Investigation of the Inherent and Processed Bitterness in Whole Wheat Foods

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Dedication

This dissertation is dedicated to my beloved parents, Bin Bin and Ping Gao.

Abstract

Although whole grain foods are considered to be a key component of a healthy diet, consumer acceptability is hindered by a number of factors including negative sensory attributes, such as excessive bitterness. This thesis investigates the compounds that contribute to bitterness in wheat bread. Ultimately this work aimed to provide a better understanding of the chemical basis of bitterness development in whole wheat foods, including characterization of the key bitter compounds and generation pathways.

Chapter 1 includes an overview of aroma and taste research literature including the basic concepts and current flavor challenges in whole grain food, with a focus on the present knowledge of bitterness in whole grains.

Chapters 2, 3, and 4 present the finding of this work regarding the chemical origins of bitterness in whole wheat foods. Whole wheat bread was studied as a food model. Crust and crumb were both examined as they have unique flavor profiles and pathways of flavor generation due to their difference in moisture content and temperature during baking. In Chapter 2 Maillard reaction product 5-(hydroxymethyl) furfural and 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one were identified as key chemical markers of the bitterness in bread crust. Using Pearson's correlation analysis, significant correlations (a= 0.01) were observed between the perceived bitterness in the crust and the quantity of 5-(hydroxymethyl) furfural (r^2 = 0.93) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (r^2 = 0.95).

Bitterness markers in bread crumb were also investigated (Chapter 3), and key bitter compounds were identified as $_{L}$-tryptophan, apigenin-C-glycosides, and 9,12,13-trihydroxy-trans-10-octadecenoic acid (pinellic acid). Pinellic acid was confirmed as the most influential bitterant in bread crumb via sensory recombination experiments. The identified bitterants were quantified in flour and during different stages of bread making, in order to elucidate the origin of bitterness in whole wheat bread crumb. $_{L}$-tryptophan and apigenin-C-glycosides were found to be intrinsic in whole wheat flour and found to degrade during the bread manufacture process, whereas pinellic acid was generated during bread production. These findings provide an improved knowledge of the origin of bitterness in whole wheat products and a basis to further develop breeding and processing strategies to reduce bitterness in whole wheat bread.

Finally in Chapter 4, the mechanism of pinellic acid generation during production of whole wheat bread was additionally investigated. Isotope labelling analysis using [$^{13}C_{18}$]linoleic acid revealed that the natural free linoleic acid in flour was the major contributor/precursor for the formation of pinellic acid which occurred in two bread-making stages: dough kneading and baking. Results suggested that generation of pinellic acid involved both enzymatic and non-enzymatic mechanistic pathways. The initial step for both pathways involved the oxidation of linoleic acid by lipoxygenase to linoleic acid hydroperoxides (LOOH). The enzymatic pathway was the main quantitative source of pinellic acid that occurred first during the hydration of the flour, which resulted in the decomposition of LOOH into pinellic acid by epoxidation and subsequent hydrolysis step. In the second stage that occurred during baking, a non-enzymatic pathway,

involving decomposition of LOOH via a free radical mechanism facilitated by transition metals was suggested to lead to formation of hydroxyoctadecadienoic acid that further react with hydroxyl radicals to form pinellic acid. The mechanistic understanding of bitter pinellic acid formation allows for targeting and controlling key factors that help minimizing bitterness generation during manufacturing of whole wheat products.

The influence of wheat class and flour storage temperature on the enzymatic formation of pinellic acid in dough was also examined in order to provide insights and ultimately develop guidelines for improved flavor quality of whole wheat products via wheat varietal selection and flour storage practices (Chapter 4). Results showed that Hard Red Spring (HRS) wheat entries generally had higher linoleic acid content and generated higher level of pinellic acid during dough kneading when compared to Hard White Winter (HWW) wheat samples. Difference in pinellic acid generation between the two classes of wheat, HRS and HWW were further noted during thermal processing. Temperature of flour storage temperature was also shown to influence the linoleic acid content in flour and subsequently the generation of pinellic acid in dough. Ambient temperature had the greatest influence on the generation of pinellic acid by increasing the free linoleic acid while maintaining lipoxygenase activity during storage. It is suggested that generation of linoleic acid hydroperoxide as a precursor for pinellic acid occurred in flour stored at ambient temperature; therefore higher pinellic acid formation was observed in the dough.

In summary, this work provided a basis to understand bitterness development in whole wheat products. The improved knowledge of key bitter markers, their origin, and

pathways of generation in whole wheat foods can provide critical insights into grain selection, potential breeding and of course processing and storage ultimately facilitating the improvment of flavor quality of whole grain foods.

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Chapter 1

Literature Review

1.1. Flavor Chemistry Overview

Reineccius defines “flavor” in his book (Reineccius 2005), as “Flavor perception is a broad multimodal cognition and process of our senses including olfaction, taste, and the somatosenses.” Historically, flavors have been studied as predominantly aroma with little attention was given to taste and somatosenses. This era of flavor research was also seen by the advancement of gas chromatography and mass spectrometry methods (e.g. GC-MS) that largely facilitated identification of aroma compounds. Efforts were focused on determination of volatile components of food and reconstitution of these volatiles to reproduce flavor. This approach was limited as only one aroma modality was taken into account. Thus, limited insight was provided and the development of solutions was hindered in order to address the flavor challenges in practical settings. Although it is challenging for sensory data and interpretation to keep pace with the accumulation of analytical data, current flavor research approaches have advanced and expanded along with improved analytical capabilities, to provide an understanding of the key compounds to flavor, taste/odor interactions, and flavor perception (Reineccius 2005). This chapter focuses on fundamental concepts of flavor development, analysis, and challenges facing flavor chemists in basic and applied research.

1.1.1. The Maillard Reaction

The Maillard reaction, also known as a type of non-enzymatic browning, has been studied for many decades due to its key role in flavor development. The Maillard reaction is a cascade of reactions initiated by a reducing sugar (carbonyl) and an amino acid (amine). The composition of the reaction products, taken the complexity of the reaction mechanisms, is influenced by many factors such as types of amino acid/sugar, pH, water activity, etc. (Steinhart 2005). Maillard reaction is known to alter aroma, taste and color of food products such as baked goods, coffee, cooked meat, etc. Moreover, Maillard reaction can affect the nutritional value of foods. For example, the Maillard reaction can utilize lysine, which is an essential amino acid, in formation of ε-Amadori compounds and also render metals such as copper and magnesium biologically unavailable via formation of melanoidin\metal complexes (Van Boekel 1998). Furthermore, Maillard reaction products (MRPs) have both toxicological and protective aspects. Numerous MRPs have been reported to be carcinogenic and mutagenic compounds (Mottram et al. 2002). However, MRPs also have been found to have antioxidant activity, antimutagenic, antibiotic and antiallergenic effects (Steinhart 2005).

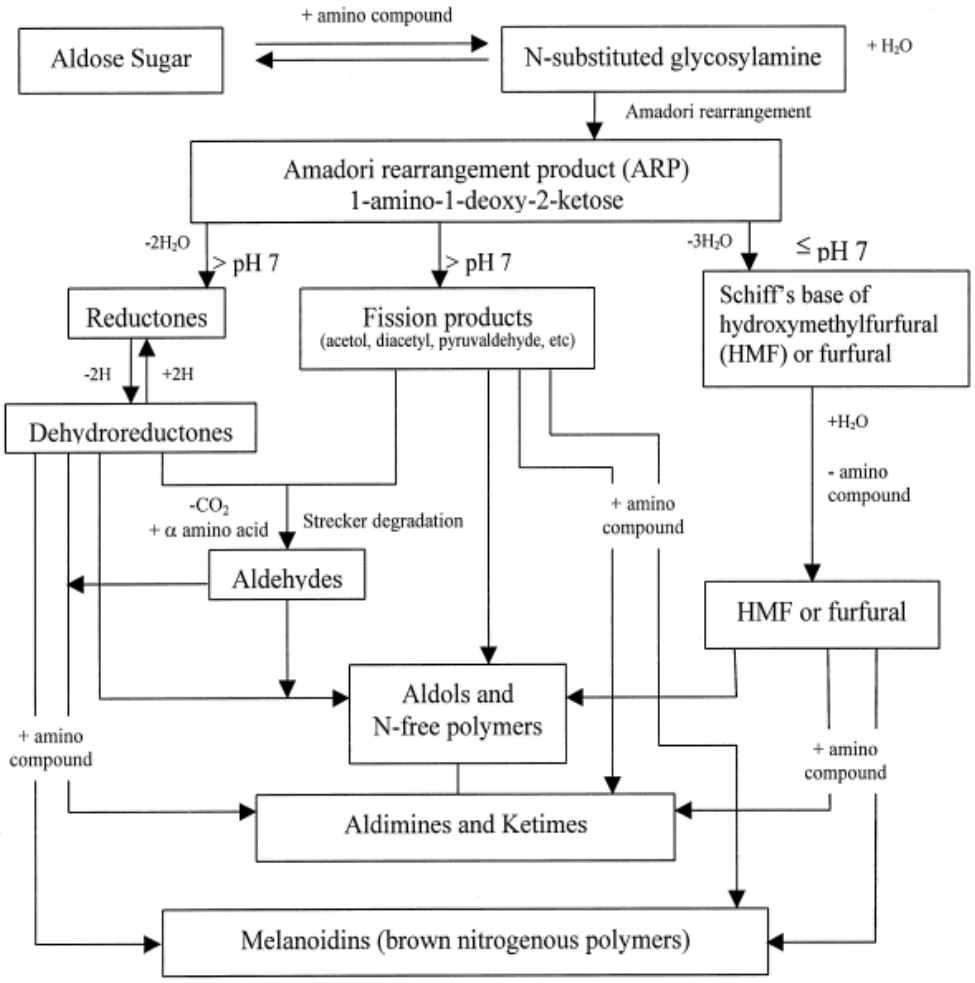

Figure 1-1. Hodge Scheme adapted from literature (Martins et al. 2000).

