandenberghe-Descamps et al., 2016). Thirdly, saliva allows oral clearance. Indeed, it can remove food debris from oral cavity after swallowing. It has been acknowledged that salivation and swallowing are the important factors to clear microorganisms and bacteria from the cavity (Spirk et al., 2019). For all saliva functions, Carpenter (2012) summarized them with a graphic, as shown in Figure 2.

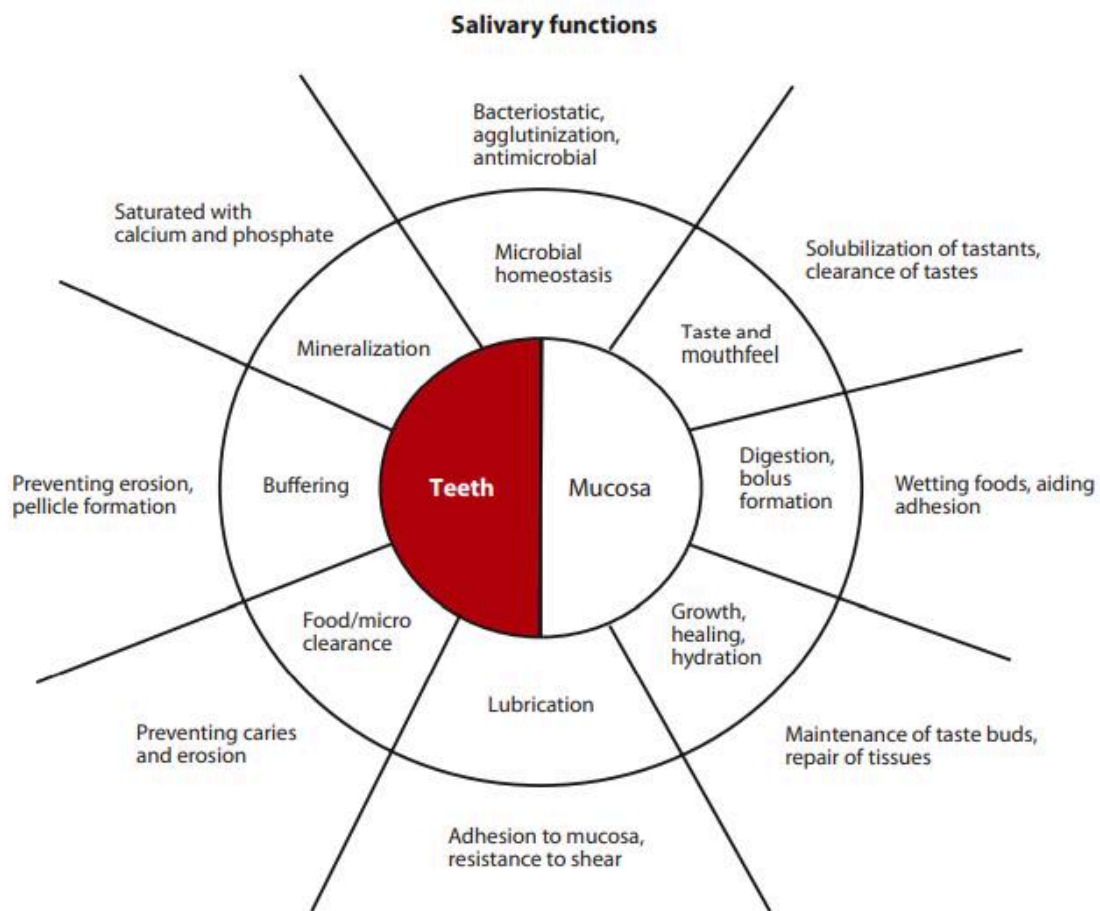


Figure 2. Schematic of main saliva functions (Carpenter, 2012).

1.4 Impact of saliva on taste perception

It has been suggested that saliva (flow rate, buffer capacity) and its composition (proteins, ions) can affect taste perception through continuously bathing oral cavity and stimulates taste receptors, thus playing a role in taste sensitivity (Fábián, Beck, Fejérdy, Hermann, & Fábián, 2015). A review collected data from literature focusing on the importance of unstimulated salivary taste substances (ions, amino acids and peptides, lipids and single sugars), on their relevant taste adaptation and their detection and recognition thresholds in humans (Feron, 2018). The author concluded that only a few salivary components like sodium, calcium and

glutamic acid may be involved in gustatory self-adaptation phenomenon. It has been shown that the concentration of taste compounds dissolved in saliva correlates better to taste perception than the initial concentration of tastants in the food material (Mosca & Chen, 2017).

For saltiness, it has been reported that higher is the salivary flow rates, lower are the sodium release and saltiness perception (Lawrence, Septier, Achilleos, Courcoux, & Salles, 2012). This could be due to the high volume of saliva that limited reabsorption of sodium within the salivary ducts. Accordingly, young healthy individuals with high salivary flows perceive less salty taste than individuals with low salivary flow (Fábián et al., 2015; Heinzerling, Stieger, Bult, & Smit, 2011). In addition, individuals with a low sodium content in saliva perceived salt-congruent aromas as more intense (Mosca & Chen, 2017).

For sourness, it has been shown that individuals with high salivary flows can neutralize the acidity of sour solutions more efficiently than individuals with low salivary flows in adults (Lugaz, Pillias, Boireau-Ducept, & Faurion, 2005). Recently, Zhang et al. (2022) reported participants with higher salivary buffering capacity perceived less sour taste with increased concentration of citric acid stimulants (Zhang, jiang, Chen & Wang, 2022). However, this is not absolutely conclusive that high salivary flow and high salivary buffer capacity can compromise the sour taste perception. Lugaz et al. (2005) indicated that subjects with high salivary flow rate compared to those with low salivary flow perceived higher sour intensity (Lugaz et al., 2005). In addition, salivary bicarbonate ions can reduce the concentration of free hydrogen ions and thereby affect sour taste (Pedersen et al., 2018).

For sweetness, it has been reported that saliva pH affects the sweetness sensitivity (Aoyama et al., 2017). Moreover, salivary proteome and glucose levels have been related to sweet taste sensitivity in young adults, subjects with higher levels of α -amylase had a lower sweetness sensitivity (Rodrigues et al., 2017).

For bitterness, there is a huge chemical diversity of bitter compounds. Some of them are phenolic compounds, which may interact with PRPs. It has been proposed that PRPs can affect bitter taste in mice, via their binding activity of bitter substances such as quinine, and then diminish the interaction of bitter substance with the taste receptor (Pedersen et al., 2018). It has been suggested that the proteolytic activity of human saliva is an important perireceptor factor associated to the sensitivity to the bitter taste of caffeine (Dsamou et al., 2012).

For umami, it has been observed that individual differences in salivary glutamate levels in stimulated whole saliva may influence perceived pleasantness of suprathreshold in monoglutamate solutions, the low glutamate levels group rated the higher monosodium glutamate (MSG) concentrations as more unpleasant (Feron, 2018; Scinska-Bienkowska et al., 2006). However, there was no difference between high glutamate levels and low glutamate levels regarding electrogustometric thresholds, rated intensity of the MSG samples and pleasantness of distilled water at the lower MSG concentrations (0.03-1.0%) (Scinska-Bienkowska et al., 2006).

For fatty taste, Mounayar et al. (2013) proposed that the proteolytic activity of human saliva plays a role in the perception of fatty (Mounayar, Septier, Chabanet, Feron, & Neyraud, 2013). Moreover, individuals with a high lipolytic activity perceived fat-congruent aromas as more intense (Mosca & Chen, 2017). Neyraud et al. (2012) suggested that composition (lipolysis, lipocalin) of saliva plays an important role in fat perception and liking (Neyraud et al., 2012). Moreover, it has been indicated that subjects overexpressing cystatin SN, cystatin D, zinc-alpha-2-glycoprotein and carbonic anhydrase 6 are sensitive to oleic acid (Mounayar et al., 2014b).

In conclusion, saliva plays all-important roles in taste and aroma sensations during food oral processing. What researchers should pay attention to are the type of saliva (stimulated or unstimulated, human or artificial) and type of stimulation (mechanical or gustatory). Additionally, the large inter-individual variability in saliva composition needs to be taken into account when interpreting saliva-related data.

1.5 Taste modalities modulate salivary composition

Some studies also investigated how taste modalities affect salivary proteome. Neyraud et al. (2006) performed proteomic analysis of human whole and parotid saliva following stimulation by different tastes, they observed that the number of proteins affected by taste stimulation increased in the order sweet < umami < bitter < acid, especially sweetness had almost no effect on saliva proteome, sour taste inducing a major increase in α -amylase (Neyraud, Sayd, Morzel, & Dransfield, 2006). A similar result regarding elevation of α -amylase concentrations after administration of citric acid in rabbit was observed (Gjorstrup, 1980). Mounayar et al. (2013) observed a significant increase in saliva antioxidant capacity and a significant decrease in lipolytic activity after oleic acid stimulation in a group with high oral sensitivity to oleic acid group compared to individual with lower sensitivity (Mounayar et al., 2013). The same research group also observed that subjects who were highly sensitive to fatty taste increased abundance of polymeric immunoglobulin receptor, rab GDP dissociation inhibitor beta, and organic acids, and decreased abundance of metabolites characteristic of mucins after stimulation of oleic acid (Mounayar et al., 2014a). Dsamou et al. (2012) indicated that subjects who are hypersensitive to bitterness have higher levels of amylase fragments, immunoglobulins, and serum albumin and/or serum albumin fragments and lower levels of cystatin SN in saliva (Dsamou et al., 2012). Another study observed that saliva proteome pattern was modified after tasting two bitter compounds (urea and quinine hydrochloride). Proteins involved in the oral cavity defense (e.g., thioredoxin, cystatin, parotid secretory proteins, etc.) or in inflammatory processes (transthyretin and transferrin) were quantitatively altered. In particular, the relative abundance of carbonic anhydrase VI also called gustin, a protein which is crucial to taste function, declined after tasting the urea solution (Quintana et al., 2009).

1.6 Saliva and aging

It is well-known that aging is often accompanied by a decrease in physical and physiological capabilities. It significantly impacts oral physiology, i.e. tooth loss, decreased muscle strength and tongue pressure, swallowing disorders and saliva modifications. Changes in saliva during ageing can be grouped into quantitative (flow rate) and qualitative (composition) properties (Vandenberghe-Descamps et al., 2018).

1.6.1 The effects of aging on salivary flow rate

The research on the effect of aging on salivary flow rate has been investigated considerably but the conclusions are contradictory. Some works reported that salivary flow rate decreases when age increase, regarding specific salivary glands or saliva type (Navazesh, Mulligan, Kipnis, Denny, & Denny, 1992; Percival, Challacombe, & Marsh, 1994; Tanida et al., 2001; Vandenberghe-Descamps et al., 2016), while other studies did not find age-related effect on salivary flow rate (Baum, Ship, & Wu, 1992; Benaryeh et al., 1986; Shern, Fox, & Li, 1993). This is probably due to the variations in the study design, saliva collection method or salivary glands secretion. Affoo et al. (2015) performed a meta-analysis from 47 studies regarding saliva and age (Affoo et al., 2015). They reported that unstimulated salivary flow and stimulated submandibular and sublingual salivary flow were significantly lower in elderly people. However, parotid and minor gland salivary flow rates did not appear to be significantly lower. These findings are consistent with the statement that aging is associated with decreased salivary flow in a gland specific manner. Moreover, they also indicated that the age-related decrease in salivary flow was not fully explained on the basis of medication or disease. Indeed, it has been also suggested that an average of 38.5% reduction of resting salivary flow and 38% reduction of stimulated salivary flow in healthy elderly people (70-92 years old) compared to young adults (22-55 years old) were independent of the dental status and drug intake (Vandenberghe-Descamps et al., 2016). Moreover, a 3-year-long longitudinal study performed on 26-90 years old healthy subjects, who did not use any medications, reported that there were no significant reductions in salivary gland function or salivary flow rate (Ship, Nolan, & Puckett, 1995). Authors concluded from this study that salivary gland dysfunction in an older person should not be considered a normal process of aging. In summary, inconsistent conclusions regarding salivary flow rate and aging are possibly due to (i) lack of details of saliva collection, (ii) limited number of subjects, (iii) no distinct age range, (iv) factors other than age-related processes in the elderly population (Xu et al., 2019). Therefore, these factors should be considered to compare results from similar studies.

1.6.2 Salivary hypofunction in the elderly people

It has been reported that saliva secretion decreases with age may cause salivary hypofunction. Salivary hypofunction is likely a symptom, the subjective complaint of dry mouth (xerostomia) (Gilbert, Heft, & Duncan, 1993). However, hyposalivation and xerostomia are two different concepts, which are not always correlated. Hyposalivation represents a decrease in the amount of saliva secreted to the oral cavity (Munoz-Gonzalez et al., 2018). It has been reported that hyposalivation is associated with difficulties in chewing and swallowing, changes in the oral mucosa, changes in nutrition, food choice, taste perception etc. (Gilbert et al., 1993;

Munoz-Gonzalez et al., 2018). A systematic literature review from 15 articles summarized the association between salivary hypofunction and food consumption including i) food oral processing (mastication/chewing, swallowing, orosensory perception), ii) food behavior (appetite and dietary intake), iii) nutritional status in elderly people (Munoz-Gonzalez et al., 2018). Authors indicated that most of the selected studies have shown that salivary hypofunction has an impact on food consumption in terms of poor chewing and swallowing ability, loss of appetite, unbalanced dietary intake and malnutrition (Munoz-Gonzalez et al., 2018).

1.6.3 The effects of aging on salivary composition

Apart from salivary flow rate, it has also been reported that the composition of saliva changed markedly during aging. However, the results are contradictory among the studies. Regarding ion content, Nassar et al. (2014) reported that the amount of Ca^{2+} decreased when comparing young and elderly groups in the case of unstimulated saliva (Nassar, Hiraishi, Islam, Otsuki, & Tagami, 2014). Nagler and Hershkovich reported that the concentration of inorganic materials (K^+ , Cl^- , P, and Ca^{2+}) increased in older individuals, which can be attributed to the reduction in saliva volume (Nagler & Hershkovich, 2005a), they also observed that the concentrations of protein content (total protein, albumin, secretory IgA) increased with aging (Nagler & Hershkovich, 2005b), while many studies agree that mucin concentration decreases with aging (Chang, Chang, Kim, Lee, & Kho, 2011; Denny et al., 1991; Navazesh, Christensen, & Brightman, 1992; Pushpass, Daly, Kelly, Proctor, & Carpenter, 2019). Maciejczyk et al. (2019) reported that antioxidant enzymes in saliva, including peroxidase, glutathione peroxidase, and catalase, decreased with age (Maciejczyk, Zalewska, & Ladny, 2019). Wang et al. (2018) performed salivary proteome profiles of subjects of different ages (young people aged between 19 to 24 years, elderly people aged 62-89 years). Results showed that age-specific differences existed in the unstimulated salivary proteome, and caused significant variations in the salivary proteome (Wang et al., 2018). Fleissig et al. (2010) also revealed differences in protein expression caused by age through proteomic analysis. In male group, the expression of eight proteins decreased with aging: β -actin, prolactin-induced protein, parotid secretory protein, calgranulin-B, cystatin-SN, phosphogluconate dehydrogenase, carbonic anhydrase, and Ig-k light chain. In female group, the expression of prolactin inducible protein and cystatin-SN decreased with aging, the expression of Ig-k light chain, β -actin and transferrin, and carbonic anhydrase were found to increase with aging, the expression α -enolase was found to increase in middle-aged individuals and decrease in the aged female group (Fleissig et al., 2010). However, Salvolini et al. (1999) evaluated the total protein content, the α -amylase activity, the sialic acid content and the calcium and phosphorus content in unstimulated whole saliva. Results showed a significant reduction in human unstimulated whole saliva sialic acid content with aging, while no difference was observed for protein composition (the total protein content, the α -amylase activity) and ion constituents (calcium, phosphorus) (Salvolini et al., 1999). With regard to PRPs change with aging, there is a paucity of research on basic PRPs and glycosylated PRPs. Acidic PRPs was investigated by Baum et al. (1982) by exploring salivary exocrine protein secretion in 220 adults. Authors did not find a change in acidic PRP secretion during ageing (Baum, Kousvelari, &

Oppenheim, 1982).

1.7 The effects of medication on saliva

In Great Britain, people over 75 years take three times more drugs than the national average used. In the United State, older people take approximately 60% of all medications that are used at the national people (Stanley L. Handelman, 1989). Apart from age-related changes of saliva, it has been demonstrated that medication intake may have a great impact on saliva. Ichikawa et al. (2011) investigated the relationships between the amount of saliva and medications in elderly individuals (79-80 years old). Their findings suggested that the volume of saliva in the elderly people is affected by the number and types of medications (K. Ichikawa et al., 2011). Other studies showed that salivary flow rate was independent of drugs intake in elderly people (Nagler & Hershkovich, 2005b; Vandenberghe-Descamps et al., 2016). However, Handelman et al. (1989) observed that elderly people (average age 86.6 ± 5.6) who took more than three drugs per day had a significantly lower stimulated salivary flow than participants who did not take any drugs (Handelman, 1989). A similar result was obtained by Gilbert et al. (1993) who indicated that saliva volume tends to decrease more in people (average age of participants was 78) who use multiple drugs than in people who use single active preparations (Gilbert et al., 1993). Johanson et al. (2015) examined the association between drug treatment and unstimulated and stimulated whole saliva in four 70-year-old Swedish cohorts during a period of 30 years. They observed that high drug consumption was associated with lower salivary flow rate in all groups (Johanson, Osterberg, Lernfelt, Ekstrom, & Birkhed, 2015). The possible reasons for the above inconsistent results are that subjects have different health status (chronic diseases or dementia e.g., Alzheimer' disease), nutritional status, body mass index (BMI), living habits (smoking, diet) etc. (Vandenberghe-Descamps et al., 2016). The effects of medications on saliva components are not as much as studies of medications on saliva flow rate. It has been indicated that antihypertensive medications significantly increased saliva amounts of K^+ , Na^+ , urea and total proteins in 30-70 year old patients (Ivanovski et al., 2015). In conclusion, medication (number or type) is an important factor in salivary secretion which should be taken into account when investigating the effects of aging on salivary properties.

1.8 Conclusion of saliva part

In this chapter, some concepts on saliva were partially demonstrated, including salivary secretion (salivary flow rate and composition) and saliva functions. The impacts of saliva properties on each taste sensation were discussed. In addition, it is well known that saliva properties could change during aging. Therefore, we described the effects of aging on saliva (flow rate and composition) in detail. Last but not least, medication consumption is a part of life for most elderly people. Therefore, it is of great importance to assess the effect of medication on saliva in elderly people. In conclusion, saliva modification is affected by normal aging is controversial, however, medication intake in elderly people should be considered when investigating the effects of aging on saliva.

2. Flavor perception and aroma

Food flavor plays an important role in food consumption. By contributing to food palatability, flavor is one of the most important driver of food intake (Yin, Hewson, Linforth, Taylor, & Fisk, 2017). Flavor perception not only consists of several sensory modalities, taste, retro-olfaction, and trigeminal sensation, resulting from the activation of sensory receptors but also of the cerebral integration of these different modalities. Flavor perception arises from the activation of receptors located in the mouth and the nose (Figure 3). Depending on their structure and properties, flavor compounds bind to chemoreceptors in the mouth (taste and trigeminal receptors) and in the nose (olfactory receptors) or increase the friction force at the surface of the oral mucosa, resulting in the activation of mechanoreceptors (Canon et al., 2018).

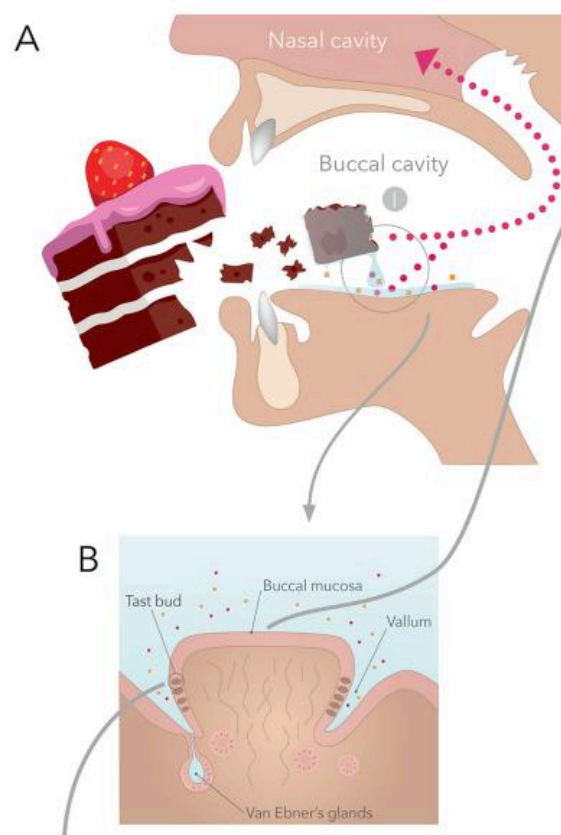


Figure 3. (A) Global view of oral mechanisms involving saliva, (B) structure of circumvallate papillae (Canon et al., 2018).

2.1 Taste perception

2.1.1 Taste buds

Taste perception corresponds to the activation of taste receptors in the taste buds, which are mainly embedded within the mucosa of the oral cavity and distributed in different regions on the tongue. A major population of the oral taste buds is found in the circumvallate papillae, which are located on the posterior tongue and are in contact with the glossopharyngeal nerve

(cranial nerve IX). Taste buds are also located at the surface of the fungiform papillae in the anterior tongue (Figure 4) (Matsuo, 2000). They are innervated by the chorda tympani nerve (a branch of the cranial nerve VII). Taste buds are also present in the position of the epithelial foldings of the foliate papillae, located on the lateral sides of the tongue, and are innervated by both nerves of branches of the chorda tympani and glossopharyngeal nerves. Many taste buds are also located on the larynx, pharynx, and epiglottis, which are in contact with the superior laryngeal branch of the vagus nerve (X) (von Molitor et al., 2021). Lastly, taste buds in the palate are innervated by the greater superficial petrosal nerve, another branch of the facial nerve (Roper, 2013).

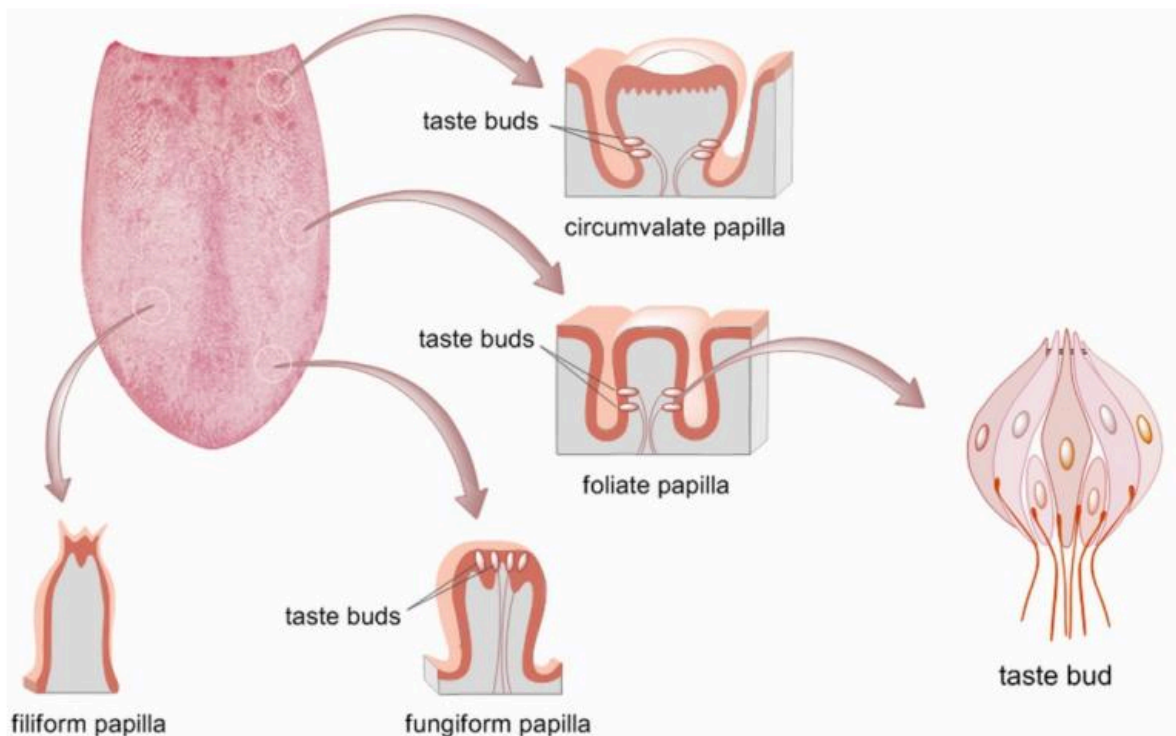


Figure 4. Taste buds occur on the tongue in three main areas, which are associated with the circumvallate, foliate and fungiform papillae (Kikut-Ligaj & Trzcielinska-Lorych, 2015).

2.1.2 Taste receptor cells (TRC)

Humans possess about 5000 taste buds. Each taste bud consists of a community of 50~100 cells that process and integrate taste information with metabolic needs. The sensitivity of the taste buds for individual taste stimuli varies extensively and depends on the type of papillae and the part of the oral cavity in which they are located. There are at least three different cell type found in mammalian taste buds: type I cells, receptor (type II) cells and presynaptic (type III) cells (Kikut-Ligaj & Trzcielinska-Lorych, 2015). They are defined by their morphology, function and expression profile: type I cells, which are considered with glia-like function, because of their role in terminating synaptic transmission; type II (receptor) cells, which express G-protein-coupled taste receptors for umami, bitter or sweet stimuli; type III cells, which transduce sour taste and make functional synapses with the afferent gustatory nerve fibers (Roper, 2013). Cells that are involved in salt (NaCl) taste sensing have not yet been

confidently identified in terms of these cell types. It has been speculated that type I cells may be in charge of the mediation of sodium transduction. The cellular basis for the taste of salt in mice has been linked to epithelial sodium channels, which may be expressed on type I taste cells. However, further research is needed to characterize the involvement of this pathway in humans (Loper, La Sala, Dotson, & Steinle, 2015).

Taste perception can be separated into five basic taste modalities, including sweetness, saltiness, umami, sourness, and bitterness (Niimi et al., 2014). Umami, sweet and bitter compounds are detected by receptors belonging to the superfamily of G-protein-coupled receptors (GPCRs). These receptors are categorized as manifesting sensitivity to the most important consumable substances, i.e. carbohydrates, proteins and the structurally diverse toxic and non-toxic bitter substances (Kikut-Ligaj & Trzcielinska-Lorych, 2015; Reichling, 2008). Sweet and umami tasting compounds are detected by GPCRs from the small family of Tas1R genes (T1R proteins), while bitter compounds are detected by 25 bitter taste receptors (TAS2Rs or T2Rs).

2.1.3 Taste modalities

a) Saltiness

Salty taste is produced by sodium chloride principally. It requires the diffusion of the Na⁺ ions through specialized membrane channels, such as the amiloride-sensitive Na⁺ channel. It has been proposed to involve the selective epithelial type sodium channel (ENaC) (Hartley, Liem, & Keast, 2019). Na⁺ is the only cation which produces a clean salt taste for humans. With Na⁺, other cations, such as Ca²⁺, K⁺, Li⁺ and NH₄⁺ also can produce a salty taste. Mineral and organic salts other than sodium chloride elicit a salty taste, but to a lower extent (Vanderklaauw & Smith, 1995). For instance, K⁺ generates a bitter or a metallic taste, which limits its application as a salt substitute in food. Cl⁻ can be replaced by the larger Br⁻ to produce salty salts; however, I⁻ salts are primarily bitter, and NaNO₃ and Na₂SO₄ are salty and bitter. Na⁺ salts with organic anions such as acetate, L-glutamate and citrate are less salty than NaCl (Frank, Gent, & Hettinger, 2001).

Salt perception plays a role in incorporating NaCl and other salts, and maintaining ion and water homeostasis, but the taste becomes unpleasant and aversive when the NaCl is too concentrated. The molecular mechanism of salty taste has yet to be completely elucidated. As detailed previously, it has been suggested that type I taste cells are responsible for detecting the salty taste, but it is not yet known which cells in the taste buds are the targets for Na⁺ stimulation. The involvement of epithelial sodium channels (ENaCs) located in taste cell membranes in fungiform papillae and amiloride sensitive vanilloid receptors (Trpv1) have been hypothesized in salt perception process (Kikut-Ligaj & Trzcielinska-Lorych, 2015).

It is accepted that stimulation of the taste cells by sodium ions is mediated by channels situated on the microvilli. Most probably, the Na⁺ enters through ENaCs situated on the lateral surface of the taste cells (Liman, Zhang, & Montell, 2014). The ENaC is part of a

highly selective epithelial heterooligomeric complex, consisting of three homologous subunits (the sodium channel, the potassium channel and the calcium channel). The complex plays a key role in the depolarization of the cell membrane, caused by accumulation of Na^+ in the taste cell, which activates migration of calcium ions to its interior. Repolarization of the taste cell is enabled by the opening of potassium channels and migration of K^+ ions out of the cell (Kikut-Ligaj & Trzcielinska-Lorych, 2015).

b) Sourness

Sour taste perception is triggered by acidic foods and substances. Numerous organic and mineral acids, such as citric acid, malic acid, tartaric acid, lactic acid, acetic acid, hydrochloride acid are responsible for sourness. It is mainly caused by the detection of protons. Peptides with a sour taste generally contain acidic amino acids such as aspartic and glutamic acids. A dipeptide with a sour taste contains at least one acidic amino acid linked to another acidic, neutral or aromatic acid.

There is currently a debate on the receptor involved in sour detection. The complete loss of gustatory nerve responses to acids occurs with the ablation of type III cells (Taruno et al., 2021). Type III cells contain a few distinct ion channels. Ion channels involve migration of hydrogen ions (H^+) through ENaCs and HCN1 and HCN4 proton channels (Sugita, 2006). Knocked out mice for PKD2L1 and PKD2L3 do not support the involvement of PKD2L1, while OTOX1 has been recently identified as a putative sour receptor (Liman & Kinnamon, 2021). A high concentration of hydrogen ions may also activate the mechanism of potassium channel blockade, which gives rise to depolarization of the cell membrane receptor (Kikut-Ligaj & Trzcielinska-Lorych, 2015). Migration of H^+ ions through all the channels augments positive intracellular potential, and depolarizes and releases neurotransmitters, which mobilizes transmission of the signals to the brain.

c) Sweetness

Sweet taste is mainly elicited by sugars (glucose, fructose, galactose, sucrose, lactose, maltose starch), while other chemical compounds such as artificial sweeteners (such as saccharin, aspartame, cyclamate, and acesulfame K), sweet amino acids and sweet proteins (such as brazzein, thaumatin, curcurin, and miraculin) can also be perceived as sweet. Sweet taste is very attractive for humans and some animals as it indicates the presence of carbohydrates, an important source of calories in food (Yoshida et al., 2013).

The sweet receptor is formed by the assembly of two GPCR subunits, named TAS1R2 (taste receptor type 1, member 2) and TAS1R3 (taste receptor type 1, member 3), which have been confirmed to be able to detect the wide chemical variety of sweet tasting compounds. TAS1R2 and TAS1R3 receptors can be also activated by some rare plant sweet-tasting proteins, such as brazzein, monellin and thaumatin (Nelson et al., 2001).

d) Umami

Umami is the meaty, mouth-filling, rich taste found in many types of seafood, seaweed, fish, meats, and mushrooms (Chaudhari, Pereira, & Roper, 2009). Umami taste is elicited by L-glutamate and aspartate and ribonucleotides (monophosphates of inosinate, inosine 5'-monophosphate and guanosine-5'-monophosphate), but also by some organic acids (e.g., lactic, succinic, and propionic acids) (Chaudhari et al., 2009).

Umami modality has been established as a basic quality of taste due to the discovery of dedicated receptors for umami compounds (Nelson et al., 2002). Receptors of the TAS1R family form functional complexes of heterodimers responsible for the recognition of sweet and umami tastes. The complex composition of TAS1R1 and TAS1R3 allows the detection of molecule responsible for umami taste (L-glutamate and monosodium glutamate, MSG) (Li et al., 2002). In addition, mGluR4 and mGluR1 receptors were identified to detect metabotropic glutamate in rat taste cells (Behrens & Meyerhof, 2011). However, their involvement in human umami perception should be investigated in the future. Chaudhari et al. (2009) proposed that umami taste detection may involve multiple receptors expressed in different subsets of taste cells. This receptor diversity may underline the complexity of the perception of umami, with different mixtures of amino acids, peptides, and nucleotides yielding subtly distinct taste qualities (Chaudhari et al., 2009).

e) Bitterness

Bitter taste is elicited by a large range of chemical compounds belonging to different chemical families: amino acids and peptides, esters and lactones, phenols and polyphenols, flavonoids and terpenes, methylxanthines (caffeine), sulfimides (saccharin), and organic and inorganic salts (Drewnowski, 2001).

On human tongue, bitter tasting molecules are recognized by a family of 25 receptor proteins (TAS2R, taste receptor type 2) expressed by the bitter taste receptor cells (Kuhn, Bufe, Batram, & Meyerhof, 2010). It has been reported that some TAS2R receptors detect only few bitter molecules, whereas others act as broadly tuned bitter receptors able to detect several bitter compounds (Shi & Zhang, 2009). Activation of TAS2R receptors is responsible for the bitter aftertaste of some compounds such as the undesirable aftertaste of the artificial sweeteners saccharin and acesulfame K.

Initially, bitter taste receptors (TAS2Rs) were considered to be clustered exclusively on the tongue, which is where their stimulation enables the perception of bitter taste. However, it has been shown that TAS2Rs are expressed in many extra-oral tissues, including the respiratory system, gastrointestinal tract, brain, reproductive tissues and airways (Foster, Roura, & Thomas, 2014).

f) Fat

Basic taste qualities are widely agreed to include sweet, sour, salty, bitter and umami. It has

been suggested that fat taste may constitute the sixth basic taste, i.e., the detection of free fatty acids (FFAs) present in food. However, the classification of fat as a basic taste is difficult to gain acceptance, largely due to the difficulties to give an exact definition on “fatty” taste and its multimodal dimension (fat receptors, aroma perception and texture). Stewart et al. (2010) reported that humans are capable of detecting FFAs in oral cavity (Stewart et al., 2010). The occurrence of dedicated receptors, expression of which have been confirmed in taste receptor cells (TRC), as well as the identification of signal transduction pathways and physiological studies prove that the taste of fat should be included to the existing canon of five fundamental flavors (Cygankiewicz, Maslowska, & Krajewska, 2014). It was shown that GPCR receptors are responsible for fat detection, these identified receptors in human are: GPR120 located in all types of taste papillae, FFAR-3 (GPR41) and FFAR-2 (GPR43) found in circumvallate and foliate papillae, and FFAR-1 (GPR40) present only in the circumvallate papillae (Stewart, Feinle-Bisset, & Keast, 2011).

2.1.4 Taste perception with aging

The taste sensory system plays a critical role in life by evaluating the quality of food components. It allows detecting and distinguishing various compounds that can be either essential nutrient for the organism or potential toxic compounds. This is of great importance for human body health. Sweet and umami tastes bring information on carbohydrate and protein contents. Bitter and sour tastes indicate the presence of potential toxic and spoiled ingredients, causing innate avoidance. The salty taste of NaCl can be attractive or aversive depending on its concentration in order to fine-tune the body electrolyte homeostasis (Taruno et al., 2021). However, there have been numerous studies indicating that taste acuity declines with age. This decline may alter food choice and diet quality, leading to risk of malnutrition. For example, a weaker perception of salty taste may induce people to season their food with excessive amounts of salt, raising the risk of cardiovascular disease (Sergi, Bano, Pizzato, Veronese, & Manzato, 2017).

2.1.4.1 Factors of taste loss in the elderly people

With aging come many physiological changes, including those in the oral and nasal cavity, such as impaired gustatory and olfactory function. It has been reported that the diminishment of taste perception usually begins at around 60 years old and becomes more severe at 70 years old (Wiriyawattana, Suwonsichon, & Suwonsichon, 2018). Taste loss is associated with multiple factors, among the causes there are the reduction in the number of taste receptors (Sergi et al., 2017). Aging-related diseases such as degenerative and cardiovascular diseases have also been reported to play considerable roles in the distortion of chemosensory functions because the medication used (Jeon et al., 2021). The lack of oral saliva is also the cause of taste impairment, since saliva plays a significant role in the oral functions such as swallowing and cleaning oral cavity, and also in taste sensitivity (Vandenbergh-Descamps et al., 2016). Indeed, saliva enables the solubilization of tastants and their transport up to taste receptors (Canon et al., 2018; Feron, 2018). Finally, saliva protects the receptor cells from damage, preserving taste acuity (Matsuo, 2000). Other factors could contribute to decrease the taste

sensitivity including zinc deficiency (Aliani et al., 2013), poor dentures that may impact mastication and then affect the general taste of foods, and poor dental hygiene that may impact taste function in the elderly (Doty, 2018). Last but not least, comorbidities and the related polypharmacy are also common causes of the age-related decline in gustatory function. Many elderly people are liable to have several chronic diseases concurrently and are forced to take different drugs simultaneously, which can affect taste acuity by impairing the function of the taste buds or the neurons involved in the transduction of flavor stimuli (Imoscopi, Inelmen, Sergi, Miotto, & Manzato, 2012).

2.1.4.2 Effect of aging on each taste perception

Numerous studies have investigated the effect of aging on taste sensation leading to conclusion with several discrepancies across studies. The reasons that could account for these discrepancies are variable, including inclusion or exclusion of confounding factors such as participants with dentures and smokers, the widely varying number of participants tested, different age ranges, the difference in populations, varying ratios of male to female, different taste compounds used and concentrations, and sensory testing methods (Methven, Allen, Withers, & Gosney, 2012). Moreover, food exposure could also affect taste sensitivity. Indeed, frequently consumption of some food may lead to a higher threshold of different taste modalities. For example, Thai population tended to have a high baseline of umami threshold due to their high consumption of monosodium glutamate (MSG) from Thai foods and seasonings (Wiriyawattana et al., 2018). Overall, the general trend of taste acuity is declining with age, however, how is the aging effect on each taste modality? And how is the extent of taste decline with aging?

Salty: Data from a systematic review performed on 23 studies about threshold and aging indicated that the NaCl taste thresholds increased with age. Similar result was found for other salt-taste compounds including potassium chloride as well as sodium salts of acetate, ascorbate, carbonate, citrate, phosphate, succinate, sulphate and tartrate (all at pH 7) (Methven et al., 2012). According to Methven's review that across studies, NaCl thresholds were found to increase between 1.4 and 6.7-fold during ageing (Methven et al., 2012). The ratio of detection threshold in elderly (70-79 years)/detection threshold in young (18-29 years) ranged from 2.7 for monosodium glutamate to 26.7 for sodium sulfate depending upon the type of Na salt (Schiffman, Crumbliss, Warwick, & Graham, 1990). KCl can also be used as a salty compound to investigate the effect of aging on salty taste perception. Mojet et al. (2003) reported that the elderly (60-75 years) perceived less intense salt taste of KCl than the young (19-33 years) did (Mojet, Heidema, & Christ-Hazelhof, 2003). Wiriyawattana et al. (2018) reported that the detection and recognition threshold values of NaCl and KCl increased significantly in middle-aged subjects (40-59 years), and they tended to increase further in the elderly, albeit not significant (Wiriyawattana et al., 2018). The magnitude of detection threshold values of NaCl and KCl increased around 2.1 folds in the elderly (above 60 years), while recognition threshold values increased to a greater extent (about 4-5 folds) compared with young people (20-39 years) (Wiriyawattana et al., 2018).

Sour: According to the meta-analysis performed by Methven et al. (2012) and across studies using citric or tartaric or acetic acids, the mean thresholds were evaluated to 0.4 mM and 0.7 mM for younger adults and older adults respectively, representing a 1.5-fold increase with age (Methven et al., 2012). This conclusion was consistent with the study from Wiriyawattana et al. (2018) (Wiriyawattana et al., 2018), who reported that the detection and recognition threshold values of citric and acetic acids in elderly (60-85 years) subjects were significantly higher than in young (20-39 years) subjects, with approximate 1.7-fold increases in both detection and recognition thresholds in the elderly group.

Sweet: Wiriyawattana et al. (2018) reported a 1.0 to 1.2 fold increases with age in threshold values evaluated on sucrose, aspartame, acesulfame-*k*. Elderly people (60-85 years) need 1.5-fold higher sucralose concentration to be able to detect and recognize its sweet taste than the young people (20-39 years) (Wiriyawattana et al., 2018). According to their systematic review, Methven et al. (2012) reported that sucrose-detection threshold increases with age from 1.2-fold to 2.6-fold, the mean threshold across the studies being 12.4 mM for younger adults and 16.8 mM for older adults, representing a 1.4-fold increase (Methven et al., 2012). Different sweet taste compounds have different effects on the increase of sweet threshold value in elderly people. Schiffman et al. (1981) evaluated a further nine sweeteners and found detection thresholds of all to increase with age from 1.5-fold (sodium cyclamate) to 4.7-fold (monellin) (Schiffman, Lindley, Clark, & Makino, 1981).

Umami: Despite the shortage of studies investigating umami perception, there is a general consensus agreement on an age-related decline in this capacity. Methven et al. (2012) reported that the mean threshold across studies was 2.5 mM for younger adults and 5.5 mM for older, representing a 2.2-fold increase (Methven et al., 2012). All these studies targeted monosodium glutamate (MSG) as reference compound for investigating umami detection threshold. Two studies using inosine monophosphate (IMP) reported 4.4-fold higher detection thresholds in older adults (1.5 mM compared with 0.3 mM) (Methven et al., 2012). Wiriyawattana et al. (2018) showed that the increases in detection and recognition threshold values with age of MSG and IMP varied in the range of 2.3-3.4 folds (Wiriyawattana et al., 2018).

Bitter: Methven et al. (2012) reported that the extent of increase for quinine detection thresholds was between 1.5-fold and 7.4-fold (Methven et al., 2012). For quinine hydrochloride the mean thresholds across four studies were 0.002 and 0.009 mM for the young and elderly adult groups respectively, representing a 4.5-fold increase. For quinine sulphate, the mean thresholds were 0.005 mM and 0.019 mM, for young and elderly groups respectively, corresponding to a 4.0-fold increase. Across four studies the mean caffeine thresholds for younger and older groups was 1.4 and 1.8 mM, respectively, representing an overall mean increase of 1.2-fold with age (Methven et al., 2012). Always on caffeine Wiriyawattana et al. (2018) found the concentration for detection and recognition threshold values in elderly (60-85 years) around 2.5-2.7 folds higher than in young (20-39 years) subjects (Wiriyawattana et al., 2018). All these results showed that different bitter compounds presented remarkable variability in the level of increase thresholds. This can be partly

explained by the effect that different bitter receptors are involved depending on the compounds and by genetic differences in subjects' ability to detect bitter taste as demonstrated for phenylthiocarbamide or propylthiouracil.

Fat: To date and as far as we know, there is no literature describing an effect of aging on fat taste perception.

2.1.5 Taste intensity with aging

Apart from detection and recognition thresholds widely investigated under aging, the effect of aging on perception of taste intensity is also important to explore. If the levels of tasting compound in foods are above detection thresholds, the perception of taste intensity at supra-threshold levels becomes extremely crucial. In the light of the meta-analysis review from Methven et al. (2012) most of studies observed that age had a significant negative impact on the intensity of perception (Methven et al., 2012). Barragan et al. (2018) also indicated that the intensity rating of all the 5 tastes (sweet, bitter, umami, salty, sour) was diminished with age, especially for bitter and sour (Barragan et al., 2018). However, the decline extent of taste intensity during aging was not frequently reported.

In general, taste sensitivity and intensity are negatively associated with aging. The extent of the decline varied among taste qualities and the types of taste compounds within the same taste quality.

2.1.6 The influence of taste loss on dietary habits

Taste perception impairment can modify people's food intake and dietary habits and, as a consequence, can influence the quality of life and health status. It is generally assumed that age-related taste loss could therefore be partly responsible for the lack of appetite observed in elderly people and thus of the modifications of food choices and food preference. For sweet taste, generally the threshold increase with age, however, one study indicated that the liking for sweet-tasting foods was even decreases with increasing age (Lampure et al., 2015). For salty taste, it was observed that elderly people who have the higher detection threshold prefer stronger salty foods (Sergi et al., 2017). In addition, enhancing the food flavor with monosodium glutamate (umami) can improve food palatability, satisfaction and salivation in elderly people who have difficulty in perceiving saltiness (van der Meij, Wijnhoven, Finlayson, Oosten, & Visser, 2015). Indeed, it has been proposed that increasing the food's flavor intensity can compensate the chemosensory perception's impairment in the elderly population (Schwartz, Vandenberghe-Descamps, Sulmont-Rosse, Tournier, & Feron, 2018). For sour and bitter taste, Duffy et al. (1995) found a lower preference for food (e.g. citrus fruits, vegetables) in elderly people with smell and taste dysfunction (Duffy, Backstrand, & Ferris, 1995). However, no direct relationship between poor sensory performance and preference for flavor-enhanced foods was observed, and the results suggested that age-associated changes in food perception was not caused by losses in sensory acuity and will not reduce the food liking of the elderly. The authors gave two explanations, the normal

deterioration of the sensory systems with age is gradual, i.e. people may continuously accustom to their attenuated perception and do not experience a decrease in food liking, or the pathways of sensory and hedonic representations do not converge in the brain and are processed by different areas in the brain (Kremer, Bult, Mojet, & Kroeze, 2007). Similarly, Schwartz et al. (2018) also concluded that food flavor re-enforcement does not significantly increase elderly's food intake (Schwartz et al., 2018) according to previous studies (Essed, van Staveren, Kok, & de Graaf, 2007; Mathey, Siebelink, de Graaf, & Van Staveren, 2001).

