# Sensory Perception of Selected Phenolic Acids Found in Whole Grains

**by**

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ABSTRACT

# SENSORY PERCEPTION OF SELECTED PHENOLIC ACIDS FOUND IN WHOLE GRAINS

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This thesis is an investigation of the sensory attributes of selected phenolic acids found in whole grains. Salivary protein-phenolic acid interactions were also investigated using SDS-PAGE to further understand possible mechanisms of astringency development in low molecular weight polyphenols. Sensory attributes of aqueous solutions of ferulic acid and vanillic acid were determined to be sour, bitter and astringent, as determined by a trained panel. Time-intensity studies on the temporal component of bitterness, sourness and astringency of phenolic acids revealed that bitterness and astringency of the phenolic acid solutions increased with repeated exposures. The precipitation of salivary proteins was not found to be required for the development of astringency of ferulic, vanillic or gallic acid. These results suggest that salivary protein binding activity may not be an accurate measure of the astringency of all polyphenols.

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# CHAPTER 1 - INTRODUCTION

Along with dietary fibre, vitamins and minerals, whole grains contain significant quantities of phytochemicals. Phenolics are a key category of phytochemicals found in grains and are thought to contribute to the flavour of cereal products. These compounds are mainly concentrated in the bran, the fibre-rich outer layers of the grain kernel (Adom, Sorrelles & Liu, 2005; Matilla, Pihlava, & Hellstrom, 2005; Kim, 2006; Dhillon, 2013). During the milling process, the bran layers are traditionally removed to produce refined flour that is thought to be more appealing to consumers (Bakke & Vickers, 2007). This is because the compounds within the bran portion are thought to impart negative taste attributes within whole grain products, including bitterness and astringency.

Recent research has demonstrated differences in the sensory properties of baked cereal products made with wheat flours milled from different coloured wheat (Challacomb, 2011; Dhillon, 2013). Upon examining differences in subclasses of phenolics, differences were found in the phenolic acid profiles of these products and their flours. This suggests that the phenolic acid profile plays a role in sensory perception of wheat based products. As consumers aim to increase whole grains in their diet, further understanding of the contribution of these compounds to the flavour of cereal products is warranted.

Most of the literature on the sensory aspects of plant phenolics focuses on those found in fruits, vegetables, wines and teas, although cereal grains contain a significant amount of the same flavonoids and phenolic acids (Maga, 1978; Herrmann, 1989; Mattila et al., 2005; Matilla et al, 2006; Matilla & Hellstrom, 2007). Furthermore, some of these compounds, for example, ferulic acid, are predominantly found in grains, and are not present in significant quantities in some

fruits and vegetables. The various classes of phenolic compounds found in grains include derivatives of hydroxybenzoic and hydroxycinnamic acids, anthocyanidins, quinones, flavanols, chalcones, flavones, and flavanones. Many of these compounds are considered to be important factors for the sensory properties of many food materials (Drewnowski & Gomez-Carneros, 2000) as they may add directly to flavour due to their inherent taste, or prevent rancid flavours by acting as antioxidants to influence lipid oxidation (Onyeneho, 1992). In fruits and beverages, flavanols (catechin, epicatechin) and their polymers (condensed tannins) are the key compounds responsible for bitter taste and astringency (Lesschaeve & Noble, 2005). The bitter and astringent sensations of these compounds, particularly the flavan-3-ols and their polymers, have primarily been investigated in wine and teas due to their high concentration in these beverages. Sensory studies of these compounds have demonstrated that, overall, larger molecules tend to be less bitter and more astringent, while smaller molecules tend to be less astringent and more bitter.

Little information exists with regards to the sensory properties of low molecular weight phenolic acids. This is likely due to the fact that they exist in much lower concentrations in most foods compared to other larger molecular weight classes of phenolics. However, phenolic acids are present in all cereal grains and have been shown to exist as free acids in wheat and triticale grains at levels equal to or above their determined flavour thresholds, and therefore may contribute to the sensory properties of their flours and cereal foods (Maga & Lorenz, 1973a; Dykes & Rooney, 2007). Phenolic acids have been shown to elicit sourness, bitterness, and astringency, however, qualitative and quantitative differences in these sensory properties have been demonstrated amongst a subset of these acids (Peleg & Noble, 1995). Furthermore, the concentrations of individual phenolic acids found in cereal grains have been shown to vary

depending on species variety, location, environmental conditions, as well as time of harvest, storage and processing conditions (Maga & Lorenz, 1973b; Maga, 1978; Hatcher & Kruger, 1997; Adom, Sorrells, & Liu, 2003; Yu, Perret, Harris, Wilson & Haley, 2003; Zhou & Yu, 2004; Mattila et al, 2005; Mpofu, Sapirstein & Beta, 2006; Fernandez-Orozco et al, 2010; Menga, Fares, Shewry et al, 2010; Toccoli, Cattivelli & Baianao, 2010). This variance in phenolic acid profiles may result in different sensory properties of cereal flours of the same species but of a different variety, location or processing condition.

As the use of whole grains in cereal products is on the rise, a greater understanding of how the individual phenolic acids present in whole grains may impact the flavours of these products is warranted. Because different grain varieties contain different phenolic acid profiles, investigating the sensory properties of each acid would provide a basis for understanding how different phenolic acid profiles might impact overall flavour.

The overall goal of this research was to explore the sensory properties of individual phenolic acids in solution using a trained descriptive panel. Understanding how these sensory properties change over repeated consumption of these acids can aid in developing wholegrain products that are acceptable to consumers. The interaction of phenolic acids with salivary proteins was also explored to further understand astringency as it relates to low molecular weight phenolics. This understanding will help to broaden our knowledge of the definition of an astringent.

# *CHAPTER 2* - LITERATURE REVIEW

## 2.1 Phenolic Acids

Phenolic acids are a subclass of secondary plant metabolites widely found in plants and plant foods. They belong to a larger category of plant metabolites termed “phenolics” due to their common structural component, a phenol (an aromatic ring bearing at least one hydroxyl group). Possessing only one such phenol unit, phenolic acids are amongst the smallest phenolics and are termed simple phenols. Phenolic acids can further be classified into two groups based on their carbon structure: derivatives of hydroxycinnamic acids and hydroxybenzoic acids. The hydroxycinnamic acid derivatives include ferulic, caffeic, sinapic, and o-, p- and m-coumaric acids. The hydroxybenzoic acid derivatives include vanillic, gallic, syringic, p-hydroxybenzoic and dihydroxybenzoic acids **(**Figure 2-1). These acids all possess one carboxylic acid (-CHO) functional group but differ in the number and position of hydroxyl and methoxyl groups on the aromatic ring (Figure 2-2).

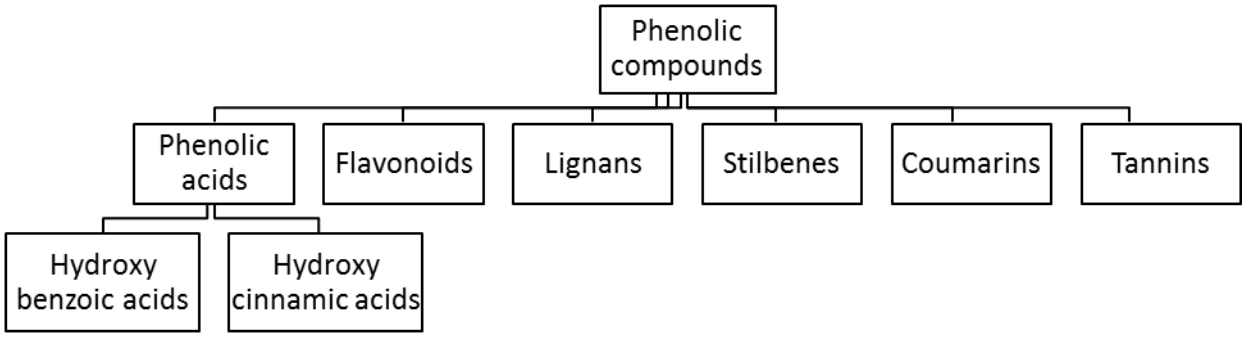


**Figure 2-1: Family of phenolic compounds**

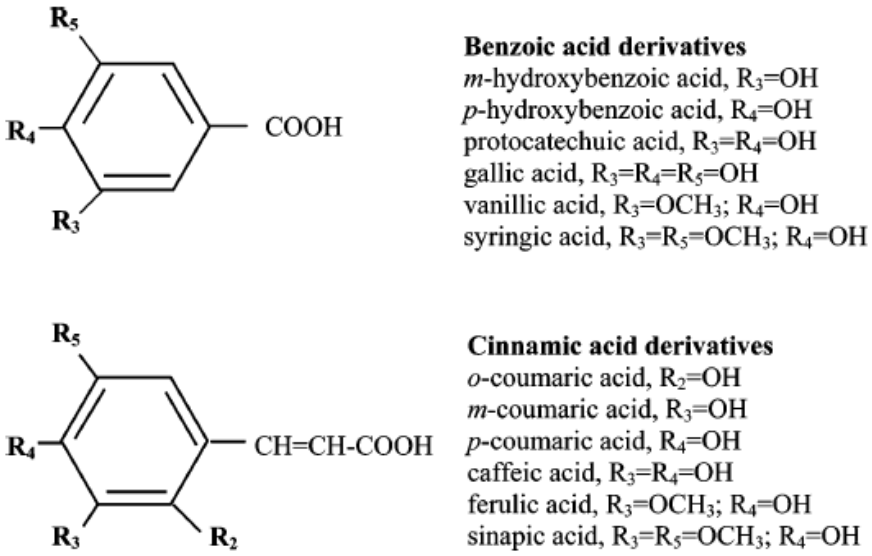


**Figure 2-2: Structures of benzoic and cinnamic acid derivatives (Mattila et al., 2005)**

When consumed in the diet, phenolic acids are thought to help reduce oxidative damage to cells by terminating free radicals that may otherwise lead to chronic cell injury. This is achieved through donation of the hydrogen atom from the hydroxyl group on the phenyl ring of the acid, and is affected by the number and proximity of the hydroxyl group(s) to the carboxylate group on the molecule (Graf, 1992; Rice-Evans, Miller & Paganga, 1996; Liu, 2004). *In vitro* studies have reported that phenolic acids demonstrate antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory effects (Shahidi & Naczk, 1995; Breinholt, 1999; Duthie et al, 2000).

Phenolic acids are dispersed throughout plant tissues in seeds, leaves, roots and stems and can exist as free acids or bound to structural components of the plant such as cell wall polymers (cellulose, proteins, lignin) (Hartley & Jones, 1976; Brett & Waldron, 1996), larger polyphenols (flavonoids) or to other low molecular weight organic molecules (glucose, quinic, maleic, or

tartaric acids) (Winter & Herrman, 1986; Klick & Herrman, 1988; Shahidi & Naczk, 1995). Most commonly, hydroxycinnamic acids exist in plants as simple esters with quinic acid or glucose, while hydroxybenzoic acids are mainly present in the form of glucosides (Figures 2-3& 2-4) (Herrmann, 1989).

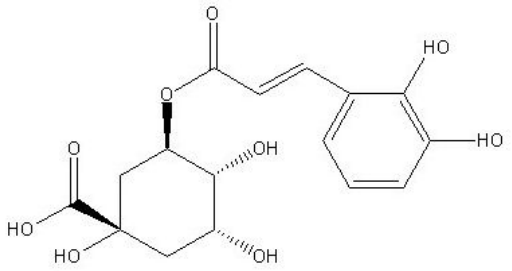


**Figure 2-3: 5-O-caffeoylquinic acid, an ester formed between caffeic acid (left) and quinic acid (right)**

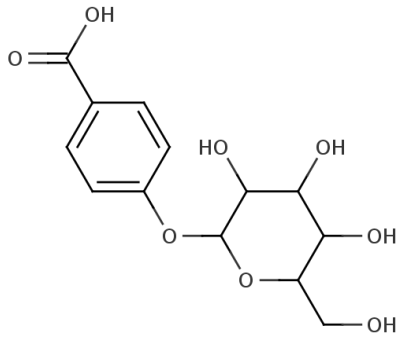


**Figure 2-4: 4-hydroxybenzoic acid 4-O-glucoside, a glucoside of 4-hydroxybenzoic acid (left) and glucose (right)**

With regard to foods, naturally occurring phenolic acids and their compounds are present in grains, tea, coffee, fruits and vegetables, nuts and spices, and account for almost one third of all dietary phenols (Herrmann, 1989; Robbins, 2003; Mattila et al, 2005; Mattila et al, 2006; Mattila & Hellstrom, 2007). Depending on the diet, the total daily consumption of phenolic acids is estimated to be between 25 mg – 1g per day (Clifford, 1999; Dykes & Rooney, 2007). The levels of individual phenolic acids within plant foods has been shown to vary with plant species and variety, location, environmental conditions, as well as time of harvest, storage and processing conditions (Hatcher & Kruger, 1997; Adom et al, 2003, Mattila et al, 2005).

As consumers aim to increase their consumption of whole grains naturally rich in phenolics, the impact of individual phenolic acids on food quality and taste requires further

examination. Furthermore, certain phenolic acids such as ferulic acid have been approved as antioxidant additives and food preservatives in countries like Japan (Graf, 1992; The Japan Food Chemical Research Foundation, 1996). Phenolic compounds in general have been described as bitter and/or astringent, but these studies mostly reference larger phenolics such as the flavonoids or tannins as they are often found in higher concentrations. While the sensory properties of many larger phenolic compounds such as the tannins and flavonoids have been described, less is known regarding the sensory properties of individual phenolic acids.

**2.2 Sensory Properties of Phenolic compounds**

The bitter taste and astringency perceived in a variety of plant-based foods and beverages has largely been attributed to the phenolic flavonoids and their polymers (proanthocyanidins or condensed tannins), as they account for approximately two-thirds of all dietary phenols (Lea & Arnold, 1978; Fisher & Noble, 1994; Scalbert & Williamson, 2000). In general, these studies define bitterness as a sharp, unpleasant taste perceived at the back of the tongue, associated with caffeine and other bitter compounds, while astringency is defined as a drying or puckering mouthfeel. Sensory studies have demonstrated that this class of polyphenols including the flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins, and isoflavones, as well as tannins are almost always bitter and astringent (Arnold & Robichaud, 1978; Arnold, Noble & Singleton, 1980; Robichaud & Noble, 1990; Rouseff, 1990; Kallithraka et al., 1997a; Thorngate & Noble, 1995; Kielhorn & Thorngate, 1999; Peleg et al, 1999; Kobue-Lekalake et al, 2007).

The sensations of bitterness and astringency elicited by flavonoids and tannins have been shown to be associated with degree of polymerization (polymer size), but also chirality (Thorngate & Noble, 1995; Kallithraka et al, 1997a), extent of galloylation, and formation of derivatives. In general, astringency increases and bitterness decreases with increasing polymer

size (Rouseff, 1990). Evaluations of cider procyanidin fractions containing oligomeric (1-5 units) and polymeric (6-10 units) procyanidins showed that while both astringency and bitterness was elicted by all fractions, astringency was predominantly associated with the polymeric fractions whereas bitterness was associated the tetrameric fractions (Lea & Arnold, 1978). A trend of increasing astringency and decreasing bitterness with increasing degree of polymerization was also demonstrated in comparisons of polymeric tannic acid and grape seed tannin to their respective monomers, gallic acid and (+) catechin in model wine solutions. It was shown that the monomers were more bitter and less astringent than their respective polymers (Robichaud & Noble, 1990). In further investigations of polymer size on the bitterness and astringency of phenolics, time-intensity evaluations of flavan-3-ol monomers, dimers and trimers revealed that, as the degree of polymerization increased, maximum bitterness intensity and duration decreased, whereas maximum astringency intensity increased (Peleg, Gacon, Schlich & Noble, 1999). This study also revealed that the bond linking the monomeric units had an influence on sensory properties; bitterness and astringency was higher in the catechin-catechin dimer linked by a (4→6) bond than the dimer linked with a (4→8) bond. Investigations have also revealed that small differences in configurations of flavonoids can result in significant differences in sensory properties; epicatechin is perceived to be more bitter and astringent than catechin, the sole difference between them being the configuration of a hydroxyl group (Thorngate III & Noble, 1995; Kallithraka, Bakker & Clifford, 1997a).

**2.3 Sensory properties of phenolic acids**

While the sensory properties of polyphenolic compounds have been extensively investigated, less is known regarding the sensory characteristics of phenolic acids. Early investigations of the phenolic content in defatted soy flour by Arai et al (1966) described the

phenolic acid fraction as having a sour, bitter, astringent and phenol-like flavour that was suggested to likely have some influence on the soybean flour flavour. Later investigations of taste thresholds of individual phenolic acids by Maga and Lorenz (1973a) referred to phenolic acids as possessing sour, bitter and astringent flavors without reference to how these taste characteristics were generated. Nevertheless, taste thresholds for aqueous solutions of the phenolic acids were determined as the point at which a definite astringency, drying or puckering occurred, suggesting that these qualities were elicited by the phenolic acid solutions. However, these sensory characteristics were generalized over all of the acids. The few studies that have considered the sensory profiles of individual phenolic acids have revealed that they can elicit a complex mixture of tastes and oral sensations over time, and that structural differences amongst the phenolic acids can confer differences in their sensory profiles. As part of a comprehensive study on the phenolic compounds in beer, Meilgaard (1975) and Dadic & Belleau (1973) describe hydroxybenzoic acid and hydroxycinnamic acids in 5% aqueous ethanol as bitter, astringent, or both. However, one consideration of these studies is that the ethanol present in the solution may have contributed to the bitterness and astringency as ethanol itself is perceived as bitter and astringent (Rouseff, 1990). Similarly, gallic acid was shown to be both bitter and astringent, evaluated in a model wine solution (Robichaud & Noble, 1990). In their review of bitter compounds, Belitz and Wieser (1985) report that, within the hydroxybenzoic acids, 2-hydroxy benzoic acid (salicylic acid) is not bitter, while 4-hydroxy-(*p*-hydroxybenzoic acid), 2,4-dihydroxy-(protocatechuic acid), 2,4,6-trihydroxy-(gallic acid), and 2,3,4-trihydroxy-(pyrogallolcarboxylic acid) benzoic acids are bitter, demonstrating that even slight structural changes can alter the sensory properties of these acids. This was echoed in later work by Peleg & Noble (1995) in which structure-sensory relationships of hydroxybenzoic acid derivatives were

investigated. It was found that aqueous solutions of hydroxybenzoic acid and its derivatives elicited complex sensations of sweetness, sourness, astringency (defined as mouth drying), bitterness and prickling, and that qualitative and quantitative differences in the sensory properties existed amongst the acids. To date, the sensory properties of the hydroxycinnamic acid derivative, ferulic acid (4-hydroxy-3-methoxycinnamic acid), and hydroxybenzoic acid derivative vanillic acid (4-hydroxy-3-methoxy benzoic acid), have not been studied. Dietary sources of ferulic acid include, but are not limited to, whole grains, coffee, orange juice, eggplant, water dropwart, cabbage, broccoli, spinach radish, potato, tomato, banana, orange, grapefruit, soy bean and peanut (Mattila et al, 2006; Mattila et al, 2007). While fruit and vegetable sources can contain anywhere from 5-7 mg ferulic acid per 100 grams of edible portion, the highest concentrations of ferulic acid are found in cereal grains (barley, maize, millet, oat, rice, rye, sorghum, wheat) and their flours, with ferulic acid being the major phenolic acid occurring in the cell walls of monocotyledons (Mattila et al, 2005; Klepacka & Fornal, 2006; Mattila et al, 2006; Mattila & Hellstrom, 2007). The estimated total dietary intake of ferulic acid through the consumption of cereals, vegetables, fruits, coffee and juices can reach 150-250 mg/day, with whole grains contributing significantly to this total (35-89 mg total FA/100 g grain) (Zhao & Moghadasian, 2008).