The classical "Hodge Scheme" (**Figure 1-1**), presented by Hodge, was the first outline of reaction mechanism of the Maillard reaction, which divides the Maillard reaction into seven steps (Hodge 1953). The initial stage of the Maillard reaction involves the formation of a Schiff base from the condensation of a carbonyl group (e.g. reducing sugar) and a free amino group (e.g. amino acids). The Schiff base is unstable and spontaneously rearranges to ketoamine, also known as the Amadori Rearrangement.

Degradation of Amadori rearrangement products (ARP) is highly pH dependent: At lower pH (<7), 1,2-enolisation is preferred via 3-deoxyglucosone pathway which leads to the formation of furfural (pentoses as source) or hydroxymethylfurfural (HMF) (hexoses as source). At higher pH (>7), degradation of ARP is via 1-deoxyglucosone pathway (2,3-enolisation), where reductones (e.g. furaneol and norfuraneol), hydroxyacetone, methylglyoxal and diacetyl are formed (Martins et al. 2000) (**Figure 1-2**). Given the high reactivity of those α-dicarbonyl compounds towards free amino groups, formation of aldehydes and a-aminoketones via Strecker degradation subsequently occurs, which gives a variety of flavor compounds pyrazines, pyrroles, thiazoles, thiophenes, furanthiols, etc. In an advanced stage, formation of nitrogenous polymers and co-polymers, known as melanoidins, leads to the brown color development of the reaction system (Namiki 1988).

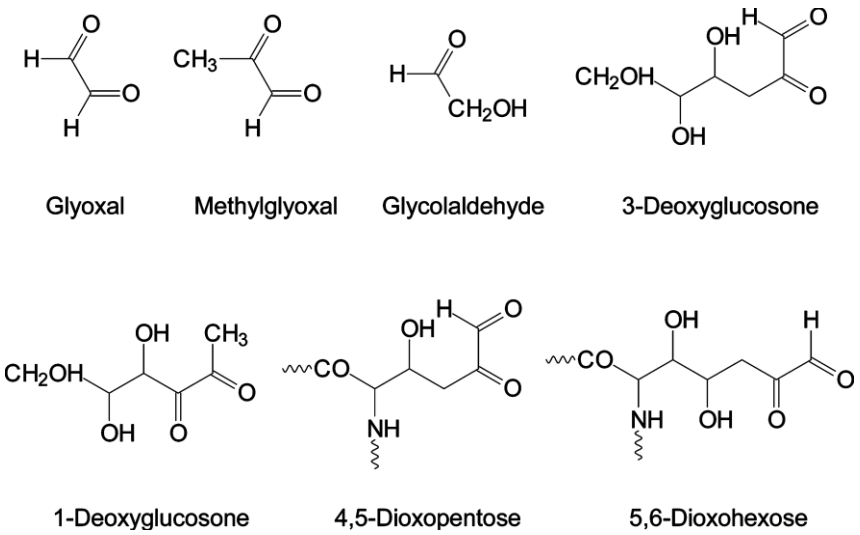

Figure 1-2. Reactive carbonyl and dicarbonyl compounds from oxidative decomposition of the Amadori rearrangement product (Zhang et al. 2008)

1.1.2. Control of the Maillard Reaction

Due to the ubiquitous nature of Maillard reaction in food and biological systems and its sensorial and nutritional impact, understanding its mechanist pathways could prove useful for the optimization of flavor and nutritional quality of food stuffs. Advancement of analytical techniques, particularly gas chromatography-mass spectrometry, has lead to the identification of many volatile compounds important to food flavor (Martins et al. 2000; Mottram & Elmore 2010), therefore allowing researchers to establish the basic understanding of the chemical pathways that lead to aroma formation. A lot of mechanistic pathways have been established utilizing model food systems, but in food products the reaction pathways are much more complex and aroma only describes one part the flavor implications of Maillard reaction. The uneven processing of food products (e.g. bread), which cause the difference in flavor profile at the surface and interior, the presence of multiple amino acid sources as well as carbohydrate sources increase the complexity of the involved mechanistic pathways, and the interaction of Maillard reaction with other food components and reactions (e.g. lipids, phenolic compounds, etc.) are a few of the challenges in understanding and controlling Maillard reaction.

Maillard – Lipid Chemistry

Lipid oxidation and the Maillard reaction are considered the two most important reactions in developing food flavors (Zamora & Hidalgo 2005). The influence of lipid oxidation products in the Maillard pathway and vice versa, suggests that both reactions

are so interrelated that they should be considered simultaneously when examining flavor development in food. Maillard reaction products with antioxidative activities that can decrease the rate of lipid oxidation have been reported (Hidalgo et al. 2006). Formation of pyrroles, the oxidized lipid/amino compound reaction products, with antioxidative activities were observed in edible oils (Hidalgo et al. 2006). Lipid oxidation products can also influence Maillard reaction by reacting with Maillard intermediates. **Figure 1-3** shows the lipid-derived intermediates, including aldehydes, ketones, alcohols, epoxides, etc., which are able to undergo aldol condensation and carbonyl-amine reaction to influence the pathway of Maillard reaction and flavor development. The flavors in cooked meat, which have been studied during the past few decades, serve as an excellent example of Maillard-lipid interactions. Mottram et al. (1983) reported the role of triglycerides and phospholipids in cooked beef aroma by removing moieties of triglycerides or phospholipids by selective solvent extraction (Mottram & Edwards 1983). It was concluded that removal of phospholipids has significant impact on the volatile profile of the cooked meat. Aliphatic aldehydes and alcohols were reported at significantly reduced levels. As heating lipid extracts alone did not result in the generation of those volatiles, lipid-Maillard interactions was considered important for the generation key precursors of meat flavors (Mottram & Edwards 1983).

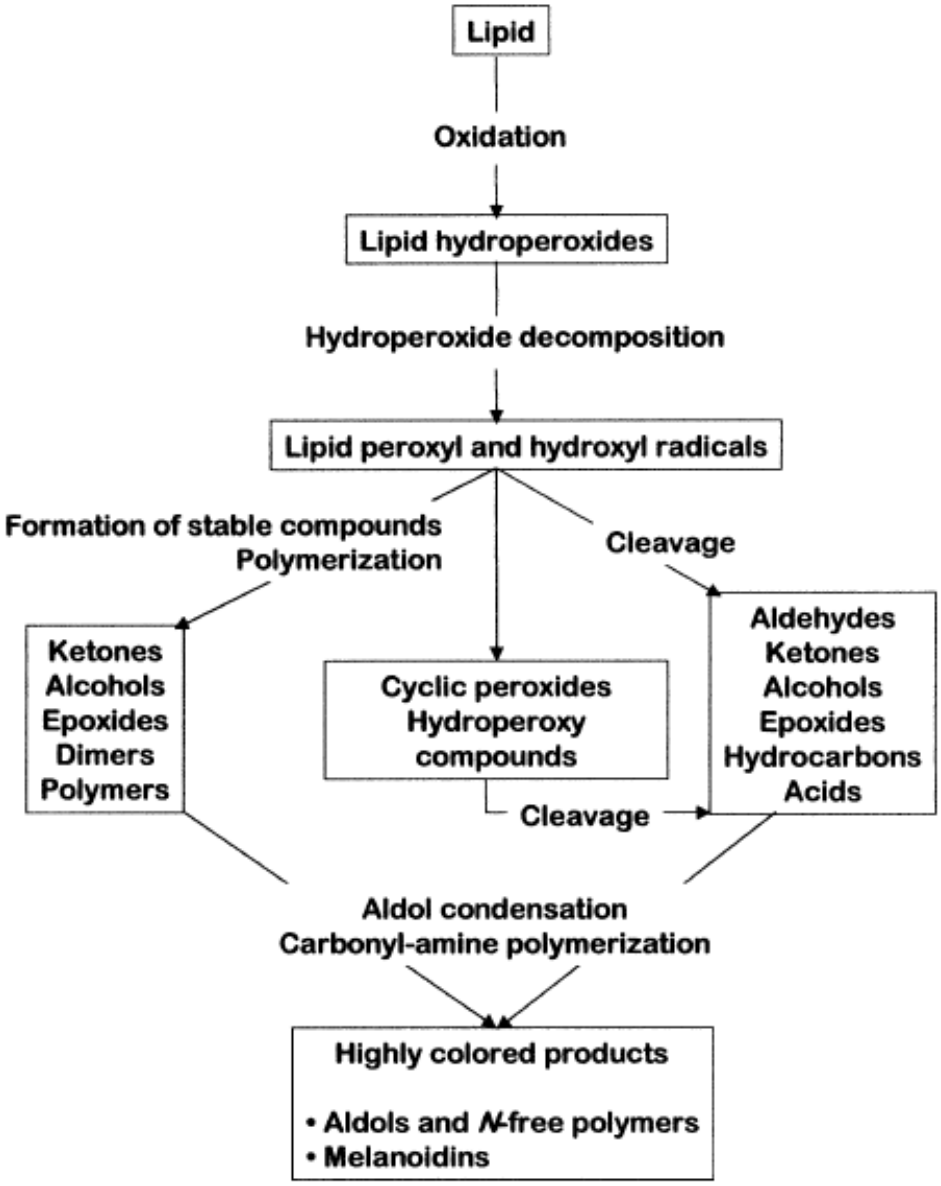

Figure 1-3. The lipid oxidation pathway (Zamora & Hidalgo 2005)

Maillard – Phenolic Chemistry

The Maillard-phenolic chemistry has been the topic of interest in whole wheat food flavors. Ferulic acid (FA) is the most abundant hydroxycinnanic acids (HCAs) in

wheat. In whole wheat flour, FA is present predominately in the bound form (Sosulski et al. 1982). Typically, <1% of the phenolic content in wheat is in the free form (Moskowitz et al. 2009), which was considered to be the reactive form related to altering Maillard chemistry and product generation. Reactivity of HCAs has been investigated in aqueous Maillard model systems and FA was shown to suppress the generation of 2-acetyl-1-pyrroline (2AP), a key aroma formed in bread (Moskowitz & Peterson 2010), by reacting with methylglyoxal, in a methylglyoxal/proline model system. Addition of HCAs to a glucose/glycine baking model to alter the mechanisms of the Maillard reaction and Maillard-type product generation was also studied (Jiang et al. 2009). Researchers in this study identified two ferulic acid-Maillard reaction products as 6-(4-hydroxy-3-methoxyphenyl)-5-(hydroxymethyl)-8-oxabicyclo[3.2.1]-oct-3-en-2-one and 2-(6-(furan-2-yl)-7-(4-hydroxy-3-methoxyphenyl)-1-methyl-3-oxo-2,5-diazabicyclo[2.2.2]oct-5-en-2-yl)acetic acid, which also indicates a Maillard-phenolic reaction mechanism underlining the flavor development of whole grain foods. As summarized, characterization of Maillard-phenolic interactions provided new mechanism of flavor development of whole grain foods.