2.2 Odor perception

2.2.1 Olfactory system

Apart from taste, odor perception plays an important role in food flavor perception as well. The ability to smell, olfaction, refers to a chemosensory process during which volatile molecules are detected by specialized sensory cells, which are called olfactory sensory neurons. These cells express protein receptors which bind to specific odorant substrates (Dan et al., 2021). The olfactory receptor neurons are bipolar cells embryologically derived from both the olfactory placode and the neural crest and form clusters within the respiratory neuroepithelium. They contain 3-50 cilia and send their unmyelinated axons through the cribriform plate to synapses in the olfactory bulb. Odorants bind to guanine nucleotide-binding (G) protein-coupled receptors (>500 coding genes but only 100-200 functional receptors) in the cilia of the olfactory receptor neurons (Attems, Walker, & Jellinger, 2015). In humans, there are over 400 olfactory protein receptors, which are primarily responsible for odor detection. Odorants are detected when they bind to receptors present in the olfactory neurons which are located in the olfactory epithelium in the dorsal aspect of the nasal cavity (Figure 5) (Lafreniere & Mann, 2009). The olfactory receptor gene family encodes 500-600 genes and is the largest in the genome (Raj et al., 2021). In humans, smell processing in the olfactory bulb is involved in experiencing emotions and memories through direct connection with the limbic system and cerebral cortex (Dan et al., 2021). Indeed, numerous researches reported olfaction has impacts on habits, social interactions, behavior, emotions, and moods (Seow, Ong, & Huang, 2016). Olfactory function is of great importance for our food choice, quality of life and daily activities, especially avoidance of environmental hazards. However, it has widely been reported that the ability to smell tends to decline with normal aging.

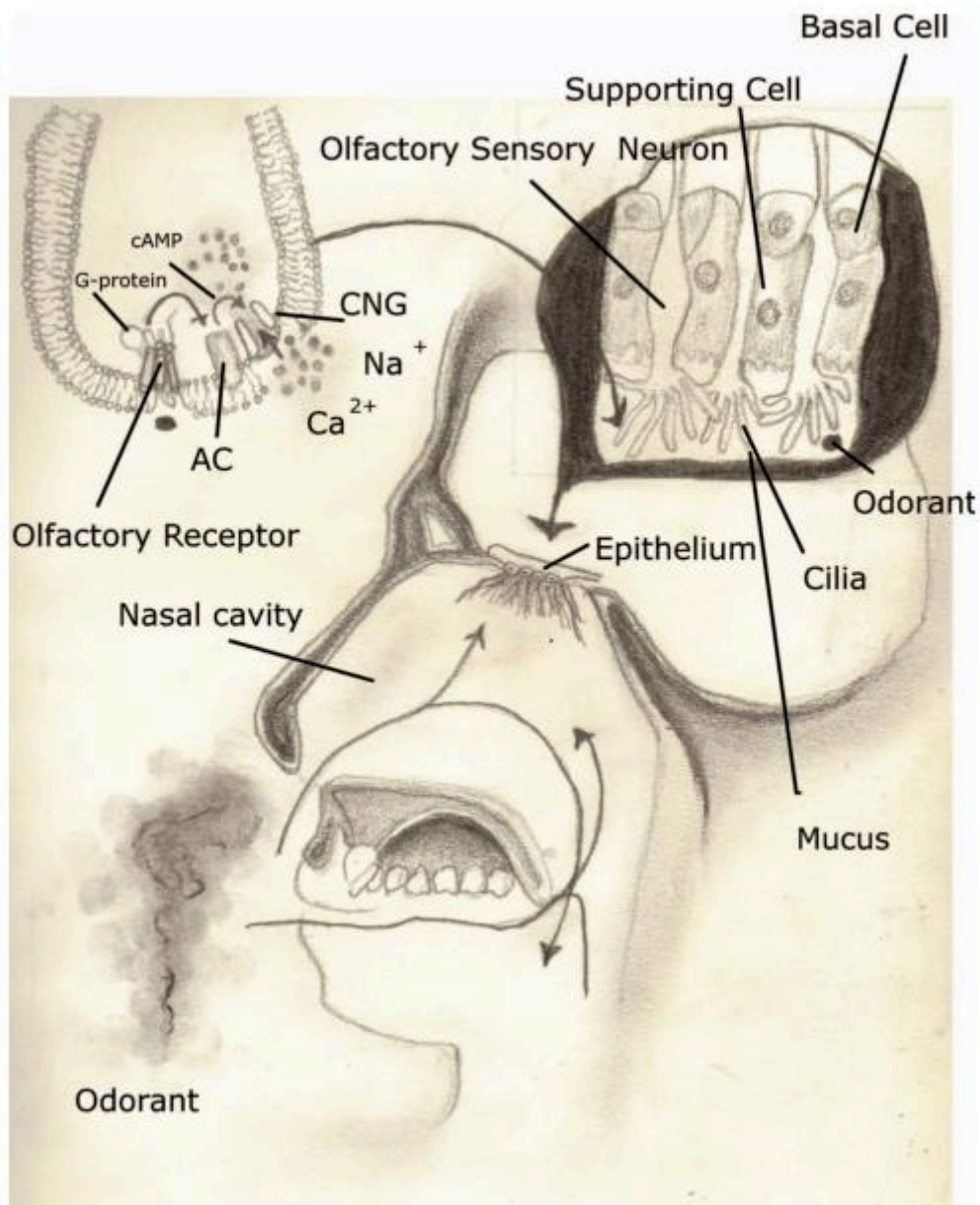


Figure 5. Odorants bind to receptor proteins present in the olfactory receptor neurons which are located in the olfactory epithelium in the dorsal aspect of the nasal cavity (Brookes, 2011).

2.2.2 Olfaction with aging

Olfactory deterioration with age has been well-documented in clinical reports, experimental and epidemiological studies. It is widely accepted that olfactory losses are far more prevalent than taste losses with age (Boyce & Shone, 2006). It has been reported that olfactory dysfunction is present in more than 50% of individuals aged between 65 and 80 years and in 62-80% of those >80 years of age (Attems et al., 2015). Zhang et al. (2017) in a meta-analysis study wanted to determine in which decade odor identification starts to decline in healthy humans. The results suggest that olfactory function deterioration starts in the fifth decade of life in healthy humans (Zhang & Wang, 2017). This information could enable clinical studies to determine when intervention should be initiated to slow or prevent the deterioration of

olfaction function.

Olfactory dysfunction can be detected by numerous olfactory tests, including psychophysical tests, e.g. odor detection (odor threshold)-the perception of odors at lowest detectable concentrations, discrimination- the nonverbal distinction of different smells, identification-the ability to name or associate an odor. The University of Pennsylvania Smell Identification Test (UPSIT) and the Sniffin' Stick test, are the most widely employed. In addition, European Test of Olfactory Capabilities (ETOC), electrophysiological, imaging tests, and psychophysiological tests can also be applied to detect age-related deficits in the olfactory systems (Attems et al., 2015; Thomas-Danguin et al., 2003).

Olfactory deficits not only affect daily activities, physical well-being, quality of life, but also can change dietary behaviors and food intake. The “anorexia of aging,” a physiological age-associated reduction in appetite and energy intake with higher prevalence of malnutrition and immune dysfunction, has been imputed partly to olfactory deficit. It has been suggested that olfactory impairments can also give rise to weight loss. Moreover, olfactory dysfunction is a contributory factor in the age-related increases in accidental gas poisonings and explosions that can endanger public safety.

2.2.3 Causes of age-related olfactory impairment

Many factors have been proposed to be associated with olfactory impairments, including age-related alterations within the nose, olfactory epithelium, olfactory bulb and higher levels of the brain that receive olfactory input (Doty & Kamath, 2014). In addition, exposed environment, age-related neurodegenerative disease and medication consumption can also affect olfactory function (Doty, 2018).

2.2.3.1 Cause related to olfactory epithelium

Olfactory receptor neurons undergo apoptosis at a baseline rate in each person. Although humans have the ability to replace these cells, this process degenerates with ageing, which results in a decline in the surface area of olfactory epithelium, along with reduced numbers of olfactory receptor neurons (Boyce & Shone, 2006). Aging can also lead to a reduction in receptors, thinning of the epithelium, alterations in olfactory receptor cells, and the replacement of olfactory with respiratory epithelia (Attems et al., 2015). Impairment of immunologic and enzymatic defense mechanisms is difficult for the maintaining of the integrity of the olfactory epithelium in older age. The response specificity of individual olfactory receptor cells reduces with aging (Doty, 2018).

2.2.3.2 Cause related to olfactory bulb

Intact sensation of olfaction depends not only on intact olfactory receptor neurons but also on intact and appropriate synapses as the signal moves centrally (Lafreniere & Mann, 2009). For example, smoking and viruses may affect the development of aberrant synapses and may

conduce to the loss of olfactory bulb mitral cells with age. In the olfactory bulb, the number of mitral cells decreases with age. It is estimated that the adult olfactory bulb contains about 60000 mitral cells at age 25 years and only 14500 at age 95 years (Lafreniere & Mann, 2009). It has been suggested that the size of the olfactory bulb and the number of its laminae decreases with age in humans, being specific in the generalized atrophy, loss of neuronal elements, and increased astroglia, secondary to damage to the olfactory epithelium (Attems et al., 2015). The bulb losses are highly due to sensory cell loss in the olfactory mucosa, accompanied with a general deterioration in central nervous system cognitive processing functions (Boyce & Shone, 2006). Moreover, it has been well documented that age-related led to changes in the volume of olfactory bulb, although this decrements may also occur under several conditions, including smoking, chronic sinusitis, multiple sclerosis, head trauma, and schizophrenia (Attems et al., 2015).

2.2.3.3 Cause related to brain region

Changes in brain regions involved in olfactory processing include a reduction in the volume of the hippocampus, amygdala, piriform cortex, and anterior olfactory nucleus (Segura et al., 2013). Age-related changes in the number, volume, and localization of islands of Calleja within the olfactory tubercle, a cortical structure receiving monosynaptic input from the olfactory bulb, may be a contributor to pathological changes in the olfactory cortex function and olfactory perception (Attems et al., 2015). In addition, anosmia per se is correlated with changes within olfaction-related structures, including the piriform and insular cortices, the orbitofrontal cortex, the medial prefrontal cortex, the hippocampus, the parahippocampal gyrus, the nucleus accumbens, the subcallosal gyrus, and the medial and dorsolateral prefrontal cortices (Attems et al., 2015). Wilson et al. (2011) also suggested that olfactory impairment in older adults is not confined to structural changes within the nose, but its aetiology is likely to be associated with higher brain structures (Wilson et al., 2011).

2.2.3.4 Cause related to Neurodegenerative Disorders

Olfactory defects occur in many age-related neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, multiple system atrophy, Huntington's disease, multiple sclerosis, and amyotrophic lateral sclerosis (Lafreniere & Mann, 2009). Alzheimer's disease is the most common neurodegenerative disorder in humans and is the major cause of dementia in the elderly. Evidence suggested that early phases of Alzheimer's disease have significant negative impacts on odor discrimination and identification (Hedner, Larsson, Arnold, Zucco, & Hummel, 2010). Olfactory loss in these patients can be seen as an early feature of the disease (Luzzi et al., 2007). Parkinson's disease is the second most common neurodegenerative disease in humans and olfactory dysfunction is a significant feature of Parkinson's disease. Masaoka and colleagues noted that it needed higher odor concentration to be recognized an odor by patients who had Parkinson's disease (Masaoka, Yoshimura, Inoue, Kawamura, & Homma, 2007).

2.2.3.5 Cause related to medication consumption

The influence of medications should also be taken into consideration. Many drugs used to treat age-related conditions, such as antihypertensive medications and statins, are known to play a major role in age-related chemosensory changes (Schiffman, Zervakis, Graham, & Westall, 2002). Schiffman et al. (2002) conducted a comprehensive discussion of these medications and diseases and stated that older adults experience an exaggerated burden of chemosensory disorders from these medications compared to younger individuals (Schiffman et al., 2002).

2.2.3.6 Cause related to physiological and oral parameters change

The retronasal perception of odorant compounds, called aroma compounds in this case, occurs during food oral processing. It has been demonstrated that the decrease in salivary secretion as well as masticatory impairment due to lack of teeth or wear of denture, could contribute to the loss of olfactory perception by impairing aroma release molecule (Munoz-Gonzalez, Feron, & Canon, 2021a; Schwartz et al., 2018). In addition, it has been suggested that the modification in salivary composition, total protein content and salivary antioxidant capacity also affect aroma release and perception. Indeed, a study performed by Munoz-Gonzalez et al. (2021b) described a correlation between retronasal aroma release and age, BMI, swallowing tongue force and salivary antioxidant capacity of the elderly participants (mean age is 74 years). Especially age, BMI and mostly salivary antioxidant capacity were the most important factors to explain interindividual variability in the retronasal aroma release from an elderly cohort (Munoz-Gonzalez, Feron, et al., 2021b). Recently, Criado et al. (2021) observed that the senior group (56-71 years), who exhibited a lower salivary flow and higher total protein content, rated more intensely and for a longer time two aroma attributes (smoky and black pepper notes) compared to the younger group (18-35 years). This seems exceptional considering that olfactory impairment is generally associated to age. Therefore, authors gave several explanations, firstly, in this study, seniors are not old enough to have olfactory impairment, second, the seniors might have a higher ability to recognize the two specific aromas compared to the younger group, third, the seniors might perceive these two odors as pleasant then they tend to rate aroma intensity higher (Criado, Munoz-Gonzalez, & Pozo-Bayon, 2021).

2.2.3.7 Cause related to environmental exposure

It has been noted that exposures to air pollution, cigarette smoke, viruses, bacteria, and other airborne xenobiotics cumulatively damage the olfactory epithelium (Doty, 2018). In addition, heavy metals and chemical toxic exposures can also result in smell loss (Lafreniere & Mann, 2009). Such exposures have more functional consequence in later years when the cumulative effects become more manifest.

2.2.3.8 Cause related to aroma specificity

To shed light on how age-related perception loss is odorant specific, Sinding et al. (2014)

investigated the difference in age-related odor perception in function of aroma molecular weights and hydrophobicity (increase of the aliphatic chain), based on the concept that the molecules would bind differently to olfactory receptors (Sinding, Puschmann, & Hummel, 2014). They found that older subjects (50-70 years) had a higher identification threshold to heavy molecules than young (18-30 years) subjects, suggesting that aroma-specific age-related loss is related to the molecular structure of individual aroma compounds. Indeed, it has been reported that odor quality changes more or less progressively depending on molecular size and hydrophobicity, expressed as length of the chain of carbon molecules attached to the functional group (Cain, de Wijk, Nordin, & Nordin, 2008). Therefore, age-related loss in odor perception is also likely due to odorant chemical structure.

2.2.4 Influence of aging on odor detection, discrimination, identification

Olfactory functions consist of 3 subtests: odor detection (threshold), discrimination, identification (Seow et al., 2016). The majority of previous studies are consistent with the conclusions that the odor detection threshold markedly increases with aging while the intensity of perception decreases. Odor identification is clearly sensitive to age-related decline too and the ability of odor quality discrimination diminishes with aging (Stevens, Cain, Schiet, & Oatley, 1989; Vantoller & Dodd, 1987). However, with rare exception, some few studies reported a larger variability in odor-detection thresholds in elderly than in young individuals and that some elderly are as sensitive in detecting odors as the most sensitive young persons (Stevens & Cain, 1987). This phenomenon was further proved by Nordin (Nordin, Almkvist, & Berglund, 2012), who conducted a study for pyridine odor detection threshold with young (20-24 years) and elderly (77-87 years) who were “successfully aged” with respect to medical health and cognitive ability. Their results showed that elderly and young adults had a very similar mean detection threshold for pyridine. Age-related loss in olfactory functioning and especially odor identification is dependent on cognitive abilities, such as recognition memory, exposure or familiarity to the odors, and vocabulary abilities (Seow et al., 2016). This theory was proposed by Xu et al. (2020) who concluded that odor sensitivity was less strongly correlated with cognitive ability than odor identification (L. Xu, Liu, Wroblewski, McClintock, & Pinto, 2020). Thereby, odor thresholds are less impacted during aging than identification ability, which decreases with cognitive abilities.

2.3 Conclusion of taste and aroma part

The taste and olfactory systems play crucial roles in food preference, food choice and food consumption in human. However, it is well known that the abilities of perception of taste and odor in elderly people are not as efficient as in young people, which could contribute to a risk of malnutrition in elderly. It appears that taste and odor perception decline with age. The causes of taste and odor perception deterioration are multi-factorial including age-related physiological and environmental factors.

3. Astringency

Astringency is an important sensory attribute that occurs during the consumption of foods and beverages containing polyphenols, especially tannins, such as vegetables, nuts, unripe fruits and berries, wine, tea, etc. It has been defined 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” by the American Society for Testing Materials (ASTM). The perception of astringency is basically a dynamic process that continuously changes and evolves, which usually takes 20 to 30 s to develop fully and to reach its maximum of intensity, and often is the last oral sensation detected (Kallithraka, Bakker, Clifford, & Vallis, 2001; Rinaldi, Gambuti, & Moio, 2012). The intensity of perceived astringency plays a crucial role in determining the acceptability of a variety of food products (Brandao, Soares, Mateus, & de Freitas, 2014). For example, the astringency of red wine is perceived as desired and even an important quality parameter at balanced levels. On the contrary, above a certain intensity, astringency is usually regarded as a disagreeable oral sensation (Soares et al., 2016). Lyman et al. (1990) have proved that astringency is a cumulative sensation that increases with repeated exposures to an astringent stimulus, suggesting that it does not involve chemoreceptors (Lyman & Green, 1990). However, the molecular mechanism at the origin of astringency is still not well understood and several hypotheses have been proposed.

3.1 Astringency is a trigeminal sensation

During the last decades, it has been demonstrated that astringency is mediated by the trigeminal nerves (Schobel et al., 2014). In human, the trigeminal system conveys sensations such as oral dryness, burning, pungency, or stinging, as well as pain, touch, pressure, and temperature (Albrecht et al., 2010; Braud & Boucher, 2020). Indeed, the trigeminal systems are both sensitive to chemical and physical stimuli. Trigeminal nerve endings are distributed throughout the oral and nasal cavity and play an important role in detecting temperature, consistency and pungency even irritants and potentially noxious chemicals of food and beverages (Hessamedin Alimohammai, 2000; Sodal, Singh, Skudutyte-Rysstad, Diep, & Hove, 2021). There is still a debate in the scientific community regarding the type of receptors which are activated by tannins.

3.2 Astringency is probably not a chemical sense

Trigeminal nerve fibers express several chemoreceptors that respond to a wide variety of chemical compounds including: transient receptor potential (TRP) channels, the acid-sensing ion channel (ASIC) (H. Ichikawa & Sugimoto, 2002), the purinergic receptor (P2X) (Spehr, Spehr, Hatt, & Wetzel, 2004), and nicotinic acetylcholine receptors (nAChR) (Hessamedin Alimohammai, 2000).

TRP channels are evolutionarily conserved integral membrane proteins and a group of biomolecules that play an imperative role in interpreting environmental stimuli. TRP channels are a class of cationic channels that act as signal transducer by altering membrane potential or intracellular calcium (Ca^{2+}) concentration (Amrita Samanta, 2018). TRP channels are grouped

into six subfamilies based on sequence homology. The canonical TRP (TRPC) subfamily contains the founding member of mammalian TRP channels. The vanilloid TRP (TRPV) subfamily is sensitive to the heat of its founding member. The melastatin-related TRP (TRPM) subfamily includes some of the few known bi-functional ion channels, which contain functional enzymatic domains. The ankyrin TRP (TRPA) subfamily consists of a single chemo-nociceptor that is a target for analgesics. The mucolipin TRP (TRPML) subfamily channels are found primarily in intracellular compartments and were discovered based on their critical role in type IV mucopolidosis (ML-IV). The polycystic TRP (TRPP) subfamily is a diverse group of proteins implicated in autosomal dominant polycystic kidney disease (ADPKD) (Amrita Samanta, 2018).

Thus, it can be hypothesized that astringency results from the activation of TRP channels by tannins. A study has reported the ability of monomers of flavan-3-ol, or their products of oxidation, to activate TRPA1 and TRPV1 (Kurogi et al., 2015; Kurogi, Miyashita, Emoto, Kubo, & Saitoh, 2012). However, considering the localization of trigeminal free nerve ending in the mucosa, it requires that tannins diffuse through the mucosa. In another study, Schobel et al. (2014) claimed that astringency perception involves the activation of G protein-coupled signaling by phenolic compounds but not transient receptor potential (TRP) channels. Moreover, astringency tends to increase with the size of tannins (Schobel et al., 2014). Tannins with a high degree of polymerization are generally more astringent than monomers of flavan-3-ols. It is unlikely that chemoreceptors can accommodate tannins with high degree of polymerization. It has also been reported that astringency perception grows over repeated exposures (Lyman & Green, 1990). This feature is not compatible with the hypothesis of the involvement of chemoreceptors.

3.3 Astringency is a physical perception

Astringency is more generally considered as a tactile sensation. It is supported by psychophysical studies that provide indirect evidence of the sensation's tactile basis. One test proved that astringency could be perceived on non-gustatory surfaces in the mouth, indicating that it is not a taste (Green, 1993). Tactile sensations involve mechanoreceptors (MRs). They are neurons located in oral mucosa and classified according to the size and character of their receptive field. Type I have small and distinct receptive fields, while type II have large, diffuse receptive fields (Bajec & Pickering, 2008). There are four main types of MRs in oral cavity: Merkel cells, Meissner cells (lamellated corpuscles), Ruffini endings, and free nerve endings. MRs are further classified depending on whether they are rapidly adapting (Meissner corpuscles) or slowly adapting (Merkel cells and Ruffini corpuscles) receptors (K. O. Johnson, 2001).

Merkel cells are present within the basal layer of mucosal epithelium and contain characteristic electron-dense granules that are located almost exclusively at the side of cytoplasm in contact with axon terminals. The lamellated corpuscles are located in the transitional area between the lamina propria and epithelial layer of the mucosa. Merkel receptors are sensitive to fine details and texture and shape. The Ruffini cylinders, which are

located in the connective tissue of the dermis, have a relatively large spindle shaped structure tied into the local collagen matrix (Johnson, 2001). Ruffini cylinders are sensitive to stretching. The free nerve endings are found in the subepithelial regions, very close to the basal laminae of mucosal epithelium (I.-s. Watanabe, 2004). Meissner corpuscles are involved in handgrip control and motion across skin (I.-s. Watanabe, 2004).

Activation of MRs is thought to result from increase of the friction forces within the oral cavity due to the alteration of the lubrication properties of the saliva and/or the mucosal pellicle due to their aggregation by polyphenols. Indeed, it has been demonstrated that tannins have the ability to alter the mucosal pellicle by generating aggregates, leading to an increase of the friction force at the surface of the oral mucosa (Ployon et al., 2018). Another study reported that the mucosal pellicle is more important than saliva in the detection of astringency (Nayak & Carpenter, 2008). The mucosal pellicle is a proteinaceous coating, whose thickness can reach 100 nm. The salivary proteins forming the mucosal pellicle are either bound by covalent and non-covalent bonds at the surface of the epithelial cells (Ployon et al., 2018). It includes salivary proteins such as MUC5B, MUC7, statherin, cystatins and IgA and also amylases and PRPs (Gibbins & Carpenter, 2013). It has been reported that the presence of the transmembrane MUC1, expressed by oral cells, improves the formation of the mucosal pellicle (Ployon et al., 2016). The main function of mucosal pellicle is to lubricate the surface of oral mucosa, and thus to prevent abrasion between surfaces, improve swallowing abilities and facilitate a normal mouthfeel (Gibbins & Carpenter, 2013).

3.4 The transmembrane MUC1, a sensor of astringency

Recently, our group came up with a new hypothesis involving MUC1. This hypothesis proposed that the aggregation of the mucosal pellicle by tannins could lead to a disruption of the 2 subunits of MUC1 and to peel away the mucosal pellicle from the cell surface. These mechanisms could both lead to an increase of the friction forces at the surface of the mucosa following the removing of the mucosal pellicle, and to the activation of a cellular pathway leading to the release of neurotransmitters activating chemoreceptors on the trigeminal free nerve endings (Canon et al., 2021).

3.5 The role of salivary proteins in astringency

In those two above mentioned hypotheses, salivary proteins are thought to play a protective role in preventing the mucosal pellicle from aggregation by tannins. Among those salivary proteins, proline-rich proteins (PRPs) have been reported having a high affinity for tannins. Indeed, it has been demonstrated that PRPs secretion can be induced by a tannin-rich diet in rodents (Carlson, Mehansho, & Ann, 1985). PRPs are considered as the first line of defense toward the deleterious effects of tannins. Indeed, tannins have the ability to interact with proteins forming soluble non-covalent complexes that aggregate due to the multi-dentate property of tannins, which allows them to form bridges between two proteins and as a result to cross-linking them. This may reduce the digestibility of plant proteins and inhibit gastrointestinal enzymes, conferring to tannins anti-nutritional properties (Jöbstl, O'Connell,

Fairclough, & Williamson, 2004).

3.6 Proline Rich-proteins

With regard to astringency sensation, PRPs seem to play a prominent role, as their presence in mammal saliva is correlated to the consumption of tannins in their diet. Moreover, PRPs have demonstrated a high affinity for tannins and in particular the ability to protect efficiently proteins (Carlson et al., 1985) or the mucosal pellicle (Ployon et al., 2018) from aggregation by tannins.

Proline-rich proteins (PRPs) account for more than 60% in weight of the total salivary peptidome. It is mainly secreted by parotid under stimulations and constitute up to 70% of parotid saliva proteins (Bajec & Pickering, 2008). PRPs are characterized by a high content in proline (25-42%), glycine (16-22%), and glutamine (15-28%) residues, which together account for 70% to 88% of all amino acids in the protein sequence (Sarni-Manchado et al., 2008). PRPs are classified in three groups according to their isoelectric point and their degree of glycosylation: acidic PRPs (aPRPs), basic PRPs (bPRPs) and glycosylated PRP (gPRPs) which account for 30%, 23% and 17%, respectively, of the total protein in parotid saliva (Bajec & Pickering, 2008). bPRPs and aPRPs show a similar C-terminal region but they differ in their N-terminal region, aPRPs are highly acidic mainly due to a high content in aspartic and glutamic acid residues in the first 30 residues. Moreover, aPRPs are usually phosphorylated in some serine residues. Regarding gPRPs, their peptide chains are close to those of bPRP, but differ by the presence of N- and O-glycosylations (Manconi et al., 2016). In these proteins, N-glycan moieties are attached to the amide group of asparagine residues whereas there is not a consensus sequence for O-glycosylation (Manconi et al., 2016).

Although all the PRPs' functions have yet to be fully elucidated, acidic PRPs are reported to bind to the surface of teeth and to be involved in the formation of the dental pellicle, as they may have a role in calcium homeostasis, but also in bacterial binding (Kamysz & Sikorska, 2010). Glycosylated PRPs participate to oral lubrication and prevent bacterial agglutination (Pascal et al., 2006). Basic PRPs are demonstrated to have anti-viral activity and a high affinity for binding tannins and thus to play a role in protecting against the harmful effects of tannins (Pascal et al., 2006). Basic PRPs have unusually extended conformations for intrinsically disorder proteins (Boze et al., 2010). Subsequently, it is proposed that this unusually extended conformations contribute to the binding of plant tannins, as the extended conformations provide a larger surface for binding (Boze et al., 2010).

It has been reported that basic PRPs (IB1-IB9) could migrate during electrophoresis in the area of 6 to 21 kDa, aPRPs could migrate in the area of 20 to 27 kDa and gPRPs molecular weight is around 66 kDa (Ramos-Pineda, Carpenter, Garcia-Estevez, & Escribano-Bailon, 2020). However, one should be cautious with the apparent molecular weight of PRPs on SDS-PAGE gels, because their particular amino acid composition, leading to an unusual low level of SDS bound to the peptidic chain. As a result PRPs are less negatively charged and migrate at a shorter distance in acrylamide gels than globular proteins do with the same

molecular weight (MW). Usually their apparent molecular weight on SDS-PAGE gel is often 1.2-1.8 times higher (molecular weight factor) than the real one (Soares et al., 2011).

The PRPs family is encoded by 6 genes: PRH1 and PRH2 encode acidic PRPs, PRB1, PRB2, PRB3, and PRB4 encode basic and glycosylated PRPs (Bennick, 2002). Each PRB gene covers four exons, the third of which is fully composed of 63-bp tandem repeats coding the proline-rich portion of the protein products. Variation in the numbers of these repeats is responsible for length differences in different alleles of the PRB genes (Padiglia et al., 2018). To be precise, PRB1 and PRB2 genes produce the non-glycosylated bPRP whereas PRB3 and PRB4 generate glycosylated bPRP (gPRPs) (Ramos-Pineda, Carpenter, García-Estévez, & Escribano-Bailón, 2019).

When it comes to secretory origin, there are differences between aPRPs and bPRPs. Indeed, aPRPs are present only in saliva and are secreted by both parotid and submandibular/sublingual glands while bPRPs are present in saliva, nasal secretions, and bronchial mucus (Igoh, Tomotake, & Doi, 2015). In saliva, bPRPs and gPRPs are secreted only by parotid glands (Padiglia et al., 2018). A further distinction between aPRPs and bPRPs is that aPRPs can be found in saliva both as intact and truncated proteoforms while bPRPs encoded by PRB1, PRB2 and PRB4 genes are detectable in saliva only as fragments of the bigger proteins and are more polymorphic than aPRPs (Manconi et al., 2016). Lately, Padiglia et al. (2018) adapted top-down liquid chromatography-mass spectrometry approach to characterize 55 new components of bPRPs family. The authors proposed that the bPRPs family can be divided into two main groups and a third minor hybrid group. Group 1 includes P-E, PKo, IB-6, Ps-1, Ps-2, P-H, P-F, P-J, and P-D. Group 2 includes IB-1, II-2, and the glycosylated bPRPs codified by PRB3 and PRB4 genes, namely, GI-1, GI-2, GI-3, GPA, II-1, and Cd-IIg. The small Group 3 is a hybrid group, which includes the two proteoforms of IB-8a, Con1- and Con1+ (Padiglia et al., 2018).

Another notable feature of PRPs is that their synthesis is induced by the presence of β -agonists and tannins. PRPs are constitutively produced at high levels in humans and mule deer (Austin, Suchar, Robbins, & Hagerman, 1989). In contrast, in rats and mice, synthesis of PRPs is induced by injection of the β -agonist isoproterenol and a tannin-rich diet (Mehansho, Butler, & Carlson, 1987). This response has been confirmed exclusively in rodent species, such as rats, mice, root voles (Juntheikki, JulkunenTiitto, & Hagerman, 1996), and Japanese wood mice (Shimada, 2006). This induction mechanism supports the importance of PRPs as a defense against dietary tannins.

As aforementioned, tannins have some detrimental effects for mammals. Growing evidence indicates that PRPs can be as a first line of defense against the harmful effects of tannins in the diet. It is thought that the major function of the proline-rich repeats is to bind and precipitate dietary tannins, thereby neutralizing their harmful actions (Baxter, Lilley, Haslam, & Williamson, 1997). The binding of tannins to PRPs may be protective and prevent astringency from developing by preventing tannins from direct interaction with the oral mucosa.

3.7 Tannins properties

Phenolic compounds that often referred to as polyphenols are an important group of secondary metabolites widespread in plants. These phenolic compounds were originally named as “tannins” because of their use in the tanning process converting hide collagen in leather (de Freitas & Mateus, 2001). Tannins are a well-known class of plant-derived food compounds with particular organoleptic properties, such as color and flavor, in particular astringency, which is perceived in red wine, tea, coffee, and other beverages. Tannins are considered to have considerable and potent health-promoting benefits. The role of tannins as antioxidants may prevent several diseases associated with oxidative stress such as cardiovascular diseases, cancers, inflammation, and others (Quideau, Deffieux, Douat-Casassus, & Pouysegou, 2011). As a consequence, tannins are of great interest for the food industry because of their well-known beneficial effects. Nevertheless, there is limited information on the daily intake of tannins because the tannin content of food cannot be measured exactly (Bennick, 2002). Some doubts remain about the formation of protein-tannin complexes as an obstacle to the nutritional bioavailability of both proteins and tannins. As a matter of fact, Serafini et al. (1997) concluded that there is a partial restoration of the antioxidant activity lost after the interactions with proteins by reducing tannin-proteins interaction (Serafini, Maiani, & FerroLuzzi, 1997).

However, a high tannin diet can also present harmful effect. Indeed, inclusion of tannin in the diet can give rise to perturbation of mineral absorption from the intestinal tract, a decrease in body weight and growth retardation and inhibition of digestive enzymes (Ahmed, Smithard, & Ellis, 1991). Tannins are of great importance for plant biology. The astringent properties of tannin may protect plant leaves by making them unpalatable to browsing animals, and the high astringency of unripe fruit results in their avoidance by herbivores until the seeds are ready to disperse (Bennick, 2002).

Tannins possess an abundance of phenolic groups. They have molecular masses in the range of 500-3000 Da, constituting a complex group of flavonoid-based polymers capable of binding and eventually precipitating proteins (Schwarz & Hofmann, 2008). Based on their structure, tannins can be divided into two groups, hydrolysable and condensed tannins (also named proanthocyanidins) (García-Estévez, Ramos-Pineda, & Escribano-Bailón, 2018). Hydrolysable tannins, which are gallic or ellagic esters of glucose, can be extracted from oak barrels or oak wood of the tanks during winemaking or aging or added via oenological tannins to wine. Considerable structural variation is introduced by additional molecules of gallic acid linked to other gallic acid moieties. As the name implies, these compounds are easily hydrolyzed in alkali, giving rise to a polyhydric alcohol and gallic acid, in the case of gallotannins, or ellagic acid, the condensation product of hexahydrodiphenic acid, in the case of ellagitannins (Bennick, 2002). Hydrolysable tannins include ellagitannins and gallotannins, giving respectively ellagic and gallic acid after acid hydrolysis. The main representative structures of ellagitannins are castalagin and vescalagin (Figure 6), whereas pentagalloylglucose (PGG) (Figure 7) and tannic acid (Figure 8) are representative

gallotannins (García-Estévez et al., 2018).

Condensed tannins are polymers of flavan-3-ol units linked by interflavan bonds from C₄ of the upper unit to C₈ and to a lesser extent C₆ of the lower unit (B-type). The basic structure of flavan-3-ol differs in the degree of hydroxylation of ring B, the stereochemistry and the presence of substituents (e.g. galloyl or glucosyl groups) (Figure 9). They can be depolymerized in hot strong acid, leading to anthocyanidin pigments and other products. Proanthocyanidin is an alternative name for condensed tannin (Figure 10) (Bennick, 2002). The degree of polymerization varies considerably from a few to more than 50 flavan-3-ol molecules. Small molecules of condensed tannins are soluble in aqueous or organic solvents, while the large polymers are insoluble (Bennick, 2002). Regarding the impact of the structural variation of proanthocyanidins on their interactions with tannins, Poncet et al. (2003) have studied the impact of the mean degree of polymerization (mDP) and percentage of galloyl group in an ethanolic solvent. They concluded that galloylation enhanced the formation of aggregates as far as monomers were concerned, but this could not be confirmed with polymers (Poncet-Legrand, Cartalade, Putaux, Cheynier, & Vernhet, 2003). The mDP had a complex effect: in a first stage, aggregation increases with mDP up to medium mDP, before decreasing in a second stage for higher molecular weight fractions.

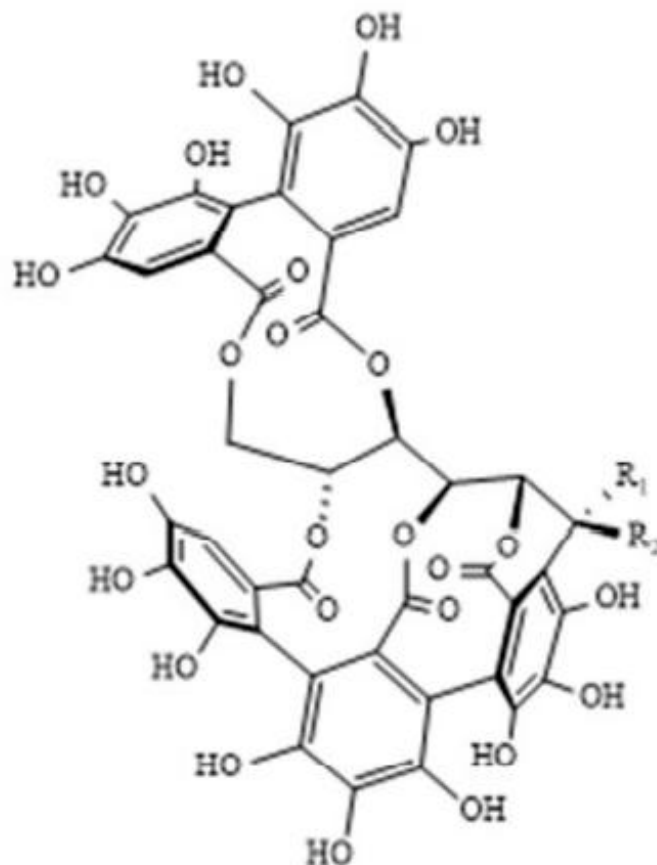


Figure 6. Castalagin R1: OH, R2: H. Vescalagin R1: H, R2: OH (García-Estévez et al., 2018).

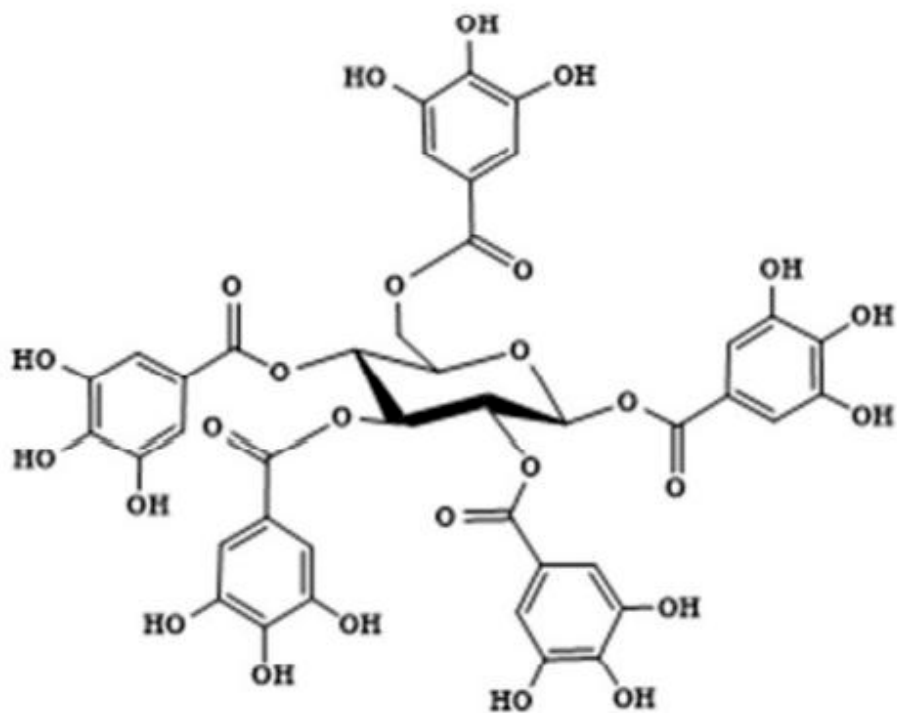


Figure 7. The structure of pentagalloylglucose (PGG) (García-Estévez et al., 2018).

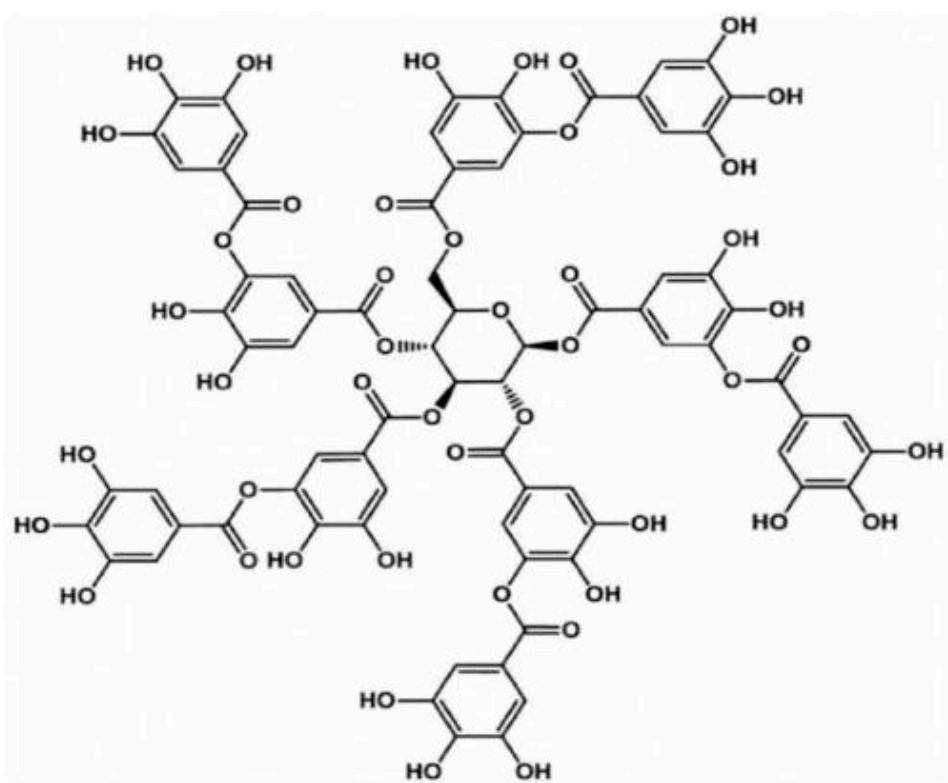


Figure 8. The structure of tannic acid (R. F. Lu et al., 2020).

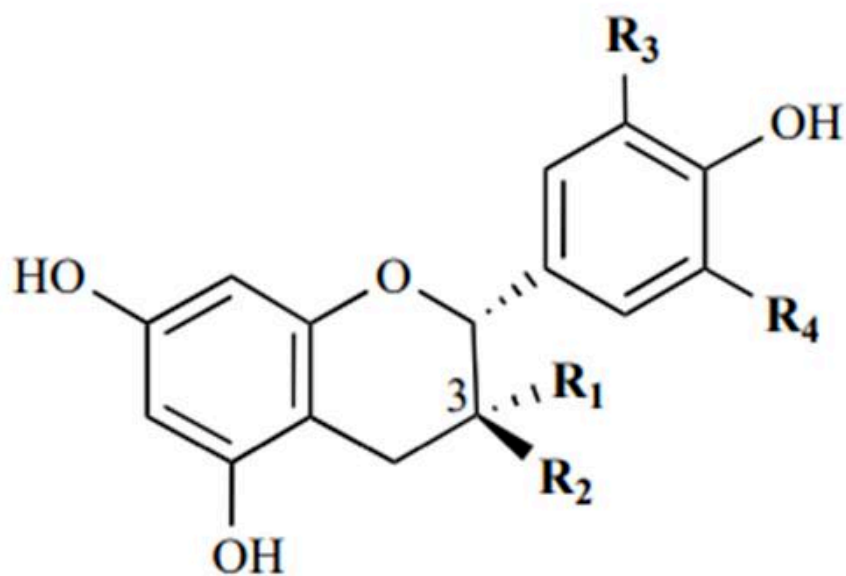


Figure 9. Flavan-3-ols (de Freitas & Mateus, 2012).

R1=H; R2=OH; R3=H; R4=OH (+)-catechin

R1=OH; R2=H; R3=H; R4=OH (-)-epicatechin

R1=H; R2=OH; R3=OH; R4=OH (+)-gallocatechin

R1=OH; R2=H; R3=OH; R4=OH (-)-epigallocatechin

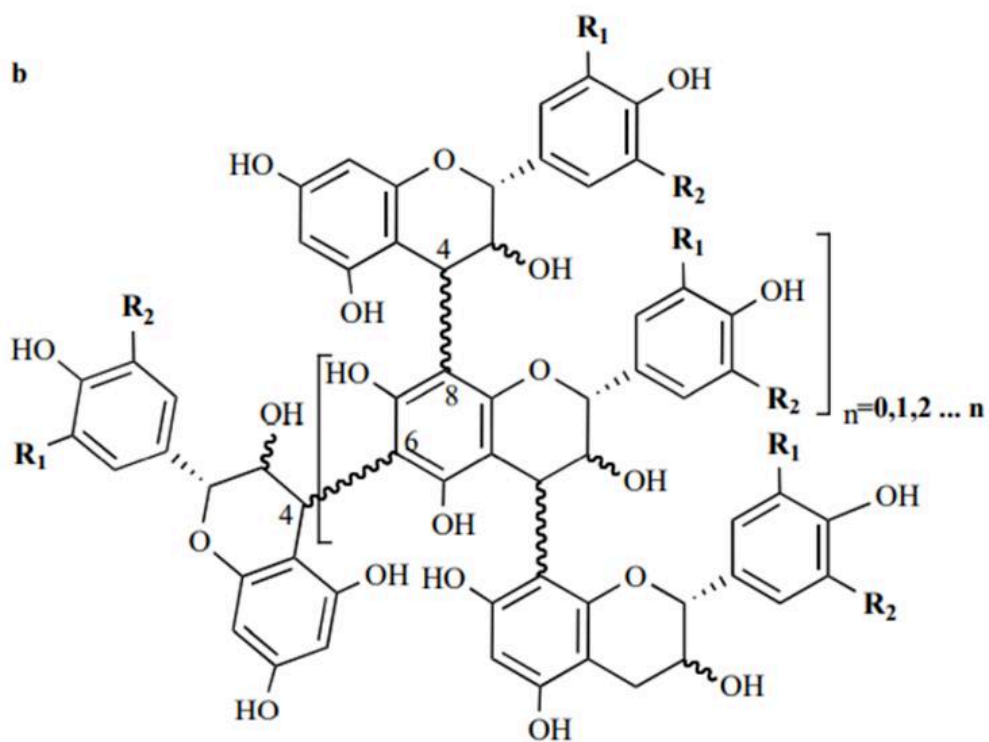


Figure 10. General structure of proanthocyanidins (de Freitas & Mateus, 2012).