Vanillic acid is also found in variable concentrations in cereal grains, although in much smaller concentrations. For example, the average concentration of vanillic acid within whole grains and their flours can range from 0.25 mg/ 100g (long grain brown rice) to 3.0 mg/ 100 g fresh weight (whole grain rye flour) (Mattila et al, 2005). Canadian wheat flours have been reported to contain up to 16 mg/kg vanillic acid, whereas American wheat and triticale have been reported to contain 34-42 mg/kg, demonstrating variation in phenolic acid content amongst

varieties. Both ferulic and vanillic acid have been demonstrated to exist as free acids in wheat and triticale grains at levels at and above their determined flavour thresholds and therefore may contribute to the sensory properties of flours and food products made from those flours (Maga & Lorenz, 1973a).

### 2.4 Perception of phenolic acids

To understand how phenolic acids contribute to taste and mouth feel, a basic understanding of taste physiology is required.

Humans can detect and differentiate between at least five basic tastes: sweet, salty, sour, bitter and umami. In order to detect taste, taste compounds must first be dissolved in saliva and transported to taste cells located at the base of the papillae of the tongue. Tastants can then either interact with taste receptors on the surfaces of these cells, leading to sweet and bitterness detection, or with ion channels, leading to salty and sour detection. As the tongue moves, spaces between the papillae expand and contract, allowing fluid to move in and out, and exposing taste receptors to new tastant molecules (Rawson & Li, 2004).

The multiple sensations elicited by phenolic acids – sourness, bitterness, astringency – allude to the complex nature in which tastants are detected in the oral cavity. Phenolic acids are both phenolic compounds, which are known to elicit bitterness and astringency, as summarized above, and also acids, which are perceived as sour.

#### 2.4.1 Sourness

Sourness perception is largely due to the detection of hydrogen ions in solution. These protons act on the taste cell in three ways: by directly entering the cell, by blocking potassium (K+) channels on the microvilli, and by binding to and opening the channels on the microvilli

that allow other positive ions to enter the cell (Rawson & Li, 2004). The accumulation of positive charges inside the taste cell depolarises the cell and leads to neurotransmitter release and sourness perception. Sensory studies have shown that both the dissociated and undissociated hydrogen ions in a solution are involved in sour perception. These studies have shown that weak organic acids can be greater in sourness intensity compared to hydrochloric acid (a strong acid) at equal pH, and that perceived sourness intensity is positively correlated with titratable acidity (Ganzalves and Kroeze, 1987; Schallengberger, 1993; Thomas & Lawless, 1995; Lawless et al, 1996; Sowalsky and Noble, 1998). This has been confirmed by physiological studies that have demonstrated that action potentials generated from taste cells in response to acidic stimuli are "dose-dependent" based on the titratable acidity of the stimulus, not the pH (Kinnamon, Dionne, & Beam, 1988; Gilbertson, Avenet, Kinnamon & Roper, 1992).

### 2.4.2 Bitterness

Bitterness is elicited by a large number of compounds with wide structural variance, and is detected by receptors located in the microvilli of the taste receptor cell. There are approximately 25 different bitter receptors, termed T2R receptors, each made up of groups of seven transmembrane proteins. When bitter compounds enter these receptors, activation of the enzyme phospholipase C results in inositol triphosphate (IP3) production and the release of calcium ions (Ca++) from internal stores. This increase in calcium ions in turn causes sodium ions to enter the cells through activation of the TRPM5 transmembrane ion channel, leading to a build-up of positive charge inside the cell, resulting in depolarization and neurotransmitter release (Rawson & Li, 2004; Engelen, 2012). The perception of bitterness is slower and longer lasting than that for tastes mediated by ion channels, such as sourness, due to the slower process of binding and unbinding of bitter compounds to receptors. This is reflected in delays of as long

as 2-7 seconds between when a bitter compound makes contact with the tongue and actual perception, and the lingering bitter aftertaste that has been demonstrated using time-intensity methods to evaluate perceived bitterness of bitter compounds (Rouseff, 1990). Bitter tastes are detected at low thresholds and are often considered unpleasant (Paulus & Reisch, 1980). This is thought to protect animals from consuming even small quantities of naturally occurring toxic compounds that taste bitter. While amides and alkaloids are some of the most bitter tasting compounds, certain amino acids, urea, fatty acids, phenols, amines, esters and some salts have also been shown to elicit bitterness (Rouseff, 1990; Engelen, 2012).

### 2.4.3 Oral Astringency

Perceptually, oral astringency is complex, involving sensations of drying and roughness perceived when oral surfaces are moved against each other, and puckering felt in the cheeks and muscles of the face (Lee & Lawless, 1991; Breslin et al, 1993; Lawless & Corrigan, 1994). Unlike the gustatory tastes sour and bitter, astringency is a tactile sensation that is not localized to any one part of the mouth and is detected by mechanoreceptors located throughout the oral cavity and transduced by free nerve endings of the trigeminal nerve (Bate-Smith, 1954; Breslin et al, 1993; Green, 1993). Oral astringency typically develops and dissipates slowly, taking 15 s or more for perception to fully develop, and builds with repeated exposure to astringent compounds over a short period of time (Guinard, Pangborn & Lewis, 1986a).

Sensory studies have demonstrated that oral astringency can be elicited by a variety of compounds, including metal salts (such as alum), dehydrating agents (ethanol and acetone), mineral and organic acids, polyphenols, and whey proteins (Haslam & Lilley, 1988; Green, 1993; Clifford, 1998; Gawel, 1998; Lee, 2010). The diversity amongst these compounds and

their properties has led to theories of differing mechanisms behind astringency development. The interaction of polyphenols with salivary proteins has long been thought to lead to the sensation of astringency.

### *Astringency of polyphenols*

The astringency of wine, teas and beer has largely been attributed to their polyphenol content, especially the flavan-3-ols and tannins. The ability of these polyphenols to bind and precipitate salivary proteins is thought to contribute to sensations of drying and roughing in the oral cavity (Haslam & Lilley, 1988). Astringency detection with regards to dietary polyphenols is thought to occur for two reasons. Firstly, the astringency of plant material is thought to be a defense mechanism of plants against predation, making the plant unpalatable (Bennick, 2002). Secondly, the binding of salivary proteins to dietary polyphenols is thought to be a protection mechanism against the anti-nutritive effects of polyphenols in the digestive tract where they may otherwise bind and inhibit important digestive enzymes, leading to decreased nutrient absorption, where by the resultant astringency is mechanism to detect these harmful compounds (Hagerman & Butler, 1981; Mehanso, Ann, Butler, Rogler & Carlson, 1987a; Lu & Bennick, 1998; Prinz & Lucas, 2000). Animal studies have demonstrated a decreased growth and body weight associated with increased tannin content of feed (Mehanso, Ann, Butler, Rogler & Carlson, 1987b).

Proteins that strongly associate with dietary polyphenols are rich in the amino acid proline, are relatively large and hydrophobic, and possess open, flexible conformations (Hagerman & Butler, 1981; Luck et al, 1994; Baxter et al, 1997; Bennick, 2002). Astringency research has largely concentrated on the interaction of polyphenols with proline-rich proteins (PRPs), a specific class of salivary proteins containing high amounts of proline because of their

ability to bind and precipitate tannins. Together with histidine-rich histatins proteins (HRPs), PRPs make up nearly 70% of all parotid salivary protein (Kauffman and Keller, 1979). Three types of PRPs are found human saliva; acidic (APRP), basic (BPRP) and glycosylated (GPRP). APRPs have been shown to bind calcium (Bennick, McLaughlin, Grey & Madapallimattam, 1981), and to bind to hydroxyapatite as part the dental pellicle, the proteinacious layer coating dental enamel (Hay, Moreno & Schlesinger, 1979; Bennick et al, 1979; Moreno et al, 1982; Bennick). BPRPs have demonstrated anti-viral activity, binding activity with certain oral bacteria (O, Sullivan et al, 1997), and a high affinity for binding tannins (Hagerman & Butler, 1981; Lu & Bennick, 1988; Mehanso et al, 1983; Kauffman et al, 1991). GPRPs contribute to the lubrication of the oral cavity, and also have demonstrated micro-organism binding activity (Hatton, Loomis, Levine & Tebak, 1985; Murray, Prakobphol, Lee, Hoover & Fischer, 1992).

Charlton et al. (2002) proposed a three stage interaction mechanism for the binding of polyphenols with salivary proteins. In the first stage of interaction, a soluble complex is first formed through hydrophobic association between the aromatic ring of the polyphenol, and the flat, open, hydrophobic surface of the proline residue of the protein (Figure 2-6). This step is thought to be followed by the formation of hydrogen bonds between the phenolic hydroxyl groups and the carboxyl group of the amino acid N-terminal of the proline ( Luck et al, 1994; Murray, Williamson, Lilley, & Haslam, 1994). If the protein is present in excess, these complexes remain soluble because each protein is bound only by a few polyphenols (Hagerman, 1989). In conditions of excess phenol, in a second step, crosslinking between the polyphenols of analogous complexes results in an insoluble complex of increased molecular mass. In the third step, further aggregation of insoluble complexes leads to precipitation of the polyphenol-protein aggregate out of solution (Figure 2-7).

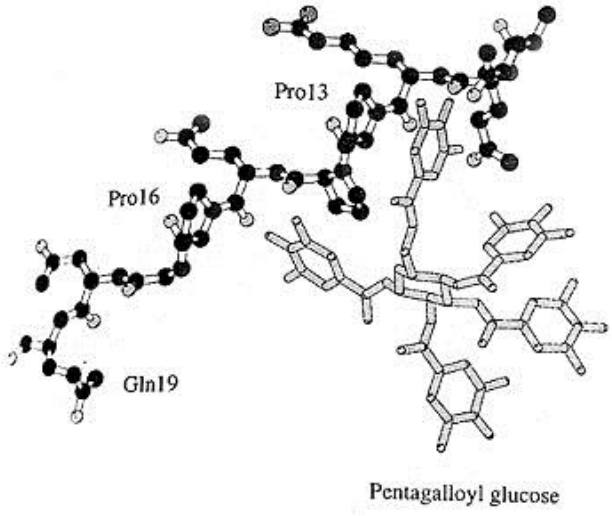


**Figure 2-5: Hydrophobic stacking between aromatic ring of polyphenol pentagalloyl glucose (PGG) (left) with proline residues (right). (Baxter, Haslam, Lilley &Williamson, 1997)**

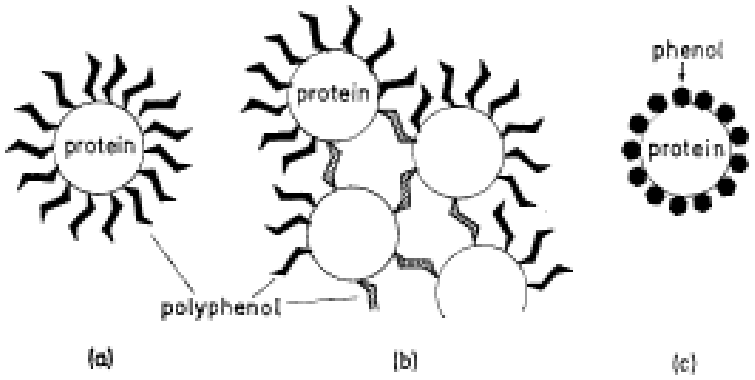


**Figure 2-6: Protein precipitation by polyphenols. (a) Polyphenol, low protein concentrations. (b) Polyphenols, high protein concentrations. (c) Simple phenols. (McManus, Davis, Lilley & Haslam, 1981)**

Although it is known that polyphenols bind with salivary proteins, the actual mechanisms behind the development of astringency in mouth are not yet fully understood. A number of popular hypotheses have been proposed related to salivary protein binding, precipitate formation, and interactions with the oral mucosa.

Much of what is known about the mechanism of astringency of polyphenols is based on studies of tannins and their ability to bind and precipitate salivary PRPs. One hypothesis is that the binding of polyphenols with salivary proteins to form insoluble complexes withdraws proteins from the salivary matrix and thus reduces its ability to lubricate oral surfaces, resulting in a perceived increase in friction between oral surfaces (Smith, 1996; Clifford, 1997; Gawel, 1997; Green, 1997). Using a Boothroyd friction tester and rheometer (Stresstech), Prinz and Lucas (2000) found *in vitro* that friction increased with the addition of tannic acid to pooled saliva, while viscosity decreased after the resultant precipitates were removed. They speculated that the mechanism by which tannic acid effects astringency perception is the thinning of saliva due to the precipitation of proteins and the subsequent decrease in lubricating properties. However, the same researchers reported a decrease in both viscosity and friction when tannic acid was mixed with pooled saliva in a later experiment (deWijk & Prinz, 2005). Alternatively, in a later publication by deWijk & Prinz (2006), it was proposed that it may be the precipitate itself that causes sensations of roughness associated with astringency, noting their experimental results indicating that salivary viscosity is not systematically related to its lubricative properties. These authors demonstrated *in vitro* that the addition of 0.5 mM of tannic acid gels saliva to form an unstructured mass due to the precipitation of protein in saliva (deWijk & Prinz, 2006). Recently, Rosetti et al (2009) also found that certain tea polyphenols (epigallocatechin-gallate) reduced salivary lubricity, while another (epicatechin) did not, although both polyphenols are perceived to be astringent.

A further hypothesis has been suggested whereby the astringent sensation is caused by a direct effect of astringents on the oral mucosa. Green (1993) suggested that astringency may be, in part, due to the binding of stimulus or stimulus-protein complexes to cells of the oral

epithelium. Guinard, Pangborn and Lewis (1986c) proposed that complexation of procyanidins with salivary proteins is followed by binding of these complexes to oral epithelial proteins, suggesting that this is the reason they observed increased astringency with repeated ingestion of wine. In their investigation of the interaction of known astringent grape seed procyanidins with oral epithelial cells, Payne et al (2009) demonstrated that astringent procyanidins do bind to oral epithelial cells, and suggested that the oral epithelium is the primary site of the astringent sensation. Additionally, some authors have proposed that salivary proteins (PRPs) within the salivary matrix may help to inhibit the mouth feeling of astringency by binding the astringent compounds before they can act on the oral mucosa (Horne et al, 2002). Work by Nayak and Carpenter (2008) also supports this hypothesis whereby astringency ratings were significantly increased after subjects rinsed their mouths with water to remove the mobile phase of saliva before the evaluation of an astringent tea solution compared to when they did not. Additionally, rinsing with the tea solution produced a visible precipitate when the subjects had rinsed their mouth with water prior to the tea, and no precipitate was visible if the prior rinsing with water was not performed. Analysis of the proteins precipitated by the tannin solution revealed proline-rich proteins and mucins, previously adherent to the mucosa. Taken together, this suggests that saliva exists in multiple phases and that certain proteins (PRPs) within the mobile phase of saliva may act as a barrier to astringency that is perceived when polyphenols interact with proteins adhered to the salivary mucosa.

While it has been demonstrated that larger and more hydrophobic polyphenols bind more effectively to proline rich peptides (Baxter, Lilley, Haslam, & Williamson, 1997; Gambuti et al, 2005), simple phenolics (resorcinol, catechol, pyrogallol) have also been shown to weakly bind model proteins such as bovine serum albumin (BSA) (McManus et al, 1981). The ability of

simple phenols to bind BSA was found to be dependent on the number and arrangement of the hydroxyl groups, with enhanced binding occurring with increased number of hydroxyl groups in the ortho- position (Figure 2-7). It was suggested that simple phenolics may contribute to astringency by forming a hydrophobic monolayer around salivary proteins, leading to their precipitation (McManus et al., 1981).

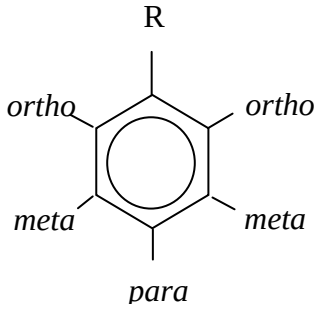


**Figure 2-7: Ortho-, meta-, para- positions of functional groups of aromatic ring**

***Astringency of Acids***

Some acids may elicit astringency by the same mechanisms as polyphenols described above. Naish et al (1993) demonstrated that chlorogenic acid (a low molecular weight polyphenol) binds with proline-rich proteins and is perceived to elicit low levels of astringency. However, sensory studies have demonstrated that acids without the suitable structure for salivary protein binding can also elicit astringency (Corrigan, Thomas & Lawless, 1995; Lawless et al., 1996) and that the astringency of acids is related to pH (Lawless et al., 1996; Sowalsky & Noble, 1998). In an investigation of the effects of pH on the perceived astringency, roughing, puckering and drying of organic acids, Lawless et al. (1996) showed that increasing the pH of equimolar solutions of acids resulted in a significant decrease in perceived astringency. These results were supported by further investigations of the effects of concentration, pH and anion species on the

perceived astringency of organic acids by Sowalski and Noble (1998). They showed that decreasing the pH of aqueous solutions of equal concentrations of acid resulted in significant increases in perceived astringency, while increasing the acid concentration under conditions of constant pH had no effect on the perceived astringency, indicating that astringency perception of organic acids is solely a function of hydrogen ion concentration. It was suggested that the inverse relationship between pH and astringency may be explained by reduced salivary lubricity due to denaturation of salivary proteins under conditions of reduced pH (Nordbo et al., 1984; Lawless et al., 1996). In their investigation of astringent-salivary protein interactions, Lee & Vickers (2010) demonstrated that hydrochloric acid was capable of precipitating low molecular weight proteins believed to include the histatins family of proteins, as well as salivary mucins.