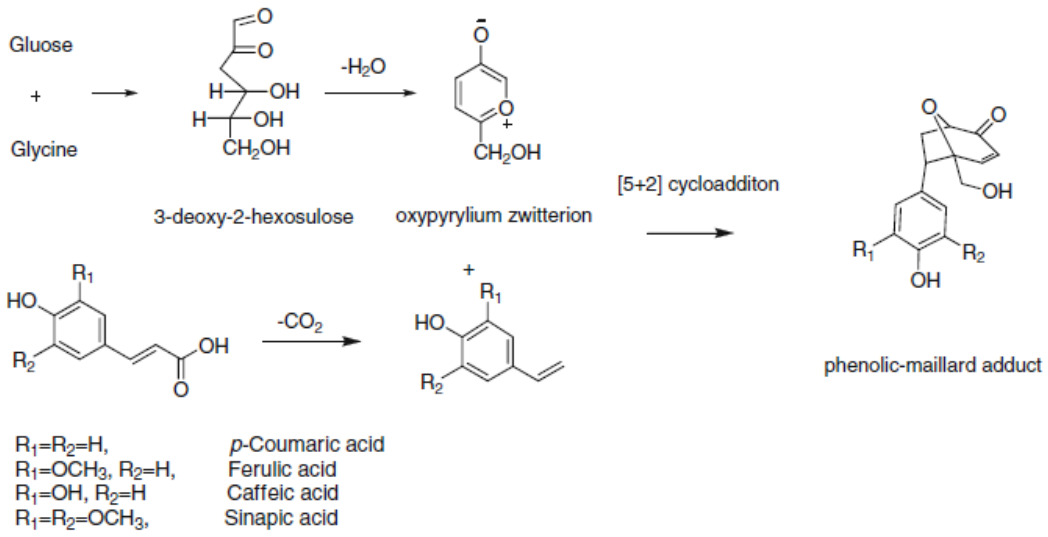

Figure 1-4. Reactivity of hydroxycinnanic acids in the Maillard reaction in a model baking system (Jiang & Peterson 2010).

Free FA liberated from bound FA during fermentation and baking has been previously quantified (Moskowitz et al. 2012) using [$^{13}C_6$]ferulic acid to estimate the FA content liberated in the whole wheat bread crust. The goal was to understand the influence of flour type, whole wheat (WWF) or refined wheat (RWF), on aroma development in bread crust. Compared to the initial free FA content measured in WWF (1.3 mg/kg), a significant amount of free FA was liberated (approximately 68 mg/kg) during the manufacturing process (fermentation and baking), which accounted for 27% of the bound phenolic content. Release of free FA from the bonded form (insoluble conjugate) in products during baking or fermentation can affect flavor development in whole wheat bread. Therefore, an equivalent concentration (68 mg/kg flour) of free FA was added to RWF prior to breadmaking, in order to investigate the influence of the

available free FA content in bread on flavor development. The addition of FA in the RWF bread formulation resulted in decreased levels of five important Maillard-derived aroma compounds: 2-acetyl-1-pyrroline, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 2-phenylethanol, 2-acetyl-2-thiazoline, and 2,4-dihyroxy-2,5-dimethyl-3(2H)-furanone. Overall, the addition of FA to the RWF resulted in a bread aroma profile similar to that of the WWF bread sample (Moskowitz et al. 2012), which further highlights the importance of Maillard-phenolic interactions in food flavor development.

1.1.3. Flavor (Aroma) Analysis

Aroma compounds elucidation has dominated flavor research for decades. Evolution of gas chromatography/olfactometry, various GC-MS techniques, as well as extraction methods for volatile analysis has greatly facilitated the discovery of the key odorants in various foods (Grosch 2001). Due to the complexity of food as a matrix, aroma active compounds are commonly present with low concentrations and low odor thresholds, creating a challenge in their extraction, separation, and quantification (Jeleń et al. 2012). Aroma compounds have a large variety of polarity, volatility and chemical character; therefore it is important to select method of extraction based upon the diversity.

Flavor analysis was categorized into three types of approaches (Jeleń et al. 2012):

1) Sensory guided flavor analysis
2) Flavor profiling. Volatile/flavor analysis to obtain their “profile” in food, followed by multivariate analysis used for authenticity testing and products classification and comparison

3) Target analysis. Compounds known as important for quality, processing and storage are monitored.

Sensory guided aroma analysis is widely used to identify compounds responsible for specific sensory attributes of food, which focus on the “key compounds” that are important to the aroma of the food. For volatile compound isolation from complex food matrices (e.g. coffee, bread crust solvent extracts), comprehensive and exhaustive extraction techniques, such as solvent-assisted-flavor-evaporation (SAFE) are usually considered (Engel et al. 1999). Development of gas chromatography–olfactometry (GC–O) in combination with GC-MS has provided fundamental tools for screening for key odorants (Van Ruth 2001; Plutowska & Wardencki 2008). The GC–O results, connecting between the composition of volatiles and the organoleptic properties of foods, are often correlated with the results of conventional sensory evaluation (Plutowska & Wardencki 2008). Recombination experiments by reconstituting key odorants to obtain the characteristic attribute is used to validate the sensory relevance of key compounds identified from sensory-guided aroma analysis, using difference detection methods (e.g. triangle method)(de Souza et al. 2006).

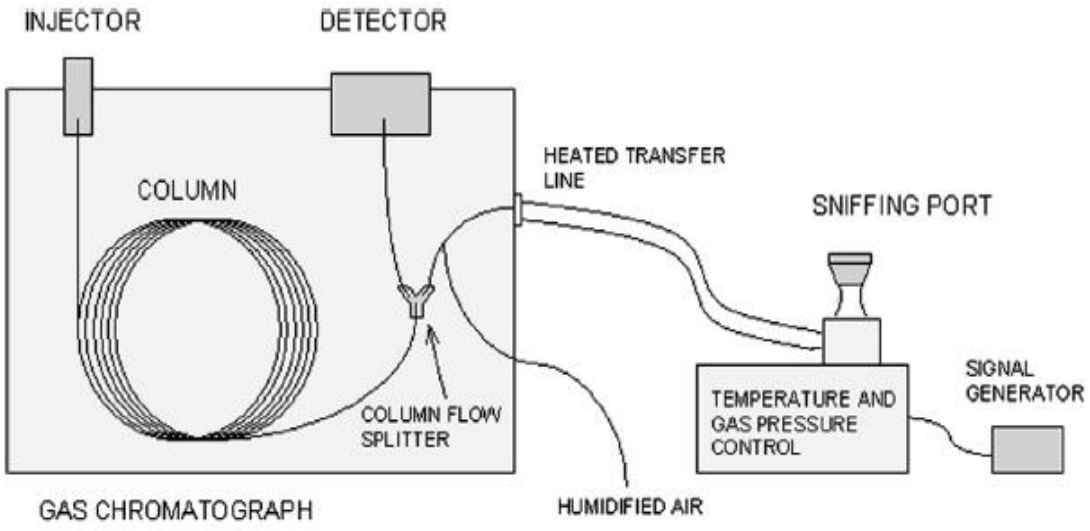

Figure 1-5. Gas chromatograph equipped with the olfactometric detector (Plutowska & Wardencki 2008).

In aroma profiling, microextraction techniques in combination with GC/MS are predominantly used in flavor analysis because it provides rapid analysis of volatile compounds compared to the traditional exhaustive extraction methods (e.g. SAFE). Microextraction techniques, mainly solid-phase microextraction (SPME) and stir bar sorptive extraction (SBSE), have been widely used for food flavor compounds analysis for the past two decades. The microextraction technique has many advantages over other aroma extraction techniques because it reduces the number of steps in sample preparation, eliminating solvents required for extraction, requiring less sample, and is adaptable to automation (Kokosa et al. 2009). However, microextraction methods, based on the partition of analytes between phases, do not always reflect the actual composition of volatiles in food matrices (Jeleń et al. 2012). Therefore, selecting the proper phase, based

on the polarity, volatility, and molecular size of the analyte, is crucial to successful compound profiling.

Target analysis in aroma research is usually aimed at compounds that are known, or suspected to be a cause of quality problems (e.g. taints and off-flavors). Methods are designed to selectively enrich analytes of interest, and improve sensitivity for analysis of compounds.

Compared to aroma-active volatiles, little attention is given to the non-volatiles in food. In recent years, this area has experienced more growth due to the advancement of non-volatile flavor active component isolation and characterization techniques leading to an increased insight regarding the importance of non-volatiles to the overall flavor perception. Advancement of liquid chromatography-mass spectrometry techniques in analyzing complex food constituents is a key driver for this change. The next section will focus on the taste research developments and challenges.