3.8 Astringency related to tannins properties

As described above, astringency is a momentous organoleptic sensory characteristic of foods rich in tannins. Many researches have investigated the relationship between astringency and the structure properties of tannins (galloyl substitution, stereochemistry and degree of polymerization (mDP)).

Low molecular weight polyphenols are not noticeably astringent probably because they do not have enough phenolic ring to cross-link salivary proteins, which limit its interaction with salivary proteins (Goldstein & Swain, 1963). It has been demonstrated that the astringent sensation can also be produced by small molecules, e.g. 5-O-caffeoylquinic acid and monomeric flavan-3-ol which are less likely to form cross-links (Kallithraka et al., 2001). Indeed, monomers of flavan-3-ols show differences in term of astringency intensity. Xu et al. (2018) quantitatively analyzed astringency of green tea infusion in terms of the concentrations of the following flavan-3-ol monomers: (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG), and nonepicatechins including (+)-catechin (C), (-)-gallocatechin (GC), (-)-catechin gallate (CG) and (-)-gallocatechin gallate (GCG) (Y. Q. Xu et al., 2018). According to the concentration-taste intensity curves of the eight catechins, at the same concentration, the astringency intensities were of the following order $ECG > EGCG > GCG > CG > EGC > EC > GC > C$. Thus, astringency intensities for catechins having a galloyl substitution were much higher than those of unsubstituted catechins at the same concentration. Among the eight catechins, the taste intensities of galloylated catechins increased with concentration faster than their non-galloylated correspondent. It is believed that the presence of a galloyl group on flavan-3-ol monomers allow them to cross-link proteins.

Regarding the stereochemistry, astringency of non-epicatechins was lower than the one of epicatechins, and they increased more slowly with concentration than those of the epicatechins (Xu et al., 2018). Time intensity sensory assays have also shown that epicatechin is more astringent than catechin (Peleg, Gacon, Schlich, & Noble, 1999; Thorngate & Noble, 1995), which indicates that the stereochemistry plays an essential role in astringency development. Ramos-Pineda et al. (2017) have proved that epicatechin with the more planar structure (due to the position of the OH in the C ring) enables a better interaction between the phenolic ring surfaces. Furthermore, owing to its more planar structure, epicatechin seems to be more easily forming hydrogen bonds between its hydroxyl groups and the polar groups of the peptide than catechin does, which enables a better interaction between the phenolic ring surfaces, which in turn contributes to a faster and more effective interaction with peptides (Ramos-Pineda et al., 2017). Regarding the degree of polymerization, the same authors observed that monomers were rated lower in astringency than the dimers or trimers, concerning only procyanidin dimers (Peleg et al., 1999). Hufnagel et al. (2008) found that the astringent threshold decreased from monomeric to dimeric and trimeric flavanols, pointing out that the more polymerized the flavanols are, the more astringent (Hufnagel & Hofmann, 2008). It has been reported that the increasing mean degree of polymerization and galloylation degree of proanthocyanidins increase astringency (Vidal et al., 2003). Because procyanidins

can act as polydentate ligand, the higher the molecular weight, the higher number of potentially binding sites in the procyanidin, which can explain the greater ability of the more polymerized tannins to interact with proteins. Concerning the highly polymerized tannins, some authors stated that maximum astringency is more likely attained by tannins of intermediate size since high polymerized tannins are too large to fitted-linked protein molecules (Lea & Arnold, 1978). It has also been demonstrated that astringency is characteristic of high molecular-weight procyanidins and increase up to about mean DP of 7 units. From this point, polyphenols start to be less soluble and thus less astringent (Lea, 1991). However, highly polymerized structures of proanthocyanidins from grape seeds (up to mDP 20) and from grape skins (up to mDP 70) have been reported to be soluble in water-alcohol solution and highly astringent (Vidal et al., 2003). In addition, the procyanidin's ability to bind PRPs also seems to increase with molecular weight (de Freitas & Mateus, 2002).

Regarding the effects of tannins concentration on astringency, there are some studies describing that the amounts of total phenols and total tannins are positively related to the astringency intensity (Cliff, King, & Schlosser, 2007; Gawel, Francis, & Waters, 2007). Medel et al. (2017) also found the type of perceived astringency was closely related to the tannin concentration by a method of temporal dominance of sensations (Medel-Maraboli, Romero, Obreque-Slier, Contreras, & Pena-Neira, 2017).

In terms of hydrolysable tannins, it seems that the interaction of salivary proteins with ellagitannins is weaker than gallotannins, and that the affinity toward proteins increases with the galloylation of the tannin, which is important in the case of gallotannins (Bacon & Rhodes, 2000). It has been reported that differences in the ellagitannin content of wines could significantly modify the astringency perception (Gonzalez-Centeno, Chira, & Teissedre, 2016). The perception of the ellagitannin astringency depends on their structure. Glabasnia and co-workers (2006) reported that the monomers, castalagin and vescalagin, are less astringent than the corresponding pentose derivatives, grandinin and roburin E. However, castalagin and vescalagin showed similar thresholds when astringency is evaluated than ellagic acid, and higher than the corresponding dimers, roburins A-D (Glabasnia & Hofmann, 2006).

In conclusion, astringency increases with the presence of a galloyl group, stereochemistry (epicatechin), the degree of polymerization, tannins concentration.

3.9 PRPs-tannin molecular interaction

It has been proposed that the interaction mechanism between tannins and PRPs can be divided into three stages as tannin concentration increases: (i) tannins bind to several sites on the free protein, (ii) the stoichiometries of the complexes increase and tannins, which are multidentate ligands, cross-link several proteins causing aggregates, (iii) the resulting multimeric aggregates grow up to precipitation (Canon et al., 2015).

3.9.1 Formation of soluble non-covalent complexes

Canon et al. (2009) investigated the detection of noncovalent complexes between IB5 and EGCG by electrospray ionization mass spectrometry (ESI-MS). Their results indicated that IB5 possesses several interaction sites to bind tannins. A maximum of 5 EgCG for 1 protein were observed in this study (Canon et al., 2009). The affinity of EgCG for the PRP IB5 has been also determined using mass spectrometry by calculating the constant of dissociation (Canon et al., 2013). In this first stage of the molecular interaction, the interaction depends on the affinity of the interactants for each other. PRP-tannins interaction is described to involve both hydrophobic effects and hydrogen bonds (Ramos-Pineda et al., 2017; Soares, Silva, et al., 2019). PRPs have open randomly coiled structures, which allow the exposure of peptide carbonyl groups to hydrogen bonding as well as the exposure of proline residues to act as binding sites for tannin by hydrophobic interaction of the aromatic portion of tannin with the pyrrolidine structure of proline (Soares et al., 2011). Indeed, Canon et al. (2015) have determined that the main binding site of tannins, whatever their structure, on the bPRP IB5 is constituted of a cluster of 5 proline (Canon et al., 2015). Thus, it is expected that protein charge how affects the affinity of tannin-protein interaction, depending on how the charge may affect the structure of the protein. When a large amount of tannins bind to the protein they induce an unfolded to folded structural transition of the unstructured bPRPs (Canon et al., 2011). It has been indicated that PRPs are considered as having a fixed number of sites that tannin can bind to. Thus different PRPs-tannin complexes are formed according to the ratio of tannin to PRPs (Brandao et al., 2014). Canon et al. (2013) found that the PRP IB5 has bound at least 3 EgCG molecules per protein to form aggregates. They also found that increasing the tannin concentration beyond the aggregation threshold (0.2 mM EgCG for 0.21 mM IB5, and 0.5 mM EgCG for 0.42 mM IB5) increases aggregate size (Canon et al., 2013).

3.9.2 Formation of aggregates

The formation of tannin - PRPs aggregates depends on the ratio of tannin bound to PRPs and the charge of the proteins. Indeed, proteins with similar charges repulse each other due to coulombic repulsions, precluding their cross-linking by tannins. As a result, pH and ionic strength are key parameters in the formation of aggregates as they impact on protein charges. If the pH is closed to the isoelectric point of a protein, the global charge of the protein is close to zero. Thus, there is less coulombic repulsion, which favors aggregation. It has been proposed that tannin-protein precipitates are more readily formed when the pH is close to the isoelectric point of the protein (Naurato, Wong, Lu, Wroblewski, & Bennick, 1999). Therefore, protein isoelectric point is also one of the factors that should be considered affecting the aggregation of PRPs by tannins depending on the pH.

3.9.3 Impact of PRP structure on the interaction

It has been indicated that different PRP families (acidic, basic, and glycosylated) for the interaction with tannins are distinct (Sarni-Manchado et al., 2008).

Basic PRPs are considered as the most effective salivary proteins to bind and scavenge

tannins. Indeed, Hagerman and Butler (1981) reported that bPRPs are the most effective protein to protect BSA from aggregation (Hagerman & Butler, 1981). Lu et al. (1998) also verified that bPRPs were very effective in complexing both condensed tannin and tannic acid (Y. Lu & Bennick, 1998). A study performed by Pascal et al. (2007) who investigated the interactions between human salivary basic PRP, IB-5 and a flavan-3-ol monomer, epigallocatechin gallate (EGCG), suggested that the interaction mechanism was dependent on protein concentration. At low protein concentrations, a three-stage mechanism was evidenced. At high protein concentrations, direct bridging between proteins and EGCG was observed, resulting in significantly lower aggregation and turbidity thresholds (Pascal et al., 2007).

Interactions between the glycosylated PRP II-1 and flavan-3-ols were studied by the same research group, who reported that gPRPs are also effective in binding tannins and compared the results to those previously obtained on the interactions between the non-glycosylated bPRP IB-5 and EGCG (Pascal, Poncet-Legrand, Cabane, & Vernhet, 2008; Pascal et al., 2007). However, the interaction between gPRP and flavan-3-ols do not necessarily result in precipitation. Finally, it has been concluded that the glycosylation of the gPRP precludes the aggregation and precipitation. It has been proposed that glycosylation of PRPs could inhibit the formation of aggregates due to steric hindrance and thus reduces tannin precipitation with regard to tannin amounts (Sarni-Manchado et al., 2008). For similar low concentrations of tannins, gPRP-tannin interactions led to complexes that remained soluble, whereas non-glycosylated PRP were precipitated. Lu et al. (1998) has also found no binding of condensed tannin to glycosylated PRP, and only a small amount of tannic acid bound to glycosylated PRP at the highest concentrations of the acid assayed, whereas deglycosylation of a gPRP gave rise to a sharp increase in tannin-precipitating, which means the carbohydrate side-chains can prevent either binding of tannins or protein cross-linking (Y. Lu & Bennick, 1998).

Several studies indicated that aPRPs were the salivary PRPs with higher affinity toward studied tannins or first interact with studied tannins compared with other salivary protein families (Brandao et al., 2014; Soares, Brandao, Garcia-Estevez, et al., 2019; Soares et al., 2011). The authors attributed this phenomenon to the structure of acidic PRPs. Due to the presence of many aspartic acid and glutamic acid residues, the acidic characteristics of aPRPs are approximately limited to the first 30 amino acids. The presence of these acid residues may favor hydrogen bonding, which may explain the high affinity of aPRPs for tannins. The rest part is basic and similarly to basic PRPs, shows repeated sequences of proline and glutamine (Brandao et al., 2014).

For all three classes of PRPs, it has been proposed that larger PRPs bind more tannins than smaller PRPs or peptide fragments (Bajec & Pickering, 2008).

3.9.4 Impact of tannins structure on the interaction

Freitas et al. (2002) reported that the binding of procyanidin with PRPs increased with its average molecular weight, and the amount of insoluble aggregates increased with the

procyanidins's degree of polymerization (de Freitas & Mateus, 2002). In addition, low-molecular weight compounds were found to trigger astringency by complexing salivary proteins without precipitation (Schwarz & Hofmann, 2008).

With regard to hydrolysable tannins, it has been demonstrated that condensed tannins have a higher affinity for PRPs than hydrolysable tannins (Bajec & Pickering, 2008). However, investigating the binding affinity of hydrolyzable tannins to PRPs, Bacon & Rhodes (2000) showed that all of the classes of PRPs (aPRP, bPRP, gPRP) interact with hydrolyzable tannins (Bacon & Rhodes, 2000). Poncet-Legrand et al. (2007) observed that catechin or epicatechin interactions with poly (L-proline) are negligible compared to those observed in the case of galloylated monomers (Poncet-Legrand, Gautier, Cheynier, & Imberty, 2007). These authors pointed out a critical role played by the galloyl moiety, which involves hydrogen bonds with the peptide thus stabilizing and strengthen the interaction. As for monomeric ellagitannins, it seems that castalagin shows a higher affinity towards salivary proteins than vescalagin, which can be related to the lower hydrophobicity of the latter (Silva et al., 2017).

The existence of mechanisms of synergy has also been pointed out. Indeed the presence of both epicatechin and catechin compounds in solution seems to produce a synergistic effect that causes them to bind more easily and efficiently to IB714 peptides (Ramos-Pineda et al., 2017). Similar results were obtained by Soares et al. (2019) who observed in particular that epicatechin: mv-3-glc mixture presents a synergic effect toward the interaction with both PRPs (aPRPs, bPRPs) when compared to individual tannins (Soares, Silva, et al., 2019). This could also explain why it is so difficult to correlate astringency of foodstuffs assessed by sensory analysis with the quantity of one polyphenol.

3.10 Astringency and individual variation

Last but not least, astringency is also associated with individual physiological properties, such as salivary flow rate, salivary protein concentration, salivary protein composition, salivary buffering capacity, saliva viscosity, etc. Ishikawa and Noble (1995) suggested that subjects with low salivary flow rated astringency higher and recorded longer duration of astringent aftertaste than subjects with high salivary flow (Ishikawa & Noble, 1995). Similar results obtained by Fischer et al. (1994) who reported that the subject group with high salivary flow rate perceived astringency intensity at a significantly lower level than the low flow rate group (Fischer U, 1994). It has been proposed that the difference of astringency perception is linked to salivary protein composition instead of salivary protein concentration (Kallithraka et al., 2001). Indeed, histatins and mucins also have been identified as potential contributors to astringency perception in humans, while their role in the underlying mechanism of this perception is still under debate (Carpenter, 2012; Gibbins & Carpenter, 2013).

Regarding the relationship between astringency and aging, so far, there is scarce research investigating the astringency perception in function of age between young adults and elderly people. However, in our work, we showed that elderly people (mean age is 75 years) have a higher astringency threshold than young people (mean age is 25.4 years) (M. Wang et al.,

2022).

3.11 Conclusion of astringency part

In this part, we systematically elaborated the possible mechanisms of astringency, including trigeminal sensation, physical perception, and the interaction of tannins and salivary proteins. A special focus was performed on proline-rich proteins (PRPs) as their presence in mammals diet appear to be correlated to tannin diet. Then we bring information of the relation between tannins and PRPs structures with interaction and aggregation mechanisms and how they influence astringency perception. Last but not least, inter-individual variations also play an important role in astringency perception. Up to now, to the best of our knowledge, the research on the effect of aging on astringency acuity is still lacking except for our published work mentioned above. Therefore, there is a need for extensive and comprehensive research on astringency inter-individual variability in particular in elderly peoples.

4. Conclusion of this part

As mentioned above, flavor perception not only consists of taste, olfaction, but also trigeminal sensation, resulting from the activation of sensory receptors in the mouth (taste and trigeminal receptors) and in the nose (olfactory receptors) or increase the friction force at the surface of the oral mucosa, resulting in the activation of mechanoreceptors. Therefore, astringency can be regarded as flavor felt in the mouth. Due to the aim of this study, i.e. the effects of aging and saliva on the astringency, we systematically demonstrated saliva properties and the changes of saliva with aging. In addition, we considered other flavor modalities (taste and olfaction) which can be impacted by saliva and aging as indicators of what can be expected regarding astringency perception and aging. Therefore, in this part, we systematically formulated saliva properties, tastes and olfactory systems, aging effects, and the possible mechanisms of astringency.

CHAPTER 2: METHODOLOGIES AND MATERIALS

Chapter 2: Methodologies and materials

1. Sensory experiment

1.1 Setting up a methodology to evaluate astringency sensitivity

In the literature, two main methods are often used for evaluating astringency sensation. They are Time intensity (T-I) and Temporal Dominance of Sensations (TDS). T-I method consists in recording one by one the intensity evolution of given attributes (W. E. Lee & Pangborn, 1986). It involves four parameters (time to maximum intensity, total duration, maximum intensity and area under the T-I curve), as shown in Figure 11. However, T-I methodology is performed only on a small number of attributes or with a limited number of products since only one attribute is evaluated at a time. Considering it is a time-consuming method and in order to obtain a more global view of the sensory properties of a food product, TDS method has been developed. It consists in identifying and rating sensations perceived as dominant as a function of time until the perception ends (Pineau et al., 2009). This method assesses the dominating sensory attribute from a number of perceived sensory features (Meillon, Urbano, & Schlich, 2009). It presents to the panelists the complete list of attributes on a computer screen. These two methods (T-I & TDS) have been used in numerous studies that describing temporality of wine sensations and identifying wine or model product quality descriptors (Ishikawa & Noble, 1995; Medel-Maraboli et al., 2017; Meillon et al., 2009; Pittari et al., 2022; Rebenaque, Rawyler, Boldi, & Deneulin, 2015). However, the aim of our work is to determine the astringency threshold of specific astringency solution (tannic acid solution), not to describe the several attributes of a solution. Therefore, these two methods are not applicable to our work.

Alternative forced choice (AFC) method is the most established one used to determine a sensory threshold. This method requires the panelist's attention which is directed to a specific sensory attribute. Panelists are asked to select a product from a pair or a triplicate that has the desired sensory attribute (McClure & Lawless, 2010). The most commonly used methods include 2-AFC or 3-AFC tests (Heikel et al., 2012; S. H. Lee, Kim, & Hong, 2022). Increasing the number of alternatives is more precise and reduces the guessing rate and therefore makes trial more informative (Vancleef et al., 2018). However, considering that astringency makes 30 s to develop fully and to reach its maximum of intensity while avoiding subject fatigue due to experiment duration (particularly for the elderlies), 2-AFC was adopted to evaluate astringency threshold in our work.

As previously indicated, astringency is a cumulative sensation that increases with repeated exposures to an astringent stimulus. In order to minimize these effects, we highlighted the rinsing procedure between sample evaluations in the sensory session. Pectin, bicarbonate and Evian water were chosen to rinse the mouth and to have the most similar oral conditions before each test pair. Sodium bicarbonate recovers pH homeostasis, and pectin removes

tannic acid from the oral mucosa due to its capacity to form complexes with tannins (Huang & Xu, 2021). This rinsing procedure was found to be efficient in wine studies for in-mouth aroma release experiments (Esteban-Fernandez, Rocha-Alcubilla, Munoz-Gonzalez, Moreno-Arribas, & Pozo-Bayon, 2016; Munoz-Gonzalez, Canon, Feron, Guichard, & Pozo-Bayon, 2019), and more recently, this procedure is also successfully applied to the study of a dynamic real-time in-vivo study coupling sensory evaluation of astringency perception and aroma release (Pittari et al., 2022). This procedure was chosen instead of other procedures, such as the milk rinsing procedure (Taladrid, Lorente, Bartolome, Moreno-Arribas, & Laguna, 2019), because of the necessity to avoid any contamination of saliva samples by food proteins. Other procedures like salty crackers was proved to be the more effective rinses in decreasing astringency than water (Colonna, Adams, & Noble, 2004), however, it is easy to form oral residues which have to be removed with more efforts than other liquid palate cleansers. In the study of Colonna et al. (2004), who used several agents for inter-stimulus-protocols (rinses) in three experiments pectin was suggested the most effectively compounds to reduce astringency in three experiments (see table 2) (Colonna et al., 2004).

In addition, it has been suggested that astringency perception is felt alongside bitterness. Hence, tannic acid was used as a component to evaluate astringency because it has been described as less bitter than other polyphenols, such as gallic acid and catechin (Robichaud & Noble, 1990), which limits the confusion between astringency and bitter taste.

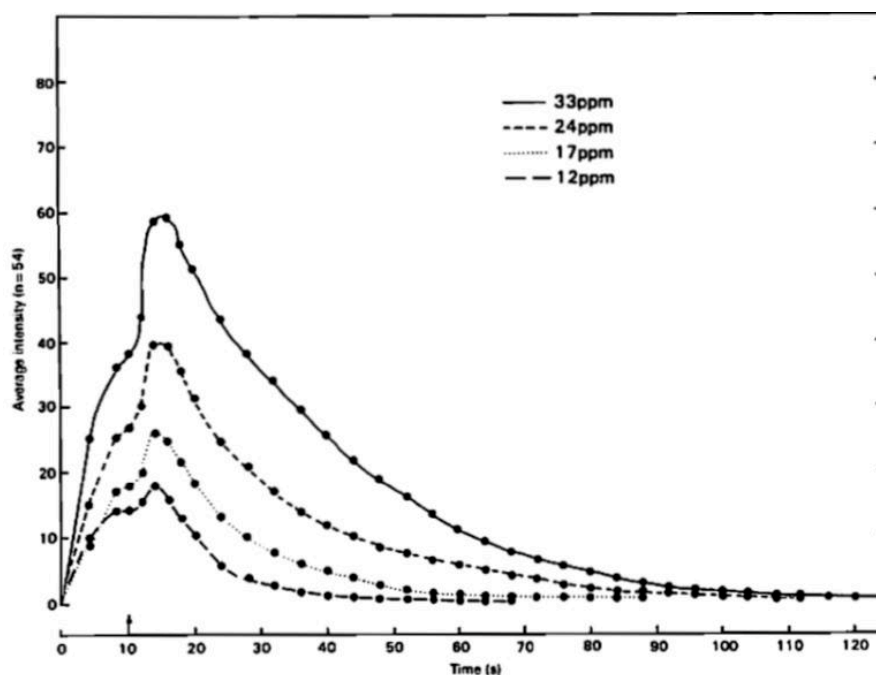


Figure 11. Average time-intensity curves for bitterness intensity of four concentrations of iso- α -acids (W. E. Lee & Pangborn, 1986).

Table 2. Concentration of rinse agents used in the three Experiments and viscosity of rinse solutions. Quoted from (Colonna et al., 2004).

Rinse	g/L	Experiment			Viscosity (cp)
		1	2	3	
Gelatin	6.00	×			1.22
Ovalbumin	4.00	×			0.85
PVP	4.00	×			0.87
Low pectin	1.00	×	×		1.41
High pectin	5.00		×	×	4.02
Low Polycose	5.00		×		1.16
High Polycose	40.00		×		0.93
Low CMC	0.01		×		1.44
High CMC	1.00		×		4.02
Crackers	na ^b			×	na ^b
De-ionised water	na ^b	×	×	×	1.00

^a × indicates which rinses were used in each Experiment

^b Not applicable

1.2 Preliminary sensory experiment

Internal panel composed of 9 subjects (Age=37 ± 13, range 22-60, 5M, 4F) was recruited from our laboratory to test the 2-AFC methodology. The session was divided into two parts. The first part was training session to be sure that subjects were able to clearly identify and differentiate astringency from bitterness and olfactory cues.

At the beginning of the testing session, panelists were asked to taste a model tannic acid solution of 1.76 g/L, in order that they have in mind a reference of an astringent product. Then, they rinsed their mouth with pectin (0.1%), bicarbonate (1%) and Evian water and waited for a 3 min break before threshold evaluation. The astringency threshold was evaluated by a 2-AFC procedure with ascending concentrations of tannic acid, which were presented four concentrations (g/L), i.e., 0.02, 0.08, 0.32 and 0.51. These concentrations were chosen referring to previous experiments performed for the European HealthSense project.

In each 2-AFC presentation, two samples were presented: a targeted sample and a control sample. Paired samples (5 mL) were presented in balanced order following a Latin square design (Williams design) at room temperature in a white plastic cup coded with the letter A or B. The testing procedure started from the lowest concentration. Panelists were given the control sample or targeted sample. They were asked to put the samples into their mouth, swirl them gently around the mouth for 30 s and then spit them out. They rinsed their mouth with pectin and waited for 1 min before evaluating the second sample. After 30 additional seconds, the panelists were asked to indicate which sample was perceived as astringent. Then, the

panelists rinsed their mouth with pectin, bicarbonate and Evian water. Each 2-AFC test was performed 3 times, the astringency sensitivity was obtained when three answers are right at the same concentration. The best estimate threshold for each subject was evaluated as the geometric mean of the three correctly answered concentration and the previous concentration. The evaluation was performed 3 times in 3 different sessions.

1.2.1 Preliminary experiment results

The mean threshold of tannic acid obtained in preliminary experiment is 0.19 ± 0.17 g/L (9 subjects). The sensitivity results were shown in Table 3. For each time of evaluation, the astringency threshold value was determined as the geometric mean of the three correctly answered concentrations and the previous lower concentration. For each subject, the astringency threshold value was calculated as the arithmetic mean of three evaluations. The mean threshold of 9 subjects was calculated as the arithmetic mean. Take subject 1 as example, the astringency threshold of the first evaluation is the geometric mean of 0.32 g/L and 0.08 g/L, the second and the third evaluation's threshold is the geometric mean of 0.02 g/L and the theoretical concentration below, i.e., $0.02/4=0.005$ g/L. Regarding the situation of subject 2, the astringency threshold of the first evaluation is the geometric mean of 0.51 g/L and the theoretical concentration above, i.e., $0.51 \times 1.58=0.80$ g/L. It is worth mentioning that the increasing tannic acid concentrations with a multiple of 4 was chosen until to 0.32 g/L, a multiple of 1.58 was chosen above 0.32 g/L. Because a multiple of 4 led to a too high concentration to challenge subjects on the detection of astringency. The mean astringency threshold of three evaluations of each subject is presented in Figure 12. We can observe a large interindividual variability and also the fact that some subjects are reproducible ($n^{\circ}4$, $n^{\circ}6$) and some are highly variable. One of the reasons can be linked to the stability of the tannic acid solution. Indeed, tannic acid can oxidize during storage and this oxidation can influence astringency. For this reason, it was decided to serve the tannic acid solutions 1 h after their preparation. Moreover, it was also decided to pay attention on the training and rinsing procedures for the final sensory experiment.

Table 3. Astringency threshold obtained from the internal 9 subjects. "0" in the table: the answer is wrong. "1" in the table: the answer is right. Red "1": astringency sensitivity level was reached when three correct answers from the same concentration were achieved. Each concentration was repeated 3 times, four ascending concentrations are presented, as shown in the first column. The evaluation was performed 3 times, rep 1: repetition 1, rep 2: repetition 2, rep 3: repetition 3.

Tannic acid	subject 1			subject 2			subject 3			subject 4			subject 5			subject 6			subject 7			subject 8			subject 9			
	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3	
C1 = 0.02g/L	0	1	1	0	0	1	0	0	1	0	0	0	0	1	0	1	1	0	1	0	1	1	1	1	0	1	0	
C1	1	1	1						0					1		0	0		1		0	1	1	1	1	1	0	
C1		1	1											1					0		0	0	0	1		1	0	
C2 = 0.08g/L	0			1	1	0	1	1	1	1	0	1	0	0	0	1	1	0	1	1	0	1	0	1	0	0	1	0
C2				0	1		1	0	0	1		0			1	1	0		1	0		1				1	1	
C2				1			1		0					1	1			1			1					1	1	
C3 = 0.32g/L	1			0		0	1	1	1	1	0	0	1			1	1		1	1		1	1		1	1	1	
C3	1						1	0	1	1	1	1	1			1	1		1	0		1	1		1	1	0	
C3	1						1		0	0	0		1			1	1		1		1				1	1	1	
C4 = 0.51g/L				0		1		1	1	1	1	1										1				1	1	
C4				0		1		1	1	1	1	1									1				1	1	1	
C4				0		1		1	1	1	1	1									1				1	1	1	

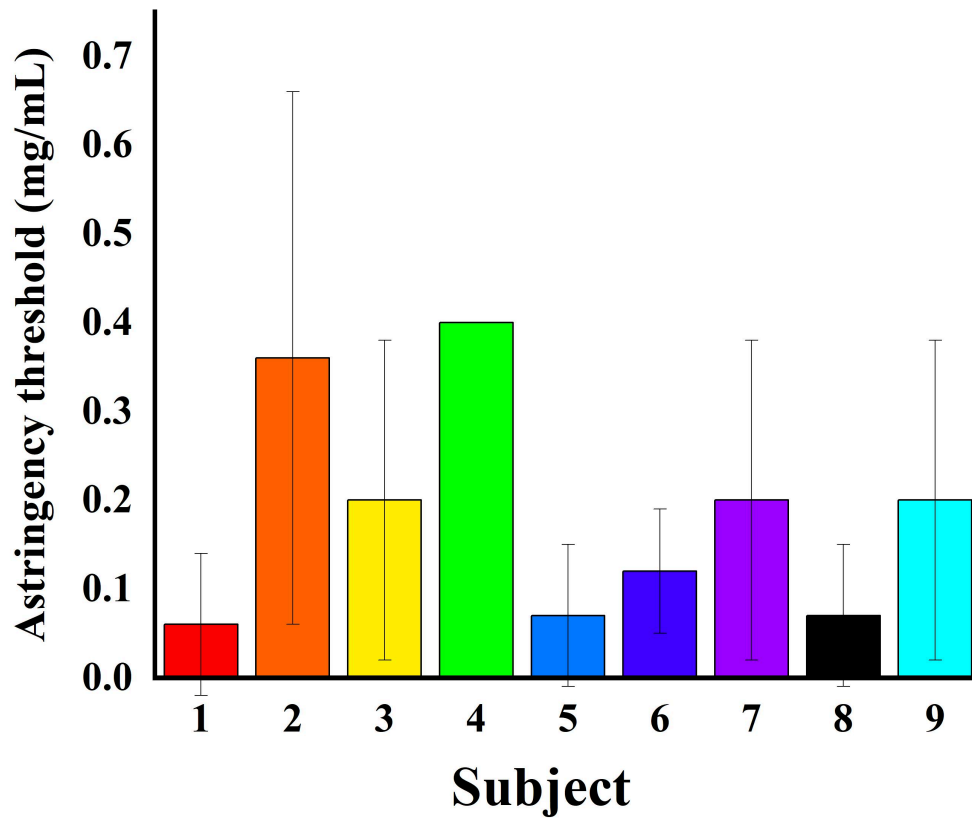


Figure 12. Mean astringency threshold of 9 subjects. X-axis represents each subject. Y-axis is astringency threshold. Whisker represents standard deviation.

At the end of the preliminary experiment a definitive methodology has been established. It is described in the “Final sensory methodology” section.

Figure 13 and Figure 14 are the diagrams of the procedures of 2-AFC and mouth rinsing.

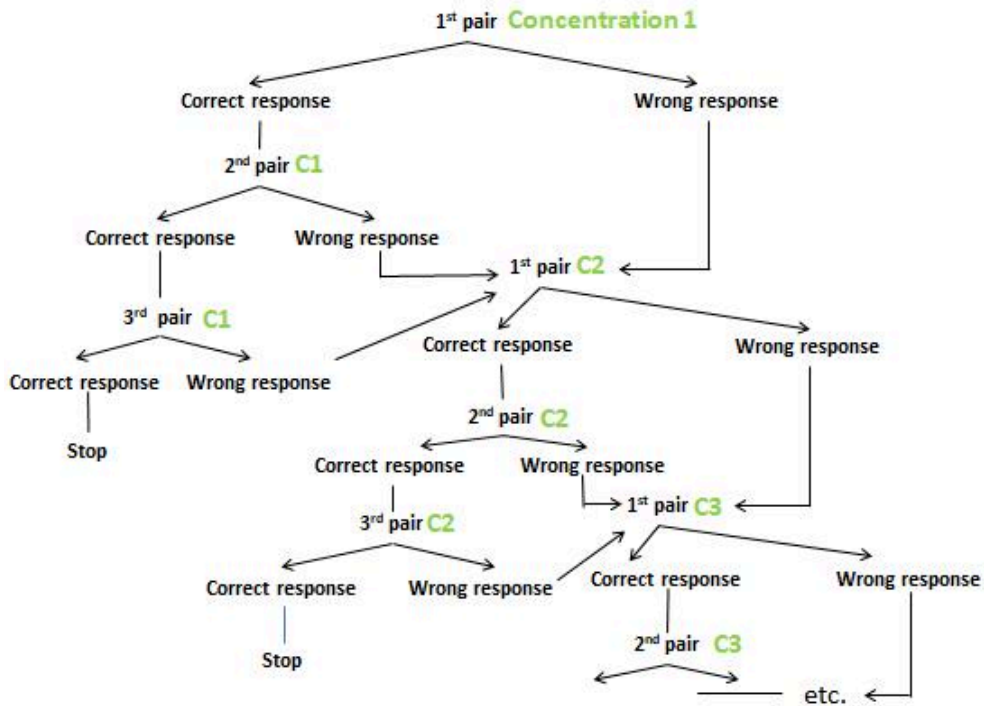


Figure 13. The procedure of 2-AFC in the sensory test. C1: concentration 1, C2: concentration 2, higher than C1, C3: concentration 3, higher than C2. Sensory test started from the lowest concentration 1. If the answer is right, subjects were given the same concentration samples. If the answer is wrong, subjects were given the higher concentration. The test was stopped when three answers were right at the same concentration.

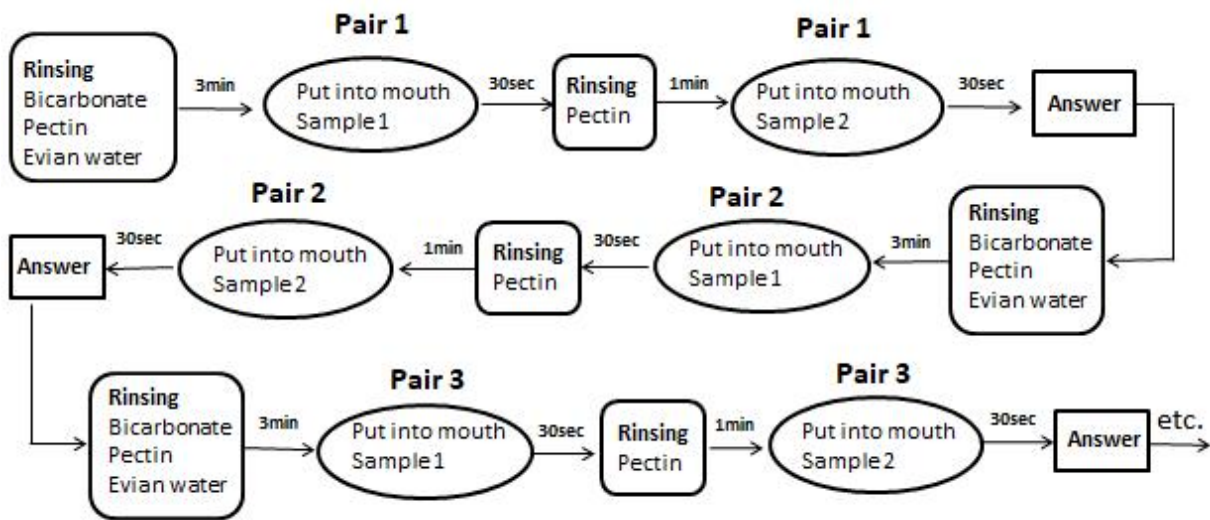


Figure 14. The procedure of rinsing mouth during sensory test. In each pair, rinsing mouth with pectin between samples. After a pair, rinsing mouth with bicarbonate, pectin and water.

1.3 Final sensory methodology

1.3.1 Materials

Solutions for rinsing consisted of 0.1% pectin (Sigma-Aldrich, France) and 1% sodium bicarbonate (Gilbert, France) dissolved in Evian water at room temperature.

Solutions for the sensory training session consisted of six taste solutions (salty, sour, sweet, bitter, umami, and astringent), whose composition is detailed in Table 4. Each solution was coded with random three-digit codes.

Solutions for astringency sensitivity evaluation consisted of four solutions with increasing tannic acid concentrations (in g/L) with a multiple of 3.05, i.e., 0.02, 0.062, 0.188, and 0.574, respectively. These concentrations were chosen on the basis of preliminary experiments performed with a small internal panel of subjects. It is worth mentioning that why we chose four concentrations. In the current sensory experiment, the maximum theoretical duration was estimated to 90 min. Increasing the number of concentrations would increase session duration significantly with possible bias due to elderly panelist fatigue. Thus four concentrations were chosen as a best compromise considering this constraint. All samples were prepared in Evian water 1 hour before testing at room temperature.

Table 4. Description of the tasting solutions used for the training session.

taste	salty	bitter	sweet	umami	sour	water	astringent	bitter	water	astringent
compose	Salt 3 g/L	Leucine 8 g/L	Sugar 15 g/L	Glutamate 0.6 g/L	Lactic acid 2 mL/L	Evian	Tannic acid 1.76 g/L	Leucine 8 g/L	Evian	Tannic acid 1.76 g/L
code	723	092	442	128	505	122	548	904	883	801

Table 5. Materials and sources of tasting samples in the sensory test.

Materials	Sources
Pectin	Sigma-Aldrich, France
Bicarbonate	Gilbert, France
Tannin acid	Sigma-Aldrich, France
Salt	Sigma-Aldrich, France
Leucine	Dolder, Rexim, France
Sugar	Sigma-Aldrich, France
Glutamate	Merck, France
Lactic acid	Fluka, France
Evian water	Evian, France

Fifty-four panelists, including 30 elderly (O) people (14 female, 16 male) (mean age is 75 years) and 24 young (Y) people (6 female, 18 male) (mean age is 25.4 years) were recruited to participate in the sensory sessions. The number of subjects was determined by a power test. Based on preliminary results described previously obtained on the internal panel, a number of at least 23 subjects per group (Y or O) were necessary to observe a difference equal to at least one standard deviation between the groups. More subjects were recruited in case of defection. All participants had good dental status. Some elderly participants took drugs with a mean

number of 2, which is considered lower than what was commonly observed in elderly population (ranging from 2.9 to 3.7 medications) (Schiffman et al., 1998).

1.3.2 Training session

The training session was divided into two parts. During the first part, subjects received 20 mL of each tasting sample in a fixed order at room temperature in plastic cups coded with random numbers. They were instructed to put the samples into their mouths, swirl the sample gently in the mouth for 30 s, spit it out and judge which taste it was. Between samples, subjects rinsed their mouth with Evian water and then waited for 1 min before the next sample. In the second part, subjects were trained for the 2-AFC procedure using astringency stimuli as described below. During both parts of the training session, there was a discussion between subjects and experimenters after each test. The objective for the experimenters was to be sure that subjects were able to (i) clearly identify and differentiate astringency from other sensory sensations, in particular bitterness, and (ii) perfectly understood the procedure of the sensory test, i.e. the 2-AFC. The whole session was conducted under red light to eliminate color differences.

1.3.3 Testing session

Astringency sensitivity evaluation was performed 3 times in 3 different testing sessions. Each whole session was conducted under red light in a sensory room equipped with individual boxes.

At the beginning of the session, panelists were asked to taste a model tannic acid solution of 1.76 g/L to be well aware of astringency. Then, they rinsed their mouth with pectin, bicarbonate and Evian water and waited for a 3 min break before threshold evaluation. The astringency threshold was evaluated by a 2-AFC procedure with ascending concentrations of tannic acid as described above in the Materials section. In each 2-AFC presentation, two samples were presented: one was the target sample, and one was the control. Each 2-AFC test was performed 3 times. Pair samples (5 mL) were presented in balanced order following a Latin square (Williams design) at room temperature in a white plastic cup coded with the letter A or B. The testing procedure started from the lowest concentration. Panelists were given the reference or stimulus sample. They were asked to put the samples into their mouth, swirl them gently around the mouth for 30 s and then spit them out. They rinsed their mouths with pectin and waited for 1 min before evaluating the second sample. After evaluating the second sample, the panelists were asked to indicate which sample was perceived as astringent. Then, the panelists rinsed their mouth with pectin, bicarbonate and Evian water before performing another 2-AFC test. Figure 13 and Figure 14 present the 2-AFC procedure and rinsing procedure respectively.

The sensitivity level was reached when three correct answers from the same concentration were achieved. The best estimate threshold for each subject was evaluated as the geometric mean of the three correctly answered concentrations and the previous lower concentration. When subjects correctly identified the lowest concentration (0.02 g/L), the geometric means

were calculated between this concentration and the theoretical concentration below, i.e., $0.02/3.05=0.0065$ g/L. In contrast, when subjects did not correctly identify the highest concentration (0.574 g/L), the geometric mean was calculated between this concentration and the theoretical concentration above, i.e., $0.574 \times 3.05=1.75$ g/L.

1.3.4 Saliva collection

All sessions were performed and saliva was collected from subjects between 2 pm and 6 pm to minimize circadian rhythms as much as possible for 3 months between the middle of November and the end of January. The subjects were instructed to avoid drinking, eating or smoking for at least 1 h before the session. Saliva was collected after the panelists had rinsed their mouths with 0.1% pectin, 1% bicarbonate and water at the start and at the end of the session. Saliva was collected by expectorating saliva into a pre-weighed tube with a cap for 5 min as described in previous study (Neyraud et al., 2012). After collection, the tubes were weighed and then stored at -80 °C. Flow rates were determined gravimetrically and expressed as grams per minute (g/min).

1.3.5 Salivary protein amount estimation

1.3.5.1 Bradford protein assay

Bradford protein assay was performed to determine the total amount of protein. This method uses the ability to bind the Coomassie Brilliant Blue G-250 dye to protein, using ionic and hydrophobic bonds. After the reaction with protein, the dye changes color in an acidic environment from brown to blue.

Saliva samples were centrifuged at 15000 g for 15 min at 4 °C before analysis. The protein concentration was determined on the supernatant using the Bradford protein assay with bovine serum albumin (BSA) used as the standard for calibration. The calibration curve is shown in Figure 15.

1.3.5.2 BSA standard range

Firstly, BSA standard range was prepared with 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1 mg/mL. Then 5 µL of each range solution was distributed into the microplate wells in triplicate with 3 blanks of 5 µL of MilliQ water. Then 250 µL of Bradford reagent was added to the wells containing the ranges and blanks. The microplate was incubated for 15 min at room temperature before reading the absorbance at 595 nm with the Victor3 microplate reader (PerkinElmer, USA).

Table 6. Gradient concentrations and absorbance of BSA.

BSA (mg/mL)	0	0.1	0.2	0.3	0.4	0.6	0.8	1
----------------	---	-----	-----	-----	-----	-----	-----	---

Absorbance	0.447	0.518	0.563	0.645	0.708	0.784	0.886	0.983
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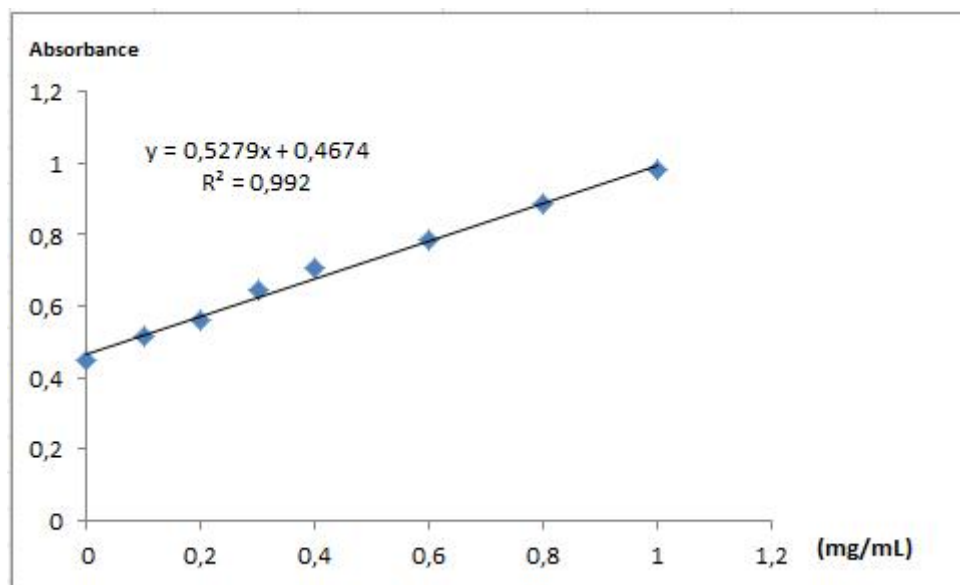


Figure 15. The calibration curve and formula of BSA. X-axis is the concentration of BSA, Y-axis is the absorbance of BSA.

1.3.5.3 Saliva samples assay

5 μ L of saliva diluted 2 folds was distributed into microplate wells in triplicate. Then 250 μ L of Bradford reagent was added to wells. The microplate was incubated for 15 min at room temperature before reading the absorbance at 595 nm with the Victor3 microplate reader (PerkinElmer, USA).

2. Biology experiment

2.1 Saliva samples sterilization

Saliva collection was described in sensory experiment part. After collection, the tubes were stored at -80 °C until analyzed. Before treating saliva samples, we conducted saliva samples sterilization in 60 °C water bath for 1 h for the sake of safety in the context of the pandemic. Then 2 mL saliva was taken from each tube into Eppendorf. The Eppendorfs were centrifuged (15000 gravitational force [g], 15 min, 4 °C) to eliminate impurities, then 50 μ L supernatant was taken from each Eppendorf into the other Eppendorf for electrophoresis and western-blot. The left supernatant of each sample was kept for Bradford, all were stored at -80 °C.