Mucins are mucoglycoproteins having protein cores with a number of oligosaccharide side chains. There are two mucins found in saliva, high molecular weight mucin glycoprotein 1 (MUC5B, >1000kDa) and lower molecular weight mucin glycoprotein 2 (MUC7, 200-250kDa) (Dawes, 2004). Aside from forming the dental pellicle, MUC5B type mucins form a thick layer over all oral surfaces to create a proteinaceous layer termed the mucosal pellicle. Mucins are also responsible for forming a viscoelasctic gel that causes the saliva matrix to be viscous. Together, with GPRPs, mucins help to accomplish the lubricating effect of saliva and play an important role in lubricating oral surfaces as part of the mucosal pellicle (Bradway et al, 1992; Fejerdy, 2007; Stokes and Davies, 2007). The precipitation of salivary mucins by acids may reduce salivary and mucosal lubricity leading to increased oral friction and astringency perception.

### 2.5 Predicting Astringency

Protein binding assays have been used to evaluate the astringency of compounds in foods and beverages, such as the astringency mucin index used to predict the astringency induced by

grape and wine phenol extracts (Monteleone et al., 2004). These assays are thought to predict astringency based on the hypothesis that the greater the protein binding activity of a compound, the greater the perceived astringency.

Protein binding assays such as SDS-PAGE can also be used to evaluate the role of protein precipitation in the development of astringency of different classes of astringents.

### 2.5.2 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method by which proteins in a sample can be separated and identified based on molecular size and charge. The identification and quantification of proteins present in saliva after being mixed with astringent solutions has been proposed as an assay to predict the astringency of a given solution based on the hypothesis that the greater the reduction in salivary protein after incubation (and subsequent centrifugation to remove formed precipitates), the more astringent the solution is predicted to be. Gambuti et al (2006) used SDS-PAGE electrophoresis of salivary proteins after binding reactions with grape tannins to demonstrate differences in protein precipitation between tannins from different sources. They demonstrated that hydrolysable tannins (at 1g/L) were capable of precipitating PRPs, lactoferrin, a-amylase. They also found that mucins were completely removed from solution after interaction with commercial grape tannin solutions (at 1g/L), indicating that mucins may also play a role in the astringency formation of tannins. Progressive reductions in band densities for lactoferrin, basic glycosylated PRPs, and α-amylase after reaction with solutions of increasing commercial tannin concentration (tannic acid, 1-10g/L) were also observed.

Lee and Vickers (2010) compared astringent-salivary protein interactions amongst different astringents by analysing differences amongst the proteins contained in the supernatant

and pellet after pooled saliva was incubated with either water (control) or alum, acid (HCl), or tannin solutions that are known to be astringent. All astringents precipitated a greater amount of low molecular weight proteins (7-17 kDa) compared to control, which were suggested to include the histatin family of proteins. Both alum and acid were found to precipitate salivary mucins (M1 and M2), while tannin and alum (but not acid) were found to precipitate PRPs. The results of this study indicate that the precipitation of PRPs is not necessary for the development of astringency as the acid solution known to be perceived as astringent did not precipitate PRPs. Additionally, the high affinity of acid and alum for mucins may suggest that the astringency perceived when these solutions are held in mouth may also be related to the interaction of alum and acid with mucins. The examination of the interaction of salivary proteins with solutions of low-molecular weight phenolic acids using SDS-PAGE may contribute to a greater understanding of the mechanisms behind astringency of low-molecular weight phenolics.

**Objectives**

Given the lack of information surrounding the sensory profiles of individual phenolic acids and the lack of understanding of the mechanisms behind the possible astringency perception of low-molecular weight polyphenols, more research is required to understand implications or strategies related to whole grain products. Therefore, the objectives of this project were to:

1) Characterize sensory properties of individual phenolic acids commonly found in whole grains
2) Determine how the sensory attributes of phenolic acids are affected with repeated consumption

3) Further understand possible mechanisms of astringency of low-molecular weight phenolic acids

# CHAPTER 3 - CHARACTERIZATION OF SENSORY PROPERTIES OF PHENOLIC ACIDS COMMONLY FOUND IN WHOLE GRAINS

## 3.1 Introduction

Phenolic compounds are ubiquitous in nature and have been shown to contribute to bitterness and astringency in foods and beverages. For instance, red wine is well known for the astringency imparted by tannins, which are large molecular weight polyphenols (Robichaud & Noble, 1990). There are however, many other phenolic compounds that we know less about from a sensory perspective and which present in a wide range of food products. In general, there is a trend toward large molecular weight polyphenolic species being more astringent and less bitter than their monomers (Robichaud and Noble, 1990; Peleg et al., 1999). There is also evidence to show that even small structural differences amongst of phenolic compounds can infer differences in the perceived intensities of their attributes, making it difficult to translate what we know about the sensory properties of one phenolic compound to others (Peleg &Noble, 1999; Lesschaeve & Noble, 2005). Research must be conducted on individual phenolic acids to understand their individual sensory profiles.

Differences amongst individual traits are thought to affect sensory perceptions. One of these traits is salivary flow, measured as the amount of saliva generated over a period of time. It is thought that individuals with greater salivary flow have higher amounts of salivary proteins available at a particular time. Although the mechanisms of astringency are not fully understood, common hypotheses suggest that decreased astringency perception in these individuals may be due to higher levels of salivary protein that prevent astringents from interacting with the oral

mucosa, which otherwise might lead to astringency development , or because the increased amounts of protein left after interactions with astringents are able to provide a greater amount of lubrication, protecting against astringency. Another trait that is known to influence sensory perception is "PROP status" (i.e. sensitivity to the bitterness elicted by 6-n-propylthiouracil or PROP). Individuals can be classified as non-, medium and super tasters based on their evaluation of the intensity of PROP and saline solutions, recorded on an LMS scale (Bartoshuk, 1980).

The objectives of the first phase of this research were to define the sensory characteristics of phenolic acids and compare those profiles to a large polyphenol, tannic acid. As well, this research will be used to determine if trends exist with respect to differences in intensity of bitterness and astringency with increasing concentration of the lower molecular weight compounds. Finally, this research will investigate whether individual characteristics of salivary flow and PROP status are associated with sensory perception of phenolic acids.

## 3.2 Methods

### 3.2.1 Materials

Equimolar aqueous solutions of phenolic acids ferulic acid (4-hydroxy-3methoxycinnamic acid), vanillic acid (4-hydroxy-methoxybenzioc acid), and propyl gallate (Sigma Aldrich, Oakville, ON) were evaluated at 1mM and 15mM (high and low) concentrations. Aqueous solutions of tannic acid were only evaluated at 1mM due to the extreme intensity of taste at 15mM which was found unsuitable to serve to the sensory panel. The 1mM solutions were selected to evaluate the samples at low concentration, and were above the recognition thresholds reported by Maga and Lorenz (1973), and Armbrister (1995). Filtered tap water (Brita) from Guelph, ON was used for preparing the standard and sample solutions, and for rinsing. Standards used as the 'high' references during sensory testing for the attributes sour,

bitter and astringent were citric acid (1.5g/l), caffeine (0.75g/l), and alum (2g/l) respectively (Sigma-Aldrich, Oakville, ON)). Alum was used as a reference standard based on the recommendation of Lee & Lawless (1990).

### 3.2.2 Sensory testing

Ethics approval for this study was obtained from the University of Guelph Research Ethics Board, (REB#10SE08) and all participants provided written informed consent. Eleven healthy, non-smoking individuals were recruited to participate in a trained panel to evaluate the phenolic solutions for the attributes of sourness, bitterness and astringency. Panelists were recruited from Guelph and surrounding area. Training was completed over 10 1-hour sessions. During that time panelists were trained to evaluate the sensory properties of the samples using standards outlined in section 2.1 and were familiarized with the magnitude estimation scale to be used during data collection.

All sample and standard solutions were prepared daily, 1 hour before testing. Samples of 10 mL were served at room temperature, in lidded 1oz plastic cups (SOLO, City) coded with 3-digit random codes. Samples were served on trays with unsalted crackers, filtered water for rinsing, and a 0.4% pectin solution (Workstead Industries, Greenfield, MA) to reduce carry-over effects in astringency evaluation between samples.

Testing took place over three consecutive days, with one 1-hour session per day. Evaluations were conducted in isolated booths illuminated with red light to mask any perceivable differences in colour amongst the samples. Panelists wore nose plugs to mask any perceivable odours in the solutions. Responses were collected using computerized data collection software (Compusense *Five* © 2008, Compusense, Guelph, ON). Fixed modulus magnitude estimation

scaling was used to measure attribute intensities. For this, panelists first tasted the standard for each attribute described in Section 3. 2.1. Each standard was given a defined intensity value of 10. After tasting the standard, each test sample was tasted in a random order and an intensity value was assigned in comparison to the standard. For example, if the standard was given a rating of 10, and the sample was half as intense as the standard, the sample would get a rating of 5. Evaluations of each attribute were conducted in the order of sour, bitter, astringent. Within each attribute, sample presentation was randomized. Panelists cleansed their palate with unsalted crackers and water and rinsed with the pectin solution between samples.

### 3.2.3 Data Analysis

In preparation for analysis, all responses were multiplied by a factor of ten and zeros were given a value of one, as per Butler et al. (1987). For each sensory property, data were then logged and analyzed for significant differences among samples using ANOVA and Tukey's HSD using SAS 9.2., p-value = 0.05.

### 3.3.3 Panelist characterization

Salivary flow and PROP status were determined for each panelist. Salivary flow was determined using the methods of Horne et al (2002). Briefly, panelists were asked to chew on a pre-weighed piece of parafilm for 1 minute while continuously expectorating into a sample cup, also pre-weighed. At the end of the 1 minute chew time, the parafilm was also spit into the cup. The weight of the expectorated saliva, cup and parafilm was recorded and the difference in weight was used as a measure of salivary flow per minute.

PROP status was determined using the one solution Prop test developed by Tepper et al., (2001). For this test, panelists were given solutions of 0.32mmol/L 6-n-propylthiouracil (Sigma)

and 0.1mol/L NaCl and were asked to rate the intensity of the solutions relative to the "strongest imaginable" taste on a 10cm labelled magnitude scale (LMS). This scale is a quasi-logarithmic scale which contains labels "equivalent to magnitude estimation".

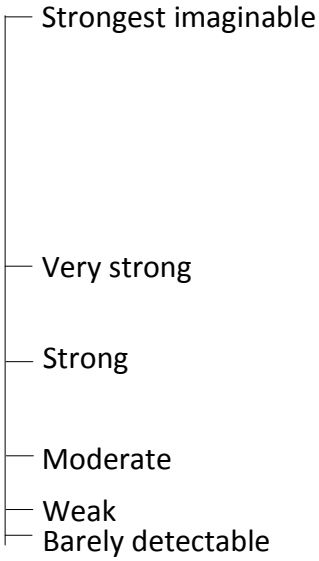


**Figure 3-1: Labelled magnitude scale (LMS) (not to scale)**

After completion of the evaluations, subjects were classified as non-tasters, medium tasters and super tasters. Those who rated the intensity of prop to be "moderate" or lower (1.02cm) on the LMS were classified as non-tasters. Any individual who classified NaCl as higher in intensity than PROP were also classified as non-tasters. Individuals were rated prop to be "very strong" or higher (1.69cm) were classified as supertasters. Individuals with scores between "moderate" and "very strong" were medium tasters (Tepper et al, 2001). A Pearson's product moment correlation

coefficient was determined to look at the relations between salivary flow rate, PROP ratings and all sensory attributes.

### 3.4 Results and Discussion

#### 3.4.1 Sample Characteristics

Significant differences in bitterness were observed amongst the samples . At 1mM, tannic acid was significantly more bitter than propyl gallate which was significantly more bitter than the two phenolic acids studied. Significant differences were also observed for astringency with tannic acid being perceived to be significantly more astringent than propyl gallate and the phenolic acids. From a sourness perspective, tannic acid was significantly more sour than ferulic acid and propyl gallate. Vanillic acid was not different to any of the other phenolic compounds studied (Figure 3.2).

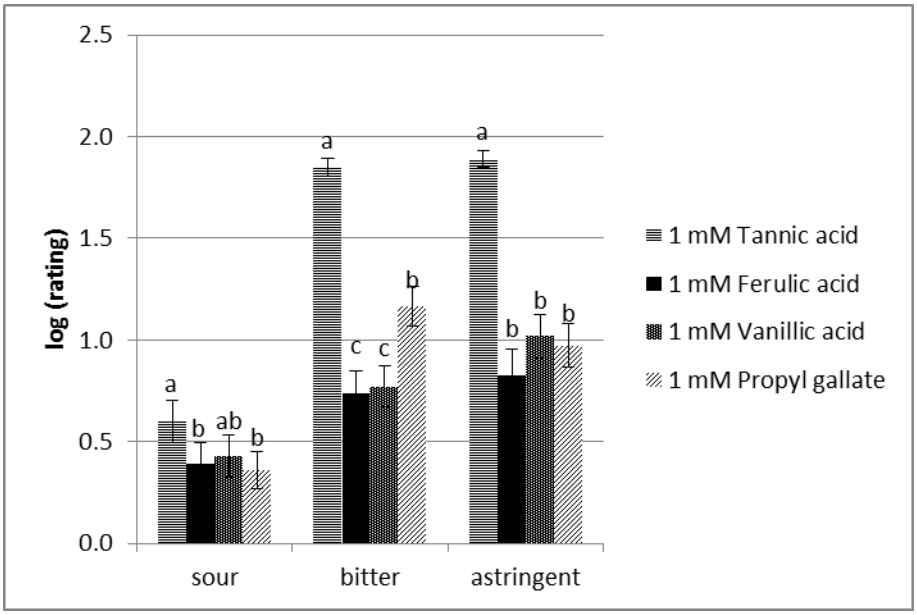


**Figure 3-2: Log ratings for sourness, bitterness, astringency of phenolic solutions at 1 mmol concentration**

The phenolic acids were also tested at 15mM. This concentration was used as it was the highest concentration at which ferulic acid was found to be soluble in the distilled water. This amount also allowed us to compare the results of this study to those of a previous study on the sensory properties of benzoic acid derivatives in which aqueous solutions were evaluated at 17mM (Peleg & Noble, 1995).

At the higher concentration, the phenolic acids were significantly more sour and more astringent than propyl gallate, while propyl gallate was significantly more bitter than the phenolic acids (Figure 3.3).

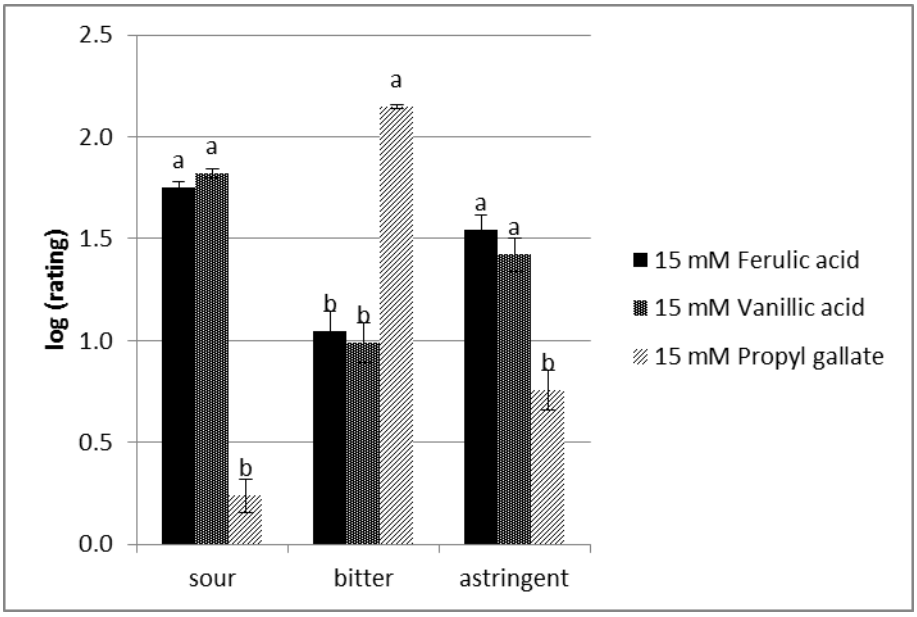


**Figure 3-3: Log rating of intensity of sourness, bitterness, astringency of phenolic acids at 15mmol (Values of bars with the same letter above them are not significantly different for the given attribute at $p \leq 0.05$)**

Each of the compounds studied elicited multiple sensations. Similar to what was observed in Peleg's work (1990), this caused the solutions to be perceived by the panelists to be mixtures of many different tastants. For the two phenolic acids, intensity of sourness, bitterness and astringency increased with increasing molarity (Figure 3-4). However, with propyl gallate,

bitterness increased with increasing molarity while sourness and astringency decreased with increasing molarity. It may be that suppression occurred in which the heightened intensity of one sensation, in this case bitterness, minimizes the perception of other sensations. Although there is little research demonstrating suppression of perception of attributes within solutions of single compounds that elicit multiple sensations, the effect of suppression has been shown in mixtures of solutions of caffeine and astringents in work by Brennan et al, (2001). Similar effects have been reported by Keast and Breslin (2002) in which, at high intensities of bitterness, sourness is suppressed.