1.2. Taste research overview

A major challenge hindering the advancement of the non-volatiles research in the last few decades was the limited analytical capacity of the liquid chromatography/mass spectrometry in analyzing complex food constituents. Due to great advancement in those analytical approaches, non-volatile research has experienced a rapid growth. Similarly to established concepts in volatile research, such as aroma activity and methods for dilution analysis, the taste activity value was recently defined, and has been mainly studied on known and quantitatively predominant compounds (e.g. amino acids, peptides).

Compounds that are not intrinsic food ingredients, but generated during food processing are largely overlooked regarding sensory activity (Hofmann, Ottinger, et al. 2004).

In addition to taste activity, researchers have focused their efforts on understanding the chemical mechanism of gustatory sensations, in the hope of bridging the gap between compound structures and taste perception. Taste transduction for taste active organic molecules includes binding at specific G-protein-coupled receptors (GPCRs) on the taste cell plasma membrane, and successful identification of some key molecules in the plethora of GPCRs has been enabled by modern molecular biochemistry techniques (Ballesteros & Weinstein 1995). More effort has been made towards the isolation and characterization of receptor proteins and other signaling molecules of transduction within taste receptors (Hofmann, Ho, et al. 2004).

1.2.1. Taste dilution analysis (TDA) and taste activity (TAV) concepts

Taste dilution analysis (TDA) has been recently developed as a screening procedure for taste-active non-volatiles in foods (Hofmann, Ottinger, et al. 2004). High-performance liquid chromatography (HPLC) or gel permeation chromatography (GPC) is commonly used as a fractionation method to separate analytes on the basis of hydrophobicity or size, respectively. The typical procedure includes serial dilutions of each of the HPLC/GPC fractions two fold sequentially with water, and evaluation of those fractions using a sensory panel and the half-tongue test in ascending (concentration) order. The dilution at which a taste difference between the diluted extract and the blank

(control) could just be detected is defined as the taste dilution (TD) factor. Higher TD values are believed to correlate with greater taste impact (Hofmann, Ottinger, et al. 2004).

In analogy to the aroma activity value, the taste activity value (TAV) is defined as the ratio between the concentration and the attribute (e.g. bitter, sweet, umani, etc.) threshold concentration of a compound in food, which can be used to evaluate the contribution of a compound to the overall attribute (Hofmann, Ottinger, et al. 2004).

This approach has been used in a variety of foods to identify key tastants in the food matrix (Frank et al. 2001a; Scharbert et al. 2004). This screening technique also led to discovery of some new taste-active compounds that are generated through food processing (Frank et al. 2001a). **Figure 1-6** shows the application of the TDA in identification of bitter-tasting substance in a Maillard reaction model mixture. An aliquot of the reaction mixture was separated by HPLC into 21 fractions, which were collected separately and freeze-dried. Fraction 19 was evaluated with the highest bitterness impact (TD factor of 512) and was identified as *1H,4H*-quinolizinium-7-olate by further purification and NMR characterization. Finally upon purification and identification of the target compounds, compounds were quantified to determine the taste activity values in the food matrix and evaluate the contribution of each compound to the overall taste attribute of the mixture (Frank et al. 2001a).

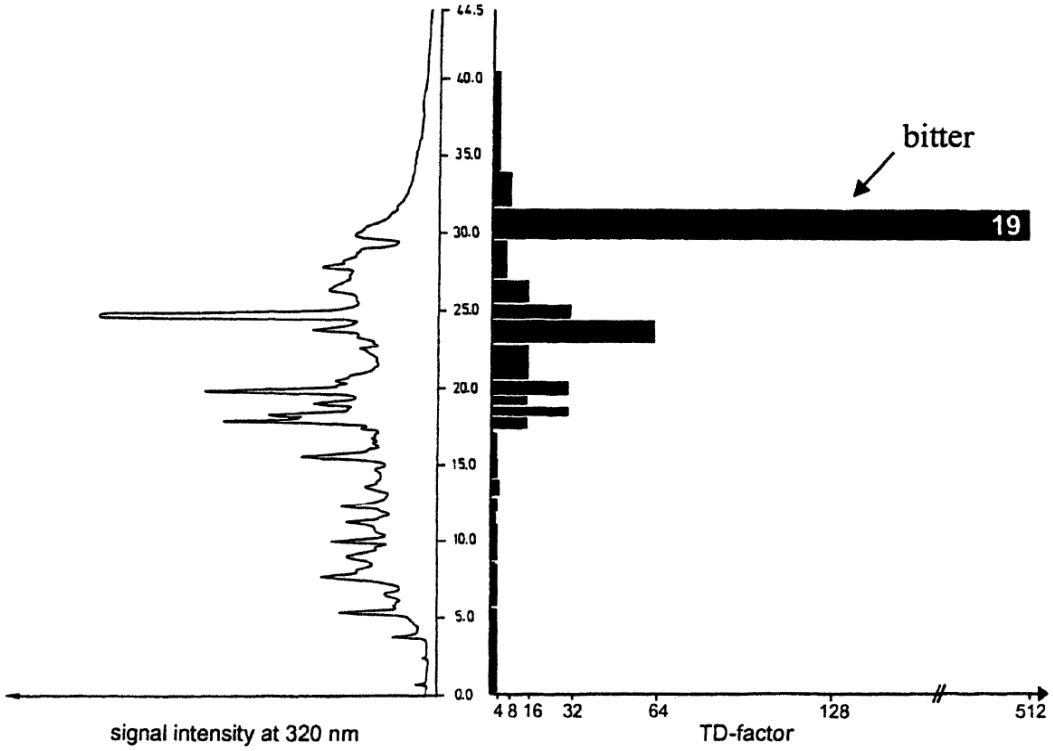

Figure 1-6. RP-HPLC chromatogram (left side) and Taste Dilution (TD)-chromatogram (right side) of the non-volatile, solvent extractables of a heated xylose/L-alanine solution (Frank et al. 2001a).

TDA represents a systematic method that requires reduced panelist training in comparison to descriptive sensory analysis, and is more numerically reproducible in screening for particular tastants of low attribute thresholds. However, for most compounds, the perceived attribute intensity is not linear to its concentration. For example, compound A may have a higher TD value for bitterness (lower bitterness

threshold) while having much lower bitterness intensity than compound B when both were at higher concentrations.

Additionally, the TAV approach only studies individual compounds, and puts those compounds out of the context of food matrix. When a tastant is present in food, many factors could contribute to its perception, such as its solubility in saliva. The forces that govern the dissolution of tastants in saliva during food mastication are complex, and some tastants are thus more likely to be extracted by saliva and to be perceived than others. In addition, the TAV approach does not often account for food matrix interactions. For example, starch has been reported to form helical inclusion complexes with different volatile flavor compounds, which limits aroma diffusion in the food matrix (Conde-Petit et al. 2006). Protein-sodium interactions have also been investigated to determine the availability of sodium in the aqueous phase and the perception of saltiness (Yucel & Peterson 2015). Therefore, a more comprehensive or representative method for taste evaluation is needed so that the tastants are evaluated in the context of perception in actual food products, when consumed.

1.2.2. Chemistry of bitter sensations

Food flavor is greatly dependent on the oral sensory systems, of which the gustatory system functions for the sense of taste. To bridge the gap between structural chemistry and human taste perception, the chemistry of gustatory sensations has been extensively studied on the mechanisms of signal transduction for the detection and discrimination of taste stimuli (Hofmann, Ho, et al. 2004). Taste transduction for taste

active organic molecules that are sweet, bitter, or umami includes binding at specific G-protein-coupled receptors (GPCRs) on the taste cell plasma membrane (Zhang et al. 2003). Identification of some key molecules in the plethora of GPCRs has been achieved. For bitter taste, a group of 30 different GPCRs, namely the T2R family, are specially expressed by taste receptor cells (Matsunami et al. 2000).

Bitter compounds in foods have diverse chemical structures (**Figure 1-7**) and belong to a wide range of chemical classes. For example bitterants can be flavonoids (e.g. naringin in fruits), alkaloids (e.g. caffeine), amino acids ($_{L}$-tryptophan), peptides, lipid diols (e.g. falcarindiol in carrots), etc. The great variety of organic molecules is explained by the heterodimeric strucutral functionality of T2R receptors, which allows them to adapt the diverse chemical structures of bitter compounds in food (Hofmann, Ho, et al. 2004).

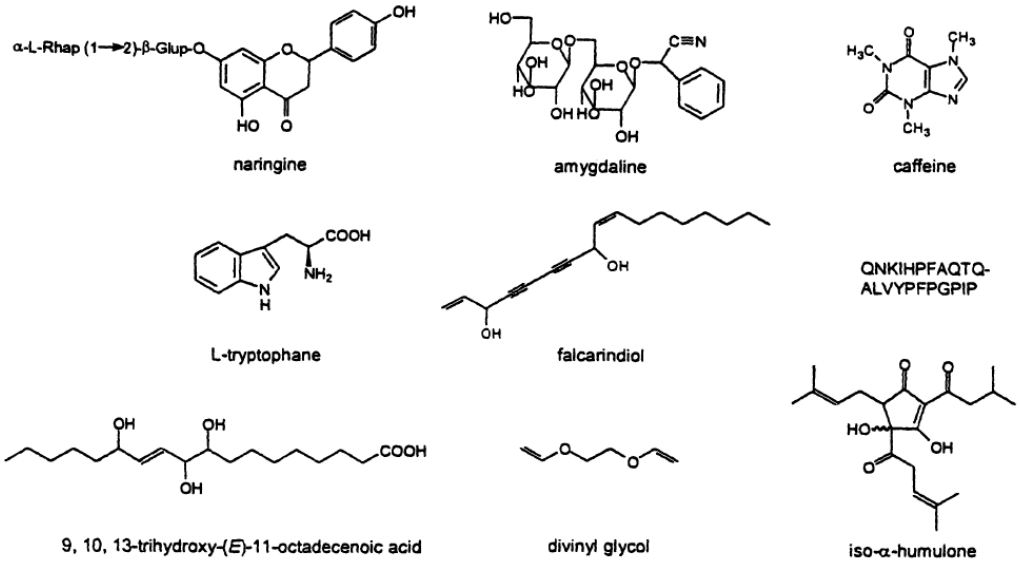

Figure 1-7. Structure diversity of bitter compounds from foods (Hofmann, Ho, et al. 2004).