2.2 Production of IB5 and II-1

IB5 is human PRP, used as a model of salivary bPRP, II-1 is a model of salivary gPRP, both of them have extendedly disordered structures and their amino acid sequences are tandem

repeated, as shown in Figure 16. They have been obtained by heterologous expression of the human gene PRB4S in the yeast *Pichia pastoris* (Pascal et al., 2006). PRB4S is a human salivary proline rich pro-protein with peptide of 220 amino acids, which presents several repeated sequences containing three to five successive proline residues and six potential glycosylation sites (Pascal et al., 2006). By heterologous expression in *P. pastoris*, large amount of recombinant PRPs are likely to be obtained with sufficient amounts to be used in the study whether they are glycosylated and cleaved by proteases or not (Pascal et al., 2006). Then the produced proteins were purified from cell culture supernatant as described below.

(a)

```

1 FLISGKPVGRRPQGGNQPQRPPPPP
26      GK PQGPPPQGGNQSQGPPPPP
47      GKPEGRPPQGRNQSQGPPPHP
68      GKPERPPPQGGNQSQGTPPPP
89      GKPERPPPQGGNQSHRPPPPP
110     GKPERPPPQGGNQSRGPPPHR
131     GKPEGPPPQEGNKRSRSAR

```

(b)

```

1 SPPG  KPQGPPQQE
14  G  NKPQGPPPP
24  G  KPQGPPPA
33  GGNPQQPQAPPA
45  G  KPQGPPPPPQ
56  GGRPPRPAQGQQPPQ

```

Figure 16. Sequences of the recombinant PRPs. (a) II-1; (b) IB5. Potential glycosylation sites are indicated in boldface type (Pascal et al., 2008).

Abbreviations:

BMGY: Buffered Medium Glycerol complex
 BMMY: Buffered Medium Methanol Tryptone
 MD: Medium Dextrose
 MeOH: Methanol
 RPM: Revolution per Minute
 YNB: Yeast Nitrogen Base

2.2.1 Seeding of the yeast strain

Medium dextrose (MD) petri dish got warm at room temperature for 30 min before

experiments under microbiological safety hood. The yeast strain was preserved in a specific medium (freezing solution composed of Bacto yeast extract, peptone, dextrose aqueous solution and glycerol solution) in a tube at -80 °C. After the tube was taken out from the freezer, a piece of frozen strain with a sterile toothpick was sampled in the tube. Then the piece of frozen strain was spread on the petri dish. After that the petri dish was incubated into the stove at 28-30 °C for 2 days for strain development.

2.2.2 Pre-culture

Sterile Erlenmeyer of 50 mL was prepared according to the number of pre-cultures. The Petri dishes (from the stove) were put under the microbiological safety hood. The volume of BMGY was prepared according to the number of Erlenmeyer. Then 10 mL of BMGY was put into each Erlenmeyer of 50 mL with the pipet-aid. A yeast colony from the MD Petri dish was taken using a sterile tip and then it was put into the Erlenmeyer of 50 mL. At last, the Erlenmeyer was put into a Multitron incubator under stirring at 225 rpm at 28 °C for 24 h.

2.2.3 Culture of 250 mL in BMGY day1

The Erlenmeyer was taken out from the incubator and then it was put under the microbiological safety hood. The cap was taken off, then we measured the optical density (OD) of pre-culture at 600 nm after a dilution at 1/100. A sterilized Erlenmeyer of 2 L was prepared, and it was put under the microbiological safety hood. The adapted volume (250 mL for 1 Erlenmeyer of 2 L) of the BMGY medium was prepared under the microbiological safety hood. The volume (V) of pre-culture to put into each Erlenmeyer of 2 L was calculated as following, i.e. $V = OD_{Final} \times Final\ Volume\ of\ the\ culture / OD_{initial} \Rightarrow 0.1 \times 250 / OD$ of the pre-culture diluted at 1/100 multiplied by 100. Then several drops of antifoam Y-30 Emulsion were added. The Erlenmeyer was put into Multitron incubator under stirring at 225 rpm at 28 °C for 24 h.

2.2.4 Changing of culture medium day2

The 2L Erlenmeyer was taken out from the incubator and it was put under microbiological safety hood. Then we measured OD 600 nm of culture diluted at 1/100. If OD is under 30, 5 mL of glycerol (1%) was added into 250 mL of culture. When OD had reached 40, the culture was put into specific centrifugation bottles ThermoFisher (250 mL). The bottles were equilibrated on a weighing machine, after that they were centrifuged at 4000 rpm at 29 °C for 10 min. The supernatant was discarded into biological waste. Then the pellets were re-suspended into 250 mL of BMMY and poured it into the Erlenmeyer. Several drops of antifoam Y-30 Emulsion were added into the Erlenmeyer which was then stirred at 225 rpm at 28 °C.

2.2.5 Incubation day2-day6

Methanol was added into the Erlenmeyer 2 or 3 times a day in order to induce the expression

of PRP by the strain because the promoter of the synthetic gene introduced into the yeast genome is activated by methanol.

2.2.6 Ending of the cultures day7

The culture in Erlenmeyer was taken out from the incubator and put into centrifuge bottles Beckman 1 L. Centrifugation was set at 15000 rpm for 30 min at 4 °C. The resulting supernatant was filtered at 2 µm, 1 µm, 0.7 µm and 0.45 µm. The filtered supernatant was dialyzed into beakers of 4L with Tris-HCl solution (50 mM, pH 8). Then the beakers were put at 4 °C for 4 h. The dialysis was repeated once. The resulting dialysate was filtered on 0.22 µm filter.

2.3 Purification of IB5 and II-1

2.3.1 Cation exchange

After dialysis, PRPs were recovered from the supernatant using cationic chromatography. Firstly, the column (XL-SP) was washed and equilibrated by 0.45 µm filtrated 50 mM Tris-HCl pH 8 buffer, flow rate was set at 0.5 mL/min. Then the dialysate was injected. After injecting the sample, a linear gradient from 0 to 100% with 1 M of NaCl buffer was used for elution of the proteins. The fractions were collected in the plastic tubes placed into the sample collector. The protein elution was monitored by measuring absorbance at 230 nm. After collecting the fractions, the column and the device were washed with water and 20% of ethanol.

2.3.2 Gel filtration

A partial separation of the PRPs was followed up by size exclusion chromatography. The column was a HiPrepSephacryl S100 26/600 bought from GE Healthcare. First, the column was equilibrated 0.45 µm filtrated acetate ammonium buffer 50 mM, pH 7.5, flow rate was set to 1 mL/min. PRPs were eluted at a flow rate of 1 mL/min and 2.5 mL of fractions were collected at 230 nm eluted peaks as PRPs do not have aromatic amino acids. Collected fractions were checked by electrophoresis. Those containing either IB5 or II-1 were lyophilized until use.

2.4 Setting up a methodology to evaluate PRP levels in saliva

Currently, PRPs analysis in saliva mostly focuses on High Performance Liquid Chromatography (HPLC) to analyze protein profile (Hay, Ahern, Schluckebier, & Schlesinger, 1994; Kallithraka et al., 2001; Soares, Silva, et al., 2019) or High Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD), which could separate different fractions at the DAD chromatographic detector outlet. Identification was then carried out by Nanoscale Liquid Chromatography coupled to tandem Mass Spectrometry (nLC-MS-MS) or Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS) and

Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF/TOF) analysis (Ramos-Pineda et al., 2017; Ramos-Pineda, Garcia-Estevez, et al., 2019; Soares, Mateus, & de Freitas, 2012; Soares et al., 2011). Some studies identified PRPs in saliva by using Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE), which could separate proteins according to their molecular weight (C. A. Lee, Ismail, & Vickers, 2012; Pascal et al., 2006). However, the aims of above studies were to identify PRPs and not to quantify the amount of PRPs in saliva. Our work aim is to determine the amount of PRPs in saliva. Therefore, it is not suitable for our work to use these methodologies. Among accessible methodologies, SDS-PAGE can be developed to be used as a methodology to assess the amount of PRP in saliva. PRPs bands have a different behaviour on the gel after staining with Coomassie Brilliant Blue R-250 and discoloration process, these bands can appear pink color instead of blue due to the particular amino acid composition (glycine, glutamic acid, glutamine and proline accounted for 70% to 90% of the total amino acids) (Beeley, J. A. et al., 1996). Therefore, PRPs are likely to have a different slope regarding the correspondence between the colour intensity and protein concentration. The principle of this method is the intensity of coloration of a band which can be correlated to the protein concentration. However, the challenge is to establish a linear correlation (calibration curve) between the colour intensity and concentration of pure PRP that will be used for quantification of PRPs in saliva. Except for SDS-PAGE, Baum et al. (1982) first developed a modified microimmunodiffusion technique using antiserum against PRP to quantify PRP in stimulated parotid glands (Baum et al., 1982).

2.4.1 SDS-PAGE PRP evaluation

Electrophoresis (SDS-PAGE) was chosen to be used as a methodology to assess the amount of PRP in saliva. As mentioned previously, the principle is the intensity of coloration of a band that can be correlated to the protein concentration. However, it was difficult to find pink color on the gel if analyzing saliva directly by SDS-PAGE. Therefore, several methods were conducted to solve this problem.

First, taking advantage of biological characteristics of tannic acid that can precipitate PRPs, 10 μL of saliva was mixed with 30 μL of tannic acid in 50% of MeOH solution. Moreover, PRPs can be chemically extracted by organic solvent (methanol, ethanol), organic acid (trifluoroacetic acid, formic acid). Thus each 200 μL of saliva was mixed with 800 μL methanol (MeOH), 800 μL of ethanol (EtOH), 200 μL of trifluoroacetic acid (TFA), 200 μL of formic acid (FA). The concentration of each reagent is shown in Table 7. All the mixtures were agitated overnight at 4 $^{\circ}\text{C}$. Supernatant and pellet of each mixture were separated after centrifugation (13000 g, 30 min, at 4 $^{\circ}\text{C}$). Both of supernatant and pellet were analyzed by electrophoresis. The parameters of SDS-PAGE are shown in Table 8. Unfortunately, there were no pink bands on the gels either supernatant or pellet. The gel scanning images are shown in Figure 17 and Figure 18.

Table 7. The concentration of each reagent used in the SDS-PAGE.

Reagent	Concentration
Tannic acid	0.5 mg/mL in 50% MeOH: 2.5 mg tannic acid into 5 mL of 50% Methanol 2.5 mg/mL in 50% MeOH: 12.5 mg tannic acid into 5 mL of 50% Methanol
MeOH	50%: 5 mL methanol into 10 mL MilliQ water
EtOH	100%
TFA	1%: 1 μ L TFA into 99 μ L MilliQ water
FA	1%: 1 μ L FA into 99 μ L MilliQ water

Table 8. Materials, parameters and corresponding information of SDS-PAGE.

Parameters	Specific information
Gel type	Precast 12% of acrylamide, 10 wells
Loading Buffer	Biorad Leamilli buffer
Voltage	200 V
Pellet loading	20 μ L buffer mixed with pellet
Supernatant loading	20 μ L buffer mixed with 20 μ L supernatant
Heating before loading	100 °C 3 min

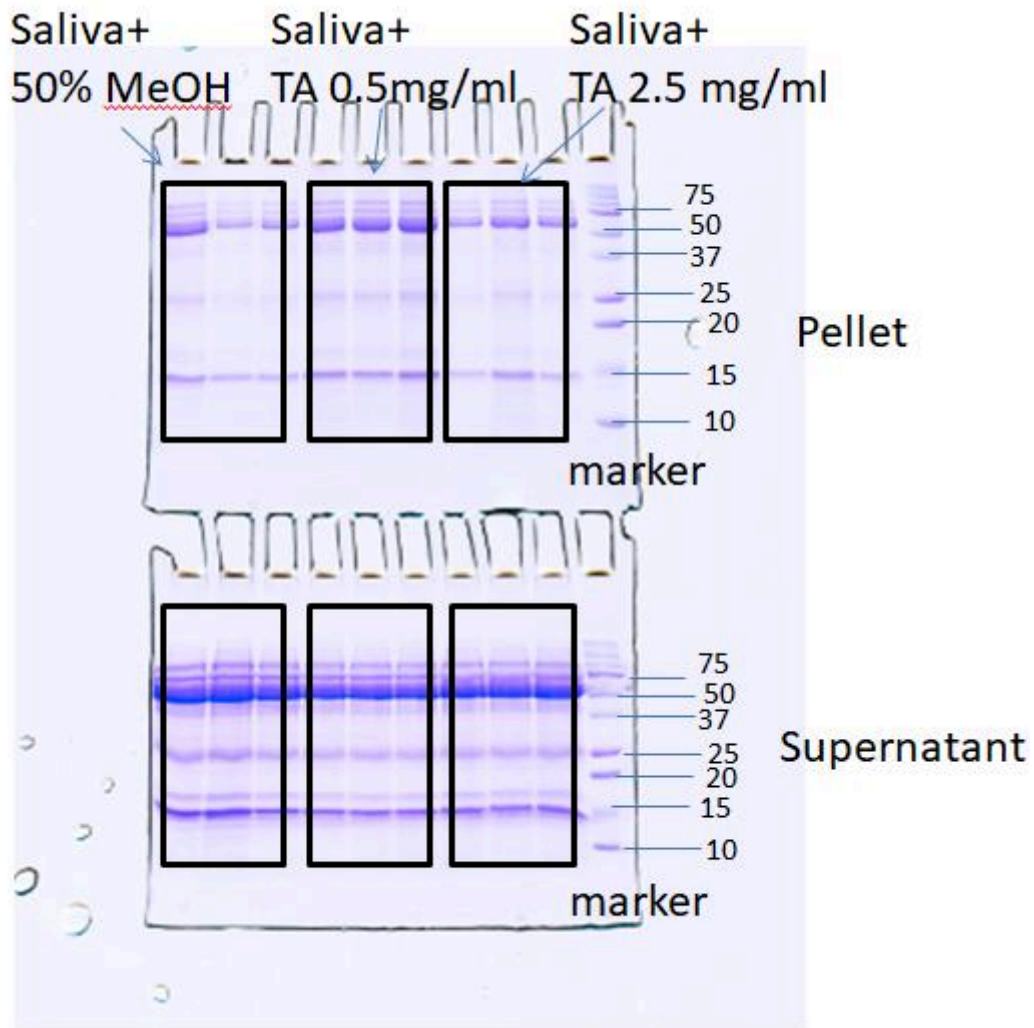


Figure 17. Gel images of supernatant and pellet separated from mixtures of 0.5 mg/mL (three lanes, corresponding to 3 repetitions, in the middle circled by the black box) and 2.5 mg/mL (the first three lanes from right circled by the black box) of tannic acid (TA). 10 μ L of saliva precipitated by 30 μ L of 50% of MeOH served as a control (the third three lanes from right circled by the black box). The top gel is pellet, the bottom gel is supernatant, lane information is the same with the top gel. The first lane (mark) from right corresponds to standardized protein markers.

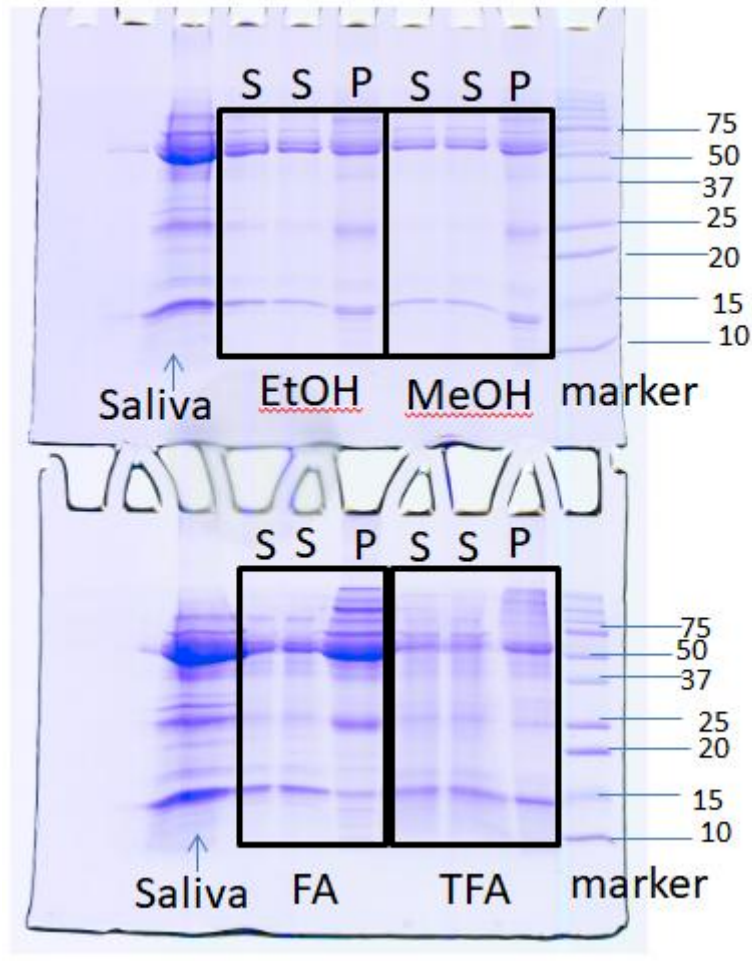


Figure 18. Gel images of supernatant and pellet separated from mixtures using MeOH (the first three lanes from right circled by the black box on the top gel), EtOH (the second three lanes from right circled by the black box on the top gel), TFA (the first three lanes from right circled by the black box on the bottom gel), FA (the second three lanes from right circled by the black box on the bottom gel). S: supernatant, two repetitions of supernatant. P: pellet. 20 μ L of saliva sample was loaded into the well with 20 μ L of buffer directly as a reference (the last lane from right). The first lane (mark) from right corresponds to standardized protein markers. The molecular weight is only presented on the bottom gel, the same with the top gel.

Second, the strategy to solve above problems was proposed. 50 μ L of saliva was concentrated by Speedvac. The concentrate was treated by precipitation ways as described above. In addition, the concentrated saliva was also analyzed by electrophoresis directly. All parameters of SDS-PAGE were the same with those mentioned above. The concentrations of precipitated reagent (tannic acid, MeOH, EtOH, TFA, FA) did not change. Results show that these methods were effective, the pink bands are visible on the gel. However, taking into consideration the time consumed using the method, concentrated saliva was analyzed directly by SDS-PAGE. The gel image of gel scanning is shown in Figure 19. The pink bands are around 25 kDa and it is likely to be non-glycosylated of II-1 (Pascal et al., 2006). bPRPs are still not visible on the gel.

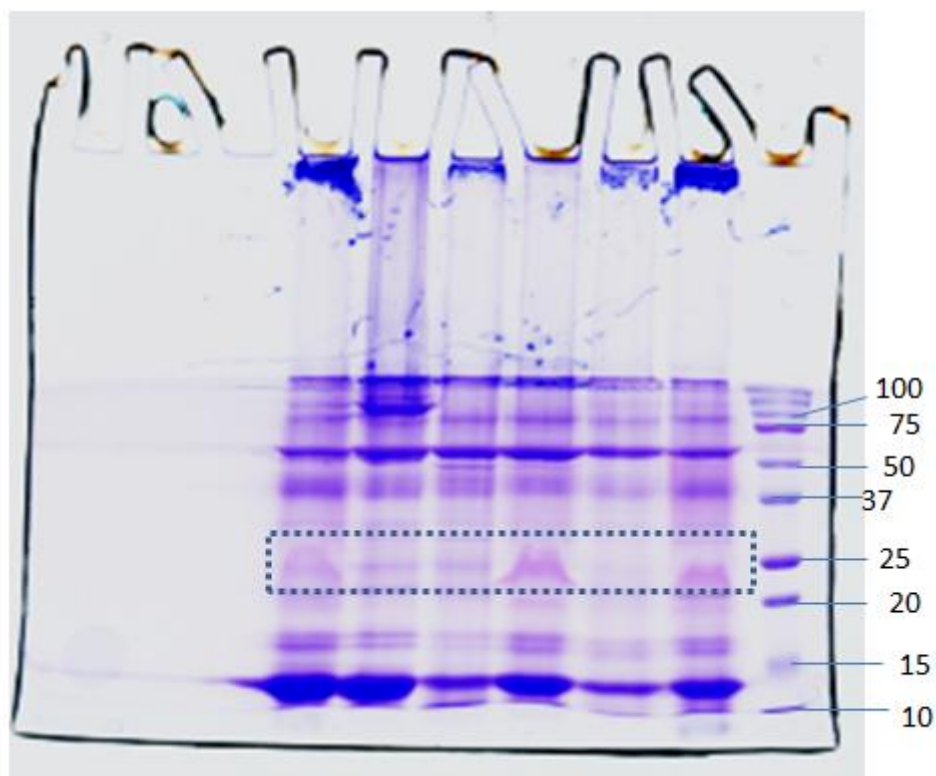


Figure 19. Gel image of concentrated saliva obtained from six subjects and analyzed by SDS-PAGE directly. Pink bands appeared on the gel, they are circled by the dotted box. The first lane (mark) from right corresponds to standardized protein markers.

Finally, western-blot approach was developed using specific antibody to detect the targeted proteins (bPRPs and gPRPs), since the color of PRPs bands on the SDS-PAGE gel was too faint to measure and in particular for the low concentration of bPRPs. This methodology is described in the next paragraph below.

2.4.2 PRP evaluation by immunochemically-western-blot

2.4.2.1 Electrophoresis

Saliva samples were dried with SpeedVac device, and each saliva sample was dissolved in 20 μ L buffer (Laemmli 2x Biorad) by vortexing for 1 min and rehydration for 1 min at room temperature. Then Eppendorf with dried saliva and buffer was heated at boiled water temperature for 3 min. After cooling down, 20 μ L aliquots were loaded on 12% commercial precast gel Biorad. Electrophoresis was run at a constant voltage of 200 V for approximately 35 min. After electrophoresis, the gels were put into MilliQ water for further analysis.

2.4.2.2 Western-blot

The parameters of the materials used in the western-blot are shown in Table 9.

Table 9. The parameters of the materials used in western-blot.

Materials	Parameters
NaCl solution	9‰: 9 g of NaCl into 1000 mL MilliQ water
PBS solution	100 mL of PBS, 0.5 mL of Tween, 900 mL of MilliQ water
NaOH solution	0.1M: 4 g of NaOH into 1000 mL MilliQ water.
Skim milk solution	8% in 9‰ of NaCl solution: 4 g of skim milk powder into 50 mL of 9‰ of NaCl solution.
Bovine serum albumin (BSA) solution	5%: 1 g of BSA powder into 20 mL of NaCl solution for antibody solutions.
Primary antibody solution	1/1000: 20 μ L of serum antibody (against IB5) into 20 mL of 5% of BSA solution in 9‰ of NaCl.
Secondary antibody	1/5000: 4 μ L of mouse anti-rabbit couple HRP (Fisher) into 20 mL of 5% of BSA solution in 9‰ of NaCl.

Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane after SDS-PAGE, the parameters of TransBlot were set as following, transfer time was 7 min, voltage was 25 V which corresponds to the program “Mixed Molecular Weight Biorad”. In the process of immunological reaction, serum antibody against IB5 was used as the primary antibody at a concentration of 1/1000. This serum has been obtained from the blood of a rabbit after full immunization procedure. On that purpose IB5 and an immunogenic protein were injected into the rabbit for 4 weeks to provoke the immune response. Mouse anti-rabbit couple HRP (Fisher) was used as the secondary antibody at a concentration of 1/5000.

We performed preliminary tests on some saliva, IB5 and II-1 samples on the same membrane. Figure 20 presents the membrane after western blot analysis. It shows that IB5, II-1 as well as bPRPs and gPRPs in saliva are visible on the membrane. From this result, it can be assumed that antibodies can detect both basic and glycosylated PRPs.

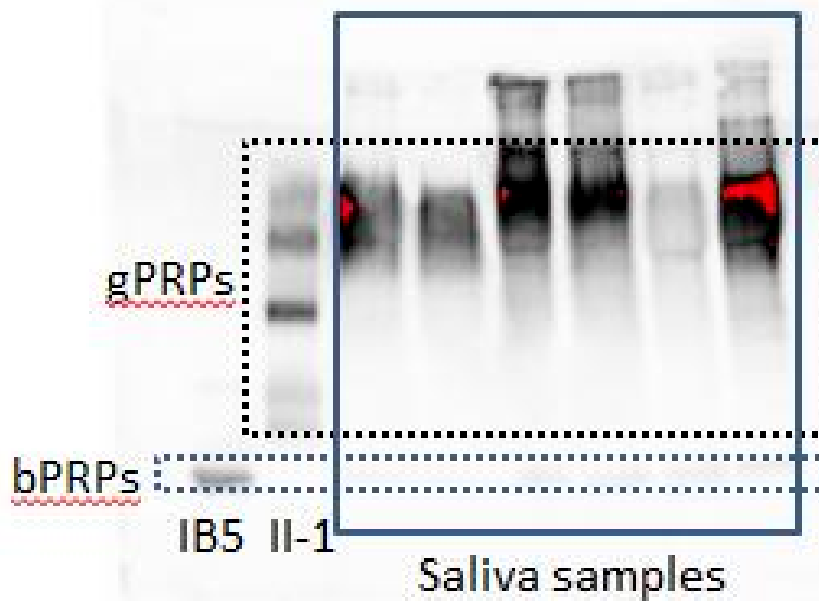


Figure 20. Image of membrane of saliva samples, IB5 and II-1 as a reference. The first lane from left is IB5, the second lane from left is II-1, the third to eighth lanes from left are saliva samples (circled by the blue box). The bands of saliva samples appeared near IB5 are regarded as bPRPs, bPRPs and IB5 are circled by the blue dotted box. The bands of saliva samples appeared near II-1 are regarded as gPRPs, gPRPs and II-1 are circled by the black dotted box.

Moreover, we check antibody specificity by studying its reactivity toward cystatin and amylase. Indeed, the molecular weight of bPRPs is similar to cystatin's molecular weight (Carpenter, 2012) and the molecular weight of amylase is within the range of the molecular weight of gPRPs (Soares et al., 2011). We tested thus pure cystatin and amylase against the antibodies used for IB5 and II-1 detection. Results showed a complete absence of reaction against cystatin and amylase ensuring the absence of cross reactions with cystatin and amylase.

i. Coloration with red Ponceau

After transferring, the membrane was rinsed with MilliQ water, then it was immersed in a red coloration Ponceau for 5 min to stain and detect proteins on the PVDF membrane. The membrane was next rinsed again with distilled water, then it was decolorized using 0.1 M NaOH solution, at last it was rinsed with water for 2-3 min.

ii. Immunological reaction

After coloration and discoloration, the membrane was rinsed with 9‰ NaCl solution two times. Blocking was performed with 8% skim milk in 9‰ NaCl for 1 h with agitation at room temperature. The membrane was rinsed with 9‰ NaCl solution two times. The membrane

was then incubated with a primary antibody at 1: 1000 in 5% BSA in 9‰ NaCl solution for 1 h at room temperature. After 2 washes in 0.05% PBS-Tween solution, incubation was performed again with 8% skim milk in 9‰ NaCl solution for 30 min with agitation at room temperature. After 2 additional washes in 9‰ NaCl solution, the membrane was then incubated with HRP-conjugated secondary antibody at 1: 5000 in 5% BSA in 9‰ NaCl solution for 1 h at room temperature. Then the membrane was rinsed with 0.05% PBS-Tween 3 times under agitation. The last step was that the membrane was rinsed in 9‰ NaCl solution.

iii. HRP colorimetric revelation following the Bio-Rad kit

Targeted proteins (PRPs) were detected by using an ECL (Clarity Enhanced Luminescence) detection system. A 1:1 mix of the 2 solutions (luminol solution and peroxide solution) was made. Membrane was immersed in ECL solution for 5 min, then the membrane was analyzed by ChemiDoc imaging system as soon as possible. Membrane was scanned with different exposure time. The best exposure time was chosen according to the pixel volume of targeted PRPs that must not be saturated.

2.5 Calibration curve of IB5 and II-1

Basic PRPs (bPRPs) levels were determined by calibration curve of IB5, glycosylated PRPs (gPRPs) levels were determined by calibration curve of II-1. The pixel volumes of the bPRPs and gPRPs bands were compared to standard curves generated for the IB5 and II-1 to yield quantitative measures for bPRPs and gPRPs. The gradient concentrations of IB5 were 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 mg/mL. The gradient concentrations of II-1 were 0.25, 1, 3, 5, 10, 25, 50 mg/mL. Both IB5 and II-1 were performed 10 repetitions. The pixel volume was determined at the critical point of pixel saturation. The ratio of pixel volume of each concentration of IB5 or II-1 to pixel volume of 3mg/ml was used as Y value in the calibration curve, each concentration of IB5 or II-1 was used as X value in the calibration curve. Similarly, the ratio of the pixel volume of each saliva to pixel volume of 3mg/ml of IB5 or II-1 as Y value to calculate the PRPs concentration in saliva according to the calibration curve. The membrane images of IB5 and II-1 are shown in Figure 21 and Figure 22. IB5 and II-1 calibration curves are shown in Figure 23 and in Figure 24 respectively. In addition, the membrane image for bPRPs of saliva sample and the membrane image for gPRPs of saliva sample are shown in Figure 25 and Figure 26.

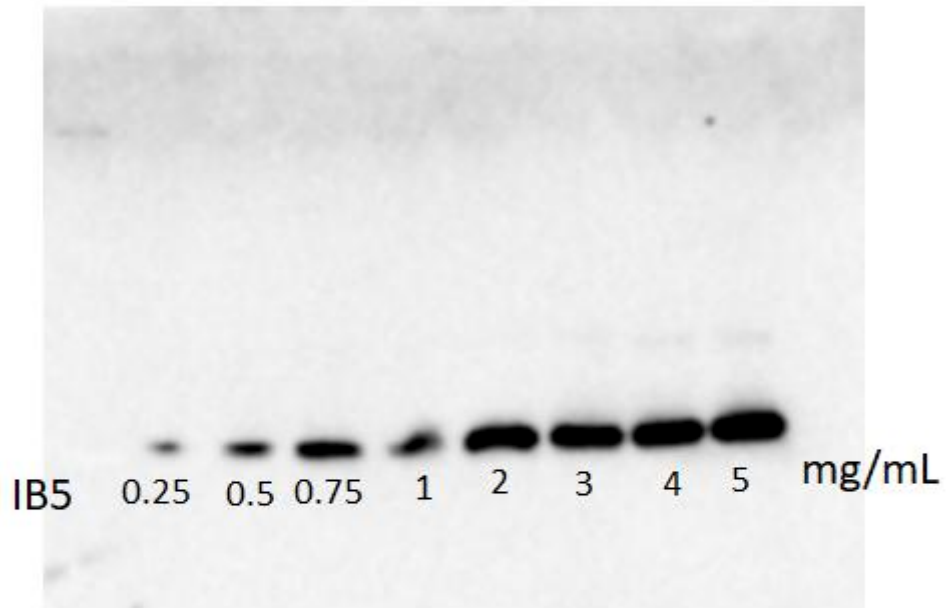


Figure 21. Membrane image of gradient concentrations of IB5. Each concentration was shown on the membrane. The higher is the concentration, the higher is the color intensity (pixel volume).

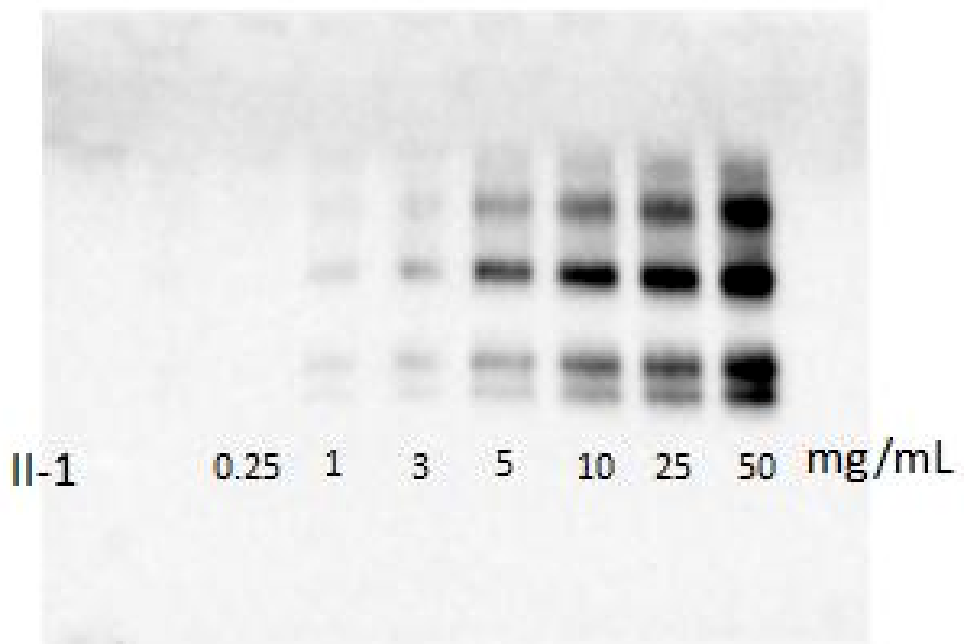


Figure 22. Membrane image of gradient concentrations of II-1. Each concentration was shown on the membrane. The higher is the concentration, the higher is the color intensity (pixel volume).

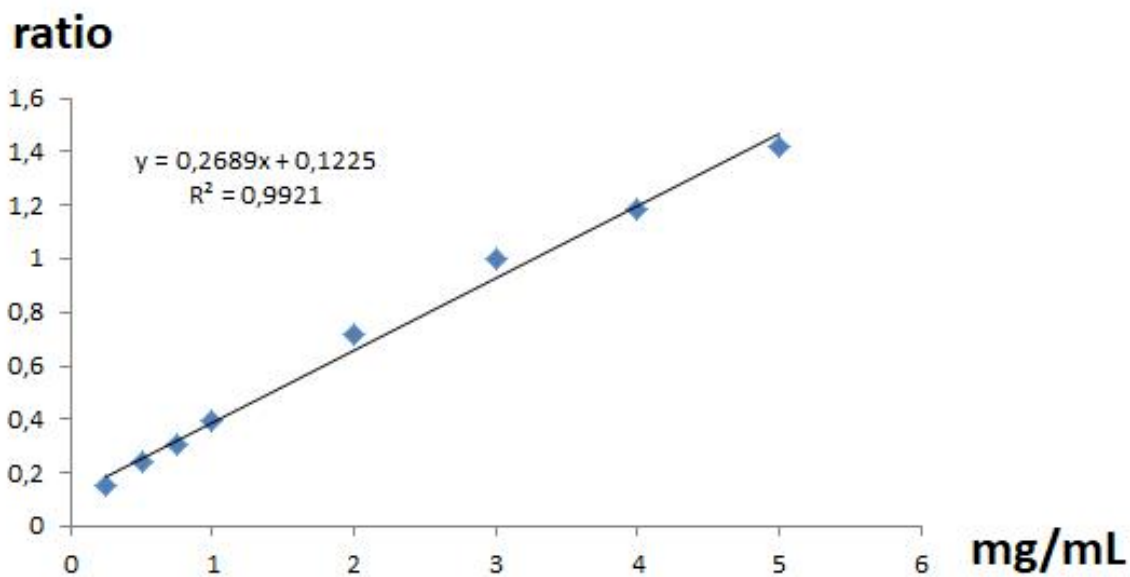


Figure 23. Calibration curve of IB5. X-axis is the concentration of IB5, Y-axis is the ratio of the pixel volume of each concentration to the pixel volume of 3 mg/mL.

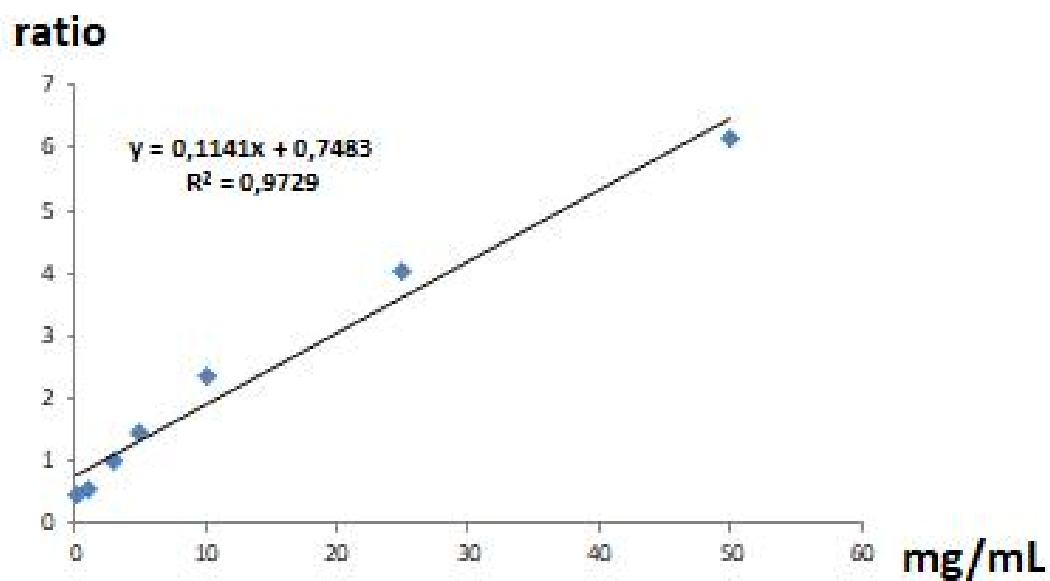


Figure 24. Calibration curve of II-1. X-axis is the concentration of II-1, Y-axis is the ratio of the pixel volume of each concentration to the pixel volume of 3 mg/mL.

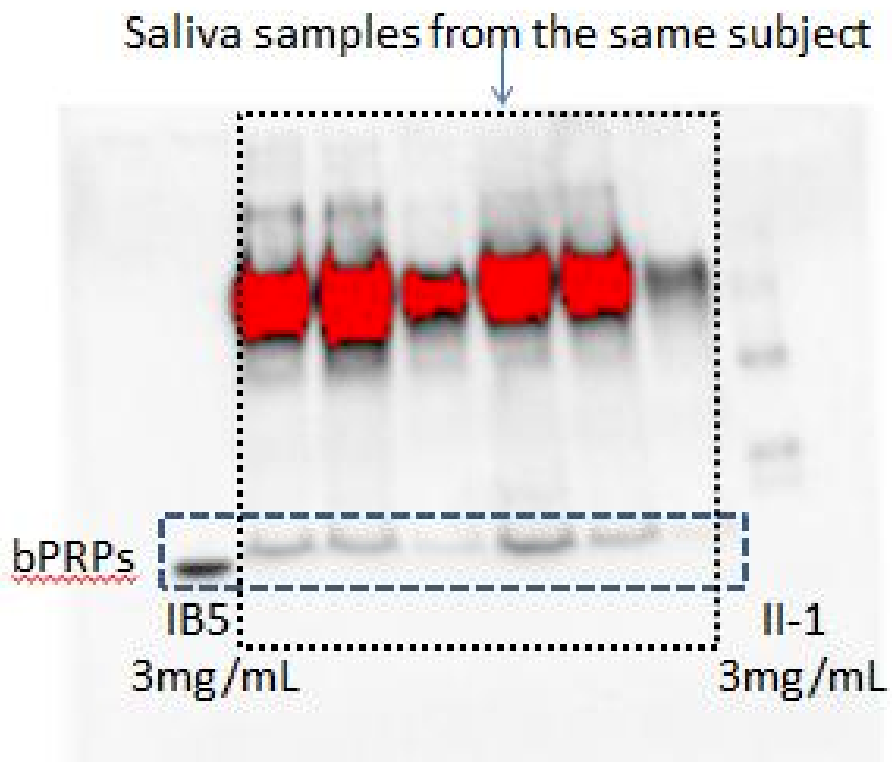


Figure 25. Membrane image for bPRPs in saliva samples from the same subjects. 3 mg/mL of IB5 (the first lane from left) and II-1 (the last lane from left) are used as references. The bands of saliva samples which appeared near IB5 are regarded as bPRPs. bPRPs in saliva samples and IB5 are circled by the blue dotted box. Saliva samples are circled by the black dotted box.

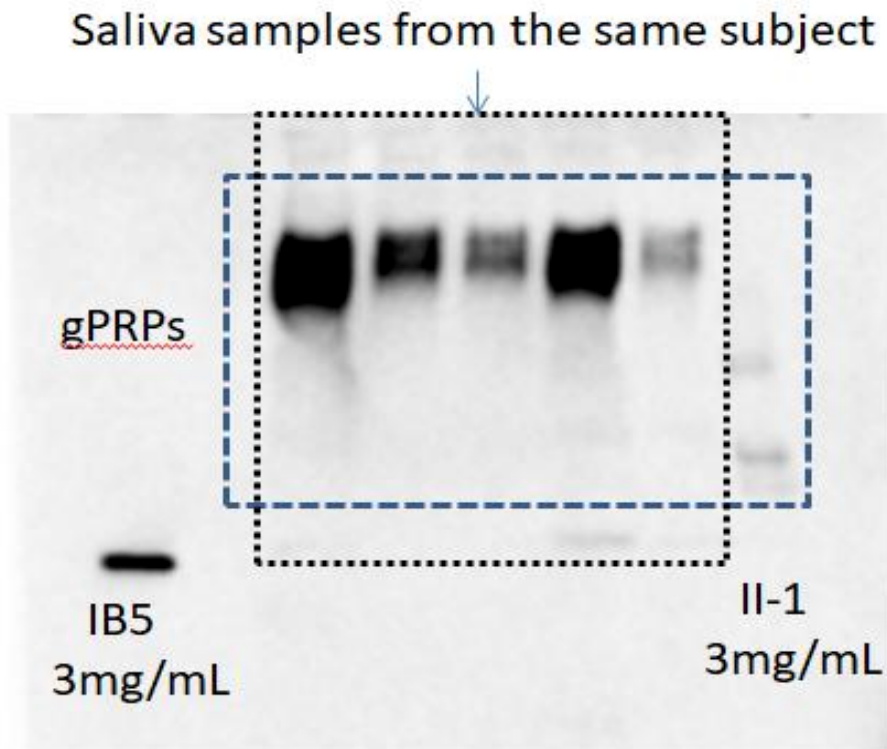


Figure 26. Membrane image for gPRPs in saliva samples from the same subjects. 3 mg/mL of IB5 (the first lane from left) and II-1 (the last lane from left) are used as references. The bands of saliva samples which appeared near II-1 are regarded as gPRPs. gPRPs in saliva samples and II-1 are circled by the blue dotted box, saliva samples are circled by the black dotted box.

2.6 Supplementary experiment--MUC5B evaluation

It has been suggested that mucin could be involved in astringency perception (Gibbins & Carpenter, 2013). MUC5B is the major mucin in saliva and the major component in mucosa pellicle. Therefore, in the supplementary experiment, we attempted to investigate MUC5B in the saliva of the young and elderly groups.

In the literature, it has been presented several methods to evaluate the level of MUC5B. Sonesson et al. (2008) assessed the MUC5B and MUC7 by carrying out an ELISA procedure using the LUM5B-2 and the LUM7-1 antiserum (Sonesson, Wickstrom, Kinnby, Ericson, & Matsson, 2008). Habotm et al. (2006) performed the purity and identity of MUC5B and MUC7 by Sepharose CL-4B column chromatography, SDS-PAGE and Western blotting methods (Habte, Mall, de Beer, Lotz, & Kahn, 2006).

Dot-blot was used in our work to detect protein-MUC5B by Bio-Dot device. Dot-blot is a technique for detecting, analyzing and identifying proteins, similar to the western blot technique but differing in that protein samples are not separated by electrophoresis but are spotted through circular templates directly onto the membrane. MUC5B is a high molecular weight mucin (>1000 kDa) (Gibbins & Carpenter, 2013), and the aim of our work is to

explore the specific protein (MUC5B). Therefore, we decided to develop a dot-blot procedure for the detection of MUC5B.

2.6.1 Dot-blot procedure

Firstly, Whatman paper and Nitrocellulose membrane were soaked in Tris-based buffer for 5 min. Then Bio-Dot device was assembled with Whatman paper and Nitrocellulose. Next, the Bio-Dot device was connected to a vacuum pump. 5 μ L of each saliva sample was finally put into membrane through the circular templates. On each membrane, we put 5 μ L of a same standard saliva sample with 3 repetitions which will serve as a reference to calculate the ratio of the pixel volume of each saliva sample to the pixel volume of this standard saliva. The vacuum pump was turned on and ran for 5 min to dry the membrane.

i. Coloration with red Ponceau

After the above procedure, the membrane was rinsed with MilliQ water, then it was immersed in a red coloration Ponceau for 5 min. Next the membrane was rinsed again with distilled water. It was then decolorized using 0.1 M NaOH solution. At last it was rinsed with ultrapure water for 2-3 min.

ii. Immunological reaction

After coloration and discoloration, the membrane was rinsed with 9‰ NaCl solution two times. Then blocking was performed with 8% skim milk in 9‰ NaCl for 30 min with agitation at room temperature. Then the membrane was rinsed with 9‰ NaCl solution two times. The membrane was then incubated with the primary antibody (made by laboratory: Floris J. Bikker, Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam, University of Amsterdam) at 1:10000 in 9‰ NaCl solution for 1h at room temperature. After 2 washes in 0.05% PBS-Tween solution, incubation was performed again with 8% skim milk in 9‰ NaCl solution for 30 min with agitation at room temperature. After 2 washes in 9‰ NaCl solution, the membrane was then incubated with HRP-conjugated secondary antibody (goat anti-mouse) at 1: 20000 in 5% BSA in 9‰ NaCl solution for 1h at room temperature. Then the membrane was rinsed with 0.05% PBS-Tween 3 times with agitation. The membrane was then rinsed in 9‰ NaCl solution two times.

iii. HRP colorimetric revelation following the Bio-Rad kit

Targeted protein (MUC5B) was detected by using an ECL (Clarity Enhanced Luminescence) detection system. A 1:1 mix of the 2 solutions (luminol solution and peroxide solution) was used. Membrane was immersed in ECL solution for 5 min, then the membrane was analyzed by ChemiDoc imaging system as soon as possible. Membrane was scanned with different exposure time, the best one was chosen according to the pixel of MUC5B which must not be saturated. The image of one membrane is shown in Figure 27.