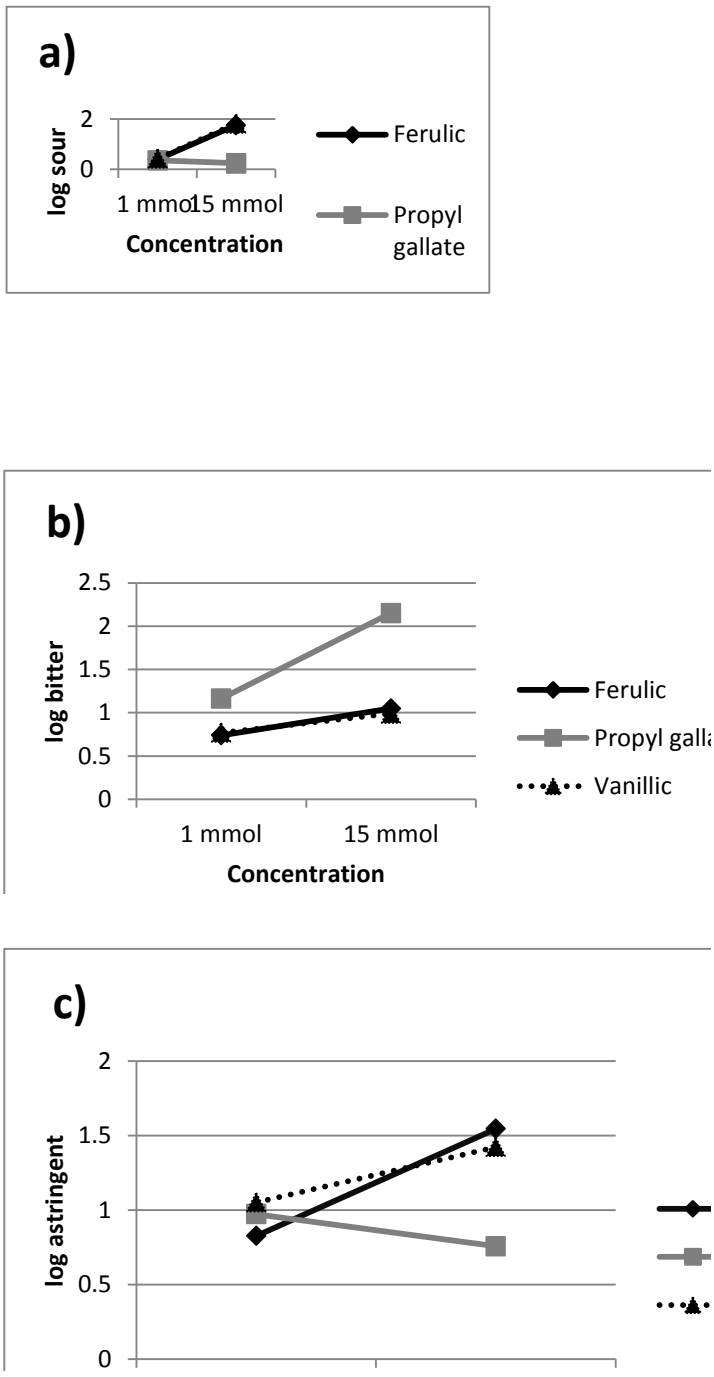


**Figure 3-4: Sensory characteristics with increasing molarity plotted by phenolic solution. a) log sour b) log bitter c) log astringent**

### 3.4.2 Panelist Characteristics

Amongst panelists, there was variability in salivary flow. Salivary flow rates varied from 0.6 to 4.2 grams per minute. This agrees with the literature that salivary flow rates can be highly variable amongst individuals (Dawes, 2004). When flow rates were correlated with bitterness, astringency, and sourness intensity perceptions, no significant relations were observed.

**Table 3-1: PROP status, mean response and standard deviation using prop one-solution test**

| Judge | Avg response | Std deviations | Status |
|---|---|---|---|
| 1 | 0.8 | .02 | Non |
| 2 | 1.1 | .009 | Non |
| 3 | 1.3 | 026 | Med |
| 4 | 1.6 | .02 | Med |
| 5 | 1.6 | .04 | Med |
| 6 | 1.7 | .04 | Super |
| 7 | 1.7 | 0.004 | Super |
| 8 | 1.8 | 0.08 | Super |
| 9 | 1.8 | 0.04 | Super |
| 10 | 1.8 | .05 | Super |
| 11 | 1.9 | .07 | Super |

There was variability amongst panelists for rating of PROP solution intensity. Within the population of panelists, there were 2 non-tasters, 3 medium tasters and 6 super tasters (Table 3.1). When PROP intensity ratings were correlated with bitterness intensity ratings, no significant relations were observed ($R= 0.01$; $p=0.77$). Bartoshuk (1993) has reported that individuals who are super tasters are more sensitive to bitterness, however, this was not observed within this population. This may be because evaluation of bitterness of PROP does not necessarily apply to evaluation of all bitter compounds. PROP intensity ratings were also not

significantly related to astringency or sourness (R=-0.20; p=.002, R=-0.19; p=0.003). Similar results were noted by Thorngate and Noble (1995) and Sowalski & Noble (1998), where *n*-Propylthiouracil status was demonstrated to have no effect on perception of either bitterness or astringency of flavanols, or the sourness or astringency of organic acids, respectively.

### 3. 5 Conclusion

Phenolic acids exhibit sensory properties of sourness, bitterness and astringency There was a trend of increasing intensity for all attributes with increasing molarity for the phenolic acids. A decrease in sourness and astringency with increasing molarity was seen for propyl gallate, likely due to taste suppression due to the high intensity of bitterness at high molarity. This also demonstrates that different compounds increase or decrease in intensity with increasing or decreasing molarity at different rates. Given that these compounds elicit tastes at different rates, and that certain taste sensations such as bitterness and intensity are known to increase with repeated consumption, it is essential to investigate the development of these tastes over time and with repeated exposures. Sensory perception of sourness, bitterness and astringency of phenolic acids does not appear to be related to salivary flow and PROP status.

# CHAPTER 4 - TIME INTENSITY SCORES FOR BITTERNESS, SOURNESS AND ASTRINGENCY OF PHENOLIC ACIDS

## 4.1 Introduction

The few studies that have considered the sensory profiles of isolated phenolic acids have revealed that they can elicit a complex mixture of tastes and oral sensations over time, and that the structural differences amongst the phenolic acids can confer differences in their sensory properties (Dadic and Belleau, 1973; Meilgaard, 1975; Belitz and Weiser, 1985; Peleg and Noble, 1995).

In Chapter 3, using a descriptive panel, both ferulic acid (a hydroxcinamic acid) and vanillic acid (a hydroxybenzoic acid) were shown to elicit sensations of sourness, bitterness and astringency. However, taste and oral sensations elicited by foods and beverages can change in intensity over time as food or beverages are manipulated, mixed with saliva and interact with oral surfaces (Lee & Pangborn, 1986). Because single scalar evaluations require judges to average their responses to arrive at a single intensity value, important information such as the rate of onset of stimulation, time of maximum intensity, and total duration of sensation, are lost. Time- intensity (TI) methods, in which judges make continuous evaluations of intensity at regularly timed intervals, generate time-intensity profiles that can expand our understanding of the perception of taste and oral sensations elicited during and after a stimulus has left the oral cavity. Time-intensity measurements are especially useful in the study of astringent compounds as this oral sensation is slower to develop and can linger for longer than other taste modalities (Guinard et al, 1986; Lawless et al, 1994).

Additionally, eating and drinking are multiphase processes in which foods are consumed bite after bite or sip after sip, in which food components come in contact with oral surfaces

repeatedly over a period of time. This is important to consider as it has been demonstrated that astringency and bitterness intensity build when multiple samples are tasted (Lesschaeve & Noble, 2005). Although phenolic acids may exist in small quantities within most foods, the perceived astringency and bitterness imparted by these phenolic acids may increase to unacceptable - or more favourable, depending on the food product - levels with repeated consumption.

The objective of this study was to investigate the sensory perception of ferulic acid and vanillic acid using a trained panel and time-intensity methods to gain an understanding of the complex mixture of tastes and oral sensations elicited by these simple phenolic compounds while in mouth and after expectoration. The effect of repeated consumption of these acids on the perception of sourness, bitterness and astringency was also investigated to further understand how they could impact the sensory characteristics of foods as they are normally consumed. Additionally, comparisons between ferulic and vanillic acids which share similar structure with the exception of a vinyl addition in the side chain may lead to further understanding of relationships between structure and sensory properties of phenolic acids.

## 4.2 Methods

### 4.2.1 Samples and Standards

Two experiments were conducted, each studying ferulic and vanillic acids. For the first experiment, ferulic acid (4-hydroxy-3-methoxycinnamic acid) and vanillic acid (4-hydroxy-3-methoxybenzoic acid) (Sigma-Aldrich, Oakville, ON) were evaluated in aqueous solutions at 6mM, and 12mM concentrations, while solutions of 1mM, 6mM and 12mM were evaluated in the second experiment. The 1mM concentration was added in the second experiment to further understand how attributes of low concentrations of the acids might change with repeated

consumption. The concentrations used were selected based on the solubility of the acids and determined thresholds, and compared to quantities in which they exist within food products. The total amount of ferulic acid in each 10ml sample of 6mM ferulic acid is approximately equal to the total ferulic acid content (bound and free) expected in the crumb portion of one whole slice of bread (28g) prepared using the AACC method from wheat flour (averaged across red and white wheat, fine and course particle size) as determined by Challacombe et al (2011). The quantity of ferulic acid present in a 10ml sample of 1mM is approximately that found in one quarter slice of the same bread. Distilled water (Fernbrook, ON) was used for preparing the standard and sample solutions, and for rinsing.

Standards used as the 'high' references for the attributes sour, bitter and astringent were citric acid (1.5g/l), caffeine (0.75g/l), and alum (2g/l), respectively (Sigma-Aldrich (Oakville, ON). Distilled water was used as a reference for absence of sensation ('none').

All solutions were prepared daily, 1 hour before testing and were stored at room temperature (25℃) wrapped in foil. Samples of 15ml were served at room temperature (25℃) in lidded 1oz plastic cups coded with randomized 3-digit codes. Samples were served on trays with unsalted crackers, distilled water and pectin (0.004g/l) solution for rinsing.

### 4.2.2 Design

A randomized complete block design was used to evaluate all samples. For experiment 1 (single-sip TI), samples were evaluated over 3 days with all 4 samples (12mM, 6mM solutions of both ferulic and vanillic acid) presented to each judge per session. For experiment 2 (sequential-sipping TI), the 6 samples were evaluated over 6 days, with 3 samples presented to each judge per session to prevent fatigue. All samples were evaluated by each judge in triplicate in both experiments.

### 4.2.3 Sensory Evaluation

Ethics approval for this study was obtained from the University of Guelph Research Ethics Board (REB#09MA10). Participants (5 male, 4 female) were recruited from Guelph, Ontario and the surrounding area, all of whom had previous training in sensory evaluation. The same participants were recruited to take part in both experiments for consistency and to minimize training time. Panelists were familiarized with the attributes and time-intensity evaluation methods prior to the formal data collection over ten 1 hour training sessions.

### 4.2.4 Data Collection

Sessions were conducted daily in isolated booths, illuminated with red light to mask any perceivable differences in colour amongst samples. Additionally, panellists wore nose plugs to mask any perceivable odours. Responses were collected using computerized data collection software (Compusense *Five* © 2008, Compusense, Guelph, ON) connected to individual monitors within the booths. Time-intensity responses were indicated on an unstructured 100 mm line scale (presented on computer screen) anchored with 'none' and 'high.' Responses were collected at 1.0 second intervals using lateral movements of the computer mouse along the line scale. Further information outlining the procedures used for each of the sensory panels is as follows:

#### *Experiment 1: Single sip time-intensity*

At each session, panelists were presented with all four samples (6mM, 12 mM ferulic acid, 6mM, 12mM vanillic acid) in randomized order. Panelists were instructed to taste the distilled water ('none') and high reference standards before beginning the test to calibrate the intensity scale, and then to rinse their mouths with cracker and distilled water and wait 3 minutes before beginning the test to minimize any carryover effects from the standards. When ready to

begin, panelists took the entire 15ml sample in their mouth and simultaneously clicked start. Judges were instructed to continuously evaluate the intensity of the sample while gently moving their tongue around their mouth to ensure the sample solution contacted all oral surfaces. At 10 seconds, a prompt on the computer screen instructed them to expectorate the sample, and panelists continued to rate the attribute until it had dissipated. A two-minute break was taken between evaluating each attribute. Sourness was always evaluated first, followed by bitterness, and then astringency. A five minute break was taken between samples to reduce carry over effect and fatigue.

***Experiment 2: Sequential sip time-intensity***

At each session, judges were presented with a total of three trays of samples, one tray at a time. Each tray contained 12 cups, divided into three rows of four cups containing 15ml of the same sample. All of the samples contained on a tray were the same, and each tray had a different sample. The first row (of 4 cups) was used to evaluate sourness, the second to evaluate bitterness and the third to evaluate astringency. Cups within each row were coded with different random numbers so that judges were unaware that samples within a row were identical (Figure 4.1).

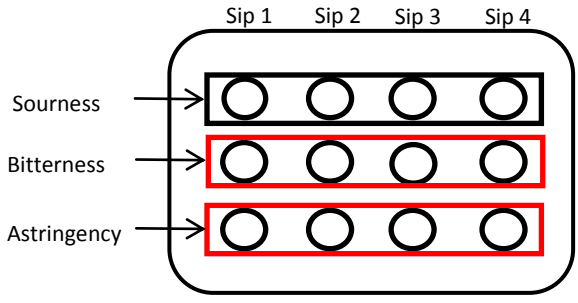


**Figure 4-1: Sample arrangement and sampling order**

As in the first experiment, at each session, panelists were instructed to taste the distilled water ('none') and high reference standards before beginning the test to calibrate the intensity scale and then to rinse their mouths with cracker and distilled water and wait 3 minutes before beginning the test to minimize any carryover effects from the standards. When ready to begin, panelists took the entire contents (15ml) of the first cup of the first row in their mouth (operationalized as "sip 1") and initiated recording their response via a computer mouse control. At 10 s, judges were prompted to expectorate and continue recording. Ten seconds later, at 20 s, judges were prompted to take the next cup in the same row (sip 2), and then to expectorate 10 s later. This continued for four sips. After expectoration of the fourth sip, judges continued rating the attribute until the sensation had disappeared. Sourness was always evaluated with the first row of sample, bitterness with the second row, and astringency with the third. A three minute break was taken between evaluation of each row in which judges were instructed to rinse with their mouths with cracker, water, and pectin rinse to minimize any carryover effects. A five minute break was taken between evaluations of trays.

### 4.2.5 Data Analysis

For experiment 1, three parameters were extracted from each of the T-I curves: maximum intensity (IMAX), time to maximum intensity (TMAX) and total duration of response (DUR). For each sensory attribute, the T-I parameters were analysed by 4-way analysis of variance (ANOVA) with interactions. Significant differences amongst means were determined using Tukey's honestly significant difference (HSD) ($p \leq 0.05$). Independent variables examined included judge, rep, acid and molarity. All statistical analysis was completed using SAS version 9.2 (SAS Institute Inc., Cary, NC).

To evaluate the information from the sequential- sip TI curves, parameters of maximum

intensity for each sip ($IMAX_n$), and time to maximum intensity for each sip ($TMAX_n$) were extracted from the data using excel command. The rate of onset ($ROI_n$) for each sip was calculated by dividing the difference between initial and maximum intensity by the time to reach max intensity for each sip [($IMAX_n$ – $IINI_n$) / $TMAX_n$]. To show the increase in attribute intensity produced by each exposure, ($IMAX_{(n+1)}$ – $IMAX_n$) was also calculated for each sip using excel command ("REAL" in ANOVA tables). The parameters extracted from the TI curves are listed in Table 4.1. The parameters were analyzed by 5-way ANOVA with interactions. Significant differences amongst means were determined using Tukey's HSD ($p< 0.05$). Independent variables included judge, rep, acid, molarity and sip.

**Table 4-1: Time-Intensity (TI) parameters extracted from repeated sip TI curves**

| | |
|---|---|
| $IMAX_n$ | Maximum intensity after each sip |
| $TMAX_n$ | Time (s) to maximum intensity after each sip |
| $IMAX_{(n+1)}$ – $IMAX_n$ | Intensity increase with each sip after the first ingestion |
| $ROI_n$ | The rate of onset for each sip: ($IMAX_n$ – $IINI_n$) / $TMAX_n$ |

## 4.3 Results and Discussion

### 4.3.1 Single-sip TI parameters (experiment 1)

The effect of acid type and molarity of solution on maximum perceived intensity, time to maximum intensity and duration of sensation for sourness, bitterness and astringency are shown in Tables 4.2 and 4.3, respectively. ANOVA results are shown in Appendix 2.1. Significant differences were perceived between acids and between molarities for maximum attribute intensities.

In agreement with other sensory studies on aqueous solutions of selected hydroxybenzoic acid derivatives (Peleg & Noble, 1995), both phenolic acids were perceived to be sour, bitter and astringent. Although structurally very similar, the sensory properties of vanillic and ferulic acid differed quantitatively, as illustrated by comparison of the maximum intensities (IMAX) for sourness and bitterness - vanillic acid was perceived to be significantly more sour than ferulic acid, and ferulic acid was perceived to be significantly more bitter than ferulic acid.

The difference in sourness intensity between the acids at equal molarity may be explained by the differences in pH of the solutions, as it has been shown that the perceived sourness intensity of acids is negatively correlated with pH (Sowalski & Noble, 1998). At both molarities, the pH of the vanillic acid solution was lower compared to the ferulic acid solution. For both acids, sourness intensity increased with increasing molarity (Table 4.3) and the corresponding decrease in pH of the solutions. Compared to the other attributes bitterness and astringency, the time to reach maximum sourness intensity was less. This is likely because the influx of ions into taste receptor cells is a faster process than the binding mechanism required for bitter taste transduction or protein interactions proposed for astringency formation.

The difference in bitterness intensities between the solutions of vanillic and ferulic acid is

not easily explained as bitterness is elicited by a wide range of molecules, with varying sizes and functional groups, wherein even small structural changes can render a bitter molecule non-bitter (Rouseff, 1990). In the case of the phenolic acids studied here, the difference in bitterness intensity may be explained by the slight structural difference - the vinyl component of ferulic acid. Since bitterness is detected via G-protein-coupled receptors located on the surface of taste cells, it may be that this slight structural difference results in a greater ability of ferulic acid to interact with the bitter receptor, leading to a higher perceived bitterness intensity. As expected, perceived bitterness intensity increased with increasing molarity for both acids.