In contrast to the activation of G-protein by bitter stimulated taste receptors, a number of artificial sweeteners were found to competitively inhibit bitter-receptor activation of G-protein, suggesting that these compounds may act as bitter inhibitors (Margolskee 2002). This concept is of extreme value for food manufactures and product developers as enhancing or inhibiting specific taste modalities can provide a scientific basis to develop food products with alternative formulations such as sugar, sodium, or fat reduction, while maintaining flavor quality. This will be a big step towards promoting the consumption of healthier foods. This approach is becoming more achievable by identifying flavor-modulating compounds which either naturally occur in food or are generated from processing.

1.3. Whole grain food consumption: The flavor challenges

Whole grain foods have been widely recognized as important components of a healthy diet. Whole grains are rich in essential nutrients such as B vitamins, minerals, polyunsaturated fatty acids, and dietary fiber. Additionally, they contain numerous physiologically active components such as phytochemicals and antioxidants that are considered beneficial to health (Liu & Adom 2007). An increasing number of epidemiological studies continue to support that consumption of whole grain can reduce the risk of several chronic diseases, which include type 2 diabetes (McKeown et al. 2002;

Liu et al. 2000), coronary heart disease (Liu et al. 1999), cancer (Slavin et al. 2011), and cardiovascular disease (Jacobs & Gallaher 2004).

USDA dietary guidelines for Americans recommends at least 6 servings of grain products per day, and at least half of the total grain (3 servings) to be consumed as whole grains (U.S. Department of Agriculture & U.S. Department of Health and Human Services 2010). During recent years, with raised awareness of health benefits of whole grain foods, consumption has been increasing. Mintel's analysis of the bread market indicates that sales of whole-grain breads has increased by 50 percent over the past five years (Mintel Oxygen 2013). However, still less than five percent of Americans consume the minimum recommended amount of whole grains, and, on average, Americans eat less than 1 ounce-equivalent daily (3 ounce is recommended). Among children and adolescents, the dietary intake of whole grains is far less than recommended. According to data collected from 1994-1996 US Department of Agriculture Continuing Survey of Food Intakes by Individuals (CSFII), 15% consumed a mean of two or more servings per day, with the average intake for children (6-11 years) 0.9 serving per day and for adolescents one serving per day (Harnack et al. 2003). The USDA recently published "Nutrition Standards in the National School Lunch and School Breakfast Programs" to align the nutrition standards with the dietary guidelines (U.S. Department of Agriculture 2012).

1.3.1. Factors that limit whole grain intake

According to the Institute of Medicine report (Institute of Medicine 2009), major factors that limit consumption of whole grain products include sensory issues of taste and texture, difficulty in identifying whole grain foods, and limited availability. From the flavor quality perspective, whole-grain products are commonly associated with bitterness, dryness, astringency, and other negative sensory attributes.

A study aiming to increase whole grain intake among children within a school-based intervention designed a sensory evaluation experiment in order to identify attributes of whole-grain foods and factors influencing intake by children, parents, and teachers (Burgess-champoux et al. 2006). Children and adults were asked a series of questions regarding grains and whole grains, including knowledge of whole grain foods, eating patterns, preferred foods, etc. Findings indicated that adults preferred cereals they perceived as more healthful with high-fiber, whole-grain, less sugar) but children preferred prefer refined, sweetened grain products. Based on the study, gradual adaptation to more whole-grain products may enhance the whole-grain acceptance over time.

Food choices and bitter taste

Food choices are largely influenced by taste (Drewnowski 2001). Generally, people like sweetness and dislike bitterness; although in certain foods (e.g. dark chocolates, cheddar cheese) and beverages (e.g. coffee, beer, wine), a certain degree of bitter tastes can help balance overall flavor and bitterness can be desirable and at times

necessary for the expected flavor profile. Humans are particularly sensitive to bitter taste of plant alkaloids, consisting of phenols and flavonoids, isoflavones, terpenes, and glucosinolates (Drewnowski & Gomez-carneros 2000). Consumer and marketing studies show that humans tend to reject foods that are perceived bitter, in spite of perceived nutritional and health value of the food. This feeling can be instinctive as plants protect themselves against predator by secreting toxins with unpleasant bitter taste (Drewnowski & Gomez-carneros 2000).

Detection thresholds for bitter taste are generally very low. For instance, bitter quinine is detected at ~ 25 µmol/L levels. In contrast, detection threshold for sucrose was on the order of 10,000 µmol/L. As a result, the food industry routinely includes debittering processes for plant-based foods (**Table 1-1**).

Table 1-1. Industrial debittering processes to remove phytonutrients from food (Drewnowski & Gomez-carneros 2000).

Phytonutrient class	Bitter compound	Food	Debittering process
Flavanones	Naringin	Grapefruit juice	Adsorption to polymers
	Naringenin	Orange juice	Passage through resins
Flavones	Tangeretin		Passage through enzyme matrix
Flavans	Catechin, epicatechin	Tea	Fermentation
		Chocolate	Removal of chocolate liquor
Triterpenes	Limonin		Use of cyclodextrin polymers
Isoflavones	Genistein and daidzein	Soy products	Selective breeding

In addition, taste masking is also an effective method utilized in order to increase food acceptability and liking rating commonly through the use of fat, sugar, and salts to mask detrimental attributes such as bitterness. A great example where taste masking has been employed effectively in soy fortified yogurt, where sweeteners (e.g. sucrose, fructose) and fruit flavors were added to decrease the perception of astringency and other negative soy flavors (Drake et al. 2001).

1.4. Bitterness in whole grains: Present knowledge

Among the sparse literature on the topic of bitterness in whole grain, a series of studies have investigated the impact of phenolic compounds on perceived bitterness in rye grains (Heiniö et al. 2003; Liukkonen et al. 2003; Heiniö et al. 2008). The studies have compared the sensory profile, including bitterness, of flour and bread made from different milling fractions of rye grains using descriptive analysis. The bran fractions were perceived as most bitter (Heiniö et al. 2003). The relative concentrations of total phenolic compounds were distributed in accordance with the bitterness of the milling fractions (Liukkonen et al. 2003). Free phenolic acids such as pinoresinol and syringic acid were suggested to impact the perceived bitterness in whole grain rye products (Heiniö et al. 2008). This correlation was based merely on statistical multivariate techniques that relate levels of chemical compounds to the intensities of sensory descriptive attributes of foods. Impacts of other non-volatile compounds on the bitterness of rye were not studied.

Bitterness in stored ground oat grain has been also investigated and attributed to oxidative changes of the lipids (Baur et al. 1977; Biermann & Grosch 1979). In these studies, 9- and 13-hydroxyoctadecanoic monoglycerides were isolated from stored oat flour and reported to have very low thresholds for bitter taste (1.1-2.2 μmol/ml) (Biermann & Grosch 1979). The identified bitter hydroxylated fatty acids were identified as enzymatic oxidation products of monolinoleate by lipoxygenase. Besides the monoglycerides, trihydroxyoctadecenoic acids, previously reported as bitter substances (Baur et al. 1977), were found to increase in oats during storage. This mechanism was suggested to involve peroxidation of linoleic acid, followed by decomposition of linoleic acid hydroperoxides. This peroxidation pathway was associated with the enzymatic activities of lipoxygenase and peroxidase (Baur et al. 1977).

Similarly to oat grains, trihydroxyoctadecenoic acids have been identified as products of the enzymatic oxidation of linoleic acid when incubating linoleic acid with a suspension of cereal (e.g. wheat, barley, rye, oats)(Graveland 1973) and leguminosae (e.g. peas)(Arens & Groseh 1974). Graveland *et al.* (1970) reported oxidation reactions of linoleic acid in wheat flour-water suspensions, in which lipoxygenase was adsorbed on gluten and oxidized linoleic acid into linoleic acid hydroperoxides, 9-LOOH and 13-LOOH (Graveland 1970). Decomposition of linoleic acid hydroperoxides into various reaction products, including bitter-tasting hydroxy fatty acids, can be either enzymatic or nonenzymatic (Gardner 1975). **Figure 1-8** shows the enzymatic pathways of linoleic acid hydroperoxides decomposition. The nonenzymatic decomposition of lipid hydroperoxides was suggested to be more predominant during food storage (Gardner 1975). The most

common nonenzymatic pathways involve free radical mechanisms that can occur under several conditions: heat, photolysis, metal catalysis, etc. Heterolytic decompositions lead to the production of corresponding hydroxy fatty acids from the hydroperoxides. (Gardner 1975).