Table 10. The parameters of the materials used in dot-blot.

Materials	Parameters
NaCl solution	9‰: 9 g of NaCl into 1000 mL MilliQ water
PBS solution	100 mL of PBS, 0.5 mL of Tween, 900 mL of MilliQ water
NaOH solution	0.1 M: 4 g of NaOH into 1000 mL MilliQ water.
Skim milk solution	8% in 9‰ of NaCl solution: 4 g of skim milk powder into 50 mL of 9‰ of NaCl solution.
Bovine serum albumin (BSA) solution	5%: 1 g of BSA powder into 20 mL of NaCl solution for antibody solutions.
Primary antibody solution	1/10000: 2 μ L of primary antibody into 20 mL of 5% of BSA solution in 9‰ of NaCl.
Secondary antibody	1/20000: 1 μ L of goat anti-mouse couple HRP (Fisher) into 20 mL of 5% of BSA solution in 9‰ of NaCl.
Tris-based buffer	24 g of Tris base, 88 g of NaCl, into 900 mL of MillQ water, adjusted pH to 7.6 with HCL solution, then added MilliQ water to 1000 mL.

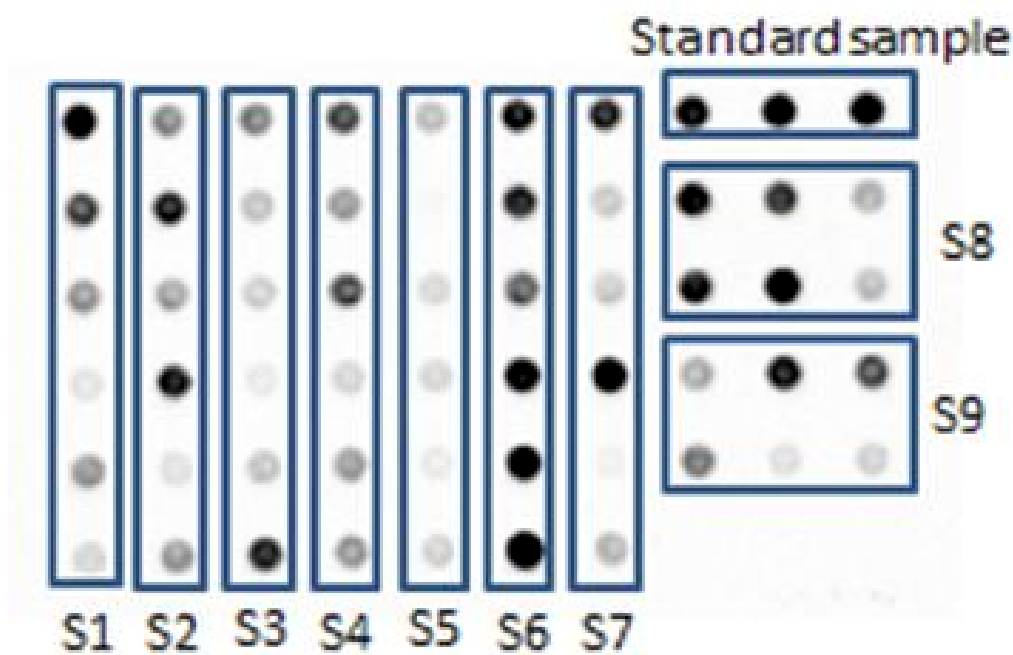


Figure 27. Image of membrane of MUC5B. S: subject. Saliva samples from the same subject were circled by the blue box. Standard saliva sample was repeated 3 times to be used reference. Pixel volume cannot be saturated.

3. Statistical Analysis

Data showed the presence of outliers for all the variables. Moreover, normality assumptions were not met for the raw data and residues. We decided to keep all the data and to not violate normality assumption. We thus performed nonparametric analyses because they are adapted

to non-normally distributed data and are more robust to the presence of outliers. Mann-Whitney U tests were performed to evaluate differences between the Y and O subjects regarding salivary parameters (flow rate, protein amount, PRPs amount and MUC5B pixel volume ratio). Wilcoxon tests were performed on the salivary parameters (flow rate, protein amount, PRPs amount and MUC5B) to evaluate differences between the start and the end of each session. Friedman ANOVA was conducted on the threshold and salivary parameter measurements to evaluate the differences between the three sessions. Spearman rank order correlations were performed for the whole group and in each group (Y and O) to evaluate the relationships between salivary parameters (flow rate, protein amounts, PRPs amount and MUC5B pixel volume ratio) and sensory parameters (threshold). The significance was set at $p < 0.05$. These tests were performed using Statistica® version 13.5.0.17 (1984-2018 TIBCO Software Inc, Palo Alto, CA, USA).

**CHAPTER 3: ASTRINGENCY SENSITIVITY TO TANNIC
ACID: EFFECT OF AGING AND SALIVA**

This chapter is based on the following article

ARTICLE I

Astringency Sensitivity to Tannic Acid: Effect of Ageing and Saliva

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Published in Molecules (2022)

Keywords: astringency; threshold; saliva; elderly; tannic acid

Abstract

Astringency is an important sensory characteristic of food and beverages containing polyphenols. However, astringency perception in elderly people has not been previously documented. The aim of the present work was to evaluate sensitivity to astringency as a function of age, salivary flow and protein amount. Fifty-four panelists, including 30 elderly people (age = 75 ± 4.2 years) and 24 young people (age = 29.4 ± 3.8 years), participated in this study. Astringency sensitivity was evaluated by the 2-alternative forced choice (2-AFC) procedure using tannic acid solutions. Whole saliva was collected for 5 min before and after the sensory tests. The results showed that the astringency threshold was significantly higher in the elderly group than the young group. No correlation was observed between the salivary protein amount and threshold value. However, a negative correlation between salivary flow and threshold was observed in the young group only. These results showed a difference in oral astringency perception as a function of age. This difference can be linked to salivary properties that differ as a function of age.

1. Introduction

Dietary polyphenols are a class of compounds present in foods and beverages, such as vegetables, nuts, unripe fruits and berries, wine, tea, etc. (Dilucia, Lacivita, Conte, & Del Nobile, 2020; Kiokias, Proestos, & Oreopoulou, 2020; Troilo, Difonzo, Paradiso, Summo, & Caponio, 2021), and they are of great interest for the food industry because of their potential beneficial effects on health, particularly for the ageing population (Chen et al., 2018; Rahman et al., 2022). In food and beverages, polyphenols, especially tannins, can elicit astringency, which is perceived as a quality parameter and desired at balanced levels depending on the food products (He, Tian, Luo, Qi, & Chen, 2015; Linne & Simons, 2017; Soares, Brandao, Guerreiro, Mateus, & de Freitas, 2020; Soares et al., 2018). In contrast, above a certain intensity, astringency is usually described as an unpleasant oral sensation (Huang & Xu, 2021), which limits the use and promotion of polyphenols at moderate levels in food despite their health benefits (Pires, Pastrana, Fucinos, Abreu, & Oliveira, 2020; Soares et al., 2020).

In 2004, the American Society for Testing and Materials (ASTM) 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” (Huang & Xu, 2021). Astringency is not confined to a particular region of the mouth but is a diffuse sensation (Kallithraka et al., 2001). Astringency is generally considered to be a tactile sensation detected through the activation of mechanoreceptors rather than a taste (Canon et al., 2021). Indeed, astringency takes 20 to 30 s to develop fully, often being the last oral sensation detected (Kallithraka et al., 2001). Although the mechanism of astringency is not yet well understood, several hypotheses have been proposed in the literature to explain astringency onset (Canon et al., 2021; Carpenter, 2012; Huang & Xu, 2021). It is most probably detected at the level of the oral mucosa (Soares et al., 2020), either by mechanoreceptors after the increase of the friction forces at the surface of the epithelial cells (Ployon et al., 2018) or by the detection of the aggregation of the mucosal pellicle by the transmembrane mucin MUC1 as recently proposed by Canon et al. (2021) (Canon et al., 2021). Salivary proteins are thought to play an important role in these two hypotheses by protecting the mucosal pellicle from aggregation by tannins. Indeed, their presence, and especially those of tannin-binding proteins, such as proline-rich proteins, decrease the perception of astringency (Glendinning, 1992; Nayak & Carpenter, 2008).

Regarding the effect of ageing on astringency perception, the literature is quite scarce, although the influence of ageing on the perception of other taste modalities has been largely documented. Longitudinal and cross-sectional studies have found that taste and smell losses tend to become noticeable after 60 years of age, with greater severity after 70 years of age (Doty et al., 1984). In 2012, a systematic review and meta-analysis showed that most of the primary studies included in the review (n=69) observed an increase in taste detection and identification thresholds and a decrease in taste intensity at the supra-threshold levels for the five basic taste modalities (bitter, salt, sour, sweet, umami) (Methven et al., 2012). However, the authors highlighted the lack of concordance among the primary studies regarding the extent of taste loss. This discrepancy between studies is probably due to significant

differences in the sensory procedures used to evaluate taste acuity (Methven et al., 2012; Mojet et al., 2001). More recently, Doty et al. (2018) (Barragan et al., 2018) evaluated a decline in the five basic taste perceptions in 1020 Caucasian European subjects (age 18–80 y/o). The study confirmed taste losses with ageing regardless of the modality. The authors also highlighted the complexity of the association between the ability to perceive a taste and the preference for the same. Moreover, beyond this overall effect of age on taste abilities, ageing is also accompanied by large inter-individual variability in olfactory performance scores and, to a lesser degree, in taste performance scores (Sulmont-Rosse et al., 2015).

Several factors can influence the extent of sensory decline during ageing (nutritional status, general health and diseases) (Sergi et al., 2017). The reasons for these sensory modifications can also be linked to changes in oral physiology with age. Indeed, in the elderly population, the cumulative effects of physiological ageing, diseases and drugs frequently impact the different aspects of oral physiology that are of great importance in taste and aroma sensitivity and thus eating behaviour (Munoz-Gonzalez et al., 2018; Schwartz et al., 2018; Sergi et al., 2017). In particular, ageing may often be accompanied by a decrease in salivary flow or changes in salivary composition (F. Xu et al., 2019), which can lead to a dry mouth or xerostomia. Hyposalivation is common among older adults due to an age-related decline in salivary gland function, and other causes include medications and systemic diseases (Iwasaki et al., 2016). Recently, Descamps et al. (2016) found an average 38.5% reduction in resting salivary flow and a 38% reduction in stimulated salivary flow in healthy elderly people compared to young adults (Vandenberghe-Descamps et al., 2016). This salivary hypofunction in elderly individuals can lead to changes in aroma, taste and textural perception, and consequently, food intake and consumption (Criado et al., 2021; Munoz-Gonzalez, Brule, Feron, & Canon, 2019; Munoz-Gonzalez et al., 2018; Spence & Youssef, 2021; F. Xu et al., 2019).

In the context of the world population becoming older and ageing well, the main objective of this study was to investigate the sensitivity to astringency as a function of age and salivary characteristics (flow and protein amount). For this purpose, a 2-alternative forced choice (2-AFC) methodology was applied to estimate astringency sensitivity in young and elderly panels while evaluating salivary flow and protein amount. Relationships between salivary flow, protein amount and sensitivity to astringency as a function of age are discussed.

2. Materials and Methods

This study was approved on 31 October 2019 by the Ethical Committee CCP Ile de France IV under the number 2019-A02434-53.

2.1 Materials

Solutions for rinsing consisted of 0.1% pectin (Sigma–Aldrich, Saint-Quentin-Fallavier, France) and 1% bicarbonate (Gilbert, France) dissolved in Evian water at room temperature.

Solutions for the sensory training session consisted of six taste solutions (salty, sour, sweet, bitter, umami, and astringent), and their compositions are detailed in Chapter 2. Each solution was coded with random three-digit codes. Solutions for astringency sensitivity evaluation consisted of four solutions with increasing tannic acid (Sigma–Aldrich, Saint-Quentin-Fallavier, France) concentrations (in g/L) with a multiple of 3.05, i.e., 0.02, 0.062, 0.188, and 0.574. These concentrations were chosen on the basis of preliminary experiments performed with a small internal panel of subjects (see Section 2.2 below). All samples were prepared in Evian water at room temperature 1 h before testing. Since potassium alum has not been allowed in sensory studies, tannic acid was used as a component to evaluate astringency because it has been described as less bitter than other polyphenols, such as gallic acid and catechin (Robichaud & Noble, 1990), and thus limits the confusion between astringency and bitter taste. This was confirmed during preliminary tests.

2.2 Sensory Analysis

Fifty-four panelists, including 30 elderly (O) people (age \geq 65 y/o) and 24 young (Y) people (age \leq 35 y/o), were recruited to participate in the sensory sessions. The panel is described in Table I-1. The number of subjects that needed to be included to find a difference between the two groups regarding astringency perception was determined by a power test (power = 0.9, significance level = 0.05, alternative = “two-sided”). The power test was based on preliminary results obtained on an internal panel (mean threshold = 0.19 ± 0.17 g/L of tannic acid, $n = 9$). At least 23 subjects per group (Y or O) were necessary to observe a difference equal to one standard deviation between the groups. More subjects were recruited in case of defection, particularly for the O group. These size groups are in line with previous studies aiming at evaluating the effect of ageing on taste perception regardless of the modalities (Methven et al., 2012; Mojet et al., 2001). Elderly and young subjects had good oral health, with a number of functional posterior units above 7 (Vandenberghe-Descamps et al., 2016). Moreover, elderly subjects were autonomous persons living at home, had no cognitive disorders (Mini Mental State Examination (MMSE) > 25) (Burns, 1998), did not have complete or half-complete dental appliances and took an average of 2 drugs per day (median = 1).

2.3 Preliminary Session

The objective of this session was to be sure that subjects were able to (i) clearly identify and differentiate astringency from other sensory sensations, in particular sourness, bitterness and olfactory cues, and (ii) perfectly understand the procedure of the sensory test, i.e., the 2-AFC to be used later.

The session was divided into two parts. During the first part, subjects received 20 mL of tasting sample in a fixed order at room temperature in plastic cups coded with random numbers. They were instructed to put the samples into their mouths, swirl the sample gently in the mouth for 30 s, spit it out and judge which taste it was. Between samples, subjects rinsed their mouth with Evian water and then waited for 1 min before the next sample. The tasting

sensations were saltiness, sweetness, sourness, bitterness and umami. Additionally, panelists were presented with tannic acid solution as an example of astringency.

In the second part, subjects were trained and familiarized for the 2-AFC procedure as described below.

During both parts of the preliminary session, there was a discussion between subjects and experimenters after each test. At the end of the session, all the panelists indicated that they were able (i) to clearly identify astringency from other sensory sensations and (ii) to perform the 2-AFC test properly.

2.4 Testing Session

All sessions were performed for 3 months between the middle of November and the end of January and between 2 and 6 p.m. to minimise seasonal and circadian rhythms as much as possible. Moreover, panelists were asked to not drink, eat or smoke 1 h before the session. The whole session was conducted under red light at room temperature in a sensory room equipped with individual boxes.

At the beginning of each session, panelists were asked to taste a model tannic acid solution of 1.76 g/L so that they could identify astringency. Then, they rinsed their mouths with pectin, bicarbonate and Evian water and waited for a 3 min break before threshold evaluation. The objective of this rinsing procedure is to perfectly clean the mouth to have the most similar oral conditions when starting each test, and thus minimize carry-over effects between sample evaluations. Sodium bicarbonate recovers pH homeostasis, and pectin removes tannic acid from the oral mucosa due to its capacity to form complexes with polyphenols (Huang & Xu, 2021). This rinsing procedure was found to be efficient in wine studies for in-mouth aroma release experiments (Esteban-Fernandez et al., 2016; Munoz-Gonzalez, Canon, et al., 2019) and, more recently, for the time sensory evaluation of astringency and aroma (Pittari et al., 2022). This procedure was chosen instead of other procedures, such as the milk rinsing procedure (Taladrid et al., 2019), because of the necessity to avoid any contamination of saliva samples by food proteins.

The astringency threshold was evaluated by a 2-AFC procedure with ascending concentrations of tannic acid. In each 2-AFC presentation, two samples were presented: a target sample and a control sample. Each 2-AFC test was performed 3 times, and the evaluation was performed 3 times in 3 different sessions. Paired samples (5 mL) were presented in balanced order following a Latin square design (Williams design) at room temperature in a white plastic cup coded with the letter A or B. The testing procedure started from the lowest concentration. Panelists were given the reference or stimulus sample. They were asked to put the samples into their mouth, swirl them gently around the mouth for 30 s and then spit them out. They rinsed their mouths with pectin and waited for 1 min before evaluating the second sample. After 30 additional seconds, the panelists were asked to indicate which sample was perceived as astringent. Then, the panelists rinsed their mouth as

described previously.

The sensitivity level was reached when three correct answers from the same concentration were achieved. The best estimate threshold for each subject was evaluated as the geometric mean of the three correctly answered concentrations and the previous lower concentration. When the subjects correctly identified the lowest concentration (0.02 g/L), the geometric means were calculated between this concentration and the theoretical concentration below, i.e., $0.02/3.05=0.0065$ g/L. In contrast, when subjects did not correctly identify the highest concentration (0.574 g/L), the geometric mean was calculated between this concentration and the theoretical concentration above, i.e., $0.574 \times 3.05=1.75$ g/L.

2.5 Saliva Collection

Whole saliva was collected after the panelists had rinsed their mouths with 0.1% pectin, 1% bicarbonate and water at the start (SFStart) and at the end (SFEnd) of the session. Saliva was collected by expectorating into a pre-weighed tube with a cap for 5 min as described previously (Neyraud et al., 2012). After collection, the tubes were weighed and then stored at -80 °C. Flow rates were determined gravimetrically and expressed as grams per minute (g/min).

2.6 Protein Amount

Saliva samples were centrifuged at 15000 g for 15 min at 4 °C before analysis. The protein concentration was determined in the supernatant using the Bradford protein assay, with bovine serum albumin (BSA) used as the standard for calibration (Neyraud et al., 2012).

2.7 Statistical Analysis

Data showed the presence of outliers for all the variables. Moreover, normality assumptions were not met for the raw data and residues. We decided to keep all the data and to not violate normality assumption. We thus performed nonparametric analyses because they are adapted to non-normally distributed data and are more robust to the presence of outliers (Abdullah, 1990; Cizek & Sadikoglu, 2020; Croux & Dehon, 2010; de Winter, Gosling, & Potter, 2016; S. M. Smith, 1995). Mann–Whitney U tests were performed to evaluate differences between the Y and O subjects regarding sensory and salivary parameters. Wilcoxon tests were performed on the salivary parameters (flow and protein amounts) to evaluate differences between the start and the end of each session. Friedman ANOVA was conducted on the threshold and salivary parameter measurements to evaluate the differences between the three sessions. Spearman rank order correlations were performed for the whole group and in each group (Y and O) to evaluate the relationships between salivary and sensory parameters. The significancy was set at $p < 0.05$. These tests were performed using Statistica® version 13.5.0.17 (1984-2018 TIBCO Software Inc, Palo Alto, CA, USA).

3. Results

3.1 Astringency Threshold

No significant differences were observed between the three sessions regarding astringency thresholds for either Group Y (young panel) (Friedman $\chi^2 = 1.13$, $p = 0.56$) or Group O (elderly panel) (Friedman $\chi^2 = 1.14$, $p = 0.56$). Therefore, we decided to merge threshold values into a unique variable.

A significant difference was observed between the Y and O groups ($Z = -2.5$, $p = 0.0110$). The O group showed a higher mean astringency threshold than the Y group (Table I-1, Figure I-1).

Table I-1. Characteristics of the young and elderly panels. SF: salivary flow.

Characteristics	Y (n=24, 18 Males/6 Females)				O (n=30, 16 Males/14 Females)			
	Mean	Median	Range	SD	Mean	Median	Range	SD
Age (years)	29.4	30	24-35	3.8	75	73.5	70-87	4.23
SF (mL/min)	0.49	0.47	0.27-0.82	0.16	0.42	0.35	0.11-0.92	0.23
Protein amount (mg/mL)	0.6	0.62	0.17-1.4	0.27	0.62	0.60	0.22-1.35	0.24
Threshold (g/L)	0.29	0.2	0.04-1.00	0.26	0.41	0.35	0.06-0.78	0.24

SD: standard deviation of the mean

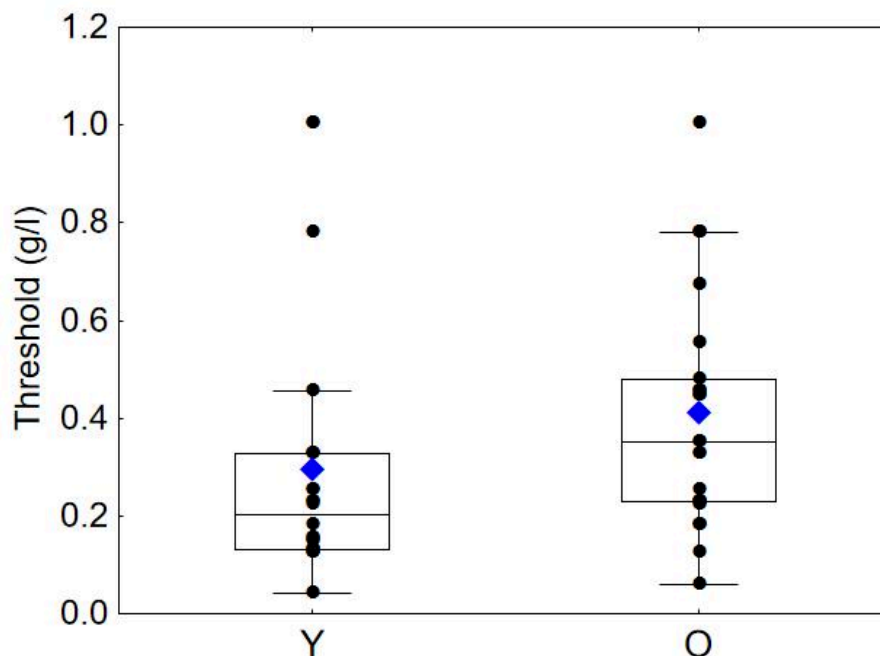


Figure I-1. Box-plot distributions of threshold values as a function of the age category (young (Y) and elderly (O)) are shown. The bottom and top of the box correspond to the 25th and 75th percentiles, respectively. The horizontal band and the blue diamond correspond to the median and the mean, respectively. The ends of the whiskers represent nonoutlier ranges. The black dot symbols correspond to individual data points.

3.2 Salivary Flow Rate and Protein Amount

No significant differences were observed between sessions regarding SFStart and SFEnd for Group Y (SFStart: Friedman $\text{Chi}^2 = 0.75$, $p = 0.68$; SFEnd: Friedman $\text{Chi}^2 = 0.75$, $p = 0.68$) or Group O (SFStart: Friedman $\text{Chi}^2 = 5.2$, $p = 0.07$; SFEnd: Friedman $\text{Chi}^2 = 1.3$, $p = 0.53$) or between the mean SFStart and mean SFEnd for Group Y (Friedman $\text{Chi}^2 = 0.68$, $p = 0.492$) or Group O (Friedman $\text{Chi}^2 = 1.49$, $p = 0.135$). For this reason, we decided to merge both variables into a unique variable, i.e., mean salivary flow (SF). SF values are presented in Figure I-2. With regard to the comparison of salivary flow rate, the SF in the O group was lower than that in the Y group but with a modest degree of evidence ($Z = 1.66$, $p = 0.09$) (Table I-1). Moreover, a larger variability was observed in the O group compared to the Y group, with the presence of outliers with a higher SF. This large inter-individual variability was previously observed in a large panel of elderly subjects and can be explained by life-style and aging factors such as diet, smoking habits, hydration status or structural changes in the salivary glands (Vandenberghe-Descamps et al., 2016).

No significant differences were observed between sessions regarding protein amount for Group Y (Friedman $\text{Chi}^2 = 1.08$, $p = 0.58$) or Group O (Friedman $\text{Chi}^2 = 2.55$, $p = 0.28$) or between the beginning and the end of the session for Group Y (Friedman $\text{Chi}^2 = 1.5$, $p = 0.91$) or Group O (Friedman $\text{Chi}^2 = 1.70$, $p = 0.19$). For this reason, we decided to merge the protein amount into a unique variable (Table I-1). Protein amounts are presented in Figure I-3, and no significant differences were observed between the Y and O groups ($Z = -0.32$, $p = 0.74$), which confirms previous results (Nagler & Hershkovich, 2005b).

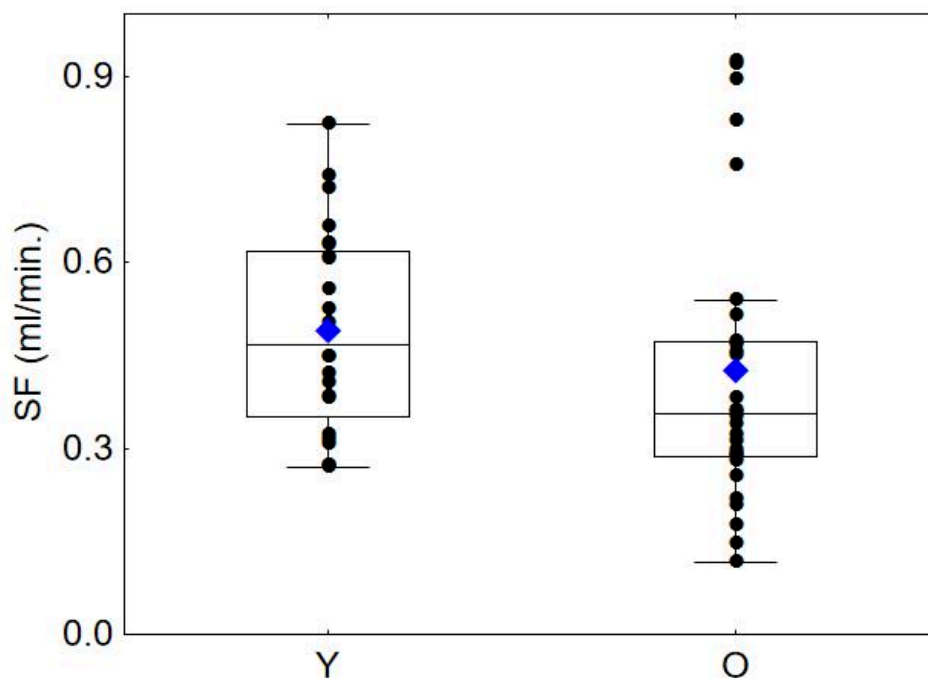


Figure I-2. Box-plot distributions of whole salivary flux as a function of age category (young (Y) and elderly (O)) are shown. The bottom and top of the box correspond to the 25th and 75th percentiles, respectively. The

horizontal band and the blue diamond correspond to the median and the mean, respectively. The ends of the whiskers represent the non-outlier range. The black dot symbols correspond to individual data points.

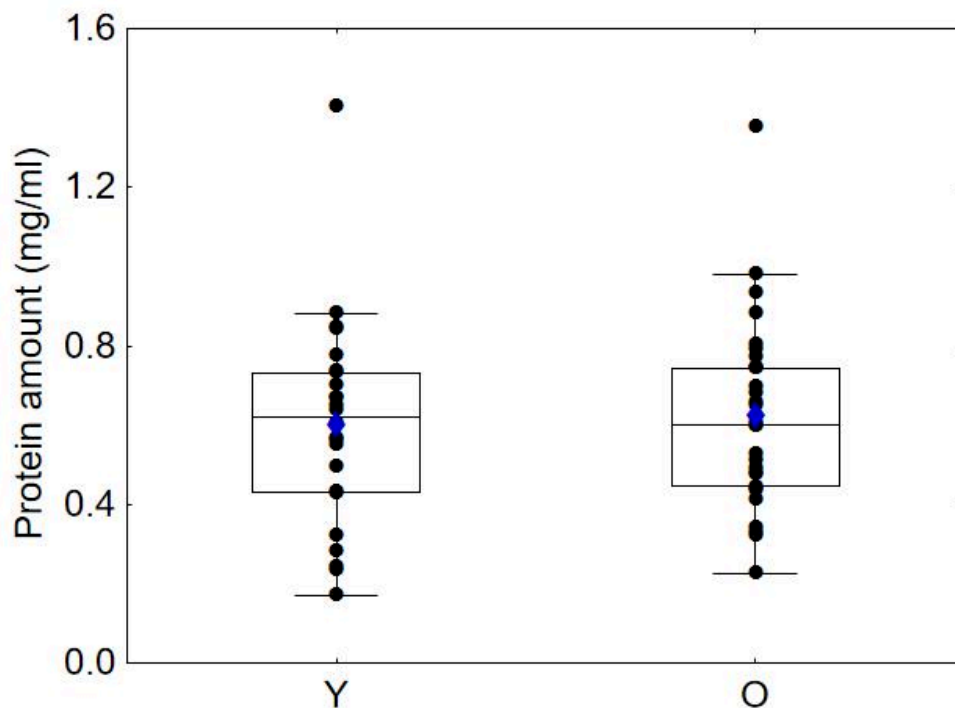


Figure I-3. Box-plot distributions of salivary protein amount as a function of age category (young (Y) and elderly (O)) are shown. The bottom and top of the box correspond to the 25th and 75th percentiles, respectively. The horizontal band and the blue diamond correspond to the median and the mean, respectively. The ends of the whiskers represent the non-outlier range. The black dot symbols correspond to individual data points.

3.3 Correlation between the Astringency Threshold and the Flow Rate and Protein Amount

The Spearman correlation between threshold and SF was not significant in the whole panel or the O group (Table I-2). However, a significant and negative correlation was observed in the young (Y) group ($r = -0.44$, $p = 0.03$), where a higher salivary flow was associated with a lower threshold (Figure I-4).

The Spearman correlation between the threshold and protein amount was not significant in the whole panel, the Y group or the O group (Table I-2).

Table I-2. Spearman correlation coefficient and p value of the astringency threshold and salivary characteristics for whole (W), young (Y) and elderly (O) panelists. SF: salivary flow.

	SF			Protein amount		
	W	Y	O	W	Y	O
Threshold	$r=-0.16$ $p=0.24$	$r=-0.44$ $p=0.03$	$r=0.14$ $p=0.47$	$r=0.19$ $p=0.16$	$r=0.18$ $p=0.39$	$r=0.19$ $p=0.30$

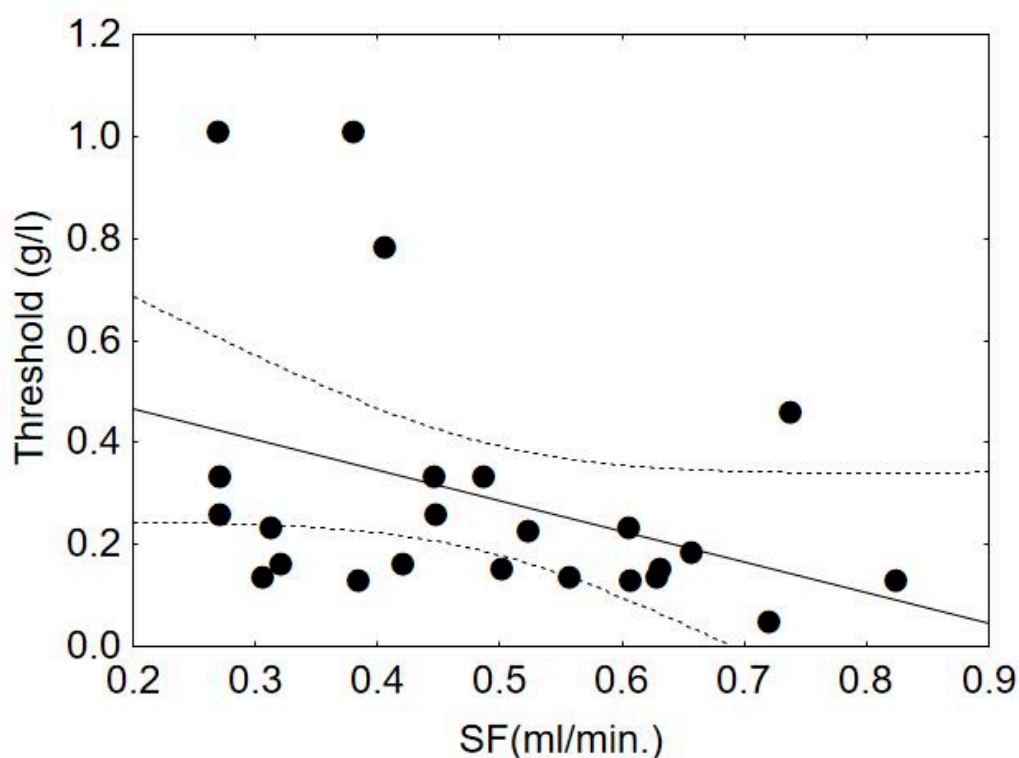


Figure I-4. Spearman correlation between astringency threshold and whole salivary flux observed in the group of young panelists. The plain line corresponds to fitted data. The dotted line corresponds to the confidence interval at 95%. The black dot symbols correspond to individual data points.

4. Discussion

In the current study, the astringency threshold was higher in elderly participants than in young participants. In other words, young adults were more sensitive to astringency than elderly adults, which confirms the findings of previous studies for other taste modalities (Barragan et al., 2018; Methven et al., 2012; Mojet et al., 2001; Sergi et al., 2017). To the best of our knowledge, this is the first study evaluating sensitivity to astringency as a function of age. In 2017, Linne and Simmons (2017) (Linne & Simons, 2017) investigated the impact of age on individual sensitivities to lingual tactile roughness in relation to sensitivity to astringent stimuli. The authors did not find a correlation between age and roughness sensitivity. However, their group was younger (21 to 60 y/o, n = 30) compared with that in our study as well as most other studies reporting taste differences as a function of age.

We found average detection thresholds of 0.2 g/L and 0.41 g/L for the Y and O groups, respectively. Using similar sensory procedures and stimuli, Linne et al. (2017) (Linne & Simons, 2017), obtained a detection threshold of 0.212 mM (0.36 g/L), which is close to our results. The increase in the detection threshold between Y and O was 1.6-fold. Similar increases on average were described for other taste modalities, such as saltiness (1.5), sourness (1.5), sweetness (1.4), umami (2.2) and bitterness (1.2 to 4.1) (Methven et al., 2012),

suggesting that astringency sensitivity loss with age is not unusual compared to these modalities.

Differences in salivary properties can explain differences in taste sensitivity. Indeed, saliva allows the transport of taste substances to the taste receptor and protects the receptors by providing growth factors for the renewal of taste buds (Matsuo, 2000; Mese & Matsuo, 2007). Some salivary components can modulate taste sensitivity (Feron, 2018). For instance, sodium and amino acid salivary concentrations can modulate the detection threshold. Salivary flow can also influence fat intensity perception and preference, and larger amounts of saliva contribute to a higher in-mouth washing of lipid emulsion when tasted (Neyraud et al., 2012).

Regarding astringency, modulation of its perception as a function of the salivary flow rate led to contradictory results. Indeed, it has been reported that subjects with low salivary flow rated astringency higher than subjects with high salivary flow (Condelli, Dinnella, Cerone, Monteleone, & Bertuccioli, 2006). Conversely, Fisher et al. (1994) and Smith and Noble (1996) did not observe a difference in intensity rating as a function of salivary flow using temporal perception experiments (Fischer U, 1994; A. K. Smith, June, & Noble, 1996). Finally, Linne et al. (2017) reported a higher sensory threshold for tannic acid in subjects with low salivary flow than in subjects with high flow (Linne & Simons, 2017).

In the present study, a positive relationship between astringency sensitivity and salivary flow was observed in the young panel only, i.e., a higher salivary flow corresponds to a higher sensitivity, which is in accordance with the results of Linne et al. (2017) (Linne & Simons, 2017). This relationship of flow rate to astringency sensitivity, as shown in Figure 4, might suggest protection through an interaction mechanism with salivary proteins rather than a simple dilution effect, as suggested in other studies (Horne, Hayes, & Lawless, 2002). However, the amount of proteins measured in saliva from the Y group was not correlated with astringency sensitivity, which is consistent with previous studies that did not observe relationships between salivary total protein content and intensity or time-intensity evaluation of astringency (Condelli et al., 2006; Guinard, ZoumasMorse, & Walchak, 1997). However, strong positive correlations of astringency time-intensity parameters with some salivary protein fractions suggested that differences regarding astringency sensitivity and salivary properties were linked to salivary protein composition rather than global protein amount (Kallithraka et al., 2001). Indeed, histatins, mucins and salivary basic proline-rich proteins (bPRPs) have been identified as potential contributors to astringency perception in humans, while their role in the underlying mechanism of this perception is still under debate (Carpenter, 2012; Gibbins & Carpenter, 2013). In particular, PRPs and histatins are described as tannin binding-proteins with a high affinity for tannins (Shimada, 2006; Soares et al., 2011). PRPs are secreted by the parotid glands, bind and scavenge tannins (Canon et al., 2015), giving them the ability to protect the mucosal pellicle against tannin aggregation (Ployon et al., 2018). Thus, bPRPs are proteins thought to play a role in astringency perception in humans (Ployon et al., 2018). In rodents, their role is much clearer as their presence in saliva increases the linking of astringent solution (Glendinning, 1992). Indeed, the secretion of PRP in the saliva of rodents is not constitutive and is induced by the diet. Moreover, rodents do not

secrete histatins (de Sousa-Pereira et al., 2013) and thus PRPs are the main tannin-binding proteins in their saliva.

We did not observe such a relationship between salivary flow and astringency sensitivity in the elderly group or a difference in protein amount between the Y and O groups, which should explain the difference in sensitivity between the two groups. However, lower salivary flow was observed in the O group than in the Y group, which is in accordance with previous studies (C. H. Smith et al., 2013; Vandenberghe-Descamps et al., 2016) and should partly explain the sensitivity differences between the two groups. Our observations suggest that the role of saliva in astringency sensitivity as a function of age should also be linked to salivary composition and, in particular, peptides and proteins. Studies on changes in salivary composition in healthy elderly individuals are relatively scarce and present poor consistency among results (F. Xu et al., 2019). Moreover, the direction of change (increase or decrease) depends on the proteins. For instance, amylase, lysozyme and IgA increase with age, while lactoferrin, glutathione, peroxidase activity and mucin levels decrease, with a large consensus for the latter (F. Xu et al., 2019). Similarly, histatin levels were also observed to decline with age (D. A. Johnson, Yeh, & Dodds, 2000), which is an interesting finding based on the possible involvement of mucins and histatins in astringency sensitivity (Canon et al., 2021; Huang & Xu, 2021). We suggest that a lower level of these classes of proteins in the saliva of the elderly population should impact astringency perception. With regard to PRP and bPRP, there is a paucity of information describing their salivary amounts during the human lifespan. Exploring salivary exocrine protein secretion in 220 adults, Baum et al. (1982) did not find a change in PRP secretion during ageing, although this study considered only acidic PRP (Baum et al., 1982).

5. Conclusion

In summary, the present study highlighted the sensory analysis of astringency perception sensitivity as a function of age and saliva from a panel formed by 30 elderly people and 24 young people using the 2-AFC method with four tannic acid concentrations. We conclude that the astringency threshold was higher in the elderly group than in the young group. In regard to the salivary flow rate and protein amount, there were no significant differences between the young and elderly groups. However, a correlation between salivary flow and threshold was observed only for young individuals, which suggests that salivary properties that influence astringency sensitivity in elderly individuals are different. Deeper characterization of salivary composition in particular regarding PRP and mucin levels in both the populations should deserve further studies.

6. Limitations

This study presents some limitations.

Although a preliminary session was provided to the training subjects on the astringency modality, bias due to other attributes, such as bitterness or olfactory cues, cannot be ignored. Regarding the latter, a nose clip was not worn during the sensory test to avoid excessive fatigue for elderly participants. The same concern guided the choice to limit the number of tannic acid concentrations to 4 to avoid excessive presentation of samples to this population.

The number of subjects was defined to be at least 23 participants in each group following a power test based on the results from a preliminary study. It is likely that this number of subjects will not permit us to capture all the variability commonly observed in the elderly population. Thus, an astringency evaluation in a larger population should be performed before generalising our findings. Moreover, we observed in both groups (Y and O) a large variability regarding the salivary flow with the presence of outliers in the O group which led to non-normally distributed data. We cannot rule out the potential effect of these outliers on the statistical results despite the use of non-parametric statistics more robust to their presence (Abdullah, 1990). However, it is important to note that sensory evaluation and salivary sampling were repeated 3 times in 3 different sessions during a short period, which ensured the good reliability of our results.

Some of the participants in the elderly group took drugs despite the low mean number (2) compared to what was commonly observed in this population (ranging from 2.9 to 3.7 medications) (Schiffman et al., 1998). Drug intake is known to cause sensory impairment, particularly in the aged population (Schiffman et al., 1998). In our study, we chose subjects who were significantly older (mean age 75 y/o) than those in the literature and thus more likely to take medication, which increased the difficulty of limiting the inclusion of drug-taking participants.

Finally, we principally included participants with good oral health based on dental observations. However, we did not check for oral microbiota impairment, a factor that is commonly observed in the elderly population and that should also affect taste sensitivity (Fluitman et al., 2021).

**CHAPTER 4: ASTRINGENCY SENSITIVITY TO TANNIC
ACID: EFFECT OF AGING AND PRPs LEVELS**

This chapter is based on the following article

ARTICLE II

Astringency Sensitivity to Tannic Acid: Effect of Ageing and PRPs levels

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Article in preparation

Keywords: astringency; threshold; bPRPs; gPRPs; elderly; tannic acid

Abstract

Previous work showed that astringency sensitivity was lower in an elderly population compared to a young one. The aim of the present work was to evaluate salivary proline-rich proteins (PRPs) levels in young and elderly group of individuals and to study their link with astringency sensitivity. Fifty-four panelists, including 30 elderly people (age=75 ± 4.2 years) and 24 young people (age=29.4 ± 3.8 years), participated in this study. Evaluation of astringency sensitivity to tannic acid and saliva sampling were performed in 3 different sessions. Basic PRPs (bPRPs) and glycosylated PRPs (gPRPs) were quantified immunochemically by western-blot analysis. The results showed that the amounts of bPRPs and gPRPs were similar between young and elderly groups. However, a positive correlation between gPRPs amount and astringency threshold was observed only in young group. A significant and negative correlation between bPRPs and astringency threshold was observed only in elderly group. This work suggests a different contribution of PRP type in astringency perception as a function of age. In the supplementary experiment, there was no difference between the young and elderly groups regarding MUC5B relative amount, and no correlation was observed between MUC5B relative amount and astringency threshold in both groups.

1. Introduction

In elderly people, the cumulative effects of physiological aging have an important impact on the different aspects of oral physiology such as loss of tooth, reduction of salivary flow, changes of salivary composition etc. These alterations may have a negative impact on food intake, with lower intake of multiple nutrients, i.e. vegetables, nuts, fish, leading to malnutrition and diet-related diseases (Vandenbergh-Descamps et al., 2018) such as cancer, vascular disease, diabetes, inflammatory, Alzheimer's disease and so on (Dainy, Kusharto, Madanijah, Nasrun, & Turana, 2018).

The protective effects of apple fruits against cardiovascular diseases (CVDs) are largely attributed to their polyphenol content, including tannins, for their impact on vascular function, inflammation, and lipid metabolism when associated with pectins (Koutsos et al., 2020). Tannins are commonly present in plant-based foods and beverages, such as vegetables, nuts, unripe fruits, red wines, teas, beers (Tarascou et al., 2010). The consumption of tannin rich-diet in mammals has been related to the presence in saliva of tannin binding salivary proteins (TBSP) (Shimada, 2006). TBSP are composed of two main families of salivary proteins, proline-rich proteins (PRPs) and histatins, while other family of salivary proteins such as mucins (Davies et al., 2014; Ployon et al., 2018) have been also reported to interact with tannins.

Consumption of these foods is often accompanied by drying, roughening and puckering sensation felt in the mouth, which corresponds to astringency sensation (Horne et al., 2002). The possible mechanisms involved in astringency perception are i): the precipitation of the proline-rich protein (PRP)-tannin aggregates in saliva reduces the salivary lubrication, which is perceived as oral friction (Canon et al., 2021). ii): direct interaction between tannins and oral epithelial cells or tannins and the salivary proteins adhered to buccal mucosal cells that form the mucosal pellicle (Canon et al., 2021; Ployon et al., 2018; Ramos-Pineda, Garcia-Estevez et al., 2019), which may involve the transmembrane MUC1 as a sensor (Canon et al., 2021). In this case, PRPs are considered to prevent astringency from tannins by protecting the mucosal pellicle from aggregation (Ployon et al., 2018) due to their ability to scavenge tannins (Canon et al., 2011).

Proline-rich proteins (PRPs), which constitute about two-thirds of proteins secreted by the human parotid glands, are characterized by a predominance of proline (25-42%), glycine (16-22%), and glutamic/glutamine (15-28%) residues (Sarni-Manchado et al., 2008). They are highly polymorphic and heterogeneous in their primary amino acid sequence, size and post-translational modifications, which could be related with their functional diversity (Ramos-Pineda, Garcia-Estevez, et al., 2019). PRPs are classified into three classes, acidic (aPRPs), basic (bPRPs), and glycosylated PRPs (gPRPs) depending on their amino acid sequence and the presence of posttranslational modifications (phosphorylation for aPRP and glycosylation for gPRP) (Bacon & Rhodes, 2000). Acidic PRPs are reported to bind calcium and to inhibit crystal growth, participating to the maintenance of calcium homeostasis in the

mouth. Glycosylated PRPs participate to the lubrication of oral cavity. They have also been reported to bind oral bacteria and tannins (Asquith et al., 1987). The main function that has been described for basic PRPs is to bind and scavenge tannins, as their presence in the saliva of mammals is linked to the consumption of tannins in their diet. Basic PRPs are thought to constitute a first line of defense against the antinutritional properties of tannins. The high affinity of PRPs for tannins is associated to their structural characteristics. PRPs are intrinsically disordered proteins, and show random coil structure except for small polyproline helix segments. It provides them an open structure with rigid elements allowing the initiation of the interaction with tannins (Canon et al., 2015). These rigid elements are surrounded by clusters of glycine and alanine residues, which provide flexible regions allowing structural rearrangements (Canon et al., 2011) and the establishment of additional hydrogen bonds (Canon, Giuliani, Pate, & Sarni-Manchado, 2010). The interaction of tannins with PRPs lead to the formation of soluble non-covalent complexes, exhibiting several stoichiometries (Canon et al., 2013). The number of binding sites appears to correspond to the number of cluster of proline (Canon et al., 2013). When at least 3 monomers of flavan-3-ols are bound per protein, PRP•tannin noncovalent complexes aggregate and grow with tannin concentration up to their precipitation (Canon et al., 2013). This mechanism is thought to impact on astringency perception, either by triggering astringency during the precipitation of the aggregates or by protecting the mucosal pellicle from aggregation. In this latter hypothesis, astringency, which is a mechanism of alarm and avoidance toward tannin-rich food, has evolved alongside with the presence of PRP in saliva, which by protecting the mucosal pellicle toward tannins. Thus, it is interesting to understand the relation between astringency threshold and the amount of PRPs in saliva as they may modulate astringency perception.