There were no significant differences in perceived astringency between vanillic and ferulic acid at equal molar concentrations. Although the mechanism of astringency of acids is unclear, it has been shown that acid solutions do elicit astringency (Lee and Lawless, 1991; Rubico and McDaniel, 1992; Peleg & Noble, 1995) and that the perceived astringency of organic acids is a function of pH, and not of acid concentration *per se* (Lawless et al, 1996; Sowalski & Noble, 1998). It has been suggested that the inverse relationship between pH and astringency may be due to alteration of salivary proteins under conditions of reduced pH, leading to a decrease in salivary viscosity and lubricative properties and increased friction between oral surfaces (Nordbo et al, 1984; Luck et al, 1994). Conditions of reduced pH may also directly affect the proteins of the oral mucosa, leading to increased friction between oral surfaces and increased astringency. More recently, it has been suggested that basal levels of polyphenol normally exist within the oral cavity, and that conditions of decreased pH allow for enhanced interaction of these polyphenols with salivary or mucosal proteins, resulting in increased perceived astringency (Seibert & Chassy, 2003). Regardless, the greater time to reach maximum astringency intensity and longer duration of sensation compared to sourness and bitterness

suggests a slower and more complex mechanism behind astringency formation. The lack of significant differences in astringency intensity between equimolar solutions of ferulic and vanillic acid may be explained by the fact that differences in pH and the size of the samples were not significant enough to lead to differences in pH within the oral cavity when mixed with saliva. However, the perceived astringency of both acids was significantly higher for the solutions of higher molarity (Table 4.3). The pH resulting from the combinations of these amounts of acid and saliva are not known.

**Table 4-2: Effect of acid on sensory properties of model solutions averaged across molarities**

| | **Mean IMAX** | | **Mean TMAX** | | **Mean DUR** | |
|---|---|---|---|---|---|---|
| | Ferulic | Vanillic | Ferulic | Vanillic | Ferulic | Vanillic |
| ***Sourness*** | | | | | | |
| Mean | 33.17b[1] | 48.36a | 11.42a | 10.53a | 23.13b | 25.75a |
| SD | 22.81 | 29.29 | 6.17 | 5.81 | 11.65 | 14.4 |
| ***Bitterness*** | | | | | | |
| Mean | 50.31a | 33.5b | 15.71a | 13.63a | 33.02a | 27.93b |
| SD | 22.61 | 25.92 | 11.04 | 8.05 | 18.62 | 16.56 |
| ***Astringency*** | | | | | | |
| Mean | 46.31a | 42.90a | 16.90a | 16.39a | 31.41a | 32.78a |
| SD | 27.31 | 23.08 | 10.18 | 10.21 | 19.04 | 20.74 |

[1]Means in a row with the same letter are NSD ($p<0.05$), n=9

**Table 4-3: Effect of molarity on sensory properties of model phenolic acid solutions averaged across phenolic acids**

| | Mean IMAX | | Mean TMAX | | Mean DUR | |
|---|---|---|---|---|---|---|
| | 12 mM | 6 mM | 12 mM | 6 mM | 12 mM | 6 mM |
| ***Sourness*** | | | | | | |
| Mean | 55.76a[1] | 25.76b | 12.05a | 9.69b | 28.71a | 14.48b |
| SD | 20.48 | 24.81 | 6.64 | 5.02 | 19.53 | 9.29 |
| ***Bitterness*** | | | | | | |
| Mean | 48.64a | 35.17b | 14.6a | 14.78a | 32.14a | 19.34b |
| SD | 25.52 | 24.16 | 9.47 | 10.01 | 28.83 | 15.94 |
| ***Astringency*** | | | | | | |
| Mean | 50.64a | 38.57b | 17.4a | 15.85a | 34.48a | 29.6b |
| SD | 23.28 | 25.85 | 11.05 | 9.15 | 21.51 | 17.75 |

[1]Means in a row with the same letter are NSD ($p<0.05$), n=42

### 4.3.2 Sequential sip TI parameters (experiment 2)

Figures 4.1, 4.2 and 4.3 show the time-intensity relationship of sourness, bitterness and astringency perception, respectively, over four sequential sips of both vanillic and ferulic acid solutions at 2 molarities. In agreement with the results from experiment 1, vanillic aid was perceived to be significantly more sour than ferulic acid, and ferulic acid was perceived to be significantly more bitter than vanillic acid. As expected, maximum sourness, bitterness and astringency intensities significantly increased with increasing molarity for both acids (Table 4.4). No significant differences between acids were seen in astringency, averaged across molarities (Table 4.5).

**Table 4-4: Effect of molarity on sensory properties of model phenolic acid solutions averaged across phenolic acids and sips**

| | **Mean IMAX** | | | **Mean TMAX** | | | **Mean ROI** | | |
|---|---|---|---|---|---|---|---|---|---|
| | 12mM | 6mM | 1mM | 12mM | 6mM | 1mM | 12mM | 6mM | 1mM |
| ***Sourness*** | | | | | | | | | |
| Mean | 38.85a[1] | 11.25b | 3.88c | 10.04a | 8.21b | 6.34c | 4.35a | 1.47b | 0.81c |
| SD | 21.40 | 10.19 | 5.06 | 3.54 | 4.15 | 4.19 | 2.93 | 1.32 | 1.00 |
| ***Bitterness*** | | | | | | | | | |
| Mean | 21.58a | 14.79b | 9.72c | 11.48a | 10.50b | 10.48b | 1.91a | 1.53b | 1.34c |
| SD | 16.40 | 11.34 | 7.62 | 5.41 | 5.32 | 5.91 | 2.04 | 1.35 | 1.61 |
| ***Astrin*** | | | | | | | | | |
| Mean | 23.69a | 16.84b | 10.51c | 13.94a | 13.01b | 12.23b | 1.88a | 1.43b | 0.96c |
| SD | 14.30 | 13.35 | 8.11 | 4.74 | 5.19 | 5.61 | 1.53 | 1.3 | 0.78 |

[1]Means in a row with the same letter are NSD ($p<0.05$), n=240

**Table 4-5: Effect of acid on sensory properties of model solutions averaged across molarities**

| | Mean IMAX | | Mean TMAX | | Mean ROI | |
|---|---|---|---|---|---|---|
| | Ferulic | Vanillic | Ferulic | Vanillic | Ferulic | Vanillic |
| ***Sourness*** | | | | | | |
| Mean | 17.06a[1] | 18.71b | 8.19a | 8.63a | 2.28a | 2.50b |
| SD | 19.25 | 21.68 | 4.22 | 4.18 | 2.41 | 2.70 |
| ***Bitterness*** | | | | | | |
| Mean | 18.61a | 12.12b | 11.26a | 10.39b | 1.84a | 1.19b |
| SD | 15.53 | 9.40 | 5.46 | 5.63 | 1.85 | 1.14 |
| ***Astringency*** | | | | | | |
| Mean | 17.33a | 16.62a | 13.19a | 12.97a | 1.48a | 1.39a |
| SD | 14.00 | 12.65 | 5.16 | 5.28 | 1.42 | 1.17 |

[1]Means in a row with the same letter are NSD ($p<0.05$), n=360

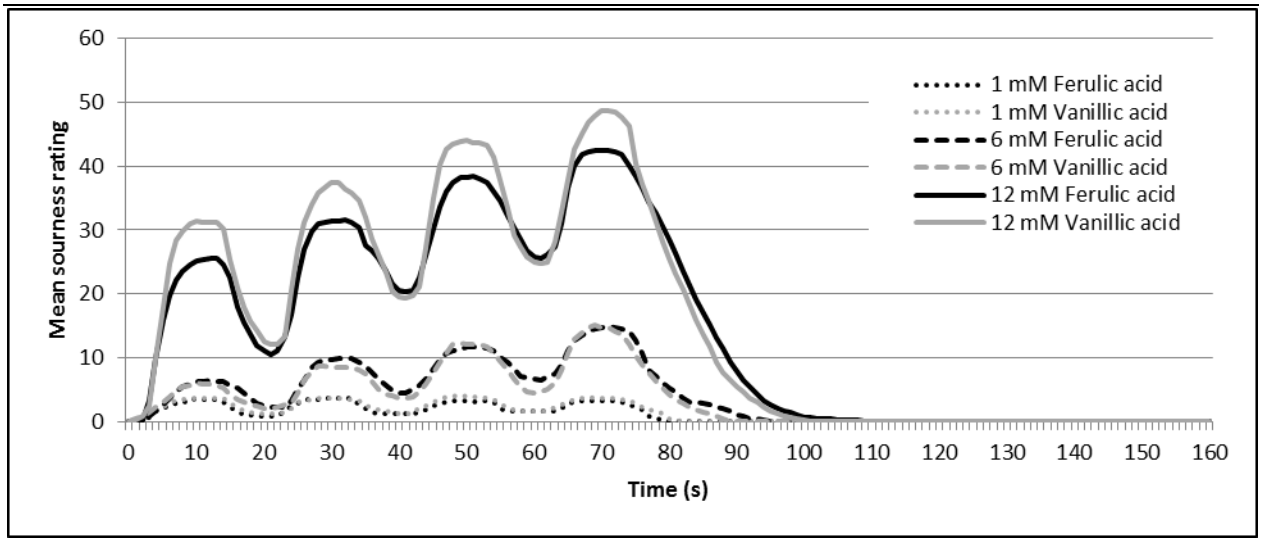


**Figure 4-2: Effect of repeated consumption on perceived sourness intensity of phenolic acid solutions at 1mM, 6mM, 12mM, measured across four sips**

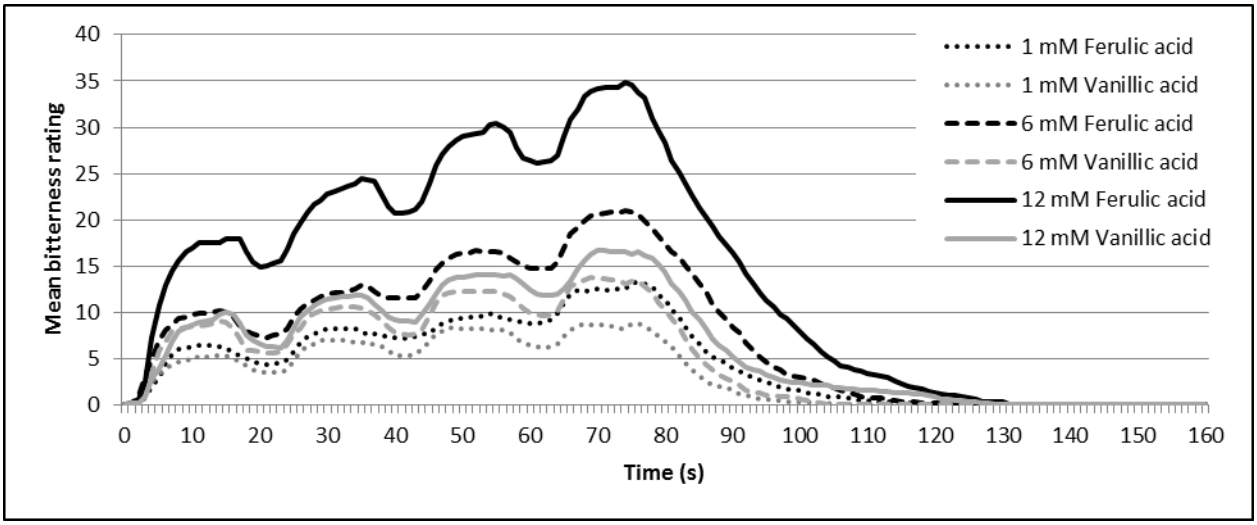


**Figure 4-3: Effect of repeated consumption on perceived bitterness intensity of phenolic acid solutions at 1mM, 6mM, 12mM, measured across 4 sips**

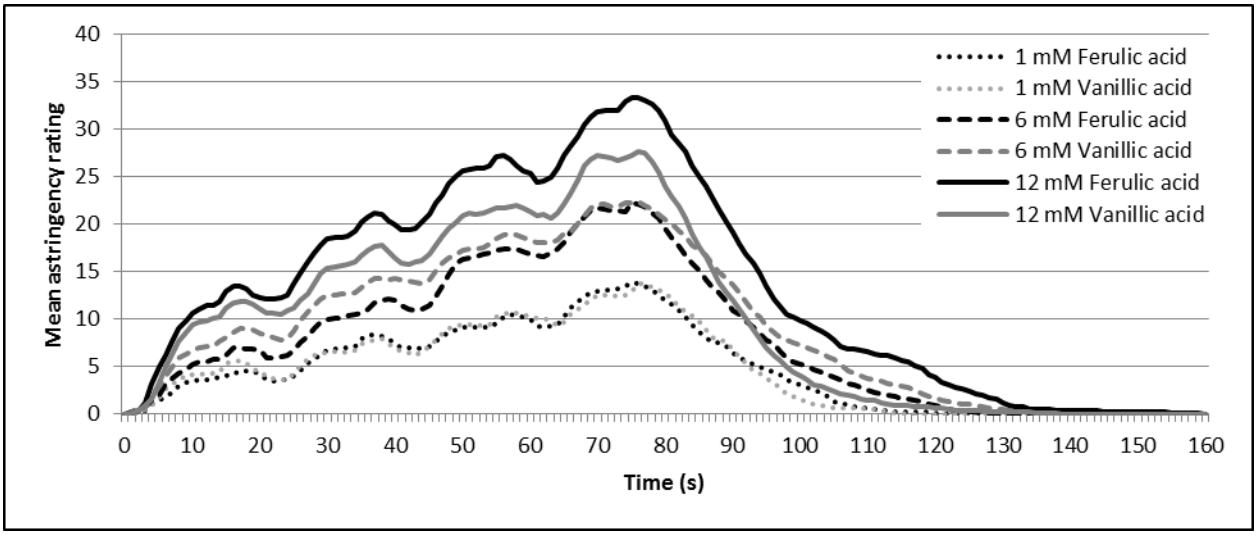


**Figure 4-4: Effect of repeated consumption on perceived astringency intensity of phenolic acid solutions at 1mM, 6mM, 12mM, measured across 4 sips**

Table 4.6 shows the differences in maximum perceived intensity (IMAX), time to maximum intensity (TMAX), rate of onset (ROI), and difference in maximum intensities between successive sips (REAL) for sourness, bitterness and astringency, amongst exposures (sips).

**Table 4-6: Effect of repeated consumption on attribute parameters averaged across molarities and acids**

| | Sip 1 | Sip 2 | Sip 3 | Sip 4 |
|---|---|---|---|---|
| ***Sourness*** | | | | |
| IMAX | 13.3a[1] | 16.5b | 19.6c | 22.0c |
| SE | 1.34 | 1.24 | 1.47 | 1.70 |
| TMAX | 9.2a | 8.8ab | 8.1bc | 7.5c |
| SE | 0.33 | 0.36 | 0.34 | 0.29 |
| ROI | 1.8a | 2.2b | 2.6c | 2.7c |
| SE | 0.18 | 0.20 | 0.23 | 0.20 |
| REAL | - | 3.2a | 3.0a | 2.3a |
| SE | - | 0.48 | 0.48 | 0.18 |
| ***Bitterness*** | | | | |
| IMAX | 12.1a | 14.6b | 17.2c | 20.18d |
| SE | 0.86 | 0.99 | 1.23 | 1.43 |
| TMAX | 10.6a | 10.4a | 10.2a | 9.5a |
| SE | 0.36 | 0.45 | 0.47 | 0.43 |
| ROI | 1.5a | 1.4a | 1.5a | 2.1b |
| SE | 0.14 | 0.10 | 0.12 | 0.18 |
| REAL | - | 2.5a | 2.5a | 2.9a |
| SE | - | 0.04 | 0.04 | 0.40 |
| ***Astringency*** | | | | |
| IMAX | 9.5a | 14.6b | 19.4c | 24.3d |
| SE | 0.56 | 0.77 | 0.97 | 1.21 |
| TMAX | 13.7a | 14.0a | 12.9a | 11.6b |
| SE | 0.37 | 0.36 | 0.42 | 0.41 |
| ROI | 0.8a | 1.1b | 1.5c | 2.2d |
| SE | 0.05 | 0.06 | 0.09 | 0.14 |
| REAL | - | 5.1a | 4.7a | 4.9a |
| SE | - | 0.36 | 0.37 | 0.38 |

[1]Means in a row with the same letter are NSD ($p<0.05$), n=179

Averaged across acids, maximum perceived sourness intensity increased significantly from sip1 (IMAX1) to sip 3 (IMAX3), then plateaued at sip 4 (IMAX4). This plateau may be explained by adaptation - a decrease or change in sensitivity to a given stimulus under conditions of continuous exposure to that stimulus or a similar one (Meilgaard, Civille and Carr, 1999). It has been shown that if a taste stimulus can be maintained, most taste sensations will disappear in a short period of time as we become adjusted to the ambient level of stimulation (Lawless & Heymann, 2010). The rate of onset of sourness increased significantly from sip 1 to 4 as the time to reach maximum intensity decreased significantly from sip 1 to sip 4. Although not significant, the difference in maximum sourness intensities for successive sips decreased from sip 2 to 4.

Averaged across acids, maximum perceived bitterness intensity increased significantly from sip1 (IMAX1) to sip 4 (IMAX4). The build-up of bitter intensity with sequential sips has been suggested in the literature (Lesschaeve & Noble, 2005), and is likely due to the length of time that bitter compounds bind to bitter receptors. With sequential sips, more bitter molecules bind to receptors while some bitter molecules from the previous sip remain bound, leading to increased bitterness perception. This is also reflected in the in the greater difference (increase) in maximum intensity with successive sips (REAL), and suggests that phenolic acids may remain bound to bitter receptors for 10 seconds or greater (the time between successive sips). Increasing the time between successive sips has been shown reduce the build-up of bitterness.

Astringency IMAX increased significantly with successive sips, as did the rate of onset (ROI) of astringency as the time to reach maximum intensity was significantly reduced in the last sip compared to the first. This is in agreement with the literature in which astringency was shown to have a carry-over effect; building upon repeated ingestion (Arnold et al, 1983; Guinard et al, 1986; Noble, 2002). If astringency of phenolic acids is due to the alteration of salivary proteins

with reduced pH, as suggested in the literature for organic acids, the observed results would be expected as with each sequential sip, more salivary proteins are altered, resulting in reduced lubricating ability and greater friction between oral surfaces, and an increase in perceived astringency. More recently it has been suggested that the astringency of polyphenols may be due to direct interaction with the proteins of the oral mucosa after the mobile phase of saliva (not adhered to the oral mucosa) has been washed away. In the case of phenolic acids, it may also be that with each successive sip and expectoration, more proteins of the oral mucosa are exposed to conditions of reduced pH, with less saliva available to buffer the incoming acidic sample, and thus greater protein denaturation or alteration occurs, leading to increased perceived astringency with each successive sip. The difference (increase) in maximum intensity of astringency with each sip was greater than that for sourness and bitterness, demonstrating the greater effect that repeated exposures has on the perceived astringency intensity of phenolic acid solutions.

### 4.4 Conclusions

To date, there is relatively little information available concerning the sensory attributes of low molecular weight phenolic acids. There is also little knowledge concerning the time course of single sipped or repeatedly sampled stimuli in model systems such as aqueous solutions, using current time-intensity methods, and few comparison of these profiles amongst different compounds have been made.