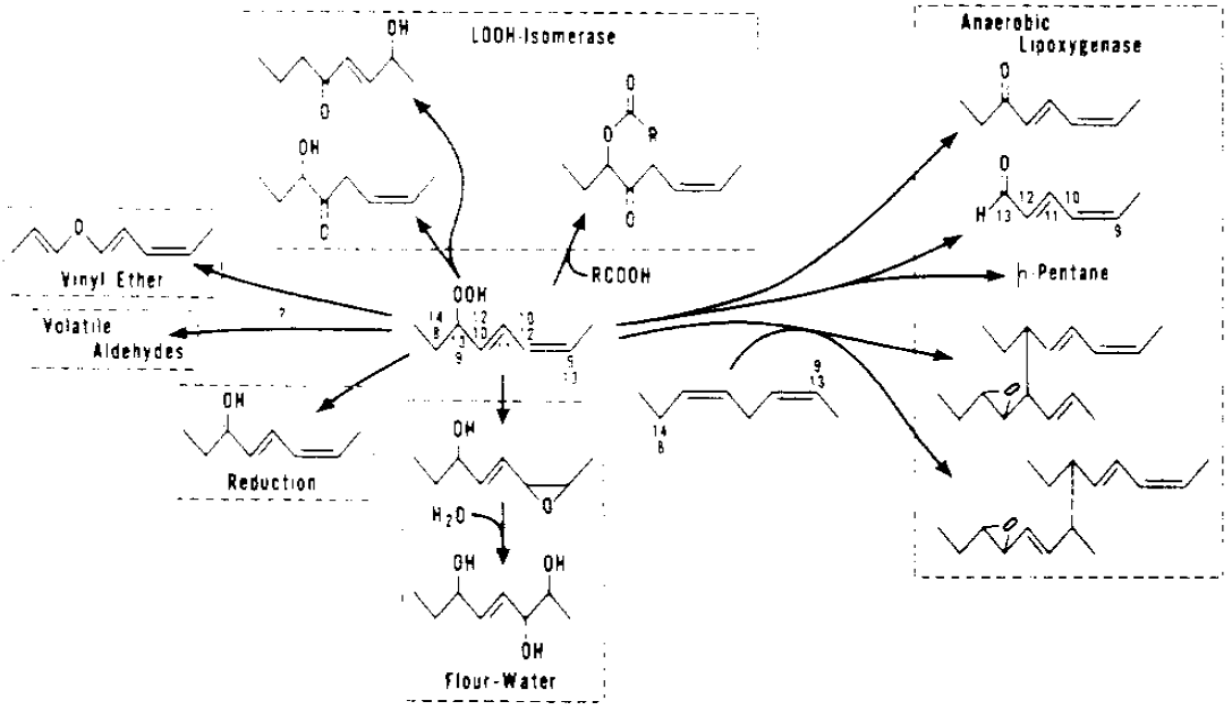

Figure 1-8. Enzymatic decompositions of linoleic acid hydroperoxide (Gardner 1975).

Bitter tastants generated from thermal food processing (e.g. Maillard reaction) have been the focus of many recent studies (Frank et al. 2001b). Characterization of Maillard-type flavor compounds has been mainly focused on volatile compounds in the past few decades. Many key Maillard-derived odorants in a variety of processed foods, such as bread crust, beef, chocolate, etc. have been successfully characterized by application of aroma extract dilution analysis (Grosch 1994). Non-volatile compounds

potentially possess taste attributes (e.g. bitterness) and taste modulation activities, and have been the focus of trending research. Taste dilution analysis (TDA) has been recently utilized for the identification of bitter tastants from the Maillard reaction model systems (Frank et al. 2003). These observed Maillard-derived bitter compounds indicate the potential of the ubiquitous Maillard reaction as a pathway of bitterness development in many types of processed foods. More specifically in wheat bread, Jiang and Peterson (Jiang & Peterson 2013) reported the Maillard reaction was a mechanism of bitterness development. Based on sensory-guided analytical fractionation techniques, eight bitter compounds were identified in bread crust. These compounds were fermentation products, including the amino acid $_{L}$-tryptophan (TRP) and its metabolite tryptophol (TRO), as well as Maillard reaction products, including TRP Amadori rearrangement product (ARP), 5-(hydroxymethyl)furfural (HMF), 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (DDMP), 4-(2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl)butanoic acid (PBA), Acortatarin A, and Acortatarin C. The proposed mechanism of generation of these bitter compounds is shown in **Figure 1-9**. The ARP is formed *via* Amadori rearrangement at the early stage of the Maillard reaction by the utilization of TRP (Hodge 1955). 5-(Hydroxymethyl)furfural (HMF) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one (DDMP) are formed *via* the 3-deoxyglucosone and 1-deoxyglucosone dehydration pathway, respectively (Hayase 2000). 4-(2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl)butanoic acid (PBA) is also a 3-deoxyglucosone degradation product, formed from 4-aminobutyric acid and 3-deoxyglucosone *via* nucleophilic intramolecular ring closure and keto/enol tautomerization (Ghiron et al. 1988). Acortatarin A and C are formed *via* the

reaction between 3-deoxyglucosone and related Strecker degradation product 1,3-dideoxy-1-amino-fructose. As a follow-up study, the chemical profile of these bitter compounds in relation to the perceived bitterness of four commercial whole wheat breads and one refined wheat bread was investigated (Bin et al. 2012). Highly significant correlations ($\alpha = 0.01$) were observed between the perceived bitterness in the crust and the quantity of 5-(hydroxymethyl)furfural (HMF, $r^2 = 0.93$) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (DDMP, $r^2 = 0.95$); for the crumb, significant correlation was observed between bitterness and $_L$-tryptophan (TRP, $r^2 = 0.91$). These findings provided a preliminary approach to the profiling of the bitter fingerprint across different whole wheat products and correlating the chemical data to sensory response.

Although the Maillard reaction was suggested to contribute to bitterness development in bread crust, the role of this reaction towards the bitterness in the entire bread is only part of the problem. Maillard reaction majorly occurs in bread crust due to increased processing temperature and dehydration during baking which creates low moisture conditions optimal for the progression of Maillard reaction. The bread crumb, consisting more than 80% of the bread mass, has higher moisture content and reaches lower processing temperature than the crust. Therefore, the crumb is far less likely to produce very high level of these bitter Maillard reaction products as compared to crust. Bitterness in whole wheat crumb may be quite distinct from the bread crust, in terms of the chemical species and generation pathways.

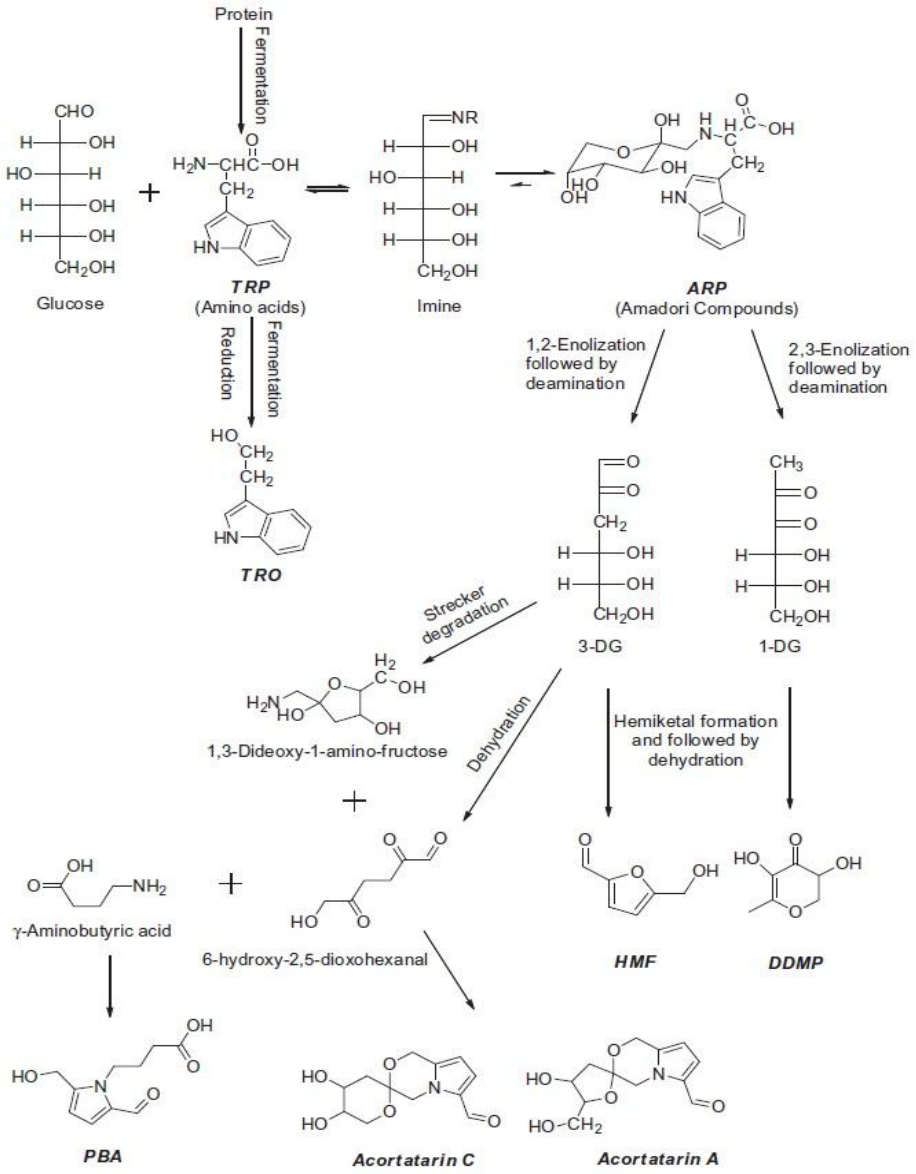

Figure 1-9. Proposed bitterness generation map in whole wheat bread crust, Jiang and Peterson(Jiang & Peterson 2013).

The above literature review summarized the main findings and limitations of the research related to bitterness in whole grains. Generation of negative flavors in whole grain foods has been poorly defined and knowledge regarding the origin of bitterness (intrinsic vs production derived) of processed whole grain foods is lacking. From taste

research perspective, there are no comprehensive studies that relate the chemical profile of whole-grains to sensory perception. Consequently the limited understanding of bitterness in whole grain foods limits the development of production strategies for flavor improvement.