Thus, the present study aims at studying the relationship between astringency sensitivity and the PRPs amount in saliva as a function of age. Indeed, aging is often accompanied by salivary composition changes, especially with a decrease of mucin and histatins concentrations (Dodds et al., 2005; F. Xu et al., 2019). Regarding PRPs, Baum et al. (1982) reported that both the total amount of aPRPs and the percentage of aPRPs of total secretory protein are not related to age (Baum et al., 1982). As far as we know, there is no literature describing the relationship between the amount of salivary PRPs, aging and astringency perception. In a previous study, we reported for the first time a difference in oral astringency threshold as a function of age. In this previous study, we investigated the correlation between astringency threshold and 2 salivary physiological parameters (salivary flow and protein amount). No significant correlation was observed, suggesting the involvement of other salivary parameters such as PRPs level.

Therefore, in this context, the main objective of this study was to measure the PRPs amount, which could be linked to astringency threshold, as a function of age. A western-blot methodology was developed to assess the amount of PRPs in saliva. Relationships between PRPs amount and astringency sensitivity as a function of age will be discussed.

In addition, we conducted supplementary experiment on MUC5B in saliva by dot-blot method. MUC5B is the major mucin in saliva, it has a high-molecular weight (>1000 kDa) with

multiple highly glycosylated covalently linked subunits (Gibbins & Carpenter, 2013). Mucins are considered to be essential lubricant of saliva due to their structure. MUC5B and MUC7 are the main mucins in saliva, while MUC5B is the main salivary protein composing the mucosal pellicle (Ployon et al., 2016). Regarding the lubricating properties of MUC5B and its abundance in saliva and the mucosal pellicle, it is also interesting to characterize the correlation between MUC5B amount in saliva and astringency threshold.

2. Materials and Methods

2.1 Saliva samples treatment

2.1.1 Sterilization of saliva samples

Saliva collection was performed as described in a previous study (M. Wang et al., 2022). After collection, the tubes were stored at -80 °C until analysis. Before treating saliva samples, we conducted saliva samples sterilization in 60 °C water bath for 1 h for the sake of safety in the context of pandemic. Then 2 mL saliva was taken from each saliva collection tube into Eppendorf which were centrifuged (15000 gravitational force [g], 15 min, 4 °C) to eliminate impurities. Then 50 µL supernatant was taken from each Eppendorf for electrophoresis and western-blot experiments, the left supernatant of each sample was kept for protein amount quantification Bradford (M. Wang et al., 2022). Both samples were stored at -80 °C.

2.1.2 Concentration of saliva samples

Evaporation of each of 50 µL saliva supernatant to dryness was conducted by SpeedVac evaporation device for 30 min to increase the final concentration of salivary PRPs.

2.2 Production of IB5 and II-1

The *Pichia pastoris* system that utilizes methanol-inducible alcohol oxidase promoter for expression of heterologous recombinant proteins has been used. As described previously described (Boze et al., 2010), IB5 and II-1 were produced during the growth phase and secreted into the culture medium. The yeast *Pichia pastoris* - PRB4S was spread on the medium dextrose (MD) petri dish. After 2 days at 28-30 °C, the yeast colony from the MD petri dish was put into glycerol medium. During the culturing, growth was evaluated by measuring the optical density at 600 nm. When the optical density had reached 40, the culture was centrifuged at 4000 rpm at 29 °C for 10 min. The supernatant was discarded into biological waste. Then the pellets were re-suspended into methanol tryptone medium. In the following days, methanol was added into the culture medium 2 or 3 times a day in order to induce the expression of PRP by the strain. After 6 days culturing, the culture medium was centrifuged at 15000 rpm for 30 min at 4 °C. After centrifugation, supernatant was kept and filtered at 2 µm, 1 µm, 0.7 µm and 0.45 µm. Then it was dialyzed into 4 L of Tris-HCl 50 mM pH 8.

2.3 Purification of IB5 and II-1

2.3.1 Cation exchange

After dialysis, PRPs were recovered from the supernatant using cationic chromatography. Firstly, the column (XL-SP) was washed and equilibrated by 0.45 μ m filtrated washing buffer (50 mM Tris-HCl, pH 8), flow rate was set to 0.5 mL/min. Then the dialysate produced by yeast strain was injected. After injecting the sample, a linear gradient from 0 to 100% with NaCl 1 M buffer was used for elution of the proteins. The fractions (3 mL) were collected in plastic tubes placed into the sample collector. The protein elution was monitored by measuring absorbance at 230 nm.

2.3.2 Gel filtration

A partial separation of the PRPs was followed up by size exclusion chromatography on a HiPrepSephacryl S100 26/600 column (GE Healthcare). The column was washed and equilibrated by 0.45 μ m filtrated acetate ammonium buffer 50 mM, pH 7.5, flow rate was set to 1 mL/min. 5 mL of PRPs solution was injected into the column. PRPs were eluted at a flow rate of 1 mL/min and collected as 2.5 mL fractions at eluted peaks at 230 nm. Collected fractions were checked by electrophoresis as described below. Those containing either IB5 or II-1 were lyophilized until use.

2.4 Electrophoresis

As described above, saliva samples were dried with SpeedVac device, and each dried saliva sample was dissolved in 20 μ L buffer (Laemmli 2x Biorad) by vortexing for 1 min and rehydration for 1 min at room temperature. Then Eppendorf with dried saliva and buffer was heated at boiled water temperature for 3 min. After cooling down, 20 μ L aliquots were loaded on 12% commercial precast gel Biorad. Electrophoresis was run at a constant voltage of 200 V for approximately 35 min until the blue had reached the bottom of the gel without exceeding it so as not to lose proteins. After electrophoresis, the gels were put into MilliQ water to wait for further analyzing.

2.5 Western-blot

2.5.1 Coloration with red Ponceau

Western blot was used for detection of bPRPs and gPRPs. Samples from the same individual were run in the same gel. After protein separation by electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane with a Trans-Blot device. TransBlot was running for 7 min, voltage was set at 25 V. After transferring, the membrane was rinsed with MilliQ water, then it was immersed in the red coloration Ponceau for 5 min, next the membrane was rinsed again with distilled water, then it was decolorized using 0.1 M

NaOH (Fluka, Germany) solution, at last it was rinsed with water for 3 min.

2.5.2 Immunological reaction

After coloration and discoloration of the membrane, it was rinsed with 9‰ NaCl (Sigma-aldrich, USA) solution two times. Blocking was performed with 8% skim milk in 9‰ NaCl for 1 h with agitation at room temperature. The membrane was then rinsed with 9‰ NaCl solution two times. It was then incubated with a primary antibody (serum from rabbit blood against IB5 after full immunization procedure, which injected IB5 and an immunogenic protein into the rabbit for 4 weeks to provoke the immune response) at 1: 1000 in 5% bovine serum albumin (BSA) (PAN-Biotech, Germany) in 9‰ NaCl solution for 1h at room temperature. After 2 successive washes in 0.05% PBS-Tween (Bio-Rad, USA) solution, incubation was performed again with 8% skim milk in 9‰ NaCl solution for 30 min under agitation at room temperature. After 2 successive washes in 9‰ NaCl solution, the membrane was incubated with HRP-conjugated secondary antibody (mouse anti-rabbit) (Invitrogen, USA) at 1: 5000 in 5% BSA in 9‰ NaCl solution for 1 h at room temperature. The membrane was rinsed with 0.05% PBS-Tween 3 times with agitation. The last step was that the membrane was rinsed in 9‰ NaCl solution.

2.5.3 HRP colorimetric revelation

Targeted protein (PRPs) were detected using an ECL (Clarity Enhanced Luminescence) (Bio-Rad, USA) detection system, a 1:1 mix of the 2 solutions (luminol solution and peroxide solution) of the kit was made. Membrane was immersed in ECL solution for 5 min, then the membrane was analyzed by ChemiDoc imaging system as soon as possible. Membrane was scanned with different exposure time, the best one was chosen according to the pixel of PRPs which must not be saturated.

2.6 PRP Quantification

Western-blot was developed to be used as a methodology to assess the amount of PRPs in saliva, the intensity of coloration (pixel volume) of a band on membrane being correlated to the protein concentration. In our study, IB5 and II-1 were chosen as references for bPRPs and gPRPs respectively, in order to establish a calibration curve on PRPs that would be used for quantification in saliva. They have been produced and purified as mentioned above. The pixel volume was determined at the exposure time when the pixel was maximum but not saturated. The gradient concentrations of IB5 were 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 mg/mL. The gradient concentrations of II-1 were 0.25, 1, 3, 5, 10, 25, 50 mg/mL. The pixel volume of the targeted protein (bPRPs and gPRPs) band was compared to standard curves generated by the IB5 and II-1 to yield quantitative measures for bPRPs and gPRPs.

After calculating, the calibration curve of IB5 was $y=0.2885x + 0.0631$, $r^2=0.9939$. The calibration curve of II-1 was $y=0.1141x + 0.7438$, $r^2=0.9729$.

2.7 Supplementary experiment –Dot blot for MUC5B

Dot-blot was adopted to detect MUC5B in saliva samples. It differs from western-blot in that targeted proteins are not separated by size but are spotted through circular templates directly onto the membrane.

Firstly, Whatman paper and Nitrocellulose membrane were soaked in Tris-based buffer for 5 min. Then Bio-Dot device was assembled with Whatman paper and Nitrocellulose. Next, the Bio-Dot device was connected with vacuum pump. All was prepared well, 5 μ L of each saliva sample finally was put into membrane through the circular templates. On each membrane, we put 5 μ L of the same standard saliva with 3 repetitions as a reference to calculate the ratio of the pixel volume of each saliva sample to the pixel volume of the standard saliva to avoid bias. The vacuum pump was turned on and ran for 5 min to dry the membrane.

After the above procedures, the procedures of coloration with Ponceau, immunological reaction and HRP colorimetric revelation were similar with western blot, as described in section 2.5. The differences with western blot were the antibodies used and concentration of antibodies. In dot-blot, the primary antibody was incubated at 1: 10000 in 5% bovine serum albumin (BSA) in 9‰ NaCl solution for 1h at room temperature. The second antibody was goat anti-mouse that incubated at 1: 20000 in 5% bovine serum albumin (BSA) in 9‰ NaCl solution for 1 h at room temperature.

2.8 Statistical analysis

Non-parametric analyses were conducted because normality assumptions were not met on raw data and on residues from ANOVA analysis. Mann-Whitney U tests were performed to evaluate differences between the young (Y) and the elderly (O) subjects regarding salivary proteins (bPRPs, gPRPs and MUC5B). Wilcoxon tests were performed on PRPs amount and MUC5B pixel volume ratio to evaluate differences between the start and the end of each session. Friedman ANOVA was conducted on PRPs amount and MUC5B pixel volume ratio to evaluate differences among the three sessions. Spearman rank order correlations were performed for the whole group and in each group (Y and O) to evaluate relationships between astringency threshold and PRPs amount, MUC5B pixel volume ratio. The significance was set at $p < 0.05$. These tests were performed using Statistica® version 13.5.0.17, 1984-2018 TIBCO Software Inc.

3. Results

Table II-1 presents the mean, the median and the standard deviation of the following parameters for the two groups: age, the threshold, the content in bPRPs and gPRPs before and after the experiments and the mean.

a) bPRPs amount

There were no differences between sessions regarding bPRPs amount for either group Y (Friedman $\chi^2 = 1.33$, $p = 0.51$) or group O (Friedman $\chi^2 = 1.87$, $p = 0.39$) or between the mean Start and mean End of sessions for the Y ($Z = 0.31$, $p = 0.75$) and O groups ($Z = 0.89$, $p = 0.37$). For this reason, we decided to merge the bPRPs amount into a unique variable, i.e., bPRPs Mean amount. With respect to the comparison of bPRPs Mean amount, no difference was observed between Y and O groups ($Z = -0.51$, $p = 0.61$) (Figure II-1). Similarly, we did not observe the differences between Y and O groups regarding bPRPs Start ($Z = -0.81$, $p = 0.42$) and bPRPs End ($Z = -0.55$, $p = 0.58$).

Table II-1. Characteristics of the young and elderly panels and PRPs amount of the two groups.

	Y (n=24)				O (n=30)			
	Mean	Median	Range	SD	Mean	Median	Range	SD
Age (years)	29.4	30	24-35	3.8	75	73.5	70-87	4.23
Threshold (mg/mL)	0.29	0.2	0.04-1.00	0.26	0.41	0.35	0.06-0.78	0.24
bPRPs Mean ($\mu\text{g/mL}$)	10.16	8.57	0.19-28.98	7.67	11.99	10.78	1.33-34.11	9.05
bPRPs Start ($\mu\text{g/mL}$)	10.20	7.18	0-29.67	8.16	12.42	10.67	1.98-31.09	8.89
bPRPs End ($\mu\text{g/mL}$)	10.11	8.77	0.23-28.29	7.53	11.56	9.42	0-37.14	9.78
gPRPs Mean ($\mu\text{g/mL}$)	352.50	217.35	51.83-1136.49	308.87	640.68	360.48	0-2299.72	659.43
gPRPs Start ($\mu\text{g/mL}$)	402.59	244.71	53.12-1242.66	397.99	819.96	472.37	0-3066.78	910.26
gPRPs End ($\mu\text{g/mL}$)	308.43	205.95	43.49-1342.03	308.23	444.79	243.98	0-1584.48	442.96

SD: standard deviation of the mean

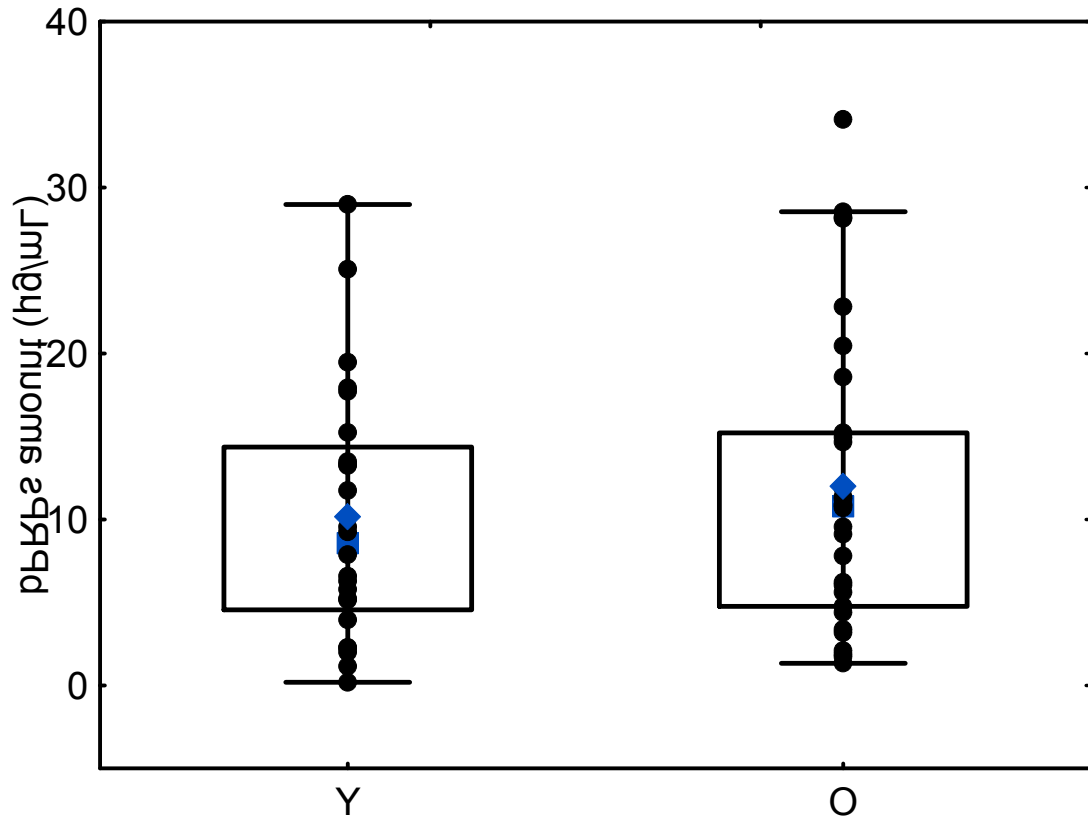


Figure II-1. Box-plot distributions of bPRPs Mean amounts in function of age category (young (Y) and elderly (O)) are shown. The bottom and top of the box correspond to the 25th and 75th percentile, respectively. The blue square and the blue diamond correspond to the median and the mean, respectively. The ends of the whiskers represent the non-outlier range. The black dot symbols correspond to individual data points.

b) gPRPs amount

No differences were observed between three sessions regarding gPRPs amount for either Y ($\text{Chi}^2 = 3.58$, $p=0.17$) or O ($\text{Chi}^2 = 3.29$, $p=0.19$). However, a significant difference was observed between the start of session (mean=819.96 µg/mL) and the end of session (mean=444.79 µg/mL) regarding gPRPs amount in O group ($Z=4.36$, $p=0.000013$); Modest difference was observed regarding gPRPs amount in Y group ($Z=1.74$, $p=0.08$) between the start of session (mean=402.59 µg/mL) and the end of session (mean=308.43 µg/mL).

However, we did not observe statistical differences between Y and O group regarding gPRPs amount for either mean value ($Z=-1.30$, $p=0.19$) (Figure II-2) or the start ($Z=-1.57$, $p=0.12$) (Figure II-3) or the end of ($Z=-0.55$, $p=0.58$) (Figure II-4) session.

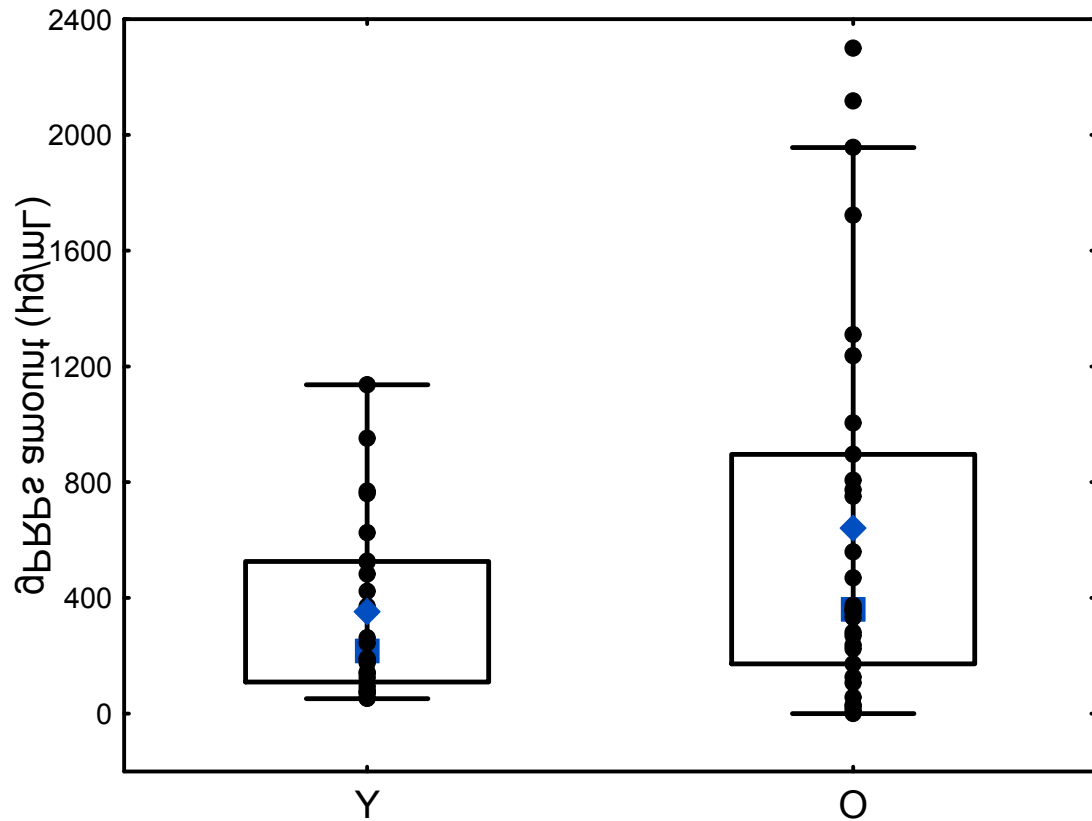


Figure II-2. Box-plot distributions of gPRPs Mean amounts in function of age category (young (Y) and elderly (O)) are shown. The bottom and top of the box correspond to the 25th and 75th percentile, respectively. The blue square and the blue diamond correspond to the median and the mean, respectively. The ends of the whiskers represent the non-outlier range. The black dot symbols correspond to individual data points.

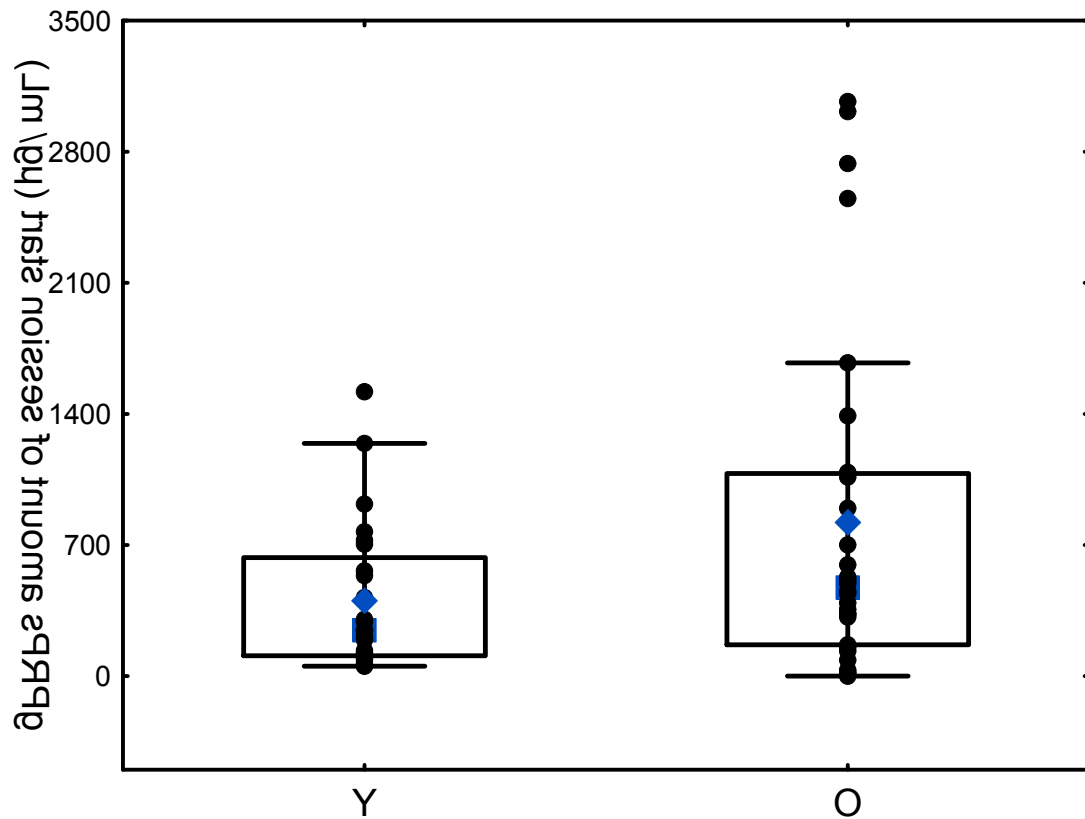


Figure II-3. Box-plot distributions of gPRPs Start amounts in function of age category (young (Y) and elderly (O)) are shown. The bottom and top of the box correspond to the 25th and 75th percentile, respectively. The blue square and the blue diamond correspond to the median and the mean, respectively. The ends of the whiskers represent the non-outlier range. The black dot symbols correspond to individual data points.

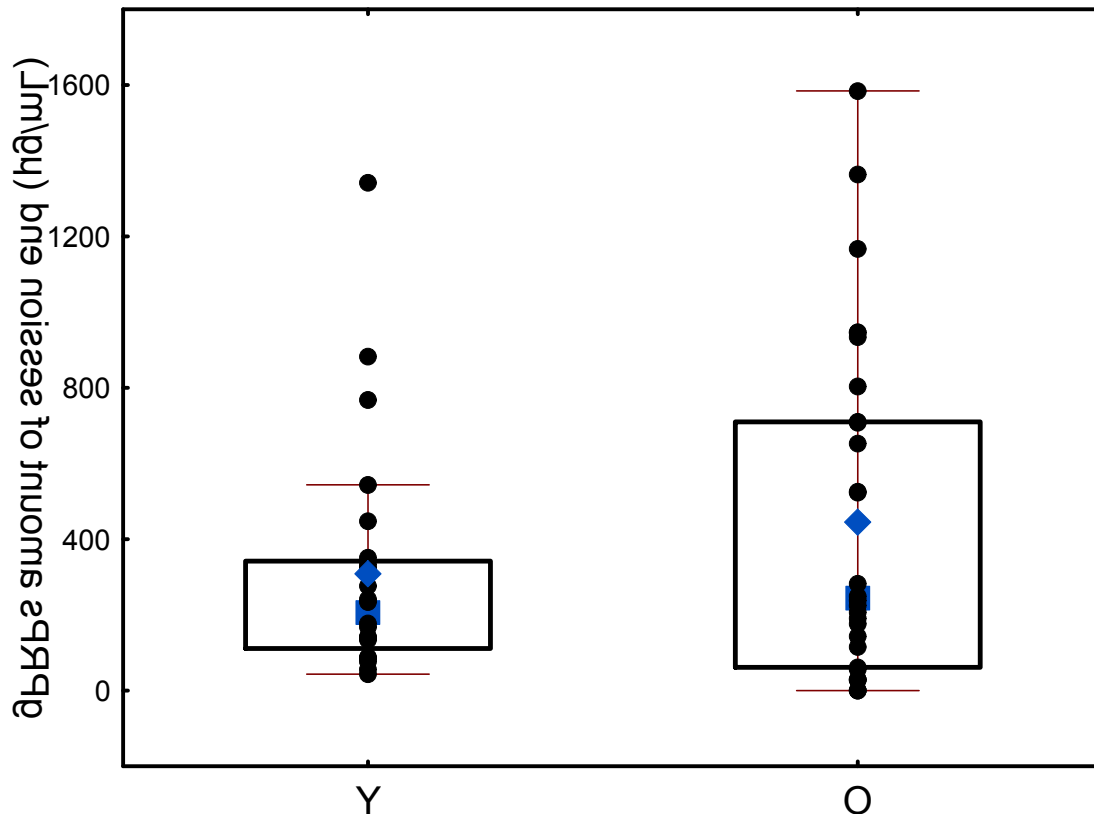


Figure II-4. Box-plot distributions of gPRPs End amounts in function of age category (young (Y) and elderly (O)) are shown. The bottom and top of the box correspond to the 25th and 75th percentile, respectively. The blue square and the blue diamond correspond to the median and the mean, respectively. The ends of the whiskers represent the non-outlier range. The black dot symbols correspond to individual data points.

c) Correlation between astringency threshold and PRPs amount

Spearman rank order correlations were performed for each group (Y and O) to evaluate relationships between threshold and bPRPs and gPRPs amount.

A significant and negative correlation was observed in the O group ($r=-0.41$, $p=0.02$) regarding mean amount of bPRPs and threshold (Figure II-5). The higher the bPRPs amount, the lower the astringency threshold. However, there was no correlation between Mean amount of bPRPs and threshold in the Y group ($r=-0.16$, $p=0.45$), Figure was not shown. Data have been shown in Table II-2.

A significant and positive correlation between gPRPs Mean amount and threshold was observed in the Y group ($r=0.49$, $p=0.01$), higher is the gPRPs amount, higher is the threshold (lower sensitivity) (Figure II-6). Similarly, there were significant and positive correlations between gPRPs Start ($r=0.46$, $p=0.02$), gPRPs End ($r=0.53$, $p=0.007$) and threshold. Figures were not shown. However, no correlation between gPRPs Mean amount and threshold was observed in the O group ($r=0.05$, $p=0.79$). Similarly, there were no significant correlations between gPRPs Start ($r=0.04$, $p=0.83$), gPRPs End ($r=-0.04$, $p=0.81$) and threshold in the O

group. Figures were not shown. Data have been shown in Table II-2.

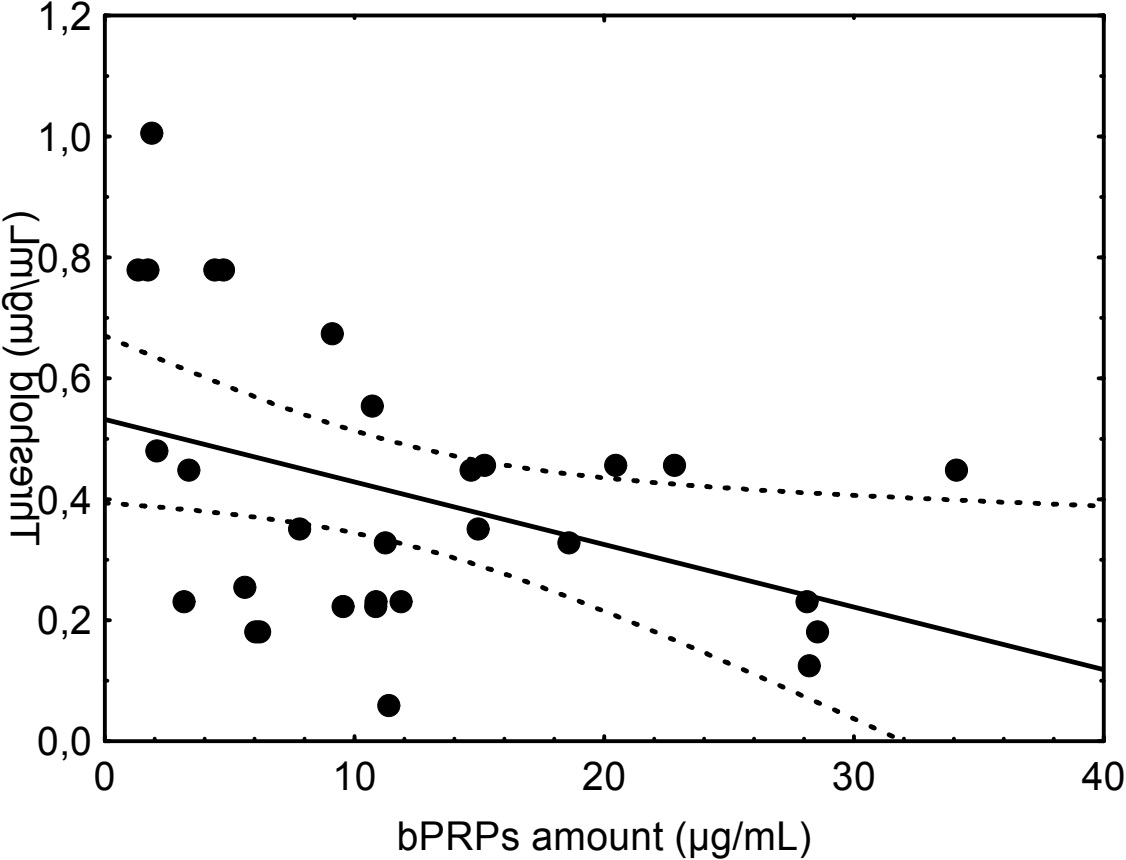


Figure II-5. Spearman correlation between astringency threshold and bPRPs Mean amount observed in the group of elderly panelists. Plain line corresponds to fitted data. Dotted line corresponds to confidence interval at 95%. Black dot symbols correspond to individual data points.

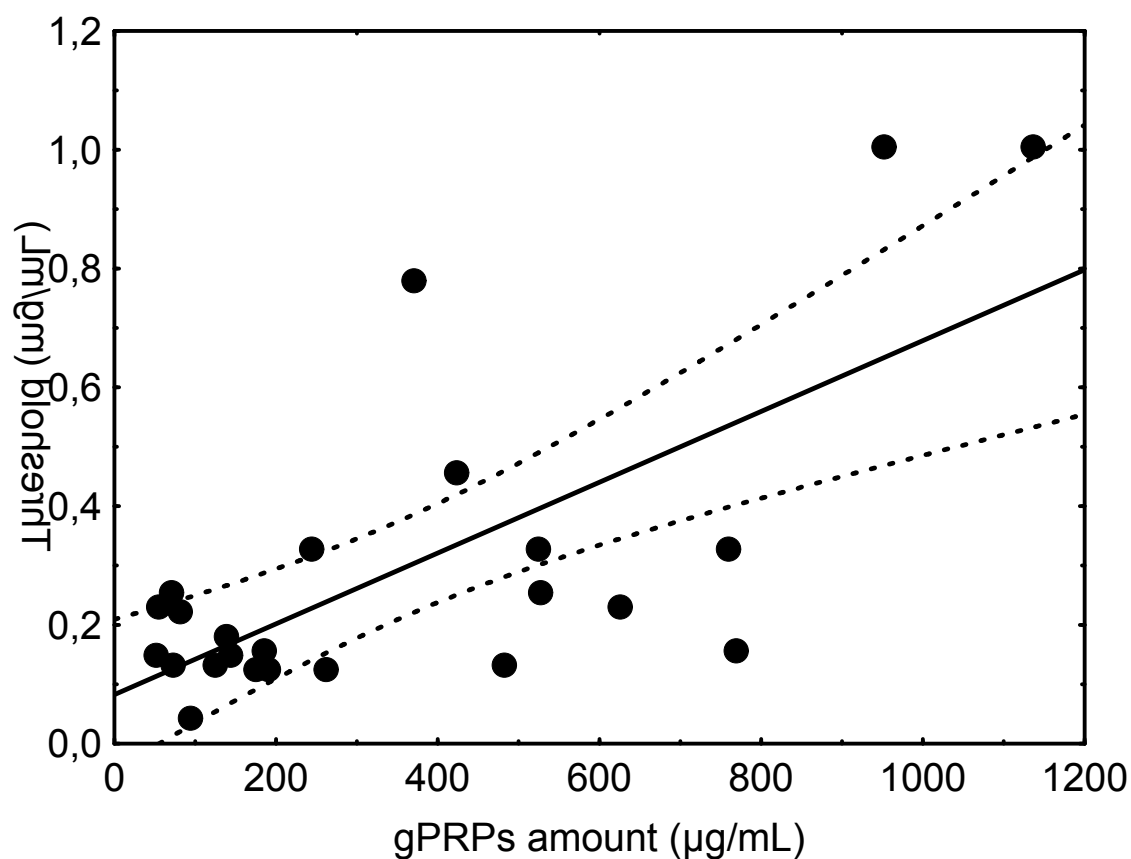


Figure II-6. Spearman correlation between astringency threshold and gPRPs Mean amount observed in the group of young panelists. Plain line corresponds to fitted data. Dotted line corresponds to confidence interval at 95%. Black dot symbols correspond to individual data points.

Table II-2. Spearman correlation coefficient (r) and p value of the astringency threshold and bPRPs and gPRPs amount for the whole (W), young (Y) and elderly (O) panelists.

	Astringency threshold		
	W	Y	O
bPRPs Mean	r=-0.19 p=0.15	r=-0.16 p=0.45	r=-0.42 p=0.02
bPRPs Start	r=-0.19 p=0.16	r=-0.22 p=0.31	r=-0.36 p=0.05
bPRPs End	r=-0.19 p=0.16	r=-0.14 p=0.52	r=-0.43 p=0.02
gPRPs Mean	r=0.34 p=0.01	r=0.49 p=0.01	r=0.05 p=0.79
gPRPs Start	r=0.32 p=0.02	r=0.46 p=0.02	r=0.03 p=0.83
gPRPs End	r=0.25 p=0.06	r=0.54 p=0.007	r=-0.04 p=0.82

d) Results on MUC5B in the young and elderly groups

Regarding the measurements of MUC5B, it is worth mentioning that it was not possible to make calibration curve as mucin are difficult to produce and purified. As mentioned above, the same standard saliva sample was put into each membrane to be as a reference, then we calculated the ratio of the pixel volume of MUC5B in saliva samples to the pixel volume of MUC5B in standard saliva sample to compare the MUC5B between the young and elderly groups. All other results were obtained by using the ratio values.

Results showed that there were no differences between sessions regarding MUC5B pixel volume ratio for either group Y (Friedman $\chi^2 = 0.58$, $p = 0.74$) or group O (Friedman $\chi^2 = 0.21$, $p = 0.9$) or between the mean Start and mean End of sessions for the Y ($Z = 0.88$, $p = 0.38$) and O groups ($Z = 0.46$, $p = 0.64$). For this reason, we decided to merge the MUC5B pixel volume ratio into a unique variable, i.e., MUC5B Mean pixel volume ratio. With respect to the comparison of MUC5B Mean pixel volume ratio, no difference was observed between Y and O groups ($Z = 1.35$, $p = 0.18$) (Figure II-7).

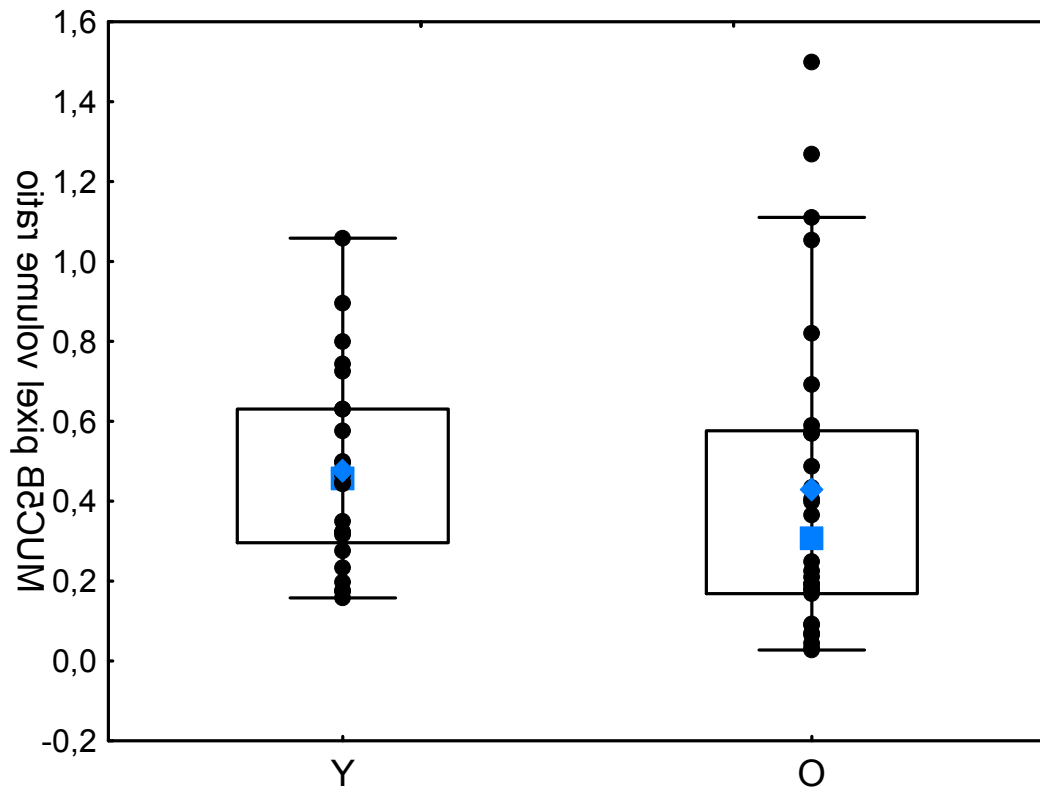


Figure II-7. Box-plot distributions of MUC5B pixel volume ratio in function of age category (young (Y) and elderly (O)) are shown. The bottom and top of the box correspond to the 25th and 75th percentile, respectively. The blue square and the blue diamond correspond to the median and the mean, respectively. The ends of the whiskers represent the non-outlier range. The black dot symbols correspond to individual data points.

Regarding the correlation between the MUC5B pixel volume ratio and astringency threshold, there were no correlations in the two groups. Data have been shown in Table II-3.

Table II-3. Spearman correlation coefficient (r) and p value of the MUC5B pixel volume ratio and astringency threshold for the whole (W), young (Y) and elderly (O) panelists.

	Astringency threshold		
	W	Y	O
MUC5B	r=-0.11	r=0.0004	r=0.07
pixel volume ratio	p=0.41	p=0.998	p=0.72

4. Discussion

In the context of the world population becoming older and ageing well, it is important to understand how flavor perception evolves over the lifetime, while considering the evolution of physiological parameters involved in flavor perception such as saliva. Indeed, the proportional volume of fat and fibrovascular tissue increases in both the parotid and submandibular glands, while the proportional volume of acini is reduced during ageing. They probably both lead to a decrease in efficiency of salivary glands which occurs in senescence (Scott, J., 1977). This study addresses a part of the question by studying the particular case of astringency. Very few researches have investigated the sensitivity to astringency as a function of age. In a previous study, we reported for the first time a difference in oral astringency perception during aging. In this previous study, the total protein concentration of saliva and salivary flow of the subjects were recorded. These salivary parameters were not linked to the difference of astringency perception between the two groups (M. Wang et al., 2022). Herein, we wanted to explore the relationship between astringency sensibility and more specific parameters such as the concentration of TBSP and in particular the one of PRPs, as they are believed to be more directly related to astringency perception.

We compared a group of young people (29.4 years old) with a group of elderly (75 years old). Young people were significantly more sensitive to astringency than the elderly. Regarding the concentrations of bPRPs and gPRPs in the saliva of young and elderly group, the mean concentration of gPRPs in the saliva of elderly group is the double of the one in the young group. However, this difference is not significant. No significant differences were also observed for the concentration of bPRPs in the two groups. A previous study has reported an increase of the bPRPs secretion in saliva as a function of age but between 3 and 15 years old. Authors attributed this difference to the processes of growth and hormonal that may impact on salivary gland function. Despite that aging has also been described to affect salivary gland function, it seems to not affect the secretion of gPRPs and bPRPs. Another study reported no significant changes with aging in the composition of parotid glands saliva, which are the only salivary glands to secrete bPRPs and gPRPs (Padiglia et al., 2018) in healthy subjects (Arjan Vissink, 1996). Baum's reported no change of aPRPs with age (Baum et al., 1982). Alterations of salivary protein secretion with aging appear to depend on the salivary protein, the salivary gland and the physiological condition (stimulated VS unstimulated). For instance, lactoferrin in stimulated parotid and stimulated submandibular/sublingual saliva increase for

both glands significantly with age. However, the opposite observation has been made for unstimulated whole saliva (Dodds et al., 2005). In stimulated parotid, lysozyme has no age-related change, while it significantly decreases with age in stimulated submandibular/sublingual saliva and increases with age in unstimulated whole saliva (Dodds et al., 2005). IgA increases with age both in stimulated parotid saliva and unstimulated whole saliva, while it has no age-related change in stimulated submandibular/sublingual saliva (Dodds et al., 2005; F. Xu et al., 2019). Regarding mucin levels, several studies have reported the decrease of their salivary concentration with age in unstimulated whole saliva as well as in stimulated saliva of mucous glands (submandibular, sublingual, and minor salivary glands) (Denny et al., 1991; Dodds et al., 2005; F. Xu et al., 2019). In the present study, there was no difference between the young and elderly group regarding the MUC5B pixel volume ratio. This discrepancy can be explained by differences in the used methodologies. In the current study, we postulated that the level of PRP or MUC5B in the supernatant of centrifuged saliva reflect the total level of saliva. The results showed that we still observed a significant interindividual variability and thus is in line with our hypothesis. However, we cannot rule out the fact that the centrifugation of the saliva may have removed a part of the salivary protein, in particular proteins with high molecular weight such as mucins or gPRPs. This could explain the absence of differences observed between the two groups. Indeed, a previous study reported that centrifugation may eliminate interindividual differences of salivary activities (Munoz-Gonzalez, Brule, Feron, & Canon, 2019).

Interestingly, we observed that gPRPs amounts were significantly lower at the session end than the session start in the elderly group; in the young group, modest difference was observed too. Literature about gPRPs amount in saliva is poor and much less is the comparison of before sensory test and after sensory test. The most possible explanation is that, during sensory test, there were many rinsing procedures, which might dilute saliva's viscosity or remove salivary film and then reduce the concentration of gPRPs in saliva. A study previously indicated that after rinsing the mouth with water, the amount of proteins within saliva significantly decreased (Nayak & Carpenter, 2008). In addition, the elderly people may have recover saliva back to its normal composition slower than the young people after external intervention as their flow is slightly lower (M. Wang et al., 2022). This observation is of particular importance in the field of astringency sensory evaluation as the role of salivary composition may be predominant in the perceived intensities of sensory attributes.