This work shows that phenolic acids are perceived as sour bitter and astringent, and that differences in intensity of these attributes can be elicited by compounds of similar structure, as demonstrated by time-intensity procedures. In both experiments 1 and 2, vanillic acid was perceived to be more sour than ferulic acid, while ferulic acid was perceived to be more bitter.

No differences in astringency were observed between the acids. For both acids, the intensity of all attributes increased with increasing concentration. With repeated exposure, maximum intensity of astringency and bitterness continued to increase significantly, while sourness intensity plateaued. This research demonstrates that even small quantities of phenolic acids can be perceived as increasingly bitter and astringent with repeated exposures. This research is novel is that no other literature has examined the sensory perception of these acids over time or with repeated exposures.

# CHAPTER 5 – *SALIVARY PROTEIN – PHENOLIC ACID INTERACTIONS*

## 5.1 Introduction

Astringency is described as drying, roughening and constricting within the oral cavity. In general, these tactile sensations have been thought to be the result of the interactions of astringent compounds with salivary proteins. The human sensory analysis of astringent compounds requiring trained panelists can be time consuming, costly and challenging. Consequently, protein/polyphenol binding assays have been proposed as rapid tools for the quantification and evaluation of astringent compounds in foods (Kallithraka et al., 2000; Monteleone et al., 2004; Wilson, 2005). These assays are based on the widely studied theory of astringency development for large polyphenols (tannins) found in grape and wine extracts (Monteleone et al, 2004). In general, with regard to high-molecular weight polyphenols, astringency is thought to be the result of the binding and/or precipitation of salivary proteins, particularly salivary proline-rich proteins (PRPs). It is thought that the greater the protein binding activity of a compound, the greater the predicted astringency of the solution. However, sensory studies have demonstrated that astringency can be elicited by a variety of compounds of diverse chemical structures, leading to different hypotheses of the mechanisms behind astringency development (Haslam and Lilley, 1988; Green, 1993; Clifford, 1998; Gawel et al, 2001). Comparisons of astringent-salivary protein interactions amongst different classes of astringents using protein binding assays have revealed that the precipitation of PRPs believed to be responsible for the astringency development of high-molecular weight polyphenols is not necessary for the development of astringency of other astringents such as acids (see section 2.4.3) (Lee & Vickers, 2010). Furthermore, they showed that both alum and acid precipitated

large-molecular weight salivary mucins, suggesting different mechanisms behind astringency development amongst different classes of astringents. Other research comparing the binding affinity of different astringents to their sensory thresholds has suggested that there is no direct relationship between oral astringency and human salivary protein binding (Schwartz & Hoffman, 2008). These authors showed that many astringent compounds exhibiting low astringency thresholds did not demonstrate any salivary protein binding activity at all, while other astringents with high sensory thresholds showed high salivary protein binding activity (Schwartz & Hoffman, 2008).

The mechanism behind astringency development of low-molecular weight phenolic acids has yet to be elucidated. All phenolic acids possess an aromatic ring, which has been shown be responsible for the hydrophobic associations of polyphenols with salivary PRPs, while the number of phenolic hydroxyl groups varies amonst the acids. The phenolic hydroxyl groups are thought to stabilize protein bindng through hydrogen bonds (see section 2.4.3). Stronger bonding occurs in compounds with two hydroxy phenolic groups (such as protocatechuic and caffeic acid) which can bind protein via a bidentate hydrogen bond than those with an isolated hydroxyphenolic group (McManus et al., 1981). Studies of the interaction of low molecular weight phenolics with bovine serum albumin (BSA) have shown that certain simple phenols (pyrogallol and resorcinol) and phenolic acids (protocatechuic acid and caffeic acid) are capable of binding to BSA protein (Bartelome et al, 2000). It was suggested that simple phenols could precipitate proteins out of solution if they were are concentrations high enough to form a hydrophobic layer on the protein surface (McManus et al., 1981). However, in their study of salivary protein-astringent interactions, Schwartz & Hoffman (2008) demonstrated that the phenolic acids protocatechuic acid (3,4-dihydroxybenzoic acid) and gallic acid had little to no

binding activity with human salivary proteins, although they did elicit oral astringeny (Peleg, 1995; Schwartz & Hoffman, 2008).

This objective of this experiment was to determine if low-molecular weight phenolic acids phenols form precipitates with salivary proteins in an effort to further understand possible mechanisms of astringency of phenolic acids.

## 5.2 Materials and Methods:

### 5.2.1 Saliva

Saliva collection took place in sensory booths (HNRU) at the University of Guelph whereby approximately 10 ml of saliva was simultaneously collected from 3 male and 3 female panelists. Panelists were asked to refrain from eating, drinking and brushing their teeth for 2 hours prior to collection, and asked to refrain from drinking tea, coffee or beer at least 8 hours prior to collection to minimize any residual oral phenolic content. Before collection, panelists were asked to rinse their mouths twice with distilled water and then to start chewing a 2 x 2 cm piece of paraffin wax. Panelists were asked to continue to chew the wax while periodically expectorating their saliva into a 15-ml tube held on ice for a duration of 5 minutes. The tubes were then centrifuged (10,000 x g, 10 min, 4℃) to remove any debris (food particles, bacteria, mucosal cells). Supernatants were then pooled and thoroughly mixed (vortex 1 min) to generate pooled whole saliva (WS). This procedure was adapted from Lee & Vickers (2010).

### 5.2.2 Astringent solutions

Astringent solutions were prepared the same day as saliva collection. Solutions of tannic acid (1g/L, 3g/L), ferulic acid (12mM), vanillic acid (12mM), and gallic acid (12mM) were prepared using distilled water (Fernbrook, ON). Tannic acid at 3g/L was used as it is known to

induce astringency (Lee & Lawless, 1991). Solutions were stored at room temperature in covered flasks until sample preparation.

### 5.2.3 Saliva-Astringent mixtures

Saliva-astringent mixtures were assayed at both a 1:1 and 2:1 ratio of astringent solution to pooled saliva (WS). Either 400ul (1:1) or 800ul (2:1) of astringent solution or water (control) was added to 400ul of WS in 1.5 ml Ependorff tubes, in duplicate. Tubes were then vortexed and held at 37℃ (body temperature) for 5 minutes. Tubes were then centrifuged (13,000g, 10 minutes) and 400ul of supernatant was then transferred to a new 1.5-ml ependorff tube and placed in liquid nitrogen. Samples were stored at -80℃ overnight before freeze drying .

### 5.2.4 SDS-PAGE

The lyophilized supernatants of the saliva-astringent mixtures were prepared for SDS-PAGE the next day. Supernatants were resolubilized with 190ul laemmli buffer and 10ul β-mercaptoethanol, placed in a water bath at 97℃ for 5 minutes, then cooled to room temperature. Samples were vortexed and 20ul aliquots were loaded onto pre-cast 10.5-14% polyacrylamide Tris-HCl gels (Bio-Rad). A pre-mixed Tris-glycine-SDS running buffer (Bio-Rad) was used. Electrophoresis was run at 70 V for 10 minutes and then increased to 150 V until the samples had migrated to the bottom of the gel. Gels were stained with Coomassie Brilliant Blue (CBB) R-250 and destained using the methods of Beeley et al. (1991). Gel images were captured and analyzed using a BioRad scanner and Gel Doc$^{TM}$ EZ software.

## 5.3 Results and Discussion

Several blue and many pink/violet stained protein bands were observed on the CBB-

stained gels. Identification of bands was based on colour and apparent molecular weight compared to published work that positively identified salivary proteins (Beeley, 1991; Becerra et al, 2003). The pink stained bands, resulting from metachromasia of CBB R-250 dye in the presence of closely spaced proline residues, were identified as PRPs. A description of the bands is given in Figure 5.1.

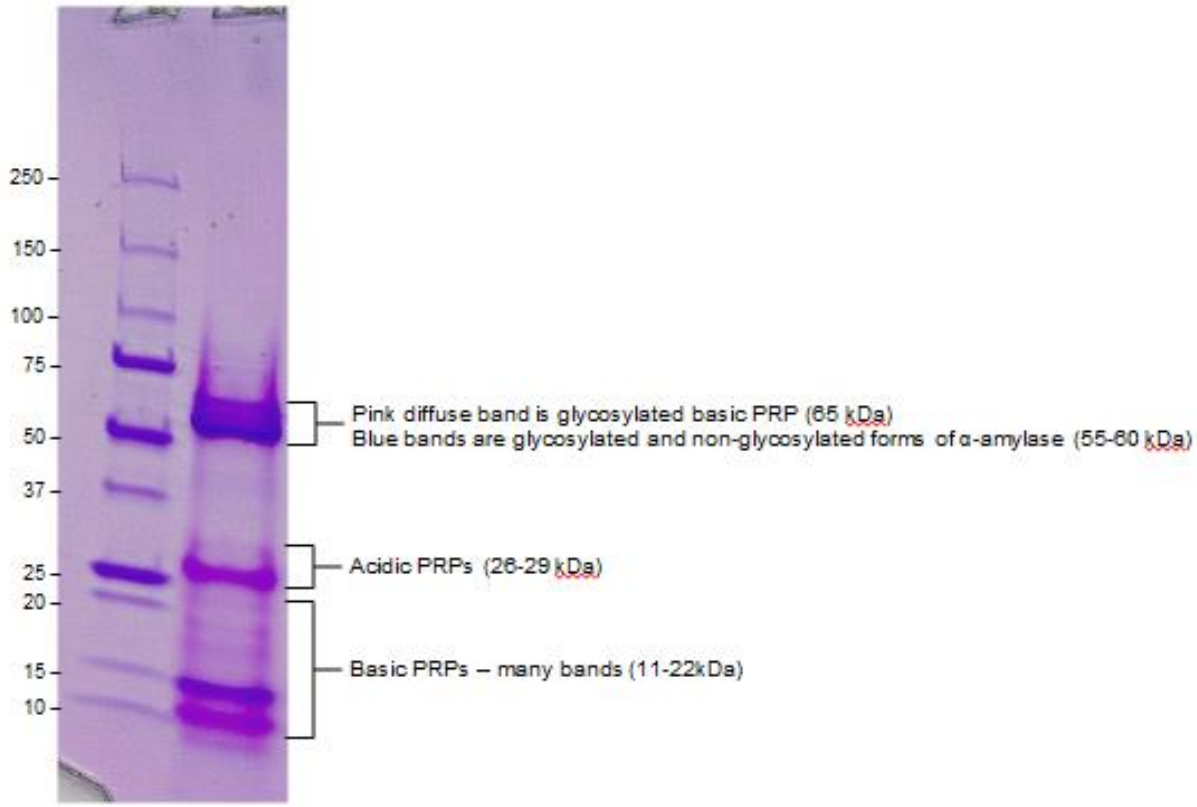


**Figure 5-1: Molecular weight standards and salivary proteins observed on the CBB stained gels.** Molecular weight standards appear on the left column with their approximate molecular weight in kilo Daltons. Salivary protein bands appear in the right column and are labelled with descriptions and approximate molecular weights. Pink/violet bands are the PRPs and blue bands are non-PRP proteins.

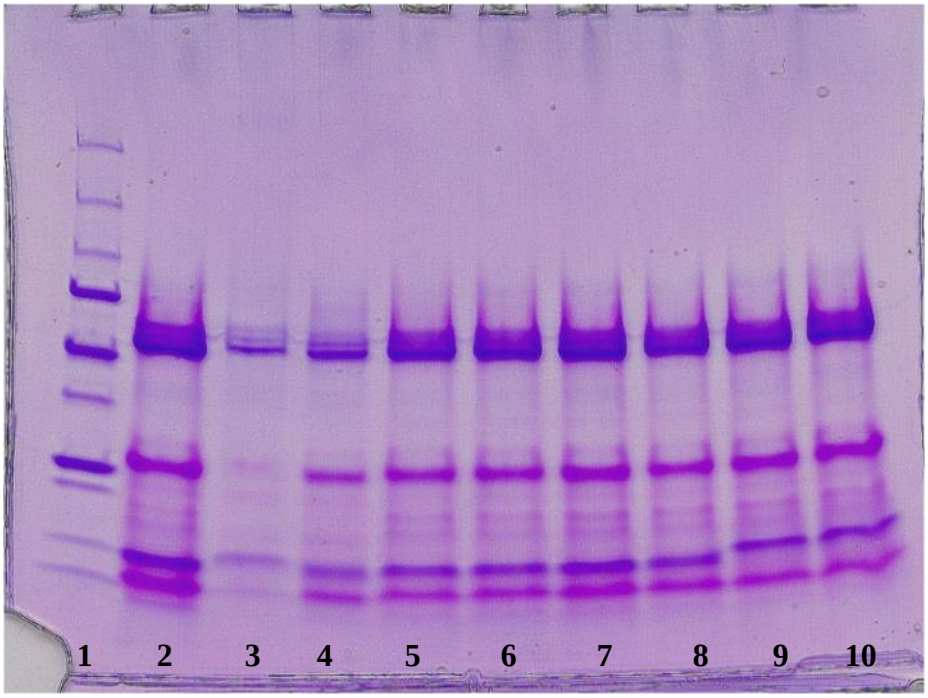


**Figure 5-2: SDS-PAGE of phenolic acids, tannic acid at 1:1 (400ul: 400ul) saliva to astringent mixtures**. Lane: 1: Protein standard, Lane 2: WS + water (control), Lane 3: WS + 3g/l tannic acid Lane 4: WS + 1g/l tannic acid, Lanes 5 & 6: WS + 12 mM ferulic acid (FA), Lanes 7 & 8: WS + 12 mM vanillic acid, Lanes 9& 10: WS + 12 mM gallic acid.

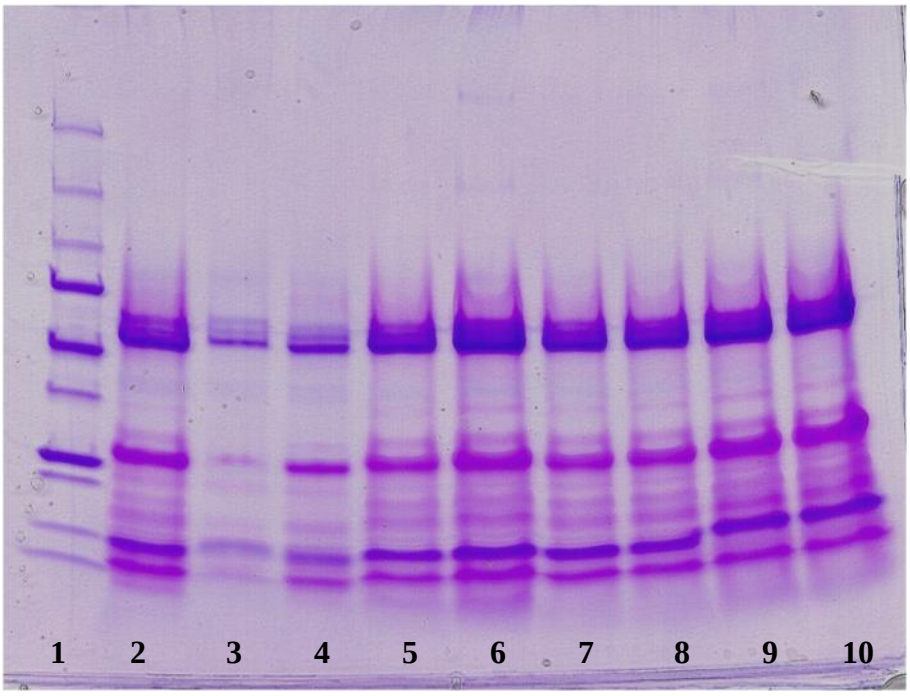


**Figure 5-3: SDS-PAGE of phenolic acids and tannic acid at 2:1 (800ul : 400ul) saliva to astringent mixtures.** Lane 1: Protein standard, Lane 2: WS + water (control), Lane 3: WS + 3g/l tannic acid  Lane 4: WS + 1g/l tannic acid, Lanes 5 & 6: WS + 12 mM ferulic acid (FA), Lanes 7 & 8: WS + 12 mM vanillic acid, Lanes 9 &10: WS + 12 mM gallic acid.

As can be seen in Figures 5.2 and 5.3, not all of the phenolic compounds tested precipitated the PRPs. In both figures, the tannic acid solutions (lanes 3 and 4) precipitated a greater amount of PRPs than did any of the other astringent solutions when compared to the control saliva: water mixture (lane 2). The bands containing the glycosylated basic (~65 kDa), acidic PRPs (26-29 kDa) and basic PRPs (11-22 kDa) were diminished in lane 4 (1g/l tannic acid), and were even more diminished or absent in lane 3 (1g/l tannic acid). This demonstrates that increased tannic acid in solution resulted in increased binding and precipitation of PRPs. These results agree with those of Gambuti et al. (2006) where tannic acid at 1g/L was demonstrated to precipitate PRPs, with progressive reductions in PRPs with solutions of

increasing tannic acid concentration. There were no visible differences between any other lanes and the control (lane 2), indicating that the low-molecular weight astringents (ferulic acid, vanillic acid and gallic acid) either did not precipitate PRPs at all or to the same extent of tannic acid.

As reported in Chapters 3 & 4, both ferulic acid and vanillic acid are perceived to be astringent at the tested concentration (12mM). The results of this experiment suggest that the precipitation of PRPs is not necessary for the development of astringency of these low-molecular weight phenolicacids. In their investigation of the binding activity of astringent compounds with salivary proteins, Shwartz and Hoffman (2008) demonstrated that phenolic acids protocatechuic and gallic acid had little to no binding activity with salivary proteins. These authors concluded that there was no direct relationship between oral astringency and human salivary protein binding amongst the compounds studied, and suggested that the interaction of astringent with the epithelial cells of the oral mucosa may be responsible for astringency development, rather than salivary protein binding and/or precipitation. It is possible that ferulic acid vanillic acid elicit astringency through a direct effect on the oral epithelial cells as well.

### *5.4 Conclusions*

These results indicate that the precipitation of PRPs is not necessary for the development of astringency of low-molecuar weight phenolics ferulic, vanillic and gallic acid. This provides support to previous literature reports. Furthermore, this implies that simple protein/polyphenol binding assays using salivary proteins may not be suitable in vitro screening tools for the reliable discovery, quantification or evaluation of all astringent compounds.