The overall goal of this project was thus to provide an improved knowledge foundation and enable the manufacture of high flavor quality whole wheat products. To accomplish this goal, this work focused on the characterization of bitter compounds in whole wheat bread, elucidation of the pathways of the formation of the identified bitter compounds, and the investigation of how intrinsic diversity (e.g. wheat varieties) and processing factors influence bitterness development in the products. The main objectives of this project were as follows:

1. Identification of compounds responsible for the bitter taste in processed whole wheat foods;
2. Development of quantitative methods of the identified bitter compounds in various matrices for analytical profiling and sensory confirmation;
3. Characterization of precursors and mechanisms of the bitterness development/degradation in whole wheat products.
4. Investigation of key production factors related to bitterness development in whole wheat foods.

The following chapters present findings regarding the composition and the origins of bitterness in whole wheat bread and provide insights on how to reduce bitterness

development by investigating the mechanisms and key production factors of generation of bitter compound. Chapter 2 and 3 focuses on characterization of key bitterness "markers" of whole wheat bread crust and crumb, respectively, with Chapter 3 presenting a novel taste evaluation approach that quantitatively determines bitter compound concentration in panelists' saliva during food mastication. Chapter 4 details the investigation of the mechanism of generation of a key bitter compound, pinellic acid, during production of whole wheat bread using stable isotope labelling study, and further addresses the influence of wheat variety and flour storage on generation of pinellic acid in whole wheat foods.

Chapter 2

Chemical Markers for Bitterness in Wheat Bread

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Synopsis

The manufacture of high-quality whole-grain products is challenged by poor flavor attributes, such as excessive bitterness. In a recent study our group characterized eight compounds that contributed to the bitterness of a whole-wheat bread sample. The goal of this study was to investigate the chemical profile of these bitter compounds in relation to the perceived bitterness of four commercial whole wheat breads and one refined wheat bread. Highly significant correlations (a= 0.01) were observed between the perceived bitterness in the crust and the quantity of 5-(hydroxymethyl)furfural (r^2 = 0.93) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (r^2 = 0.95). In the crumb, the bitterness was correlated with the amount of $_L$-tryptophan (r^2 = 0.91). In summary, both Maillard- and fermentation-derived compounds were identified as potential chemical markers to predict bitterness of whole-wheat bread.

Introduction

Whole grain foods are considered to be a key component of a healthy diet. According to the dietary guidelines for Americans (U.S. Department of Agriculture & U.S. Department of Health and Human Services 2010), the United States Department of Agriculture recommends that at least half of total grain intake should be whole grains.

These guidelines are based on an increasing amount of epidemiological studies supporting whole grain consumption can reduce the risk of several chronic diseases, such as coronary heart disease (Liu et al. 1999), cardiovascular disease (Jacobs & Gallaher 2004), type 2 diabetes (McKeown et al. 2002; Liu et al. 2000), and cancer (Jacobs et al. 1998), as well as help with weight maintenance. However, less than 5 percent of Americans consume the minimum recommended amount of whole grains. Increasing the intake of whole grains for consumers has been a challenge. Compared to refined grain formulated products, factors that limit consumption of whole grain products include a lack of awareness of the health benefits, generally higher prices, and perceived inferior flavor (Kantor et al. 2001). From the flavor quality aspect, whole grain products are commonly associated with bitter, grainy, and other negative sensory attributes. A study on consumer liking of refined and whole wheat bread showed that consumers prefer refined grain bread over whole grain bread when both were made using equivalent ingredients and procedures (Bakke & Vickers 2007), indicating that flour composition (refined versus whole grain) does have a significant impact on the acceptability of wheat bread. However, limited knowledge of the related off-flavor compounds, such as those that contribute to bitterness (Jiang & Peterson 2013), and related pathways has been reported, making it challenging to pinpoint the molecular basis and mechanistic details for the inferior palatability of whole grain foods.

Jiang and Peterson (Jiang & Peterson 2013) recently used a sensory-guided analytical fractionation technique to characterize the primary bitter compounds in a whole wheat bread crust sample. They identified eight bitter compounds of which two

were fermentation products, the amino acid L-tryptophan (TRP) and its metabolite tryptophol (TRO) (**Figure 2-1**). The remaining six compounds were Maillard reaction products: TRP Amadori rearrangement product (ARP), 5-(hydroxymethyl)furfural (HMF), 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (DDMP), 4-(2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl)butanoic acid (PBA), Acortatarin A and Acortatarin C. Amino acid TRP is one of the primary bitter tastants and is approximately half as bitter as caffeine(Mitteilungen 1965). Metabolism of TRP during the fermentation process give rise to tryptophol (TRO) (Sapis & Ribéreau-Gayon 1969), a compound commonly found in wine as a secondary product of alcoholic fermentation (Sapis & Ribéreau-Gayon 1965).

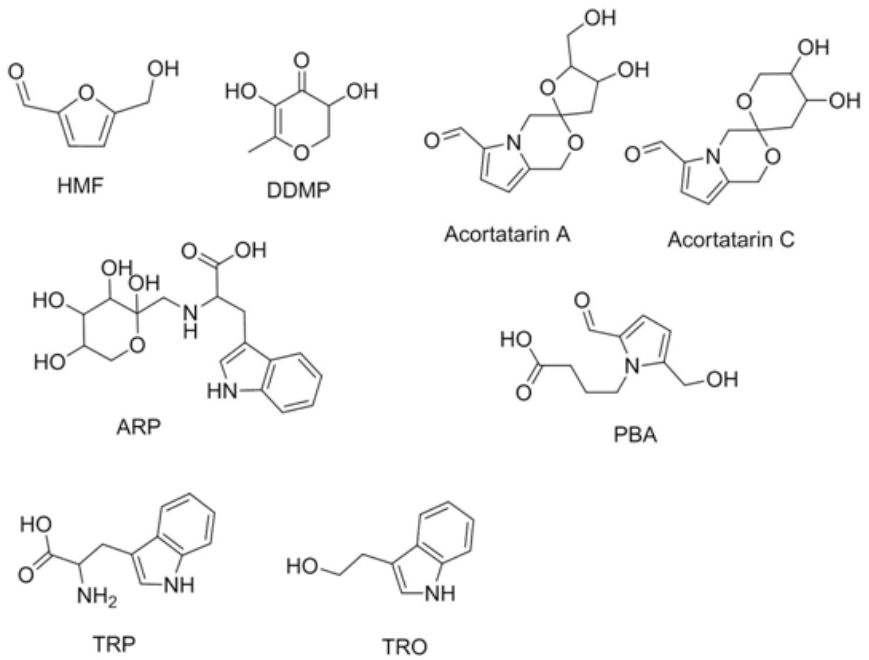

Figure 2-1. Chemical structures of eight bitter compounds identified from whole grain bread crust adapted from Jiang and Peterson(Jiang & Peterson 2013); L-tryptophan (TRP), tryptophol (TRO), TRP Amadori rearrangement product (ARP), 5-

(hydroxymethyl)furfural (HMF), 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (DDMP), 4-(2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl)butanoic acid (PBA)

The other six compounds are generated from the Maillard reaction (Nursten 2005). The tryptophan Amadori rearrangement product (ARP) (Yaylayana & Huyghues-Despointes 1994) is formed via Amadori rearrangement(Hodge 1955) at the early stage of the Maillard reaction by the utilization of TRP. 5-(Hydroxymethyl)furfural (HMF) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one (DDMP) are formed *via* the 3-deoxyglucosone and 1-deoxyglucosone dehydration pathway, respectively (Cutzach et al. 1997). 4-(2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl)butanoic acid (PBA) is also a 3-deoxyglucosone degradation product, formed from 4-aminobutyric acid and 3-deoxyglucosone *via* nucleophilic intramolecular ring closure and keto/enol tautomerization (Tressl et al. 1993). Acortatarin A and C were reported for the first time in bread and as Maillard reaction products. It was proposed that the formation of Acortatarin A and C was from the reaction between 3-deoxyglucosone and related Strecker degradation product 1,3-dideoxy-1-amino-fructose (Ghiron et al. 1988).

The main objective of this study was to correlate the relative concentrations of the eight previously identified bitter compounds to the perceived bitterness of the crust and crumb regions of commercial whole wheat and refined wheat formulated bread samples. The comparison of commercial samples investigates how ubiquitous these compounds are in wheat bread and to identify chemical markers of bitterness in related products.

Materials and Methods

Commercial Breads. Arnold Soft Family 100% whole wheat (AW), Brownberry 100% whole wheat (BW), Sara Lee Soft & Smooth 100% whole wheat (SW), Pepperidge Farm 100% whole wheat (PW), Wonder Classic refined wheat (WR) breads were purchased from a local grocery store.

Chemicals. Ethanol (Absolute, HPLC Grade) and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (98-100%, puriss) was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Water was purified through Barnstead Nanopure Diamond water purification system (Thermo Scientific, Dubuque, IA, USA).

Sample Preparation for Liquid Chromatography – Mass Spectrometry (LC – MS) Analysis. Five grams of bread crust or crumb were extracted with 20% aqueous ethanol (50 mL) spiked with internal standard caffeine (5 mg/kg) for 8 h (agitation speed 500 rpm). The extract was centrifuged, filtered, the permeate was pooled, the ethanol removed under vacuum, and the sample was then freeze-dried. The residue from the freeze-dried sample was re-extracted with 25 mL of methanol: water (50:50 v/v) and a five mL aliquot was passed through a 500 mg preconditioned C18 cartridge (Supelco, Bellefonte, PA, USA) and eluted with 2 mL of methanol for further sample clean up. The methanol and eluent were pooled, concentrated under vacuum, and filtered through 0.20 µm Nylon syringe filter (Millex, Billerica, CA, USA) prior to LC-MS analysis. All samples were prepared in duplicate.