When we look at the elderly group, a significant and negative correlation between bPRPs and astringency threshold was observed (Figure II-5): the sensitivity to astringency increases with the concentration of bPRPs. This result appears in contradiction with previous results. A previous study has reported an increase of astringency of a tea solution after removing saliva from the oral cavity of the subjects (Nayak & Carpenter, 2008). It is also in disagreement with the hypothesis that PRPs protect the mucosal pellicle from tannin aggregation (Ployon et al., 2018). At the opposite, it is in agreement with the hypothesis postulating that the precipitation of PRP•tannin aggregates are at the origin of astringency (Bajec & Pickering, 2008). In this hypothesis, this precipitation may induce astringency either by the activation of external

sensors, which may be MUC1 (Canon et al., 2021), or by decreasing the lubrication properties of saliva.

When we look at the young group, a significant and positive correlation between gPRPs and astringency threshold was observed (Figure II-6), higher is the gPRPs amount, higher is the threshold (lower sensitivity). Therefore, we may speculate that gPRP plays a protective role and prevents astringency by binding tannin before its interaction with the mucosal pellicle, as previously proposed (Ployon et al., 2018). Indeed, there are several studies (Y. Lu & Bennick, 1998; Sarni-Manchado et al., 2008) reporting that the glycosylation of human PRPs favors the stability of soluble complexes by preventing the formation of aggregates and thus precipitation. This mechanism is attributed to the carbohydrate chain of glycosylated PRPs that provide steric hindrance for protein-protein cross-linking by tannins. As a result, tannin•protein noncovalent complexes does not form aggregation, increasing their solubility and possibly the number of tannins bound per proteins. Canon et al. (2013) have previously reported that 3 molecules of EgCG per bPRP are required to form aggregates (Canon et al., 2013), this number may be higher with gPRP.

This work reports for the first time a difference in astringency perception as a function of age, while there was no difference regarding bPRPs, gPRPs and MUC5B concentrations between the young and elderly groups, which cannot explain the difference in astringency threshold between the two groups. As indicated above, one of the limits of the present study is that salivas have been centrifugated, which may have impacted the concentration of the different proteins and especially those with a high molecular weight. However, a correlation between PRP concentration and astringency sensibility was observed at the group scale (bPRPs for the elderly group and gPRPs for the young group). At the same time, it is observed the diminution of gPRPs after several rinsing procedures in the elderly group, leading to gPRPs concentration closer to the young group. It has been previously suggested that the ability of subjects to maintain constant saliva characteristics can modulate astringency sensitivity, subjects capable of maintaining constant saliva characteristics after both mechanical and chemical stimulation were less sensitive to astringent stimuli (Dinnella, Recchia, Fia, Bertuccioli, & Monteleone, 2009). One should have also in mind that bPRPs and gPRPs are in competition to interact with tannins. Therefore, the decrease of gPRPs in the saliva of elderly may favor bPRP•tannin interaction and thus their aggregation and precipitation. Thus, it can be postulated that the interaction between gPRPs and tannins govern the different mechanisms. As a result, the decrease of gPRPs in elderly saliva favor interaction with bPRPs, explaining that in elderly astringency is correlated with bPRPs, while in the young group it is with gPRPs. Regarding the opposite correlation, it suggests that different mechanisms may involve the PRPs depending on their structure. Basic PRPs forms aggregates with tannins that may be detected at the mucosal pellicle level by external sensor such as MUC1(Canon et al., 2021), while gPRPs, which are less sensitive to aggregation play a protective role by scavenging tannins and preventing the mucosal pellicle from aggregation. These two mechanisms could occur simultaneously in both groups.

One should have also in mind that tannins are able to interact with all protein, despite that

some of them have a higher affinity. Thus, PRPs and MUC5B are two variables among the others. Considering the numerous different proteins in saliva, it could be difficult to have a clear idea of the impact of only one variable.

5. Conclusion

In summary, the present work reported a decrease of the sensitivity to astringency with ageing. Western-blot methodologies were developed to explore the concentration of PRP and MUC5B in saliva and have been applied to a panel of 30 elderly people and 24 young people. Results indicate no significant difference between the two groups. Thus, the concentration of PRP and MUC5B in saliva did not explain the difference of astringency sensitivity between the two groups. Interestingly, a correlation between PRP concentration and astringency sensibility was observed at the group scale (bPRPs for the elderly group and gPRPs for the young group). Therefore, different mechanisms could occur at the same time and respective importance may evolve as a function of age. Considering that other salivary proteins may also interact with tannins, further analysis should be carried out in the future to take into account other salivary proteins that could affect astringency perception.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

Chapter 5: General discussion and conclusion

The aim of this work was to investigate the sensitivity to astringency as a function of age and salivary parameters (flow rate, protein amount and composition).

In this part, we will start the discussion by the sensory experiment, which explore the salivary flow rate, protein amount and astringency threshold of subjects, and whether there is a correlation between flow rate, protein amount and astringency threshold. Then the objectives and results of biology experiment will be discussed, including PRP levels in function of age and the relationships between astringency threshold and PRPs levels. Finally, we will give a conclusion and perspective of future work.

1. Astringency sensitivity: effect of aging and saliva

The sensory experiment and corresponding objective, results and discussion were presented in detail in chapter 3.

As mentioned previously, the five basic tastes (salty, sweet, sour, bitter, umami) perception could be affected by aging. How aging affects astringency perception, what is the difference of astringency perception between young and elderly people, the literature regarding this aspect is quite scarce. Saliva has been reported to have an impact on taste perception, especially the reduced secretion of saliva (hyposalivation) can alter flavor release and perception (Munoz-Gonzalez, Brule, Martin, Feron, & Canon, 2021). Therefore, we set up a methodology (2-alternative forced choice) to explore the astringency threshold in young and elderly group while measuring different salivary properties (flow rate and protein amounts). 24 young people (29.4 ± 3.8 years) and 30 elderly people (75 ± 4.2 years) were recruited in the sensory experiment. The number of subjects recruited in each group was based on a power test according to the preliminary experiment. The following main results were obtained:

- 1) No significant difference of salivary flow rate between young and elderly group. But flow rate in the elderly group was lower than that in the young group with a modest degree.
- 2) No significant difference of total salivary protein concentration between young and elderly group.
- 3) A significant difference of astringency threshold between young and elderly group was observed. The elderly group showed a higher mean astringency threshold than the young group.
- 4) The study reported no correlation between salivary flow rate and astringency threshold in the elderly group. A significant and negative correlation was observed in the young group, higher the salivary flow, lower astringency threshold.

- 5) No correlation between salivary protein concentration and astringency threshold in both young and the elderly group was observed.

Regarding the changes of salivary flow rate with aging, as mentioned previously, the results from literature are not consistent. The alteration of saliva type (unstimulated saliva, stimulated saliva) and saliva secreted from salivary glands (submandibular/sublingual, parotid) with aging differ across studies (Affoo et al., 2015). The variability might be due to different factors: i) saliva collection method, ii) saliva collection duration and circadian rhythm, iii) stimulus type and intensity, iv) subject's number and age range. In addition, one crucial factor corresponding to inter-individual variability should be kept in mind. In our work, the collected saliva should be regarded as whole saliva (between unstimulated and stimulated saliva) instead of unstimulated because it was collected after rinsing mouth with pectin, bicarbonate and water. This can explain the absence of differences between young and elderly. Indeed, it is likely that pectin and bicarbonate can have an effect on salivary flow. Although salivary flow rate was lower in the elderly people, it did not reach a level of significance. The dissimilarity to our result with those of Vandenberghe et al. (2016) (Vandenberghe-Descamps et al., 2016), who showed an average 38.5% reduction of resting salivary flow in the healthy elderly people, can be probably due to the fact that the number of subjects participated in experiment is different. In their study, 93 young adults (20-55 years) and 84 elderly people (70-92 years) were recruited. However, only 24 young adults and 30 elderly people were included in our experiment, based on a power test to evidence a difference in astringency sensitivity only. It is possible that this number of subjects was not enough to fully explain the difference between individuals. Indeed, a power test performed on the data described by (Vandenberghe-Descamps et al., 2016) shows that a minimum of 32 subjects in both group are necessary to observe a difference regarding salivary flow. This result is coherent with the p value (0.09) observed in our study. Moreover, we found that there were 4 outliers of salivary flow rate with a higher flow than the mean flow rate in the elderly group. It may have reduced the gap with the young group. However, in their study, 13% of the participants were suffering from hyposalivation (resting salivary flow less than 0.1 mL/min). As described previously, this large inter-individual variability is probably due to life style and aging factors, such as diet or smoking habits and hydration status, structural changes in the salivary glands, neurophysiological changes or ageing-related events (Vandenberghe-Descamps et al., 2016).

Regarding protein amount in saliva, our result is in line with Salvolini et al. (1999) who did not find significant difference of total protein content in human unstimulated whole saliva with aging (Salvolini et al., 1999). Benaryeh et al.(1986) (Benaryeh et al., 1986), Aguirre et al.(1987) (Aguirre, Levine, Cohen, & Tabak, 1987) and Dodds et al. (2005) (Dodds et al., 2005) reported that total protein in parotid saliva did not change with age. However, in Dodds's study, total protein in submandibular/sublingual saliva decreased significantly with aging (Dodds et al., 2005). This means that the changes in protein contents with aging are also related to the different salivary glands, which produce salivas that have different composition. Indeed, although there is no consensus regarding the changes in saliva properties of specific salivary glands, from the data showed in literature, parotid gland fluid secretions are more

inclined to remain stable with age. Studies of submandibular gland secretions with age are not as consensual. It has been suggested that submandibular/sublingual gland functions seem to be more vulnerable to various perturbations than are parotid gland functions (Baum et al., 1992). In addition, it is worth mentioning that protein concentration changes with aging might be dependent on flow rate (Dodds et al., 2005). Indeed, Nagler et al. (2005) reported that protein concentrations increased with aging while salivary flow rate reduced with aging. They attributed this observation to a higher concentration of salivary components due to a reduced salivary flow (Nagler & Hershkovich, 2005b). However, from a clinical standpoint, the total quantity of the salivary components secreted into the oral cavity is more important (Nagler & Hershkovich, 2005b). In our work, there is no age-related change in the salivary flow rate, no age-related change in the total protein concentration. Accordingly, there is no age-related change in the total protein amount.

Regarding the astringency threshold comparison between young and elderly people, the available literature is extremely poor. As far as we know, a study investigated the impact of age on individual sensitivities to lingual tactile roughness in relation to astringent stimuli sensitivity and did not find a correlation between age and roughness sensitivity (Linne & Simons, 2017). In our study, as the result presented in chapter 3, the threshold in the elderly group is higher than that in the young group. It means young people are more sensitive to astringency than the elderly people. Indeed, as mentioned previously, aging has a negative effect on taste acuity due to multiple factors. Among these factors, saliva cannot be neglected. In fact, it has been widely suggested that saliva plays a key role in taste perception. However, the exact mechanisms of how saliva modulates taste sensation are still needed to be further researched. Currently, salivary components and salivary flow are considered to play an important role in taste sensations. Therefore, salivary flow is considered as the first parameter to be involved in astringency evaluation. The difference of astringency threshold between young and elderly people can be partly explained by the difference of salivary flow between the two groups, because of a lower flow rate in elderly group even though the difference did not reach a significance.

The relationship between astringency threshold and salivary flow was found only in the young group, a higher salivary flow corresponding to a higher sensitivity (lower threshold). However, no relationship was observed in the elderly group. Thus, the mechanism by which salivary flow influences astringency sensitivity is different as a function of age. Even though it is difficult to explain this mechanism, some hypothesis can be formulated. Indeed, when comparing the astringency sensitivity across studies, salivary flow rate should be considered. Put aside the age effect and considering the young group only, it could be suggested that a higher flow rate corresponds to a high protein content in saliva, and then a higher astringency sensitivity is caused by the interaction of salivary protein with tannic acid. However, there are no relationships between astringency threshold and protein amount in both young and elderly group. This is in accordance with Kallithraka et al. (2001), who suggested that astringency was not correlated with the total protein content. The authors also indicated that the concentration of individual proteins might be more important for astringency (Kallithraka et al., 2001). Therefore, salivary component proline-rich proteins (PRPs) were investigated to

explain the difference of astringency threshold between young and elderly group. Indeed, PRPs have a high affinity for tannins, and seem to be involved in the adaptation to a tannin-rich diet (Canon et al., 2021). In addition, the literature regarding the amount of age-related change in PRPs is scarce except (Baum et al., 1982), who reported acidic PRPs in parotid saliva did not change with aging.

2. Astringency sensitivity: effect of aging and PRPs levels

The biological experiment and corresponding objective, results and discussion have been presented in detail in chapter 4.

In the previous chapter, we showed that astringency sensitivity is lower in an elderly group than in young one. However, it was not linked to the salivary flow and total protein concentration. Therefore, in this chapter, we mainly focused on the evaluation of salivary proline-rich proteins (PRPs) amount in young and elderly groups of individuals and to link them to astringency sensitivity. Therefore, a western-blot methodology was developed to explore the PRPs amount in saliva. IB5 and II-1 were used as reference of bPRPs and gPRPs, respectively. The main result obtained in this chapter were:

- 1) No significant difference between young and elderly group regarding both bPRPs and gPRPs.
- 2) A significant reduction in gPRPs at the end of session compared with the beginning of session in the elderly group, lower gPRPs at the end of session than that at the beginning of session in the young group but with a modest degree of evidence.
- 3) A significant and negative correlation between bPRPs amount and astringency threshold in the elderly group, a positive correlation between gPRPs amount and astringency threshold in the young group.

Regarding the bPRPs and gPRPs amounts between young and elderly group, to the best of our knowledge, there is no literature on how these two classes of PRPs change with aging. However, it is not surprising to obtain these results, which are in line with some studies showing that there are no age-associated alterations in salivary proteins including α -amylase, IgA, lactoferrin, lysozyme and acidic PRPs (Aguirre et al., 1987; Baum et al., 1982; Fox et al., 1987). What is different from our study is that these proteins are from parotid glands following stimulation. In our work, we studied whole saliva. Nevertheless, some studies showed that salivary proteins decrease with aging including histatins, mucins and lysozyme, those proteins being excreted from stimulated submandibular/sublingual salivary glands (Dodds et al., 2005). It is worth mentioning several studies reported that mucin concentration decreases with age (F. Xu et al., 2019). We cannot compare the results obtained in our work on bPRPs and gPRPs to what is described in the literature on other proteins. Nonetheless, according to these results obtained from different studies, it can be assumed that salivary

proteins change with aging is related to the type of secretory glands. bPRPs and gPRPs are secreted only by parotid gland.

Regarding the amount of gPRPs between the start of the session and the end of the session, the results obtained from our study are very new. The gPRPs concentration significantly reduced at the end of the session in comparison to the start of the session in the elderly group. The amount of gPRPs at the end of the session was lower than at the start of the session in the young group, even though it did not reach significance. As explained in chapter 4, the most possible reason is that gPRPs was removed with salivary film after several rinsing procedure. The ability of saliva characteristics returning back to normal composition might be poorer in the elderly group than in the young group (Figure 28). This hypothesis should be further studied to test the ability of gPRPs to return to normal levels after intervention in function of age. Dinnella et al. (2010) reported that gPRPs significantly decreased after tasting tannic acid solutions. However, this phenomenon happened in high astringency responding group (Dinnella, Recchia, Vincenzi, Tuorila, & Monteleone, 2010). They attributed it to the aggregation of gPRPs and tannic acid. In our study, it happened in low astringency sensitive group. The possible reason is that gPRPs also interact with tannic acid. However, it does not form aggregation but soluble complexes, and thus does not induce a strong astringency sensation. It is worth mentioning that the concentrations of tannic acid between the two studies are different. In their study, tannic acid concentration was set to 3 g/L. In our study, the maximum concentration of tannic acid was 0.574 g/L.

The difference in astringency threshold between the two groups cannot be explained by differences in bPRPs and gPRPs levels between the two groups. Indeed, it has been suggested that astringency is a complex sensation and it is likely that multiple mechanisms are occurring simultaneously. PRPs seem not to be the only mechanism in astringency development (Gibbins & Carpenter, 2013). It is worth noting that PRPs amounts can only explain the astringency threshold at the group scale (bPRPs for elderly and gPRPs for young). This is a very new result regarding the astringency mechanism as a function of age. As explained in chapter 4, perhaps the combinations of bPRPs and gPRPs with tannic acid have different competitive mechanisms in the young and elderly groups. In the young group, gPRPs play a primary role in interaction with tannic acid to prevent astringency perception. In the elderly group, bPRPs first interact with tannic acid to provoke astringency perception due to gPRPs decrease (Figure 29). Actually, three classes of PRPs can be competitive for binding tannins. Some results indicated that bPRP precipitated a higher quantity of tannins compared to that of the other PRPs (Y. Lu & Bennick, 1998). Some other studies observed that condensed tannins interact first with acidic PRPs (Soares et al., 2011). Our explanation is thus speculative and needs of course further investigations about this complex mechanism.

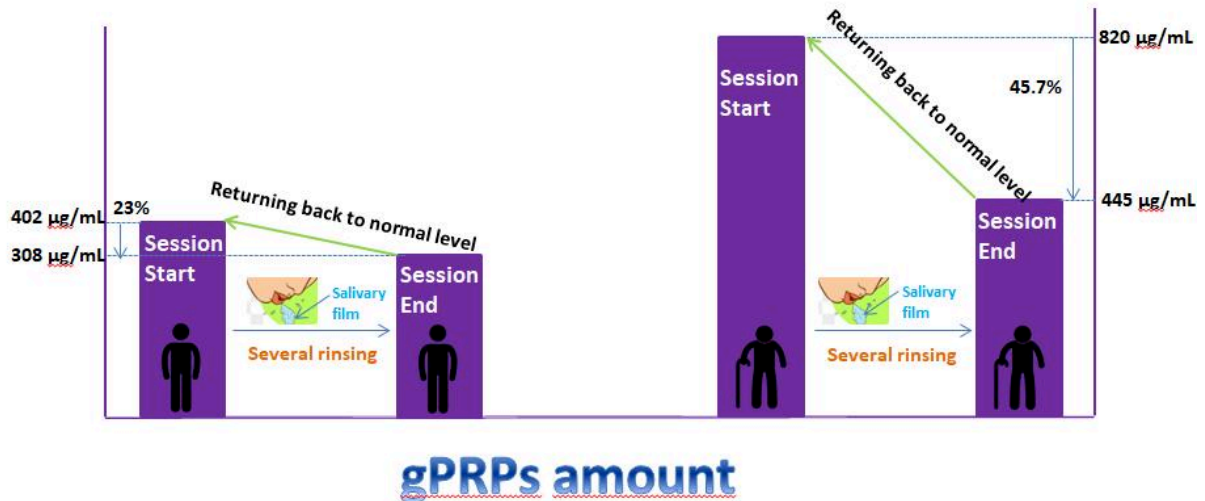


Figure 28. Illustration of mechanistic hypothesis of gPRPs reduction after rinsing and the time of returning back to normal level in function of age.

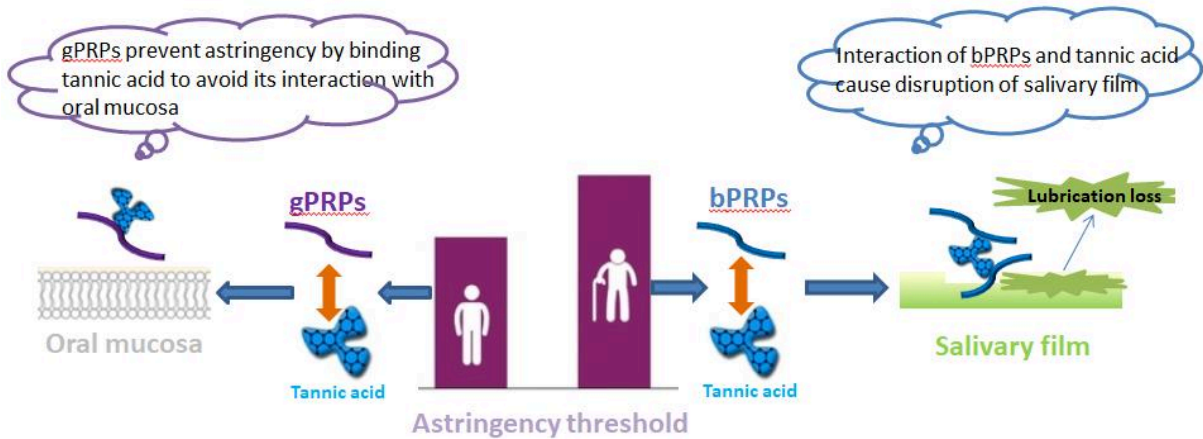


Figure 29. Illustration of mechanistic hypothesis of astringency involved in PRPs type in function of age.

3. Conclusion and Perspective

In conclusion, this thesis was divided into two parts (sensory experiment and biological experiment). In sensory experiment, 2-AFC method was developed to investigate astringency threshold/sensitivity and salivary properties (flow rate, total protein content) as a function of age. It allowed us for the first time to find a difference in astringency sensitivity in function of age, astringency threshold was higher in the elderly group than in the young group. However, this difference was not linked to the salivary flow rate and total protein content. For this reason, biological experiment was conducted by developing western-blot methodology to explore salivary proteins levels especially proline-rich protein (PRPs). However, there were no significant differences between the young and the elderly group in regard to PRPs levels. The difference of astringency threshold was not linked to PRPs levels too.

In addition, some interesting results were observed. Salivary properties can explain astringency threshold at the group scale. In the young group, salivary flow rate and gPRPs amount have shown a positive correlation with astringency threshold. In the elderly group, bPRPs amount has shown a negative correlation with astringency threshold.

To explain the difference in astringency threshold in the two groups, further studies should be done including investigation of histatins levels, saliva pH, buffer capacities, viscosity, rheology etc.

It is worth highlighting that individual variations, such as individual physiological variations of gland functionality, life style and habits, daily diets etc., are the large factors in the development of astringency. Therefore, it is important to have a survey on subjects' daily diet and food preference. Indeed, it has been suggested that taste sensitivity can be affected by food intake and its frequency.

This work can also provide inspirations when designing plant-based food for elderly people. Last but not least, it is worth mentioning that astringency is still mysterious sensation due to complicated mechanisms. As a consequence, there is a need to continue research in this field in order to develop new food products enriched in polyphenol adapted to the nutritional and sensory requirements of older people.

RÉSUMÉ SUBSTANTIEL

Introduction générale

La proportion de personnes âgées dans le monde augmente rapidement. Dans la première moitié du siècle actuel, la population mondiale de 60 ans et plus devrait augmenter de plus de trois fois pour atteindre près de 2.1 milliards d'ici 2050 (Assad-Bustillos et al., 2019). Chez la population âgée, les effets cumulatifs du vieillissement physiologique, des maladies et des médicaments affectent souvent différents aspects de la physiologie buccale, qui jouent un rôle clé dans le comportement alimentaire (Vandenberghe-Descamps et al., 2018). Par exemple, le vieillissement s'accompagne souvent d'une diminution du flux salivaire ou de changements dans la composition salivaire (F. Xu et al., 2019). Certains composés salivaires peuvent stimuler continuellement les récepteurs du goût (Canon et al., 2018), et la salive a un rôle de base pour dissoudre et délivrer les molécules sapides aux récepteurs du goût localisés au niveau des papilles gustatives (Laguna et al., 2021). Par conséquent, il a été proposé qu'une réduction de la sécrétion salivaire peut entraîner une diminution de la sensibilité au goût. En outre, il a également été rapporté que la salive peut affecter la libération et la perception des arômes (Canon et al., 2018). À l'exception de l'impact de la salive sur le goût et la perception des arômes, le vieillissement en soi a un impact sur la perception du goût et des arômes. En effet, les diminutions de la perception du goût et des arômes liées à l'âge ont été bien documentées (Doty, 2018; Doty & Kamath, 2014; Methven et al., 2012; Sergi et al., 2017; Zhang & Wang, 2017). Comme mentionné ci-dessus, le vieillissement est souvent associé à une dégradation de l'état de santé bucco-dentaire, où la perte de dents, la diminution de la force musculaire et de la pression de la langue et la réduction du flux salivaire sont parmi les principaux facteurs responsables des difficultés alimentaires et de la perte de plaisir alimentaire chez les personnes âgées (Assad-Bustillos et al., 2019; Laguna, Hetherington, Chen, Artigas, & Sarkar, 2016; Vandenberghe-Descamps et al., 2016), ce qui pourrait augmenter le risque de diminution de la consommation alimentaire, conduisant à la malnutrition et à d'autres maladies, par exemple l'augmentation du stress oxydatif chez les personnes âgées.

Les composés polyphénoliques sont connus pour être des piègeurs efficaces des espèces réactives de l'oxygène et peuvent également fonctionner indirectement comme antioxydants (Koleckar et al., 2008). Par conséquent, la supplémentation des aliments en polyphénols pourrait être une stratégie pour maintenir un bon état de santé général, en particulier chez les personnes âgées. Cependant, les polyphénols peuvent être à l'origine de la sensation d'astringence, qui correspond à une sensation tactile de sécheresse ressentie dans la bouche (Canon et al., 2021). Cependant, la littérature concernant l'effet du vieillissement sur la perception de l'astringence est assez rare. Comme mentionné ci-dessus, il a été largement rapporté que la salive a un impact sur la perception sensorielle. Par conséquent, l'objectif de ce travail était d'étudier la perception de l'astringence en fonction de l'âge en tenant compte des propriétés salivaires (débit salivaire, quantité de protéines, niveaux de PRP).

Chapitre 1 : Etat de l'art

1. Salive

1.1 Propriétés salivaires

La salive est un liquide physiologique qui joue un rôle important dans la cavité buccale et la transformation orale des aliments. Elle est sécrétée dans la cavité buccale par les glandes parotides, sous-maxillaires, sublinguales et salivaires mineures (Feron, 2018). La salive peut être classée comme stimulée, qui est sécrétée principalement par la glande parotide et non stimulée, qui est sécrétée principalement par les glandes sous-maxillaires et sublinguales (Mosca & Chen, 2017). Le débit quotidien moyen de la salive globale varie entre 1 et 1.5 L (Humphrey & Williamson, 2001). Les contributions en pourcentage des différentes glandes salivaires au cours de l'écoulement non stimulé sont les suivantes: 20% de la parotide, 65% de la sous-maxillaire, 7% à 8% de la sublinguale et moins de 10% pour de nombreuses glandes mineures (Humphrey & Williamson, 2001). Avec la stimulation, la glande parotide augmente sa contribution à l'ensemble du volume de salive de la bouche de 10% (Dawes, 2008). Le débit salivaire non stimulé est d'environ 0.1 à 0.5 mL/min, le débit salivaire stimulé est d'environ 1 à 5 mL/min (Boehm et al., 2020). Lorsque le débit salivaire non stimulé est inférieur à 0.1 mL/min ou que le débit de salive stimulé par la mastication est inférieur à 0.7 mL/min, il est considéré comme indicateur d'une hyposalivation (Pedersen et al., 2018). La salive se compose principalement de 99% d'eau (un fluide non newtonien car la viscosité diminue avec l'augmentation du cisaillement), de près de 3000 protéines et peptides, d'électrolytes et de petites molécules (Canon et al., 2018; Dawes, 2008). Plus de 95% des protéines salivaires proviennent des principales familles de protéines salivaires, qui comprennent les protéines riches en proline (PRP) acides, basiques et glycosylées, l'amylase, les mucines (MUC5B et MUC7), les cystatines, les histatines et la statherine (Helmerhorst & Oppenheim, 2007). Les fonctions de la salive sont multiples, c'est-à-dire la lubrification, l'homéostasie microbienne, la fonction tampon, la minéralisation, la digestion, le goût, la clairance buccale et autres (Feron, 2018; Mosca & Chen, 2017).

Il a été suggéré que la salive (débit, capacité tampon) et sa composition (protéines, ions) peuvent affecter la perception du goût. Certains composés salivaires peuvent stimuler continuellement les récepteurs du goût, conduisant à un mécanisme adaptatif ayant un impact sur la sensibilité au goût (Canon et al., 2018; Feron, 2018). Les chercheurs devraient ainsi faire attention au type de salive (stimulée ou non stimulée, humaine ou artificielle) et au type de stimulation (mécanique ou gustative). De plus, la grande variabilité interindividuelle concernant les propriétés de la salive doit être prise en compte lors de l'interprétation des données liées à la salive.

1.2 L'effet du vieillissement sur la salive

Il est bien connu que le vieillissement s'accompagne souvent d'une diminution des capacités

physiques et physiologiques. Il a un impact significatif sur la physiologie buccale, c'est-à-dire la perte de dents, la diminution de la force musculaire et de la pression de la langue, les troubles de la déglutition et les modifications de la salive. Les changements dans la salive au cours du vieillissement peuvent être regroupés en propriétés quantitatives (débit) et qualitatives (composition) (Vandenberghe-Descamps et al., 2018). La recherche sur l'effet du vieillissement sur le débit salivaire a été considérablement étudiée mais les conclusions sont contradictoires. Certains travaux ont rapporté que le débit salivaire diminue lorsque l'âge augmente, ceci en ce qui concerne les glandes salivaires spécifiques ou le type de salive (Navazesh, Mulligan, et al., 1992; Percival et al., 1994; Tanida et al., 2001; Vandenberghe-Descamps et al., 2016). Alors que d'autres études n'ont pas trouvé d'effet liée à l'âge sur le débit salivaire (Baum et al., 1992; Benaryeh et al., 1986; Shern et al., 1993). Affoo et al. (2015) ont effectué une méta-analyse à partir de 47 études concernant la salive et l'âge (Affoo et al., 2015). Ils ont rapporté que le flux salivaire non stimulé et le flux salivaire sous-maxillaire et sublingual stimulé étaient significativement plus faibles chez les personnes âgées. Cependant, les débits salivaires des glandes parotides et des glandes mineures ne semblaient pas être significativement plus faibles (Affoo et al., 2015). Une étude a rapporté qu'une réduction moyenne de 38.5% du flux salivaire au repos et de 30% du flux salivaire stimulé chez les personnes âgées en bonne santé (70-92 ans) par rapport aux jeunes adultes (22-55 ans) étaient indépendantes de l'état dentaire et de la prise de médicaments (Vandenberghe-Descamps et al., 2016). Affoo et al. (2015) ont également suggéré que le vieillissement est associé à une réduction du flux salivaire qui ne peut pas être entièrement expliquée par la prise de médicaments ou la maladie (l'état dentaire n'a pas été exploré) (Affoo et al., 2015). Les auteurs ont conclu que les raisons possibles de ces résultats incohérents sont la structure des glandes salivaires, c'est-à-dire la perte de cellules acineuses, la réduction du volume acineux et la perte de tissu sécrétoire avec détérioration neurophysiologique (Affoo et al., 2015; Vandenberghe-Descamps et al., 2016; F. Xu et al., 2019). En outre, l'hypofonction salivaire est un problème courant chez les personnes âgées (Gilbert et al., 1993; Munoz-Gonzalez et al., 2018).

Il a également été rapporté que la composition de la salive peut changer de manière marquée au cours du vieillissement. Cependant, les résultats sont contradictoires entre les études. Nagler et Hershkovich (2005) ont rapporté que la concentration de matières inorganiques (K^+ , Cl^- , P et Ca^{2+}) a augmenté chez les personnes âgées, ce qui peut être attribué à la réduction du volume de salive (Nagler & Hershkovich, 2005a). Ils ont également observé que les concentrations de composants organiques comme les protéines totales, l'albumine, les IgA sécrétoires augmentaient avec le vieillissement (Nagler & Hershkovich, 2005b). Cependant, Nassar et coll. (2014) ont rapporté que la quantité de Ca^{2+} diminuait en comparant les groupes jeunes et âgés dans le cas de la salive non stimulée (Nassar et al., 2014). Pour les composants organiques, de nombreuses études s'accordent à dire que la concentration de mucine diminue avec le vieillissement (Chang et al., 2011; Denny et al., 1991; Navazesh, Mulligan, et al., 1992; Pushpass et al., 2019). Pour d'autres protéines, comme l' α -amylase, les IgA, la lactoferrine ou le lysozyme, le changement avec l'âge dépend du type de salive et de glande (Aguirre et al., 1987; Baum et al., 1982; Dodds et al., 2005; Fox et al., 1987). En ce qui concerne le changement des PRP avec le vieillissement, il y a un manque de recherche sur les

PRP de base et les PRP glycosylés, les PRP acides ont été étudiés par Baum et al. (1982) en explorant la sécrétion de protéine exocrine salivaire chez 220 adultes, ils n'ont pas trouvé de changement dans la sécrétion acide de PRP pendant le vieillissement (Baum et al., 1982).

1.3 Les effets des médicaments sur la salive chez les personnes âgées

Outre les changements de la salive liés à l'âge, il a été démontré que la prise de médicaments peut avoir un grand impact sur la salive. Ichikawa et coll. (2011) ont étudié les relations entre la quantité de salive et de médicaments chez les personnes âgées (79-80 ans). Leurs résultats suggèrent que le volume de salive chez les personnes âgées est affecté par le nombre et les types de médicaments chez les personnes âgées (K. Ichikawa et al., 2011). Stanley et coll. (1989) ont observé que les personnes âgées (âge moyen de 86.6 ± 5.6 ans) qui prenaient plus de trois médicaments par jour avaient un flux salivaire stimulé significativement plus faible que les participants qui ne prenaient aucun médicament (Stanley L. Handelman, 1989). Un résultat similaire a été obtenu par Gilbert et coll. (1993) qui ont indiqué que le volume de salive a tendance à diminuer davantage chez les personnes qui utilisent plusieurs drogues que chez les personnes qui utilisent des préparations actives uniques, l'âge moyen des participants était de 78 ans (Gilbert et al., 1993). Les effets des médicaments sur les composants de la salive ne sont pas aussi documentés que les études portant sur l'effet des médicaments sur le débit de la salive. Il a été indiqué que les médicaments antihypertenseurs augmentaient significativement les quantités de salive de K^+ , Na^+ , d'urée et de protéines totales chez les patients âgés de 30 à 70 ans (Ivanovski et al., 2015). En conclusion, les médicaments (nombre ou type) sont un facteur important dans la sécrétion salivaire qui doit être pris en compte lors de l'étude des effets du vieillissement sur les propriétés salivaires.

2. Goût et arôme

La saveur des aliments joue un rôle important dans la consommation alimentaire. En contribuant à l'appétence des aliments, la saveur est l'un des facteurs les plus importants de la prise alimentaire (Yin et al., 2017). La saveur est définie comme « l'interprétation psychologique d'une réponse physiologique à un stimulus physique » (Noble, 1996). Par conséquent, la perception des saveurs se compose non seulement de plusieurs modalités sensorielles, du goût, de la rétro-olfaction et de la sensation trigéminale, résultant de l'activation des récepteurs sensoriels, mais également de l'intégration au niveau cérébrale de ces différentes modalités. La perception de la saveur provient de l'activation de récepteurs situés dans la bouche et le nez (Canon et al., 2018). Selon leur structure et leurs propriétés, les composés aromatiques se lient aux chémorécepteurs dans la bouche (récepteurs du goût et du nerf trijumeau) et dans le nez (récepteurs olfactifs) ou augmentent la force de frottement à la surface de la muqueuse buccale, entraînant l'activation des mécanorécepteurs (Canon et al., 2018).

2.1 Perception du goût

La perception du goût correspond à l'activation des récepteurs du goût dans les papilles gustatives, qui sont principalement intégrés dans la muqueuse de la cavité buccale et répartis dans les différentes régions de la langue. Une population importante de papilles gustatives orales se trouve dans les papilles circumvallées. Les papilles gustatives sont également situées à la surface des papilles fongiformes dans la langue antérieure et dans la position des plis épithéliaux des papilles foliées (Matsuo, 2000). Les humains possèdent environ 5000 papilles gustatives. Chaque papille gustative se compose d'une communauté de 50 à 100 cellules qui traitent et intègrent l'information gustative. La sensibilité des papilles gustatives aux stimuli gustatifs individuels varie considérablement et dépend du type de papilles et de la zone de la cavité buccale dans laquelle elles se trouvent. Il existe au moins trois types de cellules différents dans les papilles gustatives des mammifères : les cellules de type I, les cellules réceptrices (type II) et les cellules présynaptiques (type III) (Kikut-Ligaj & Trzcielinska-Lorych, 2015). Elles sont définies par leur morphologie, leur fonction et leur profil d'expression : les cellules de type I, qui sont considérées comme ayant une fonction glie, en raison de leur rôle dans la fin de la transmission synaptique ; les cellules de type II (récepteurs), qui expriment les récepteurs gustatifs couplés aux protéines G pour les stimuli umami, amers ou sucrés; les cellules de type III, qui transmettent le goût aigre et créent des synapses fonctionnelles avec les fibres nerveuses gustatives afférentes (Roper, 2013). Les cellules impliquées dans la détection du goût du sel (NaCl) n'ont pas encore été identifiées avec certitude en termes de types de cellules. Il a été spéculé que les cellules de type I pourraient être en charge de la médiation de la transduction du sodium. La base cellulaire du goût du sel chez la souris a été liée aux canaux sodiques épithéliaux, qui peuvent être exprimés sur les cellules gustatives de type I. Cependant, d'autres recherches sont nécessaires pour caractériser l'implication de cette voie chez l'homme (Loper et al., 2015). La perception du goût peut être séparée en cinq modalités gustatives de base, y compris le sucré, le salé, l'umami, l'acide et l'amertume (Niimi et al., 2014). Les composés sapides responsables des saveurs umami, sucré et amer sont détectés par les récepteurs couplés aux protéines G: Les composés au goût sucré et umami sont détectés par les RCPG de la famille des gènes Tas1R (protéines T1R), et probablement d'autres gènes également. Les récepteurs classés comme récepteurs du goût (TASR) montrent une sensibilité aux composés nutritionnels les plus importants en terme de consommation, à savoir les glucides, les protéines et les substances amères, toxiques et non toxiques et structurellement diverses (Kikut-Ligaj & Trzcielinska-Lorych, 2015; Reichling, 2008). Le goût salé est produit principalement par le chlorure de sodium. Il nécessite la diffusion des ions Na^+ à travers des canaux membranaires spécialisés, tels que le canal Na^+ sensible à l'amiloride. Il a été aussi proposé une implication du canal sodique de type épithélial sélectif (ENaC) (Hartley et al., 2019). La disparition complète des réponses nerveuses gustatives aux acides se produit avec l'ablation des cellules de type III (Taruno et al., 2021). Les cellules de type III contiennent quelques canaux ioniques distincts. Les canaux ioniques impliquent la migration des ions hydrogène (H^+) à travers les ENaC et les canaux protoniques HCN1 et HCN4 (Sugita, 2006).

2.1.1 Modalités gustatives

a) Saveur salé

Le goût salé est produit principalement par le chlorure de sodium. Il nécessite la diffusion des ions Na^+ à travers des canaux membranaires spécialisés, tels que le canal Na^+ sensible à l'amiloride. Il a été proposé l'implication du canal sodique de type épithélial sélectif (ENaC) (Hartley et al., 2019). La perception du sel joue un rôle dans la consommation de NaCl et d'autres sels, et dans le maintien de l'homéostasie des ions et de l'eau, mais le goût devient désagréable et aversif lorsqu'il est trop fort. Le mécanisme moléculaire du goût salé n'a pas encore été élucidé. Comme détaillé précédemment, il a été suggéré que les cellules gustatives de type I sont responsables de la détection du goût salé, mais on ne sait pas encore quelles cellules de la papille gustative sont les cibles de la stimulation Na^+ . L'implication des canaux sodiques épithéliaux (ENaC) situés dans les membranes cellulaires du goût dans les papilles fongiformes et les récepteurs vanilloïdes sensibles aux amilorides (Trpv1) a été suggérée dans le processus de perception du sel (Kikut-Ligaj & Trzcielinska-Lorych, 2015).

b) Saveur acide

La perception de la saveur acide est déclenchée par des aliments et des substances acides. De nombreux acides organiques et minéraux, tels que l'acide citrique, l'acide malique, l'acide tartrique, l'acide lactique, l'acide acétique, l'acide chlorhydrate sont responsables de l'acidité. Il y a actuellement un débat sur le récepteur impliqué dans la détection de la saveur acide. La perte complète des réponses nerveuses gustatives aux acides se produit avec l'ablation des cellules de type III (Taruno et al., 2021). Les cellules de type III contiennent quelques canaux ioniques distincts. Les canaux ioniques impliquent la migration des ions hydrogène (H^+) à travers les ENaC et les canaux protoniques HCN1 et HCN4 (Sugita, 2006). Ainsi, la saveur acide peut résulter de l'intégration de multiples voies.

c) Saveur sucrée

La saveur sucrée est principalement provoquée par les sucres (glucose, fructose, galactose, saccharose, lactose, amidon maltose), tandis que d'autres composés tels que les édulcorants artificiels (tels que la saccharine, l'aspartame, le cyclamate et l'acésulfame K), les acides aminés sucrés et les protéines sucrées (telles que la brazzéine, la thaumatine, la curcurine et la miraculine) peuvent également être perçus comme sucrés. La saveur sucrée est très attractive pour les humains et certains animaux car elle indique la présence de glucides, une source importante de calories dans les aliments (Yoshida et al., 2013). Le récepteur au goût sucré est formé par l'assemblage de deux sous-unités GPCR, nommées TAS1R2 (récepteur du goût de type 1, membre 2) et TAS1R3 (récepteur du goût de type 1, membre 3), qui ont été décrits pour être en mesure de détecter une grande variété chimique de composés au goût sucré, y compris les acides D-aminés, les glucides (tels que le fructose, le glucose, le saccharose) et les édulcorants artificiels (tels que l'aspartame, la saccharine, le cyclamate) et édulcorants naturels (p. ex. stévia). Les récepteurs TAS1R2 et TAS1R3 peuvent également être activés par certaines protéines végétales rares au goût sucré, telles que la brazzéine, la monelline et la thaumatine (Nelson et al., 2001).

d) Umami

L'umami est un goût que l'on trouve dans de nombreux aliments comme les fruits de mer, les algues, poissons, viandes et champignons (Chaudhari et al., 2009). Le goût umami est provoqué par le L-glutamate et l'aspartate et les ribonucléotides (monophosphates d'inosinate, d'inosine 5'-monophosphate et de guanosine-5'-monophosphate), mais aussi par certains acides organiques (par exemple, les acides lactique, succinique et propionique) (Chaudhari et al., 2009). La modalité sensorielle umami a été établie comme une saveur primaire en raison de la découverte de récepteurs dédiés pour la détection des composés umami (Nelson et al., 2002).

e) Amertume

La sensation d'amertume est provoquée par une large gamme de composés chimiques appartenant à différentes familles: acides aminés et peptides, esters et lactones, phénols et polyphénols, flavonoïdes et terpènes, méthylxanthines (caféine), sulfimides (saccharine) et sels organiques et inorganiques (Drewnowski, 2001). Sur la langue humaine, les molécules au goût amer sont reconnues par une famille d'environ 25 protéines réceptrices (TAS2R, récepteur du goût de type 2) situées dans les cellules réceptrices du goût amer (Kuhn et al., 2010).

f) Saveur gras

Les qualités gustatives de base sont largement acceptées pour inclure le sucré, l'acide, le salé, l'amer et l'umami. Il a été suggéré que le goût gras pourrait constituer le sixième goût de base, c'est-à-dire la détection d'acides gras libres (FFA) présents dans les aliments. Cependant, la classification des lipides alimentaires en tant que goût de base est encore en débat.

2.1.2 L'effet du vieillissement sur la perception du goût

De nombreuses études indiquent que l'acuité gustative diminue avec l'âge. Ce déclin peut altérer le choix des aliments et la qualité de l'alimentation, entraînant une diminution de l'appétit et un risque de dénutrition. Par exemple, une perception plus faible du goût salé peut inciter les gens à assaisonner leurs aliments avec des quantités excessives de sel, ce qui augmente le risque de maladie cardiovasculaire (Sergi et al., 2017). La perte de goût est associée à de multiples facteurs, la réduction du nombre de récepteurs du goût, le manque de salive buccale, la carence en zinc, de mauvaises prothèses dentaires, une mauvaise hygiène dentaire, la prise de médicaments (Aliani et al., 2013; Canon et al., 2018; Doty, 2018; Feron, 2018; Sergi et al., 2017). Selon une revue systématique de la littérature réalisée à partir de 23 études primaires portant sur le seuil de goût et le vieillissement, chaque seuil de goût augmente avec l'âge, avec cependant une amplitude différente en fonction de l'attribut sensoriel (Methven et al., 2012). Rocio et al. (2018) ont également indiqué que l'indice d'intensité des 5 goûts (sucré, amer, umami, salé, aigre) diminuait avec l'âge, en particulier pour l'amer et l'acide (Barragan et al., 2018).

2.2 Perception des odeurs

La capacité de sentir, l'olfaction, fait référence à un processus chimiosensoriel au cours duquel des molécules volatiles sont détectées par des cellules sensorielles spécialisées, appelées neurones sensoriels olfactifs. Ces cellules expriment des récepteurs protéiques qui se lient à des substrats odorants spécifiques (Dan et al., 2021). Chez l'homme, il existe plus de 400 protéines réceptrices olfactives, qui sont principalement responsables de la détection des odeurs. Les odorants sont détectés lorsqu'ils se lient aux récepteurs présents dans les neurones olfactifs qui sont situés dans l'épithélium olfactif de la cavité nasale (Lafreniere & Mann, 2009).

2.2.1 L'effet du vieillissement sur la perception des odeurs

La détérioration de la capacité olfactive avec l'âge a été bien documentée dans les études cliniques, les études expérimentales et épidémiologiques. Il est largement admis que les pertes olfactives sont beaucoup plus fréquentes que les pertes de goût avec l'âge (Boyce & Shone, 2006). De nombreux facteurs ont été associés à ces déficiences olfactives, y compris des altérations de la cavité nasale liées à l'âge, l'épithélium olfactif, le bulbe olfactif et à un niveau plus central (Doty & Kamath, 2014). En outre, l'exposition à l'environnement, les maladies neurodégénératives liées à l'âge et la consommation de médicaments peuvent également affecter les fonctions olfactives (Doty, 2018).