# CHAPTER 6 – *CONCLUSIONS AND RECOMMENDATIONS*

Differences in phenolic acid profiles amongst cereal grains and their flours may result in flavour differences amongst baked products made from these flours. However, to date, there is relatively little information concerning the sensory attributes of low molecular weight phenolic acids on their own, nevermind in complex food matrices. There is also little information concerning the time course of single sipped or repeatedly sampled stimuli in model systems such as aqueous solutions, using current time-intensity methods, and little comparison of these profiles amongst different compounds.

The objectives of this research were to characterize the sensory properties of individual phenolic acids commonly found in whole grains to further understand how they might contribute to the flavour of whole grain products, and how these attributes change over time and with repeated consumption. Using a descriptive panel, this work revealed that phenolic acids ferulic and vanillic acid are perceived to elicit a combination of multiple sensory attributes; sour, bitter and astringent, and that differences in the perceived intensity of these attributes amongst acids (Chapter 2). Vanillic acid was perceived to be more sour than ferulic acid, and ferulic acid was perceived to be more bitter than vanillic acid, while no differences in astringency were seen between acids. This suggests that small structural differences between compounds of otherwise similar structure can result in differences in their sensory perception. For both acids, intensity of attributes increased with increasing concentration. With repeated exposures, the perceived intensity of astringency and bitterness of the acids continued to increase. This suggests that even small quantities of phenolic acids can be perceived as increasingly bitter and astringent with

repeated exposures. No associations were found between salivary flow or PROP status and the perception of sourness, bitterness and astringency of phenolic acids.

The interaction of phenolic acids with salivary proteins was explored to further understand astringency as it relates to low molecular weight phenolics. It was found that the precipitation of PRPs is not necessary for the development of astringency of low-molecuar weight phenolics ferulic, vanillic and gallic acid. This also suggests that simple salivary protein/polyphenol binding assays may not be suitable in vitro screening tools for the reliable discovery, quantification or evaluation of all astringent compounds.

This research is novel is that no other literature has examined the sensory perception of these individual phenolic acids over time or with repeated exposures. Understanding of the sensory attributes of individual phenolic acids can aid in developing wholegrain products that are acceptable to consumers.

***Proposed ideas for future research***

1. The sensory perception of combinations of phenolic acids at levels found in whole grain flours and baked products should be investigated to further understand how combinations of these acids may contribute to the flavour of whole grain products.

2. The sensory evaluation of baked products containing varying levels of added phenolic acids would provide further understanding of how phenolic acids might interact with other ingredients in the baked product matrix and contribute to flavour.

3. The effect of increasing the time between sequential sample evaluations on the perceived sensory attributes of phenolics would aid in further understanding of the temporal aspects of sourness, bitterness and astringency formation.

4. Future research examining the sub-qualities of astringency perceived amongst varying phenolic compounds and other astringents using multidimensional scaling techniques could help to identify possible associations between subqualities of astringency and different mechanisms of astringency formation.

5. Protein binding assays examining differences in protein precipitation amongst phenolic and non-phenolic astringents matched for astringency intensity would aid in further understanding of protein interactions involved in astringency formation

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# APPENDICES

## APPENDIX A: Consent forms for sensory analysis

### A.1. Consent to participate in study REB#10SE048

**CONSENT TO PARTCIPATE IN RESEARCH**

**Sensory panel evaluation of low molecular weight polyphenols**

**You are asked to participate in a research study conducted by Allison Langfried and Lisa Duizer (supervisor) from the Department of Food Science at the University of Guelph.**

We are looking for people who are interested in being trained in sensory evaluation to help Allison Langfried with study the flavours of phenolic compounds commonly found in fruits and cereal grains. The funding for this Master's thesis research is being provided by an NSERC Discovery grant.

**If you have any questions or concerns about the research, please feel free to contact Lisa Duizer, Faculty member in the Department of Food Science, phone: (519) 824-4120 ext 53410.**

**PURPOSE OF THE STUDY**
The purpose of this study is to understand how astringency and bitterness relate to low molecular weight polyphenols

**PROCEDURES**
If you volunteer to participate in this study, you will be asked to do the following things:

**1. Screening Questionnaire:**

At the top of each questionnaire is the information you provided when first contacted regarding your interest in the study, including your contact, personal health information and availability. Before starting the second portion of the questionnaire, please ensure all the information in the above sections is accurate and updated. The remainder of the questionnaire contains information regarding your food habits and knowledge of food flavours. During panel training, you will be asked to describe the taste and flavours of solutions and food products. Your answers to the food habits and flavour sections will assist us in evaluating your ability to successfully complete the training.

**2. Sensory Screening:**

Once we have confirmed that you will not suffer any adverse reactions to the products you will be testing, we will invite you to attend two tasting sessions. The testing sessions will take approximately two hours over two days. During the sessions you will be asked to taste solutions containing sodium chloride (salt), caffeine (bitter), alum (astringent). You will be tested on your ability to differentiate between the tastes/sensations and the strength of taste/sensations of these solutions.

**Based on availability and scores from the screening tests, we will be inviting selected individuals to further participate in the study who are both available to attend all training sessions and who have good ability to differentiate between tastes as well as rate strength of tastes/sensations upon repetition.**

**3. Training:**

If selected, you will be invited to participate in sensory training. You will be trained to evaluate solutions having different taste characteristics. The training process is expected to take 20 sessions of 1 hour each. These sessions will be held 5 days per week (Monday-Friday). The time for the sessions will be confirmed after screening is complete.

Training involves meeting as a group to taste, describe and evaluate the perceived tastes and in-mouth sensations of various standard solutions. We will work with you to define descriptors and teach you how to evaluate the solutions using these descriptors. Some training will involve practicing and using computers in sensory booths to make and record your evaluations.

You will be provided with the ingredients of the solutions/products you will be evaluating each day before you begin the evaluation process. You will be asked to taste solutions and then to spit them out in the cups provided. You will not be asked to not taste any solution/product that you have an allergy or food sensitivity to.

4. **Testing:**

After you have successfully completed the training process, you will be asked to evaluate solutions and products for the sensory characteristics in which you were trained using a computer for data entry.

Testing sessions will take one hour each day for three days

You will be compensated $10.00 for each training session you attend. If you cannot attend a tasting session, please let us know and we will reschedule a tasting session for you.

**POTENTIAL RISKS AND DISCOMFORTS**

You will be tasting solutions and food products which contain the following ingredients (may be subject to change, a full list of ingredients will be provided before every testing):

**Training Solutions:** You will be asked to taste different solutions to determine your ability to discriminate between the following tastes: sour, bitter, astringent. These solutions will contain citric acid, alum, quinine monohydrochloride dehydrate, aluminum potassium sulphate, tannic acid, water.

**Testing Solutions/products:** You will be asked to taste different solutions/products containing phenolic acids. Phenolics are compounds produced by plants and function in their reproduction, growth, defense and coloration. Phenolic compounds are commonly consumed in the human diet in the form of red fruits, vegetables, teas, coffee and cereal grains. Phenolics may provide health benefits associated with reduced risk of chronic diseases through their role as antioxidants.

The solutions you will be tasting contain 8 phenolic acids that are present in wheat flours; p-hydroxybenzoic acid, salicylic acid, vanillic acid, syringic acid, p-coumaric acid, caffeic acid, ferulic acid and sinapic acid.

You will also be asked to taste cereal products containing water, wheat flour, water, sugar, yeast, vegetable oil (canola and/or soybean oil), salt, (may contain eggs).

You will NOT be asked to swallow any of the training or testing solutions.

Additionally, you will be cleansing your palate with unsalted Premium Plus crackers and filtered water. At intervals you will also be asked to rinse and spit out a pectin solution to alleviate bitterness and astringency.

On each training day a complete list of solution/product ingredients will be provided.

If you are uncomfortable tasting any of these ingredients, please do not take part in this study.

## POTENTIAL BENEFITS TO PARTICIPANTS AND/OR SOCIETY

Information collected in this study will help the scientific community to better understand the sensory characteristics of low molecular weight polyphenols and their contribution to the perceived tastes of cereal products containing the phenolic acids of interest.

## PAYMENT FOR PARTICIPATION

You will receive $10 compensation per session completed. Payments will be made at the end of the training phase and at the end of the testing phase of the study.

**CONFIDENTIALITY**

Every effort will be made to ensure confidentiality of any identifying information that is obtained in this study. All data collected will be encrypted and stored on a password protected computer in a locked room that can only be accessed by the researcher and advising faculty. The data will be analyzed to generate statistical results.

**PARTICIPATION AND WITHDRAWL**

You can choose whether to participate in this study or not. If you choose to participate, you may withdraw at any time without consequence. You may exercise the option of removing your subset of data from the study data. You may also refuse to answer any questions you don't want to answer and still remain in the study if it is safe to do so. The investigator may withdraw you from the research if circumstances arise that warrant doing so.

**RIGHTS OF THE RESEARCH PARTICPIANTS**

You may withdraw your consent at any time and discontinue participation without penalty. You are not waiving any legal claims, rights, or remedies because of your participation in this research study. This study has been reviewed and received ethics clearance through the University of Guelph Research Ethics Board. If you have any questions regarding your rights as a research participant, contact:

**Research Ethics Coordinator**
**University of Guelph**
**437 University Centre**
**Guelph, ON  N1G 2W1**

**Telephone: (519) 824-4120, ext. 56606**
**E-mail: sauld@uoguelph.ca**
**Fax: (519) 821-5326**

**SIGNATURE OF RESEARCH PARTICIPANT**

I have filled in the questionnaire for allergies and I have read the ingredient listings for the solutions/products that I will be tasting. I am not allergic or sensitive to any of the listed items.

I have read the information provided for the study "Sensory evaluation of low molecular weight polyphenols" as described herein.

My questions have been answered to my satisfaction and I agree to participate in this study. I have been given a copy of this form.

_____ _____

Name of Participant (please print) Date

**SIGNATURE OF WITNESS**

_____
Name of Witness (please print)

_____ _____
Signature of Witness Date

**A:2. Consent form to participate in study (REB#09MA10)**

## CONSENT TO PARTCIPATE IN RESEARCH

### Interaction of low molecular weight polyphenols with salivary proteins

**You are asked to participate in a research study conducted by Allison Langfried and Lisa Duizer (supervisor) from the Department of Food Science at the University of Guelph.**

We are looking for people who are interested in participating in study to further understand the interaction of food components (phenolic acids) with salivary proteins ( REB#09MA10). The funding for this Master's thesis research is being provided by an NSERC Discovery grant.

**If you have any questions or concerns about the research, please feel free to contact Lisa Duizer, Faculty member in the Department of Food Science, phone: (519) 824-4120 ext 53410.**

**PURPOSE OF THE STUDY**

The purpose of this study is to understand how phenolic acids interact with salivary proteins to further understand possible mechanisms of astringency perception of low molecular weight polyphenols.

**Please do not participate if you are any taking medications, smoke or have any health concerns.**

**PROCEDURES**

If you agree to participate in this study, you will be asked to produce a sample of your saliva for analysis of salivary protein interaction with phenolic acid solutions.

You will be asked to abstain from consuming foods and beverages rich in phenolics (coffee, tea, beer, whole grains) for at least hours prior to sample collection, and to abstain from having any food or drink (excluding water) 2 hours prior to sample collection.

**Saliva collection:**

You will be asked to chew on a piece of Parafilm without swallowing to evoke saliva, and to evacuate the generated saliva into a sampling vial, then secure the lid on the sample vial. A total of approximately 10 ml saliva is needed from each participant.

**Analysis:**
All collected samples will be pooled together for analysis. Phenolic acid solutions will be mixed with the samples and analyzed for interaction of the phenolic acids with salivary proteins using SDS-PAGE and turbidity measurements.
Collection will take one half hour (or less) and you will be compensated $15.00.

## POTENTIAL RISKS AND DISCOMFORTS

You will be asked to chew a piece of Parafilm (2cm x 2cm). This is an inert plastic that poses no potential risk.

## POTENTIAL BENEFITS TO PARTICIPANTS AND/OR SOCIETY

Information collected in this study will help the scientific community to better understand the interaction of low molecular weight food components (phenolics) with salivary proteins. The results of this study will contribute to the understanding of astringency and other sensory characteristics of low molecular weight phenolics and their contribution to the perceived tastes of cereal products containing the phenolic acids of interest.

## PAYMENT FOR PARTICIPATION

You will receive $15 compensation for you participation. Payments will be made at the end of the sample collection.

## CONFIDENTIALITY

Every effort will be made to ensure confidentiality of any information that is collected in this study. Immediately after samples are collected they will be pooled together for analysis– no data will be able to be linked to any individual participant and all participant information will be kept confidential.

## RESULTS

Upon study completion you will receive an email from Allison Langfried (alangfri@uoguelph.ca) that will provide a link to a summarized copy of the results.

## PARTICIPATION AND WITHDRAWL

You can choose whether to participate in this study or not. If you choose to participate, you may exercise the option of withdrawing your sample from the study before the samples have been pooled (within 24 hours of collection) after which your sample cannot be withdrawn. You may also refuse to answer any questions you don't want to answer and still remain in the study if it is safe to do so. The investigator may withdraw you from the research if circumstances arise that warrant doing so.

**RIGHTS OF THE RESEARCH PARTICPIANTS**

You are not waiving any legal claims, rights, or remedies because of your participation in this research study. This study has been reviewed and received ethics clearance through the University of Guelph Research Ethics Board. If you have any questions regarding your rights as a research participant, contact:

**Research Ethics Coordinator**
**University of Guelph**
**437 University Centre**
**Guelph, ON  N1G 2W1**

**Telephone: (519) 824-4120, ext. 56606**
**E-mail: sauld@uoguelph.ca**
**Fax: (519) 821-5326**

**SIGNATURE OF RESEARCH PARTICIPANT**

I have filled in the questionnaire for allergies and I have read the ingredient listings for the solutions/products that I will be tasting. I am not allergic or sensitive to any of the listed items.

I have read the information provided for the study "Sensory evaluation of low molecular weight polyphenols" as described herein.

My questions have been answered to my satisfaction and I agree to participate in this study. I have been given a copy of this form.

_____ _____

Name of Participant (please print) Date

**SIGNATURE OF WITNESS**

_____

Name of Witness (please print)

_____ _____

Signature of Witness Date

## APPENDIX B: ANOVA tables from Chapter 4

**Appendix B-1: (Experiment 1) ANOVA table for sensory attributes**

| Parameter | Source | Df | Type III SS | F-value | P-value |
|---|---|---|---|---|---|
| Sourness IMAX | JUDGE | 8 | 8067.852 | 4.82 | 0.0001 |
| | ACID | 1 | 4446.75 | 21.27 | <.0001 |
| | MOL | 1 | 24994.9 | 119.53 | <.0001 |
| | REP | 2 | 17641.24 | 42.18 | <.0001 |
| | JUDGE*MOL | 8 | 1566.852 | 0.94 | 0.4938 |
| | JUDGE*ACID | 8 | 806.3333 | 0.48 | 0.864 |
| | JUDGE*REP | 16 | 7070.426 | 2.11 | 0.0199 |
| | ACID*MOL | 1 | 8.89815 | 0.04 | 0.8373 |
| | ACID*REP | 2 | 930.3889 | 2.22 | 0.1172 |
| | MOL*REP | 2 | 2317.019 | 5.54 | 0.0063 |
| Sourness TMAX | JUDGE | 8 | 1406.506 | 12.4 | <.0001 |
| | ACID | 1 | 10.66271 | 0.75 | 0.3899 |
| | MOL | 1 | 116.0792 | 8.19 | 0.0061 |
| | REP | 2 | 25.87548 | 0.91 | 0.4081 |
| | JUDGE*MOL | 8 | 286.7008 | 2.53 | 0.0215 |
| | JUDGE*ACID | 8 | 53.58767 | 0.47 | 0.8697 |
| | JUDGE*REP | 16 | 391.9453 | 1.73 | 0.0717 |
| | ACID*MOL | 1 | 0.639343 | 0.05 | 0.8327 |
| | ACID*REP | 2 | 20.83431 | 0.73 | 0.4847 |
| | MOL*REP | 2 | 9.857343 | 0.35 | 0.708 |
| Sourness DUR | JUDGE | 8 | 7600.36 | 39.89 | <.0001 |
| | ACID | 1 | 181.4142 | 7.62 | 0.0081 |
| | MOL | 1 | 2233.781 | 93.79 | <.0001 |
| | REP | 2 | 874.15 | 18.35 | <.0001 |
| | JUDGE*MOL | 8 | 945.6992 | 4.96 | 0.0001 |
| | JUDGE*ACID | 8 | 343.2413 | 1.8 | 0.099 |
| | JUDGE*REP | 16 | 1221.907 | 3.21 | 0.0008 |
| | ACID*MOL | 1 | 0.006936 | 0 | 0.9865 |
| | ACID*REP | 2 | 44.79895 | 0.94 | 0.3972 |
| | MOL*REP | 2 | 192.8947 | 4.05 | 0.0234 |

| | | | | | |
|---|---|---|---|---|---|
| Bitterness IMAX | JUDGE | 8 | 23419.33 | 10.77 | <.0001 |
| | ACID | 1 | 3256.009 | 11.97 | 0.001 |
| | MOL | 1 | 1625.565 | 5.98 | 0.0175 |
| | REP | 2 | 34.38889 | 0.06 | 0.9388 |
| | JUDGE*MOL | 8 | 4741.185 | 2.18 | 0.0423 |
| | JUDGE*ACID | 8 | 19090.07 | 8.78 | <.0001 |
| | JUDGE*REP | 16 | 5076.444 | 1.17 | 0.3211 |
| | ACID*MOL | 1 | 52.08333 | 0.19 | 0.6633 |
| | ACID*REP | 2 | 440.3519 | 0.81 | 0.45 |
| | MOL*REP | 2 | 141.6852 | 0.26 | 0.7715 |
| Bitterness TMAX | JUDGE | 8 | 4257.301 | 13.31 | <.0001 |
| | ACID | 1 | 36.33668 | 0.91 | 0.3447 |
| | MOL | 1 | 30.80209 | 0.77 | 0.384 |
| | REP | 2 | 2.737031 | 0.03 | 0.9664 |
| | JUDGE*MOL | 8 | 352.2118 | 1.1 | 0.377 |
| | JUDGE*ACID | 8 | 610.18 | 1.91 | 0.0777 |
| | JUDGE*REP | 16 | 1602.006 | 2.5 | 0.0062 |
| | ACID*MOL | 1 | 1.209889 | 0.03 | 0.8626 |
| | ACID*REP | 2 | 98.06252 | 1.23 | 0.3014 |
| | MOL*REP | 2 | 131.4271 | 1.64 | 0.2028 |
| Bitterness DUR | JUDGE | 8 | 20540.39 | 50.45 | <.0001 |
| | ACID | 1 | 156.8526 | 3.08 | 0.0848 |
| | MOL | 1 | 33.06659 | 0.65 | 0.4237 |
| | REP | 2 | 12.12158 | 0.12 | 0.888 |
| | JUDGE*MOL | 8 | 1391.107 | 3.42 | 0.003 |
| | JUDGE*ACID | 8 | 3856.587 | 9.47 | <.0001 |
| | JUDGE*REP | 16 | 852.9284 | 1.05 | 0.4255 |
| | ACID*MOL | 1 | 0.3366 | 0.01 | 0.9355 |
| | ACID*REP | 2 | 22.50883 | 0.22 | 0.8023 |
| | MOL*REP | 2 | 17.68595 | 0.17 | 0.841 |

| | | | | | |
|---|---|---|---|---|---|
| Astringency IMAX | JUDGE | 8 | 37319.17 | 16.39 | <.0001 |
| | ACID | 1 | 54.89815 | 0.19 | 0.6622 |
| | MOL | 1 | 4218.75 | 14.82 | 0.0003 |
| | REP | 2 | 4726.222 | 8.3 | 0.0007 |
| | JUDGE*MOL | 8 | 973.8333 | 0.43 | 0.8997 |
| | JUDGE*ACID | 8 | 2874.352 | 1.26 | 0.2809 |
| | JUDGE*REP | 16 | 3484.278 | 0.77 | 0.7163 |
| | ACID*MOL | 1 | 1.12037 | 0 | 0.9502 |
| | ACID*REP | 2 | 142.5185 | 0.25 | 0.7793 |
| | MOL*REP | 2 | 1164.222 | 2.05 | 0.1386 |
| Astringency TMAX | JUDGE | 8 | 6519.539 | 43.09 | <.0001 |
| | ACID | 1 | 0.675558 | 0.04 | 0.8508 |
| | MOL | 1 | 107.8938 | 5.7 | 0.0205 |
| | REP | 2 | 294.6815 | 7.79 | 0.0011 |
| | JUDGE*MOL | 8 | 473.7666 | 3.13 | 0.0057 |
| | JUDGE*ACID | 8 | 114.0302 | 0.75 | 0.6444 |
| | JUDGE*REP | 16 | 453.6577 | 1.5 | 0.1352 |
| | ACID*MOL | 1 | 0.074845 | 0 | 0.9501 |
| | ACID*REP | 2 | 77.41288 | 2.05 | 0.1393 |
| | MOL*REP | 2 | 77.29893 | 2.04 | 0.1397 |
| Astringency DUR | JUDGE | 8 | 26970.95 | 42.89 | <.0001 |
| | ACID | 1 | 148.6975 | 1.89 | 0.1748 |
| | MOL | 1 | 865.7821 | 11.01 | 0.0016 |
| | REP | 2 | 182.9605 | 1.16 | 0.3202 |
| | JUDGE*MOL | 8 | 1840.335 | 2.93 | 0.0088 |
| | JUDGE*ACID | 8 | 521.2153 | 0.83 | 0.5812 |
| | JUDGE*REP | 16 | 1289.135 | 1.02 | 0.447 |
| | ACID*MOL | 1 | 26.73009 | 0.34 | 0.5623 |
| | ACID*REP | 2 | 48.91967 | 0.31 | 0.7339 |
| | MOL*REP | 2 | 372.6811 | 2.37 | 0.1033 |

**Appendix B-2: (Experiment 2) ANOVA table for sensory attributes**

| Parameter | Source | Df | Type III SS | F-value | P-value |
|---|---|---|---|---|---|
| Sourness IMAX | JUDGE | 9 | 26812.2 | 34.3 | <.0001 |
| | ACID | 1 | 728.0545 | 8.38 | 0.0039 |
| | MOL | 2 | 162620.6 | 936.28 | <.0001 |
| | SIP | 3 | 7771.143 | 29.83 | <.0001 |
| | REP | 2 | 388.9958 | 2.24 | 0.1074 |
| | JUDGE*ACID | 9 | 2395.331 | 3.06 | 0.0013 |
| | JUDGE*MOL | 18 | 30135.62 | 19.28 | <.0001 |
| | JUDGE*SIP | 27 | 5142.842 | 2.19 | 0.0005 |
| | JUDGE*REP | 18 | 3907.359 | 2.5 | 0.0006 |
| | ACID*MOL | 2 | 1451.461 | 8.36 | 0.0003 |
| | ACID*SIP | 3 | 38.9915 | 0.15 | 0.9299 |
| | ACID*REP | 2 | 1719.461 | 9.9 | <.0001 |
| | MOL*SIP | 6 | 5233.962 | 10.04 | <.0001 |
| | MOL*REP | 4 | 837.0102 | 2.41 | 0.0482 |
| | SIP*REP | 6 | 141.2084 | 0.27 | 0.9505 |
| Sourness TMAX | JUDGE | 9 | 749.9492 | 6.79 | <.0001 |
| | ACID | 1 | 40.55322 | 3.31 | 0.0696 |
| | MOL | 2 | 872.2436 | 35.56 | <.0001 |
| | SIP | 3 | 260.7842 | 7.09 | 0.0001 |
| | REP | 2 | 119.4262 | 4.87 | 0.008 |
| | JUDGE*ACID | 9 | 63.42558 | 0.57 | 0.8183 |
| | JUDGE*MOL | 18 | 866.2288 | 3.92 | <.0001 |
| | JUDGE*SIP | 27 | 569.7354 | 1.72 | 0.0143 |
| | JUDGE*REP | 18 | 395.7846 | 1.79 | 0.0234 |
| | ACID*MOL | 2 | 87.94614 | 3.59 | 0.0284 |
| | ACID*SIP | 3 | 42.98568 | 1.17 | 0.3212 |
| | ACID*REP | 2 | 22.71282 | 0.93 | 0.3968 |
| | MOL*SIP | 6 | 51.01343 | 0.69 | 0.6552 |
| | MOL*REP | 4 | 67.75924 | 1.38 | 0.2392 |
| | SIP*REP | 6 | 40.51918 | 0.55 | 0.7696 |

| | | | | | |
|---|---|---|---|---|---|
| Sourness ROI | JUDGE | 9 | 431.7209 | 27.61 | <.0001 |
| | ACID | 1 | 12.49876 | 7.19 | 0.0076 |
| | MOL | 2 | 1281.03 | 368.67 | <.0001 |
| | SIP | 3 | 48.64663 | 9.33 | <.0001 |
| | REP | 2 | 12.60082 | 3.63 | 0.0273 |
| | JUDGE*ACID | 9 | 24.599 | 1.57 | 0.1202 |
| | JUDGE*MOL | 18 | 503.714 | 16.11 | <.0001 |
| | JUDGE*SIP | 27 | 114.8252 | 2.45 | <.0001 |
| | JUDGE*REP | 18 | 109.8864 | 3.51 | <.0001 |
| | ACID*MOL | 2 | 3.448282 | 0.99 | 0.3714 |
| | ACID*SIP | 3 | 2.286722 | 0.44 | 0.7254 |
| | ACID*REP | 2 | 34.59619 | 9.96 | <.0001 |
| | MOL*SIP | 6 | 70.21464 | 6.74 | <.0001 |
| | MOL*REP | 4 | 25.73837 | 3.7 | 0.0055 |
| | SIP*REP | 6 | 11.87398 | 1.14 | 0.3382 |
| Sourness REAL | JUDGE | 9 | 2228.118 | 14.17 | <.0001 |
| | ACID | 1 | 0.370734 | 0.02 | 0.8842 |
| | MOL | 2 | 2266.674 | 64.88 | <.0001 |
| | SIP | 3 | 1225.082 | 23.38 | <.0001 |
| | REP | 2 | 44.74349 | 1.28 | 0.2786 |
| | JUDGE*ACID | 9 | 95.91513 | 0.61 | 0.7889 |
| | JUDGE*MOL | 18 | 2383.918 | 7.58 | <.0001 |
| | JUDGE*SIP | 27 | 1010.162 | 2.14 | 0.0008 |
| | JUDGE*REP | 18 | 253.6831 | 0.81 | 0.6933 |
| | ACID*MOL | 2 | 2.132514 | 0.06 | 0.9408 |
| | ACID*SIP | 3 | 107.6269 | 2.05 | 0.1052 |
| | ACID*REP | 2 | 8.619466 | 0.25 | 0.7814 |
| | MOL*SIP | 6 | 971.8145 | 9.27 | <.0001 |
| | MOL*REP | 4 | 112.3301 | 1.61 | 0.1707 |
| | SIP*REP | 6 | 97.68754 | 0.93 | 0.4712 |

| | | | | | |
|---|---|---|---|---|---|
| Bitterness IMAX | JUDGE | 9 | 11403.22 | 20.55 | <.0001 |
| | ACID | 1 | 7579.022 | 122.9 | <.0001 |
| | MOL | 2 | 17004.72 | 137.88 | <.0001 |
| | SIP | 3 | 7147.5 | 38.64 | <.0001 |
| | REP | 2 | 950.1694 | 7.7 | 0.0005 |
| | JUDGE*ACID | 9 | 15942.87 | 28.73 | <.0001 |
| | JUDGE*MOL | 18 | 9075.086 | 8.18 | <.0001 |
| | JUDGE*SIP | 27 | 5301.611 | 3.18 | <.0001 |
| | JUDGE*REP | 18 | 5346.053 | 4.82 | <.0001 |
| | ACID*MOL | 2 | 4841.669 | 39.26 | <.0001 |
| | ACID*SIP | 3 | 1138.611 | 6.15 | 0.0004 |
| | ACID*REP | 2 | 304.0361 | 2.47 | 0.0858 |
| | MOL*SIP | 6 | 754.025 | 2.04 | 0.0588 |
| | MOL*REP | 4 | 1595.222 | 6.47 | <.0001 |
| | SIP*REP | 6 | 54.875 | 0.15 | 0.9894 |
| Bitterness TMAX | JUDGE | 9 | 5561.382 | 33.48 | <.0001 |
| | ACID | 1 | 162.3708 | 8.8 | 0.0031 |
| | MOL | 2 | 151.4046 | 4.1 | 0.017 |
| | SIP | 3 | 271.2083 | 4.9 | 0.0023 |
| | REP | 2 | 73.01277 | 1.98 | 0.1393 |
| | JUDGE*ACID | 9 | 1267.489 | 7.63 | <.0001 |
| | JUDGE*MOL | 18 | 815.6908 | 2.46 | 0.0008 |
| | JUDGE*SIP | 27 | 1155.434 | 2.32 | 0.0002 |
| | JUDGE*REP | 18 | 698.9267 | 2.1 | 0.005 |
| | ACID*MOL | 2 | 22.82883 | 0.62 | 0.5392 |
| | ACID*SIP | 3 | 80.18337 | 1.45 | 0.2278 |
| | ACID*REP | 2 | 9.637971 | 0.26 | 0.7703 |
| | MOL*SIP | 6 | 89.11537 | 0.8 | 0.5665 |
| | MOL*REP | 4 | 29.1556 | 0.39 | 0.8124 |
| | SIP*REP | 6 | 157.1477 | 1.42 | 0.2049 |

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| Bitterness ROI | JUDGE | 9 | 198.0033 | 17.14 | <.0001 |
| | ACID | 1 | 64.39494 | 50.17 | <.0001 |
| | MOL | 2 | 119.9307 | 46.71 | <.0001 |
| | SIP | 3 | 45.56651 | 11.83 | <.0001 |
| | REP | 2 | 21.55255 | 8.39 | 0.0003 |
| | JUDGE*ACID | 9 | 83.30446 | 7.21 | <.0001 |
| | JUDGE*MOL | 18 | 84.13735 | 3.64 | <.0001 |
| | JUDGE*SIP | 27 | 144.5948 | 4.17 | <.0001 |
| | JUDGE*REP | 18 | 55.5533 | 2.4 | 0.001 |
| | ACID*MOL | 2 | 63.7337 | 24.83 | <.0001 |
| | ACID*SIP | 3 | 18.16787 | 4.72 | 0.0029 |
| | ACID*REP | 2 | 5.657189 | 2.2 | 0.1113 |
| | MOL*SIP | 6 | 20.04168 | 2.6 | 0.017 |
| | MOL*REP | 4 | 21.46639 | 4.18 | 0.0024 |
| | SIP*REP | 6 | 4.354065 | 0.57 | 0.7581 |
| Bitterness REAL | JUDGE | 9 | 2262.624 | 22.1 | <.0001 |
| | ACID | 1 | 518.5014 | 45.59 | <.0001 |
| | MOL | 2 | 321.2583 | 14.12 | <.0001 |
| | SIP | 3 | 1074.282 | 31.49 | <.0001 |
| | REP | 2 | 19.075 | 0.84 | 0.4328 |
| | JUDGE*ACID | 9 | 1192.29 | 11.65 | <.0001 |
| | JUDGE*MOL | 18 | 646.1306 | 3.16 | <.0001 |
| | JUDGE*SIP | 27 | 1101.593 | 3.59 | <.0001 |
| | JUDGE*REP | 18 | 151.5639 | 0.74 | 0.7701 |
| | ACID*MOL | 2 | 46.73611 | 2.05 | 0.129 |
| | ACID*SIP | 3 | 174.7153 | 5.12 | 0.0017 |
| | ACID*REP | 2 | 21.88611 | 0.96 | 0.3826 |
| | MOL*SIP | 6 | 139.9972 | 2.05 | 0.0571 |
| | MOL*REP | 4 | 48.49167 | 1.07 | 0.3725 |
| | SIP*REP | 6 | 39.24722 | 0.58 | 0.7503 |

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| Astringency IMAX | JUDGE | 9 | 27223.78 | 61.32 | <.0001 |
| | ACID | 1 | 81.50191 | 1.65 | 0.1991 |
| | MOL | 2 | 20055.84 | 203.29 | <.0001 |
| | SIP | 3 | 21528.04 | 145.47 | <.0001 |
| | REP | 2 | 1706.249 | 17.29 | <.0001 |
| | JUDGE*ACID | 9 | 1662.499 | 3.74 | 0.0001 |
| | JUDGE*MOL | 18 | 11144.62 | 12.55 | <.0001 |
| | JUDGE*SIP | 27 | 6073.315 | 4.56 | <.0001 |
| | JUDGE*REP | 18 | 4189.331 | 4.72 | <.0001 |
| | ACID*MOL | 2 | 980.3634 | 9.94 | <.0001 |
| | ACID*SIP | 3 | 112.2575 | 0.76 | 0.5177 |
| | ACID*REP | 2 | 23.46344 | 0.24 | 0.7884 |
| | MOL*SIP | 6 | 1592.704 | 5.38 | <.0001 |
| | MOL*REP | 4 | 190.0772 | 0.96 | 0.4271 |
| | SIP*REP | 6 | 267.5246 | 0.9 | 0.4915 |
| Astringency TMAX | JUDGE | 9 | 4401.858 | 28.88 | <.0001 |
| | ACID | 1 | 6.70019 | 0.4 | 0.5296 |
| | MOL | 2 | 316.9064 | 9.36 | 0.0001 |
| | SIP | 3 | 509.9743 | 10.04 | <.0001 |
| | REP | 2 | 40.58924 | 1.2 | 0.3024 |
| | JUDGE*ACID | 9 | 459.8247 | 3.02 | 0.0016 |
| | JUDGE*MOL | 18 | 749.1918 | 2.46 | 0.0008 |
| | JUDGE*SIP | 27 | 1110.575 | 2.43 | <.0001 |
| | JUDGE*REP | 18 | 664.4689 | 2.18 | 0.0034 |
| | ACID*MOL | 2 | 7.460289 | 0.22 | 0.8024 |
| | ACID*SIP | 3 | 72.20032 | 1.42 | 0.2356 |
| | ACID*REP | 2 | 26.2633 | 0.78 | 0.461 |
| | MOL*SIP | 6 | 53.93447 | 0.53 | 0.785 |
| | MOL*REP | 4 | 46.04538 | 0.68 | 0.6062 |
| | SIP*REP | 6 | 72.45374 | 0.71 | 0.6392 |

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| Astringency ROI | JUDGE | 9 | 232.4827 | 42.81 | <.0001 |
| | ACID | 1 | 1.500173 | 2.49 | 0.1154 |
| | MOL | 2 | 99.90497 | 82.79 | <.0001 |
| | SIP | 3 | 171.0728 | 94.51 | <.0001 |
| | REP | 2 | 15.17296 | 12.57 | <.0001 |
| | JUDGE*ACID | 9 | 18.30261 | 3.37 | 0.0005 |
| | JUDGE*MOL | 18 | 90.02944 | 8.29 | <.0001 |
| | JUDGE*SIP | 27 | 91.75489 | 5.63 | <.0001 |
| | JUDGE*REP | 18 | 29.25223 | 2.69 | 0.0002 |
| | ACID*MOL | 2 | 6.339936 | 5.25 | 0.0055 |
| | ACID*SIP | 3 | 4.184192 | 2.31 | 0.0752 |
| | ACID*REP | 2 | 0.233758 | 0.19 | 0.8239 |
| | MOL*SIP | 6 | 17.12109 | 4.73 | 0.0001 |
| | MOL*REP | 4 | 2.438464 | 1.01 | 0.4014 |
| | SIP*REP | 6 | 7.012613 | 1.94 | 0.0729 |
| Astringency REAL | JUDGE | 9 | 2591.083 | 23.48 | <.0001 |
| | ACID | 1 | 52.9041 | 4.31 | 0.0382 |
| | MOL | 2 | 711.4093 | 29.01 | <.0001 |
| | SIP | 3 | 3283.136 | 89.25 | <.0001 |
| | REP | 2 | 122.3973 | 4.99 | 0.0071 |
| | JUDGE*ACID | 9 | 140.4362 | 1.27 | 0.2485 |
| | JUDGE*MOL | 18 | 695.9145 | 3.15 | <.0001 |
| | JUDGE*SIP | 27 | 1322.949 | 4 | <.0001 |
| | JUDGE*REP | 18 | 445.3839 | 2.02 | 0.0076 |
| | ACID*MOL | 2 | 29.72167 | 1.21 | 0.2983 |
| | ACID*SIP | 3 | 25.72875 | 0.7 | 0.5526 |
| | ACID*REP | 2 | 6.894051 | 0.28 | 0.755 |
| | MOL*SIP | 6 | 318.572 | 4.33 | 0.0003 |
| | MOL*REP | 4 | 14.42928 | 0.29 | 0.8818 |
| | SIP*REP | 6 | 70.48036 | 0.96 | 0.453 |