2.2.2 L'effet du vieillissement sur la détection des odeurs, la discrimination, l'identification

Les fonctions olfactives se composent en 3 niveaux : détection des odeurs (seuil), discrimination, identification (Seow et al., 2016). Il a été suggéré que le seuil de détection des odeurs augmentait considérablement avec le vieillissement ainsi qu'une diminution de l'intensité de perception. L'identification des odeurs et à la capacité de discrimination de la qualité des odeurs nettement sont également clairement sensibles et diminuées avec le vieillissement (Stevens et al., 1989; Vantoller & Dodd, 1987).

3. Astringence

L'astringence est un attribut sensoriel important qui se produit lors de la consommation d'aliments et de boissons contenant des polyphénols, en particulier des tanins, tels que des légumes, des noix, des fruits et des baies non mûrs, du vin, du thé, etc. Il a été défini comme « l'ensemble des sensations dues au resserrement, à la contraction et assèchement des tissus de l'épithélium buccal à la suite de l'exposition à des substances telles que les aluns ou les tanins » par l'American Society for Testing Materials (Ramos-Pineda, Garcia-Estevez, et al., 2019). A ce jour, le mécanisme moléculaire à l'origine de l'astringence n'est pas bien compris, plusieurs hypothèses ont été proposées. L'astringence est une sensation trigéminal (Schobel

et al., 2014). L'astringence n'est probablement pas un sens chimique (Schobel et al., 2014). L'astringence est une perception physique (Ployon et al., 2018) avec la protéine transmembranaire MUC1 jouant la fonction de capteur de cette perception (Canon et al., 2021). On pense que les protéines salivaires jouent un rôle protecteur dans la prévention de l'agrégation de la pellicule mucoale par les tanins. Parmi ces protéines salivaires, les protéines riches en proline (PRPs) ont été identifiées comme ayant une forte affinité pour les tanins et étant considérées comme une première ligne de défense contre les effets nocifs des tanins dans l'alimentation. Elles jouent ainsi un rôle protecteur dans la prévention de l'astringence en empêchant les tanins d'interagir directement avec la muqueuse buccale (Pascal et al., 2006).

3.1 Astringence liée aux propriétés des tanins

Il a été rapporté que l'astringence apportée par des catéchines ayant une substitution galloyle étaient beaucoup plus élevées que celles des catéchines non substituées à la même concentration. L'intensité gustative des catéchines galloylées augmentait avec leur concentration plus rapidement que leur correspondante non galloylée (Y. Q. Xu et al., 2018). La stéréochimie joue un rôle essentiel dans le développement de l'astringence. En effet, des tests sensoriels d'intensité temporelle ont également montré que l'épicatéchine est plus astringente que la catéchine (Peleg et al., 1999; Thorngate & Noble, 1995). En ce qui concerne le degré de polymérisation, plus les flavanols sont polymérisés, plus ils sont astringents (Hufnagel & Hofmann, 2008). Il a été rapporté que le degré moyen croissant de polymérisation et le degré de galloylation des proanthocyanidines augmentent la sensation d'astringence (Vidal et al., 2003). En ce qui concerne les effets de la concentration de tanins sur l'astringence, certaines études ont décrit que les quantités de phénols totaux et de tanins totaux sont positivement liées à l'intensité de l'astringence (Cliff et al., 2007; Gawel et al., 2007).

3.2 Interaction moléculaire PRP-tanin

Il a été proposé que le mécanisme d'interaction entre les tanins et les PRP puisse être divisé en trois étapes à mesure que la concentration de tanin augmente: (i) les tanins se lient à plusieurs sites sur la protéine libre, (ii) les stœchiométries des complexes augmentent et les tanins se lient à plusieurs protéines formant des agrégats, (iii) les agrégats multimériques résultants se développent jusqu'à la précipitation (Canon et al., 2015). L'interaction PRP-tanins est décrite comme impliquant à la fois des effets hydrophobes et des liaisons hydrogène (Ramos-Pineda et al., 2017; Soares, Brandao, Garcia-Estevez, et al., 2019; Soares et al., 2011). Il a été indiqué que les PRP sont considérés comme ayant un nombre fixe de sites auxquels le tanin peut se lier. Ainsi, différents complexes PRP-tanin sont formés en fonction du rapport tanin/PRP (Brandao et al., 2014). La formation d'agrégats tanin - PRPs dépend du rapport tanin/PRP et de la charge des protéines. Par conséquent, le pH et la force ionique sont des paramètres clés dans la formation des agrégats car ils ont un impact sur les charges protéiques (Naurato et al., 1999).

3.3 Impact de la structure PRP sur l'interaction

Les PRP basiques sont considérés comme les protéines salivaires les plus efficaces pour se lier et piéger les tanins (Hagerman & Butler, 1981; Y. Lu & Bennick, 1998; Pascal et al., 2007). En fait, les gPRP sont également efficaces pour lier les tanins. Cependant, l'interaction entre le gPRP et les tanins n'entraîne pas nécessairement des précipitations. Il a été conclu que la glycosylation du gPRP empêche l'agrégation et la précipitation (Sarni-Manchado et al., 2008). Certaines études ont indiqué que les aPRP étaient les PRP salivaires ayant une affinité plus élevée pour les tanins par rapport aux autres familles de protéines salivaires (Brandao et al., 2014; Soares, Brandao, Garcia-Estevez, et al., 2019; Soares et al., 2011). Cela est dû à la présence de nombreux résidus d'acide aspartique et d'acide glutamique, les caractéristiques acides des aPRPs étant approximativement limitées aux 30 premiers acides aminés. La présence de ces résidus acides peut favoriser la liaison hydrogène, ce qui peut expliquer la forte affinité des aPRP pour les tanins (Brandao et al., 2014). Pour les trois classes de PRP, il a été proposé que les PRP plus gros lient plus de tanins que les PRP ou les fragments de peptides plus petits (Bajec & Pickering, 2008).

3.4 Impact de la structure des tanins sur l'interaction

Il a été rapporté que la liaison des tanins aux PRP augmente avec le poids moléculaire et le degré de polymérisation (de Freitas & Mateus, 2002). Les tanins condensés ont une affinité plus élevée pour les PRP que les tanins hydrolysables (Bajec & Pickering, 2008). Cependant, les tanins hydrolysables sont également efficaces pour interagir avec toutes les classes de PRP (aPRP, bPRP, gPRP) (Bacon & Rhodes, 2000). Il a été rapporté que la fraction galloyle joue un rôle clé dans l'interaction, qui implique des liaisons hydrogène avec le peptide, stabilisant et renforçant ainsi l'interaction (Poncet-Legrand et al., 2007).

3.5 Astringence et variation individuelle

Enfin, l'astringence est également associée à des propriétés physiologiques individuelles, telles que le débit salivaire, la concentration en protéines salivaires, la composition en protéines salivaires, la capacité tampon salivaire, la viscosité de la salive, etc. Certaines études ont indiqué qu'il existe une corrélation négative entre le débit salivaire et l'intensité de l'astringence (Fischer U, 1994; Ishikawa & Noble, 1995).

En ce qui concerne la relation entre l'astringence et le vieillissement, jusqu'à présent, il existe peu de recherches sur la perception de l'astringence en fonction de l'âge entre les jeunes adultes et les personnes âgées. Cependant, dans notre travail, nous avons obtenu le résultat que les personnes âgées (l'âge moyen est de 75 ans) ont un seuil d'astringence plus élevé que les jeunes (l'âge moyen de 25.4 ans) (M. Wang et al., 2022).

Chapitre 2 : Méthodologie

1. Analyse sensorielle

1.1 Mise en place d'une méthodologie pour évaluer la sensibilité à l'astringence

Dans la littérature, deux méthodes principales sont souvent utilisées pour évaluer la sensation d'astringence. Il s'agit du temps-intensité (T-I) et de la dominance temporelle des sensations (TDS). Ces deux méthodes (T-I & TDS) ont été utilisées dans de nombreuses études qui décrivent la temporalité des sensations du vin et identifient les descripteurs de qualité du vin ou de produits modèles (Ishikawa & Noble, 1995; Medel-Maraboli et al., 2017; Meillon et al., 2009; Pittari et al., 2022; Rebenaque et al., 2015). Cependant, le but de nos travaux est de déterminer le seuil de détection d'une solution d'astringence spécifique (solution d'acide tannique), et non de décrire les différents attributs d'une solution complexe. Par conséquent, ces deux méthodes ne sont pas applicables à notre travail. La méthode par Choix Alternatif Forcé (AFC) est la plus utilisée pour déterminer un seuil sensoriel. Cette méthode nécessite l'attention du panéliste qui est dirigée vers un attribut sensoriel spécifique. Les panélistes sont tenus de sélectionner le produit présente l'attribut sensoriel souhaité à partir d'une paire ou d'un triplé (McClure & Lawless, 2010). Compte tenu de la durée de l'expérience et de la fatigue du sujet (en particulier chez les personnes âgées), le 2-AFC a été adopté pour évaluer le seuil de perception à l'astringence dans notre travail.

L'astringence est essentiellement un processus dynamique qui change et évolue continuellement et qui a des effets de report (Rinaldi et al., 2012). Afin de minimiser ces effets, une procédure de rinçage qui utilise de la pectine, du bicarbonate et de l'eau d'Evian pour se rincer la bouche et nettoyer la bouche a été optimisée et utilisée entre les évaluations d'échantillons durant la session sensorielle. Cette procédure de rinçage s'est avérée efficace dans de nombreuses études (Esteban-Fernandez et al., 2016; Munoz-Gonzalez, Brule, et al., 2019; Pittari et al., 2022). Dans notre étude, l'acide tannique a été utilisé parce qu'il est décrit comme moins amer que les autres polyphénols, et limite ainsi la confusion entre l'astringence et le goût amer (Robichaud & Noble, 1990).

1.2 Expérimentation sensorielle préliminaire et expérimentation sensorielle formelle

L'expérimentation sensorielle préliminaire a été menée avec un panel interne composé de 9 sujets recrutés dans notre laboratoire. Une première partie était une séance d'entraînement pour s'assurer que les sujets étaient capables de comprendre parfaitement la procédure du test sensoriel, c'est-à-dire le 2-AFC à utiliser plus tard. Des solutions d'acide tannique à quatre concentrations de 0.02, 0.08, 0.32 et 0.51 g/L ont été présentées lors de la séance d'essai. Après trois séances d'essai, le seuil moyen de 9 sujets d'acide tannique a été évalué à 0.19 ± 0.17 g/L. Selon les résultats obtenus à partir de l'expérience préliminaire, le nombre de sujets nécessaires pour trouver une différence entre deux groupes en ce qui concerne la perception

de l'astringence a été déterminé par un test de puissance ((puissance = 0.9, niveau de signification = 0.05, alternative = « bilatéral. Un nombre d'au moins 23 sujets par groupe (jeunes ou personnes âgées) était nécessaire pour observer une différence égale à un écart-type entre les deux groupes. Sur la base des résultats expérimentaux préliminaires du seuil des panélistes, les solutions pour l'évaluation de la sensibilité à l'astringence consistaient en quatre solutions avec des concentrations croissantes d'acide tannique (en g/L) selon un multiple de 3.05, c'est-à-dire 0.02, 0.062, 0.188 et 0.574, respectivement.

Cinquante-quatre panélistes, dont 30 personnes âgées (O) (l'âge moyen est de 75 ans) et 24 jeunes (Y) (l'âge moyen est de 25.4 ans), ont été recrutés pour participer aux séances sensorielles. La procédure comprenait également une séance d'entraînement pour s'assurer que les sujets étaient capables d'identifier et de différencier clairement l'astringence des autres sensations sensorielles, en particulier l'acidité, l'amertume et les attributs olfactifs. Lors de cette séance d'entraînement, 20 mL de chaque échantillon de dégustation ont été mis en bouche et après 30 s, recraché et l'attribut sensoriel a été décrit. Lors de la séance d'essai, le seuil d'astringence a été évalué par une procédure 2-AFC avec des concentrations croissantes d'acide tannique. Dans chaque présentation 2-AFC, deux échantillons ont été présentés: l'un était l'échantillon cible et l'autre était le témoin. Chaque test 2-AFC a été effectué 3 fois. Le niveau de sensibilité a été atteint lorsque trois réponses correctes de la même concentration ont été obtenues. En outre, la salive des sujets avant et après la séance de test a été collectée et la teneur en protéines a été mesurée par le test de protéines de Bradford. Les sujets sont venus à 3 séances correspondant donc à 3 répétitions.

2. Expérience de biologie

2.1 Production d'IB5 et II-1

IB5 et II-1 ont été utilisés comme référence des bPRPs et des gPRPs et pour explorer la quantité de bPRPs et de gPRPs. Ils peuvent être obtenus par l'expression hétérologue du gène humain PRB4S dans la levure *Pichia pastoris* (Pascal et al., 2006). Les protéines produites ont été purifiées à partir d'un surnageant de culture cellulaire par chromatographie liquide (échange de cations et filtration sur gel).

2.2 Évaluation au niveau PRP

2.2.1 Mise en place d'une méthodologie pour évaluer les niveaux de PRP dans la salive

Actuellement, la technique d'analyse des PRPs dans la salive se concentre principalement sur la chromatographie liquide à haute performance (HPLC) pour analyser le profil des protéines, ou la chromatographie liquide à haute performance-détection de réseau de diodes (HPLC-DAD) chromatographie liquide à l'échelle nanométrique couplée à la spectrométrie de masse en tandem (nLC-MS-MS) ou à la chromatographie liquide-électropulvérisation-spectrométrie de masse (LC-ESI-MS) pour séparer différentes

fractions et les identifier (Hay et al., 1994; Kallithraka et al., 2001; Ramos-Pineda et al., 2017; Ramos-Pineda, Garcia-Estevez, et al., 2019). Notre objectif de travail est d'évaluer les niveaux de PRP dans la salive et non d'identifier les PRPs. Baum et al. (1982) ont d'abord développé une technique de microimmunodiffusion modifiée en utilisant un antisérum contre les PRPs pour les quantifier dans les glandes parotides stimulées (Baum et al., 1982). Dans notre étude, le western-blot a été développé en utilisant des anticorps spécifiques pour détecter des protéines ciblées (bPRPs et gPRPs) afin d'évaluer la quantité de PRPs. Le principe est une corrélation entre l'intensité de coloration sur une membrane et la concentration en protéines. Selon la courbe d'étalonnage IB5 et II-1, on peut ainsi calculer la quantité de bPRPs et de gPRPs. En outre, nous avons également développé une procédure par dot-blot pour quantifier la teneur MUC5B dans le groupe des personnes jeunes et des personnes âgées.

2.3 Analyse statistique

Les données ont montré la présence de valeurs aberrantes pour toutes les variables. De plus, les hypothèses de normalité n'ont pas été respectées pour les données brutes et les résidus des Anovas. Nous avons cependant décidé de conserver toutes les données et de ne pas déroger aux contraintes de normalité de distribution. Nous avons donc effectué des analyses non paramétriques car elles sont adaptées à des données non distribuées normalement et sont plus robustes à la présence de valeurs aberrantes. Les tests U de Mann-Whitney ont été effectués pour évaluer les différences entre les sujets Y (jeunes) et O (âgés) en ce qui concerne les paramètres salivaires (débit, quantité de protéines, quantité de PRPs et rapport de volume de pixels MUC5B). Des tests de Wilcoxon ont été effectués sur les paramètres salivaires (débit, quantité de protéines, quantité de PRPs et MUC5B) pour évaluer les différences entre le début et la fin de chaque séance. Une ANOVA de Friedman a été réalisée sur les mesures de seuil et de paramètres salivaires pour évaluer les différences entre les trois séances. Des corrélations de Spearman ont été effectuées pour l'ensemble du groupe et dans chaque groupe (Y et O) afin d'évaluer les relations entre les paramètres salivaires (débit, quantités de protéines, quantité de PRP et rapport de volume de pixels MUC5B) et les paramètres sensoriels (seuil). Le niveau de significativité a été fixée à $p < 0.05$.

Chapitre 3 : Sensibilité à l'astringence à l'acide tannique : effet du vieillissement et de la salive

1. Introduction

L'astringence est une caractéristique sensorielle importante des aliments et des boissons contenant des polyphénols, qui présentent un grand intérêt pour l'industrie alimentaire en raison de leurs effets bénéfiques potentiels sur la santé, par exemple les effets anticancéreux et l'activité antioxydante, en particulier pour la population vieillissante. Cependant, l'astringence est une sensation sensorielle controversée. Dans certains aliments, comme le vin rouge, cette sensation est souhaitée à des niveaux équilibrés, étant même un paramètre de qualité important. D'autre part, dans d'autres aliments, des niveaux élevés d'astringence ne sont pas appétissants, comme dans le cas des fruits, des jus de fruits et du thé (Soares et al., 2016). L'astringence a été définie comme le complexe de sensations dues au rétrécissement, à la contraction et à l'assèchement de l'épithélium à la suite de l'exposition à des substances telles que l'alun ou les tanins (Huang & Xu, 2021). En ce qui concerne l'effet du vieillissement sur la perception de l'astringence, la littérature est très rare, bien que l'influence du vieillissement sur la perception d'autres modalités gustatives ait été largement documentée. Le vieillissement s'accompagne souvent d'une diminution du flux salivaire ou de changements dans la composition salivaire. L'hypofonction salivaire chez les personnes âgées peut entraîner des changements dans la perception de l'arôme, du goût et de la texture des aliments, et par conséquent, l'apport et la consommation de nourriture (Munoz-Gonzalez, Brule, et al., 2019; Munoz-Gonzalez et al., 2018; F. Xu et al., 2019).

Dans un contexte de vieillissement de la population mondiale, l'objectif principal de cette étude était d'étudier la sensibilité à l'astringence en fonction de l'âge le lien avec des caractéristiques salivaires (débit et teneur en protéines). À cette fin, une méthodologie 2-AFC a été appliquée pour estimer la sensibilité à l'astringence dans les panels de sujet jeunes et âgés tout en évaluant le flux salivaire et la quantité de protéines. Les relations entre le flux salivaire, la quantité de protéines et la sensibilité à l'astringence en fonction de l'âge sont discutées.

2. Matériel et méthodes

2.1 Matériel

Les solutions de rinçage étaient constituées de 0.1% de pectine (Sigma-Aldrich, Saint-Quentin-Fallavier, France) et de 1% de bicarbonate (Gilbert, France) dissous dans de l'eau d'Evian à température ambiante. Les solutions pour la séance d'entraînement sensoriel consistaient en six solutions gustatives (salées, acides, sucrées, amères, umami et astringentes).

Les solutions pour l'évaluation de la sensibilité à l'astringence consistaient en quatre solutions faites avec des concentrations croissantes d'acide tannique (Sigma-Aldrich, Saint-Quentin-Fallavier, France) (en g/L) selon un multiple de 3.05, soit 0.02, 0.062, 0.188 et 0.574 g/L.

2.2 Analyse sensorielle

Cinquante-quatre panélistes, dont 30 personnes âgées (O) (âge ≥ 65 ans) et 24 jeunes (Y) (âges ≤ 35 ans), ont été recrutées pour participer aux séances sensorielles. Tous les sujets avaient une bonne santé bucco-dentaire.

2.3 Séance de test

Les panélistes ont été priés de ne pas boire, manger ou fumer 1 heure avant la session. Au début de chaque séance, les panélistes ont été invités à goûter une solution modèle d'acide tannique de 1.76 g/L afin de pouvoir identifier la sensation d'astringence. Ensuite, ils se sont rincés la bouche avec de la pectine, du bicarbonate et de l'eau d'Evian et ont attendu une pause de 3 minutes avant l'évaluation du seuil. Le seuil d'astringence a été évalué par une procédure 2-AFC. Dans chaque présentation 2-AFC, deux échantillons ont été présentés : un échantillon cible et un échantillon témoin. Les panélistes ont reçu l'échantillon de référence ou de stimulus (chacun de 5 mL) à partir de la concentration la plus faible. On leur a demandé de mettre les échantillons dans la bouche, de les agiter dans la bouche pendant 30 s, puis de les cracher. Ils se sont rincés la bouche avec de la pectine et ont attendu 1 minute avant d'évaluer le deuxième échantillon. Après 30 s, les panélistes ont été invités à indiquer quel échantillon était perçu comme astringent. Ensuite, les panélistes se sont rincés la bouche avec de la pectine, du bicarbonate et de l'eau d'Evian. Chaque test 2-AFC a été effectué 3 fois, le niveau de sensibilité a été atteint lorsque trois réponses correctes de la même concentration ont été obtenues. Le seuil de la meilleure estimation pour chaque sujet a été évalué comme la moyenne géométrique des trois concentrations correctement répondues et de la concentration inférieure précédente. L'évaluation a été réalisée 3 fois en 3 séances différentes.

2.4 Collecte de salive et quantité de protéines

La salive globale a été recueillie après que les panélistes se soient rincés la bouche avec 0.1% de pectine, 1% de bicarbonate et de l'eau au début (SFStart) et à la fin (SFEnd) de la session. La salive a été recueillie en expectorant dans un tube pré-pesé pendant 5 min. Après la collecte, les tubes ont été pesés puis stockés à -80 °C. Les débits ont été déterminés gravimétriquement et exprimés en grammes par minute (g/min).

2.5 Quantité de protéines

Les échantillons de salive ont été centrifugés à 15000 g pendant 15 min à 4 °C avant l'analyse. La concentration en protéines a été déterminée dans le surnageant par la méthode de Bradford, avec l'albumine sérique bovine (BSA) utilisée comme étalon.

2.6 Analyse statistique

Des analyses non paramétriques ont été effectuées pour analyser les données. Les tests Mann-Whitney U ont été effectués pour évaluer les différences entre les sujets jeunes et âgés en ce qui concerne les paramètres sensoriels et salivaires. Des tests de Wilcoxon ont été effectués sur les paramètres salivaires (débit et quantités de protéines) pour évaluer les différences entre le début et la fin de chaque séance. Une ANOVA de Friedman a été réalisée sur les mesures de seuil et de paramètres salivaires pour évaluer les différences entre les trois séances. Des corrélations de l'ordre de classement de Spearman ont été effectuées pour l'ensemble du groupe et dans chaque groupe (jeunes et personnes âgées) afin d'évaluer les relations entre les paramètres salivaires et sensoriels. La signification a été fixée à $p < 0.05$.

3. Résultats

3.1 Seuil d'astringence

Aucune différence significative n'a été observée entre les trois séances en ce qui concerne les seuils d'astringence pour le groupe Y (panel jeune) (Friedman $\text{Chi}^2 = 1.13$, $p = 0.56$) ou le groupe O (panel des personnes âgées) (Friedman $\text{Chi}^2 = 1.14$, $p = 0.56$). Par conséquent, nous avons décidé de fusionner les valeurs seuils dans une variable unique.

Une différence significative a été observée entre les groupes Y et O ($Z = -2.5$, $p = 0.0110$). Le groupe O a montré un seuil d'astringence moyen plus élevé que le groupe Y.

3.2 Débit salivaire et quantité de protéines

Aucune différence significative n'a été observée entre les sessions concernant SFStart et SFEnd pour le groupe Y (SFStart: Friedman $\text{Chi}^2 = 0.75$, $p = 0.68$; SFEnd: Friedman $\text{Chi}^2 = 0.75$, $p = 0.68$) ou groupe O (SFStart: Friedman $\text{Chi}^2 = 5.2$, $p = 0.07$; SFEnd : Friedman $\text{Chi}^2 = 1.3$, $p = 0.53$) ou entre la moyenne SFStart et la moyenne SFEnd pour le groupe Y (Friedman $\text{Chi}^2 = 0.68$, $p = 0.492$) ou le groupe O (Friedman $\text{Chi}^2 = 1.49$, $p = 0.135$). Pour cette raison, nous avons décidé de fusionner les deux variables en une variable unique, c'est-à-dire le flux salivaire moyen (SF). En ce qui concerne la comparaison du débit salivaire, le SF dans le groupe O était inférieur à celui du groupe Y, mais avec un niveau de preuve modeste ($Z = 1.66$, $p = 0.09$).

Aucune différence significative n'a été observée entre les séances concernant la quantité de protéines pour le groupe Y (Friedman $\text{Chi}^2 = 1.08$, $p = 0.58$) ou le groupe O (Friedman $\text{Chi}^2 = 2.55$, $p = 0.28$) ou entre le début et la fin de la séance pour le groupe Y (Friedman $\text{Chi}^2 = 1.5$, $p = 0.91$) ou le groupe O (Friedman $\text{Chi}^2 = 1.70$, $p = 0.19$). Pour cette raison, nous avons décidé de fusionner la quantité de protéines en une variable unique. Aucune différence significative n'a été observée entre les groupes Y et O ($Z = -0.32$, $p = 0.74$), ce qui confirme

des résultats précédents.

3.3 Corrélation entre le seuil d'astringence et le débit et la quantité de protéines

La corrélation de Spearman entre le seuil et le flux salivaire n'était pas significative dans l'ensemble du panel ou dans le groupe des personnes âgées. Cependant, une corrélation significative et négative a été observée dans le groupe jeune ($r = -0.44$, $p = 0.03$), où un flux salivaire plus élevé était associé à un seuil plus bas.

La corrélation de Spearman entre le seuil et la quantité de protéines n'était pas significative dans l'ensemble du panel, le groupe des jeunes ou le groupe des personnes âgées.

4. Conclusions

Nous concluons que le seuil d'astringence était plus élevé dans le groupe des personnes âgées que dans le groupe des jeunes. En ce qui concerne le débit salivaire et la quantité de protéines, il n'y avait pas de différences significatives entre les groupes jeunes et âgés. Cependant, une corrélation entre le flux salivaire et le seuil n'a été observée que chez les jeunes individus, ce qui suggère que les propriétés salivaires qui influencent la sensibilité à l'astringence chez les personnes âgées sont différentes. Une caractérisation plus approfondie de la composition salivaire, en particulier en ce qui concerne les niveaux de PRPs et de mucines dans les deux populations, devrait mériter des études plus approfondies.

Chapitre 4 : Sensibilité à l'astringence à l'acide tannique : effet du vieillissement et des PRPs

1. Introduction

Des travaux antérieurs ont montré que la sensibilité à l'astringence était plus faible chez une population âgée que chez une population jeune. Cependant, elle n'est pas liée au flux salivaire. Par conséquent, dans ce chapitre, nous nous sommes principalement concentrés sur l'évaluation des niveaux dans la salive de protéines riches en proline (PRPs) dans un groupe d'individus jeunes et âgés et de les lier à la sensibilité à l'astringence. En effet, il a été suggéré que l'astringence est déclenchée par l'interaction et la précipitation des protéines salivaires par les tanins alimentaires, en particulier les protéines riches en proline (PRPs) (Soares et al., 2016).

Les protéines riches en proline (PRPs) constituent environ les deux tiers des protéines sécrétées par les glandes parotides humaines. Les PRPs sont caractérisés par une teneur élevée en résidus de proline (25-42%) et cette famille est divisée en 3 classes, i.e. basiques (bPRPs), acides (aPRPs) et glycosylées (gPRPs) (Soares, Brandao, Guerreiro, Mateus, & de Freitas, 2019). La forte affinité des PRPs pour les tanins est associée à leurs caractéristiques structurales. Il a été suggéré que les bPRPs sont les protéines salivaires les plus réactives envers les tanins. Des travaux récents ont conclu que l'aPRPs, les gPRPs sont également réactifs envers les tanins alimentaires (Soares et al., 2011). IB5 est un modèle de PRP salivaire de base, II-1 est un modèle de PRP glycosylés. Elles peuvent être produits par l'expression hétérologue du gène humain PRB4S dans la levure *Pichia pastoris* (Canon et al., 2015).

En ce qui concerne l'effet du vieillissement sur la quantité de PRPs, seuls les aPRPs dans les glandes parotides stimulées ont été étudiés par Baum et coll. (1982), qui ont rapporté que la quantité totale d'aPRPs et le pourcentage d'aPRPs de protéine dans la salive parotidienne ne sont pas liés à l'âge (Baum et al., 1982). Par conséquent, dans ce contexte, l'objectif principal de cette étude était d'étudier les propriétés salivaires (niveaux de PRPs), qui pourraient être liées au seuil d'astringence, en fonction de l'âge. Une méthodologie par western-blot a été développée pour évaluer les niveaux de PRPs dans la salive. Les relations entre les niveaux de PRPs et la sensibilité à l'astringence en fonction de l'âge seront discutées.

En outre, nous avons mené une expérience supplémentaire concernant le niveau de MUC5B dans la salive par une méthode de dot-blot. MUC5B est la principale mucine dans la salive. MUC5B est également la principale protéine composant la pellicule muqueuse à la surface de la muqueuse orale (Canon et al., 2021). Il a été suggéré que l'un des mécanismes possibles de l'astringence est l'interaction directe entre le tanin et la pellicule muqueuse. En outre, il a été indiqué que l'interaction des composés astringents avec les mucines adsorbées pourrait jouer

un rôle important dans la perception de l'astringence (Ployon et al., 2018). Par conséquent, le niveau de MUC5B dans la salive, qui pourrait être lié au seuil d'astringence, a été évalué dans ce travail.

2. Matériel et méthodes

2.1 Traitement des échantillons de salive et production d'IB5 et II-1

Après le prélèvement de salive, les échantillons de salive ont été stérilisés au bain-marie à 60 °C pendant 1 h par souci de sécurité dans le contexte de la pandémie liée au Covid. Ensuite, ils ont été centrifugés pendant 15 minutes à 4 °C et le surnageant de chaque salive a été conservé.

IB5 et II-1 ont été produits par le système *Pichia pastoris* qui utilise le promoteur de l'alcool oxydase inductible au méthanol pour l'expression de protéines recombinantes hétérologues. Les protéines produites ont été purifiées à partir d'un surnageant de culture cellulaire par chromatographie liquide (échange de cations et filtration sur gel).

2.2 Électrophorèse et western-blot

Après avoir chargé un gel d'électrophorèse des échantillons de salive avec un tampon (Laemmli 2x Biorad), l'électrophorèse a été exécutée à une tension constante de 200 V pendant environ 35 minutes. Après séparation, les protéines ont été transférées sur une membrane PDVF à l'aide d'un dispositif Trans-Blot. Le dispositif fonctionnait pendant 7 min, la tension était réglée à 25 V. Après le transfert, la membrane a été rincée à l'eau MilliQ, après avoir été immergée dans une coloration rouge Ponceau pendant 5 min, elle a été décolorée à l'aide d'une solution de NaOH de 0.1 M, enfin elle a été rincée à l'eau pendant 2-3 min.

La membrane a été rincée avec une solution de 9‰ NaCl deux fois. Le blocage des sites non spécifiques a été effectué avec du lait écrémé à 8% dans 9‰ NaCl pendant 1h avec agitation à température ambiante. La membrane a ensuite été rincée avec une solution de 9‰ NaCl deux fois. Elle a ensuite été incubée avec un anticorps primaire à 1 : 1000 dans 5% d'albumine sérique bovine (BSA) dans une solution de NaCl à 9‰ pendant 1h à température ambiante. Après 2 lavages dans une solution PBS-Tween à 0.05%, une incubation a été effectuée à nouveau avec du lait écrémé à 8% dans une solution de NaCl à 9‰ pendant 30 min avec agitation à température ambiante. Après 2 lavages dans une solution de NaCl à 9‰, la membrane a été incubée avec un anticorps secondaire conjugué HRP à 1 : 5000 dans 5% de BSA dans une solution de NaCl à 9‰ pendant 1h à température ambiante. La membrane a été rincée avec 0.05% de PBS-Tween 3 fois avec agitation. La dernière étape a consisté à rincer la membrane dans une solution de NaCl 9‰.

Enfin, la révélation colorimétrique a été réalisée. Les protéines ciblées (PRPs) ont été

détectées à l'aide d'un système de détection ECL (Clarity Enhanced Luminescence), puis la membrane a été analysée par le système d'imagerie ChemiDoc.

Le volume en pixels de la bande des protéines ciblées (bPRPs et gPRPs) a été comparé aux courbes standard générées par l'IB5 et le II-1 afin de produire des mesures quantitatives pour les bPRPs et les gPRPs.

2.4 Expérience supplémentaire – Dot blot pour l'évaluation de MUC5B

Tout d'abord, le papier Whatman et la membrane de nitrocellulose ont été trempés dans un tampon à base de Tris pendant 5 minutes. Ensuite, le dispositif Bio-Dot a été assemblé avec du papier Whatman et de la nitrocellulose. L'appareil Bio-Dot a été connecté à une pompe à vide. 5 µL de chaque échantillon de salive ont été déposés sur la membrane dans des puits. Dans chaque expérimentation, nous mettons aussi 5 µL d'une même salive standard avec 3 répétitions comme référence pour calculer le rapport entre le volume de pixels de chaque échantillon de salive et le volume de pixels de la salive standard. La pompe à vide a fonctionné pendant 5 minutes pour sécher la membrane.

Les procédures de coloration au rouge Ponceau, de réaction immunologique et de révélation colorimétrique étaient similaires avec celles utilisées pour le transfert western-blot, comme décrit à la rubrique 2.2. Les différences avec le transfert Western étaient les anticorps utilisés et la concentration d'anticorps. Les membranes ont été incubées en présence de l'anticorps primaire utilisé à 1: 10000 dans de l'albumine sérique bovine (BSA) à 5% dans une solution de NaCl à 9‰ pendant 1h à température ambiante puis avec le deuxième anticorps dilué à 1: 20000 dans 5% d'albumine sérique bovine (BSA) dans une solution de NaCl à 9‰ pendant 1h à température ambiante.

2.4 Analyse statistique

Des analyses non paramétriques ont été effectuées. Des tests U de Mann-Whitney ont été effectués pour évaluer les différences entre les sujets jeunes (Y) et âgés (O) en ce qui concerne les protéines salivaires (bPRPs et gPRPs). Des tests Wilcoxon ont été effectués sur la quantité de PRP et le rapport de volume de pixels MUC5B pour évaluer les différences entre le début et la fin de chaque session. Une ANOVA de Friedman a été menée sur la quantité de PRPs et le rapport de volume de pixels MUC5B pour évaluer les différences entre les trois sessions. Des corrélations de Spearman ont été effectuées pour l'ensemble du groupe et dans chaque groupe (Y et O) afin d'évaluer les relations entre le seuil d'astringence, la quantité de PRP et le rapport de volume de pixels MUC5B. Le niveau de significativité a été fixé à $p < 0,05$.

3. Résultats

a) Niveaux des bPRPs

Il n'y avait aucune différence entre les séances concernant le niveau des bPRPs pour le groupe Y (Friedman $\chi^2 = 1.33$, $p = 0.51$) ou le groupe O (Friedman $\chi^2 = 1.87$, $p = 0.39$) ou entre le début moyen et la fin moyenne des sessions pour les groupes Y ($Z = 0.31$, $p = 0.75$) et O ($Z = 0.89$, $p = 0.37$). Pour cette raison, nous avons décidé de fusionner le niveau des bPRPs en une variable unique, c'est-à-dire le niveau moyen des bPRPs. En ce qui concerne la comparaison de la quantité moyenne de bPRPs, aucune différence n'a été observée entre les groupes Y et O ($Z = -0.51$, $p = 0.61$). De même, nous n'avons pas observé les différences entre les groupes Y et O en ce qui concerne les bPRPs Start ($Z = -0.81$, $p = 0.42$) et bPRPs End ($Z = -0.55$, $p = 0.58$).

b) Montant des gPRPs

Aucune différence n'a été observée entre trois séances en ce qui concerne la quantité de gPRPs pour Y ($\chi^2 = 3.58$, $p = 0.17$) ou O ($\chi^2 = 3.29$, $p = 0.19$). Cependant, une différence significative a été observée entre la fin de la session (moyenne = 444.79 $\mu\text{g/mL}$) et le début de la session (moyenne = 819.96 $\mu\text{g/mL}$) en ce qui concerne la quantité de gPRPs dans le groupe O ($Z = 4.36$, $p = 0.000013$); Une différence modeste a été observée en ce qui concerne la quantité de gPRPs dans le groupe Y ($Z = 1.74$, $p = 0.08$) entre le début de la séance (moyenne = 402.59 $\mu\text{g/mL}$) et la fin de la session (moyenne = 308.43 $\mu\text{g/mL}$).

Cependant, nous n'avons pas observé de différences statistiques entre les groupes Y et O en ce qui concerne le niveau des gPRPs pour la valeur moyenne ($Z = -1.30$, $p = 0.19$) ou le début ($Z = -1.57$, $p = 0.12$) ou la fin de la session ($Z = -0.55$, $p = 0.58$).

c) Corrélation entre le seuil d'astringence et le montant des PRPs

Des corrélations de Spearman ont été effectuées pour chaque groupe (Y et O) afin d'évaluer les relations entre le seuil et les niveaux de bPRPs et de gPRPs.

Une corrélation significative et négative a été observée dans le groupe O ($r = -0.41$, $p = 0.02$) en ce qui concerne le niveau moyen et le seuil des bPRPs, plus élevé est le niveau des bPRPs, plus bas est le seuil de perception de l'astringence. Cependant, il n'y avait pas de corrélation entre la quantité moyenne de bPRPs et le seuil dans le groupe Y ($r = -0.16$, $p = 0.45$).

Une corrélation significative et positive entre la quantité moyenne et le seuil des gPRPs a été observée dans le groupe Y ($r = 0.49$, $p = 0.01$), plus élevée est la quantité de gPRPs, plus élevé est le seuil (sensibilité plus faible).

De même, il y avait des corrélations significatives et positives entre le début des gPRPs ($r = 0.46$, $p = 0.02$), la fin des gPRPs ($r = 0.53$, $p = 0.007$) et le seuil. Cependant, aucune corrélation entre la quantité moyenne et le seuil des gPRPs n'a été observée dans le groupe O ($r = 0.05$, $p = 0.79$). De même, il n'y avait pas de corrélations significatives entre le niveau des gPRPs en début de séance ($r = 0.04$, $p = 0.83$), celui la fin de séance ($r = -0.04$, $p = 0.81$).

et le seuil dans le groupe O.

d) Résultats sur MUC5B dans les groupes jeunes et âgés

Les résultats ont montré qu'il n'y avait aucune différence entre les sessions en ce qui concerne le rapport de volume de pixels MUC5B pour le groupe Y (Friedman $\text{Chi}^2 = 0.58$, $p = 0.74$) ou le groupe O (Friedman $\text{Chi}^2 = 0.21$, $p = 0.9$) ou entre le début et la fin des sessions pour les groupes Y ($Z = 0.88$, $p = 0.38$) et O ($Z = 0.46$, $p = 0.64$). Pour cette raison, nous avons décidé de fusionner le rapport de volume de pixels MUC5B en une variable unique, c'est-à-dire le rapport de volume de pixel moyen MUC5B. En ce qui concerne la comparaison du rapport de volume de pixels moyen MUC5B, aucune différence n'a été observée entre les groupes Y et O ($Z = 1.35$, $p = 0.18$).

En ce qui concerne la corrélation entre le rapport de volume de pixels MUC5B et le seuil d'astringence, il n'y avait aucune corrélation dans les deux groupes.

4. Conclusion

En résumé, le présent travail a mis en évidence, en utilisant une méthodologie western-blot pour explorer la quantité de PRP dans la salive à partir d'un panel formé par 30 personnes âgées et 24 jeunes, que la différence de seuil d'astringence entre le groupe des jeunes et celui des personnes âgées n'était pas liée au montant des PRPs. Fait intéressant, nous avons trouvé une corrélation positive entre la quantité de gPRPs et le seuil d'astringence uniquement dans le groupe jeune. Une corrélation significative et négative entre les bPRPs et le seuil d'astringence n'a été observée que dans le groupe des personnes âgées. Par conséquent, nous pourrions tirer la conclusion d'une contribution différente du type de PRPs dans la perception de l'astringence en fonction de l'âge. En outre, il n'y a pas de différence dans le rapport de volume de pixels MUC5B entre les groupes jeunes et âgés, ce qui n'est pas lié à la différence de seuil d'astringence.

Chapitre 5 : Discussion générale et conclusion

1. Discussion

L'objectif de ce travail était d'étudier la sensibilité à l'astringence en fonction de l'âge et des caractéristiques salivaires (flux, quantité de protéines et composition).

Dans l'expérience sensorielle, le résultat a montré que le seuil d'astringence dans le groupe âgé était significativement plus haut que dans le groupe jeune. Cependant, cette différence n'était pas liée au flux salivaire et à la quantité de protéines. Pour cette raison, la quantité de PRPs a été explorée dans des échantillons de salive de sujets jeunes et âgés. En effet, de plus en plus de preuves indiquent que les PRPs sont des protéines salivaires qui peuvent interagir avec les tanins alimentaires pour déclencher la sensation d'astringence. Toutefois, la différence de seuil d'astringence n'était pas non plus liée au niveau salivaire des PRPs.

Le vieillissement joue un rôle important dans la perception du goût, il est généralement considéré comme ayant un effet négatif sur la perception du goût. Plusieurs facteurs peuvent influencer l'importance du déclin sensoriel pendant le vieillissement (état nutritionnel, état de santé général et maladies). Les raisons de ces modifications sensorielles peuvent également être liées à des changements dans la physiologie orale avec l'âge. En effet, chez la population âgée, les effets cumulatifs du vieillissement physiologique, des maladies et de la prise de médicaments ont fréquemment un impact sur les différents composants de la physiologie orale qui sont d'une grande importance dans la sensibilité au goût et donc dans les choix et le comportement alimentaire. En particulier, le vieillissement peut souvent s'accompagner d'une diminution du flux salivaire ou de modifications de la composition salivaire, ce qui peut entraîner une sécheresse de la bouche ou une xérostomie. L'hyposalivation chez les personnes âgées peut entraîner des changements dans la perception de l'arôme, du goût et de la texture, et par conséquent, l'apport et la consommation de nourriture. Cependant, la littérature sur l'effet du vieillissement sur la sensation d'astringence est quasi inexistante. Pour autant que nous sachions, il s'agit de la première étude évaluant la sensibilité à l'astringence en fonction de l'âge. Les sujets jeunes étaient plus sensibles à l'astringence que les personnes âgées, ce qui est conforme aux études précédentes sur les modalités gustatives. Pour la modulation de la perception de l'astringence en fonction des propriétés salivaires, dans nos travaux, une relation positive entre la sensibilité à l'astringence et le flux salivaire a été observée uniquement dans le panel de sujets jeunes, ce qui est cohérent avec certaines études et en contradiction avec d'autres. Probablement en raison de différentes méthodes de collecte de la salive, du traitement de la salive (centrifugation ou non) et de différents types de salive. Cependant, aucune corrélation n'a été observée entre le seuil d'astringence et la quantité de protéines dans le groupe jeune et le groupe âgé, ce qui est conforme à une conclusion selon laquelle la sensibilité à l'astringence était liée à la composition en protéines et non à la quantité de protéines (Kallithraka et al., 2001).

En ce qui concerne les niveaux de PRPs, il n'y avait pas de différence entre le groupe des jeunes et des personnes âgées. Ceci est conforme à l'étude précédente sur les changements des aPRPs avec le vieillissement (Baum et al., 1982). Cependant, les changements dans la composition salivaire chez les personnes âgées en bonne santé sont protéines spécifiques. De nombreuses études ont indiqué que le niveau de mucine diminue avec l'âge. Pour d'autres protéines, telles que l'amylase, le lysozyme, les IgA et la lactoferrine, la direction des changements (augmentation ou diminution) n'est pas cohérente (F. Xu et al., 2019). Dans notre étude, une corrélation positive entre la quantité de gPRPs et le seuil d'astringence n'a été observée que dans le groupe jeune. La corrélation négative entre les bPRPs et le seuil d'astringence n'a été observée que dans le groupe des personnes âgées. Cela suggère une contribution différente en fonction de la famille de PRPs dans la perception de l'astringence en fonction de l'âge.

La différence de seuil d'astringence entre le groupe des jeunes et des personnes âgées n'était pas liée au débit, à la quantité de protéines et à la quantité de PRPs. Il a été indiqué que les histatines sont également efficaces pour lier les tanins. Le pH de la salive, les capacités tampons, la viscosité, la rhéologie, etc. peuvent également affecter la perception de l'astringence.

En outre, la question de la variabilité interindividuelle ne doit pas être ignorée, comme les variations physiologiques individuelles liées à la fonctionnalité des glandes, le mode de vie et les habitudes, les régimes alimentaires, etc. Ces facteurs devraient être pris en compte à l'avenir lors d'une étude plus approfondie sur l'astringence.

2. Conclusion

L'objectif du présent travail était d'évaluer la sensibilité à l'astringence en fonction de l'âge, du flux salivaire, de la quantité de protéines et de la quantité de PRPs. Cinquante-quatre panélistes, dont 30 personnes âgées (âge = 75 ± 4.2 ans) et 24 jeunes (âge = 29.4 ± 3.8 ans), ont participé à cette étude. La sensibilité à l'astringence a été évaluée par la procédure 2-AFC en utilisant des solutions d'acide tannique. La salive entière a été recueillie pendant 5 minutes avant et après les tests sensoriels. Les PRPs ont été quantifiées immunochimiquement par une analyse par Western-Blot. Les résultats ont montré que le seuil d'astringence était significativement plus élevé dans le groupe des personnes âgées que dans le groupe des jeunes. Aucune corrélation n'a été observée entre la quantité de protéines salivaires et la valeur de seuil. Cependant, une corrélation négative entre le flux salivaire et le seuil n'a été observée que dans le groupe jeune. Les niveaux de bPRPs et de gPRPs étaient similaires entre les groupes de personnes jeunes et de personnes âgées. Cependant, une corrélation positive entre la quantité de gPRPs et le seuil d'astringence n'a été observée que dans le groupe jeune. Une corrélation significative et négative entre les bPRPs et le seuil d'astringence n'a été observée que dans le groupe des personnes âgées. Ce travail suggère une contribution différente du type de PRPs dans la perception de l'astringence en fonction de l'âge.

Ces résultats ont montré une différence dans la perception de l'astringence orale en fonction de l'âge. Cela peut fournir des indications et leviers lors de la conception d'aliments à base de plantes pour les personnes âgées.

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