Liquid Chromatography – Mass Spectrometry. Samples were analyzed on a Waters ACQUITY UPLC system interfaced with a Quattro Premier XE™ mass spectrometer (Waters, Milford, MA, USA). Two µL were injected on an ACQUITY UPLC BEH C18 1.7 µm column (2.1 mm × 50 mm) (Waters, Milford, MA, USA) at 25ºC. The mobile phase was maintained at a flow rate of 388 µL/min using binary solvent system of 0.1% formic acid in water (A) and methanol (B). The elution gradient started at 10% B (0-1 min), linearly increased to 50% B (1-8 min), held at 100% B (8-9 min), decreased to 10% B (9-10 min), and held at 10% B (10-20 min). The Quattro Premier XE™ mass spectrometer was equipped with an electrospray ionization probe and the operational parameters were: +ve ion mode, source temperature was 110°C; desolvation temperature was 400°C; and the capillary voltage was 3.0 kV. Data was collected in multiple reaction-monitoring (MRM) mode. The ion transitions for the eight bitter compounds along with the cone voltages and collision energies were as follows: HMF, 109 ← 127, cone 30V, collision 10V; DDMP, 109 ← 145, cone 40V, collision 16V; TRO, 144 ← 162, cone 20V, collision 12V; TRP, 146 ← 205, cone 15V, collision 17V; Acortatarin A and C, 218 ← 254, cone 30V, collision 7V; ARP, 349 ← 367, cone 45V, collision 12V. The dwell time was 0.1s for each compound; the inter-channel delay was 0.02s and inter-scan delay was 0.1s. All samples were analyzed in duplicate.

The accuracy of the detector response for the targeted bitter compounds was assessed by a recovery experiment based on the analysis of mixtures of the samples isolates instead of spiked compounds, due to insufficient quantities of the pure chemical standards needed for standard recovery experiments. Four samples (two of crust and two of crumb)

containing different amounts of the target compounds were chosen as references and analyzed in duplicate. Then, four of their 1:1 mixtures (SW crust:PW crust, SW crumb:PW crumb, SW crust:SW crumb, PW crust:PW crumb) were also analyzed by LC-MS-MRM. The accuracy of the instrumental response was then estimated via the calculation of the signal recovery through the following equation:

Signal recovery for compound i in mixture A(%)=[Measured response of i in the mixture A/(averaged response of i in the two corresponding unmixed samples)]×100.

Sensory Analysis. A trained sensory panel, ages 22 to 45, consisting of two males and five females, evaluated bitterness in bread samples. All evaluations were conducted with nose-clips. Panelists were trained to evaluate bitter taste intensity using caffeine solutions as references. Three reference levels were provided to panelists (0.03, 0.08, and 0.15% of caffeine w/w in water), corresponding to a bitterness intensity ratings of 2, 5 and 10, respectively (Meilgaard et al. 1997). Judge's performance was evaluated by a general linear model analysis of variance test with sample, panelist, and their interaction as variation factors. For the evaluation of the bread, 5 grams of crust and crumb samples were presented to panelists in cups labeled with 3-digit codes and were evaluated in duplicate by each panelist. Water and unsalted crackers were used as palate cleansers.

Statistical Analysis. The sensory data was analyzed by ANOVA with Tukey's pairwise mean comparison to determine differences in bitterness perception among samples, significance was established at p-value $\leq$ 0.05. Pearson's correlation analysis was conducted between the analytical and sensory data. Statistical analysis was performed with Prism (V. 5.04, GraphPad Software Inc.)

Results and Discussion

Sensory evaluation of commercial whole wheat and refined wheat breads

The perceived bitterness intensity of the crust and the crumb portions of the five commercial wheat breads (4 whole-wheat and 1 refined wheat) are shown in **Figure 2-2**. Among the whole-wheat bread samples, there was variation in bitterness ratings within both the crust and crumb. For the crust, two of the whole wheat samples (AW and SW) were significantly higher in bitterness intensity (approximately 2-fold) in comparison to the other whole wheat samples (BW and PW) and refined wheat sample (WR). Furthermore, both the AW and SW whole wheat samples reported significantly higher bitterness in the crust (versus the crumb) while the opposite trend was observed for the BW and PW samples. Bitterness in the crumb however was higher in the whole wheat samples in comparison to the refined sample.

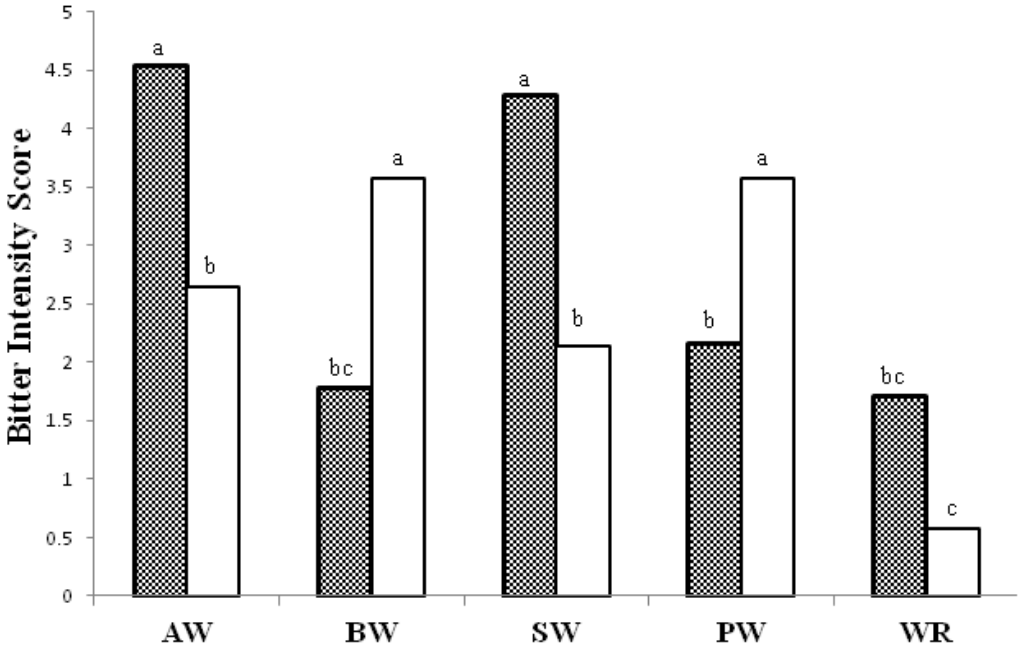

Figure 2-2. Mean sensory ratings of the bitterness intensity in crust ■ and crumb □ of wheat bread; Arnold Soft Family 100% whole wheat (AW), Brownberry 100% whole wheat (BW), Sara Lee Soft & Smooth 100% whole wheat (SW), Pepperidge Farm 100% whole wheat (PW), and Wonder Classic refined wheat (WR). Bitterness score of 2, 5, and 7 were corresponded to caffeine standard solutions 0.03%, 0.08%, and 0.096% w/w in water, respectively. Different letters (a-c) mean significantly different (p-value < 0.05).

Analytical measurement of bitter compounds among commercial whole wheat and refined wheat breads.

All eight compounds were detected in all five samples suggesting they are common to wheat bread. The quantitative accuracy of the LC/MS technique used to monitor the relative concentrations of the analytes was evaluated (see **Table 2-1**) and overall good agreement between the expected and measured responses were found in the recovery experiment carried out with mixture samples. In general, recoveries are between

90 and 113% and only the four mixtures for TRP had slightly higher % recoveries (119-125%), indicating the instrumental methods were accurate enough to make an assessment of the involvement of these compounds in the perceived bitterness differences. The relative concentrations of the eight bitter compounds in the five commercial wheat bread samples are reported in **Figure 2-3** (fermentation products) and **Figure 2-4** (Maillard products). For the fermentation products, the concentration of TRP in each crust sample was lower than that of the crumb as would be predicted due to a more rapid utilization of this amino acid in the crust by the Maillard reaction during the baking process. The concentration of TRO (fermentation product of TRP) was evenly distributed between crust and crumb, however the enzymatic conversion of TRP to TRO during bread dough fermentation appeared to be negligible in two of the BW and PW samples.

Table 2-1. Accuracy assessment of the LC/MS-MRM peak area responses for monitored bitter compounds.

	Samples[b]							
Compounds[a]	SW Crust	PW Crust	SW Crumb	PW Crumb	Crust 1:1 Mix SW:PW	Crumb 1:1 Mix SW:PW	SW 1:1 Mix Crust: Crumb	PW 1:1 Mix Crust: Crumb
TRP	959	7183	24294	42693	4857[c] (119%[d])	39495 (118%)	15735 (125%)	29803 (120%)
TRO	71087	637	82277	740	38551 (108%)	46933 (113%)	81375 (106%)	718 (104%)
ARP	878	3720	377	639	2425 (105%)	540 (106%)	642 (102%)	2208 (101%)
HMF	246551	67830	626	138	156046 (101%)	390 (102%)	124609 (101%)	38094 (112%)
DDMP	676	265	-	-	433 (92%)	-	320 (95%)	126 (95%)

PBA	1301	2135	-	-	1930 (112%)	-	687 (106%)	1161 (109%)
Acortatarin A	1076	623	-	-	830 (102%)	-	534 (101%)	303 (97%)
Acortatarin C	1231	730	-	-	1034 (106%)	-	634 (103%)	394 (108%)

a = $_{L}$-Tryptophan (TRP), tryptophol (TRO), TRP Amadori rearrangement product (ARP), 5-(hydroxymethyl)furfural (HMF), 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (DDMP), 4-(2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl)butanoic acid (PBA); b = LC/MS isolate for Sara Lee Soft & Smooth 100% whole wheat (SW) and Pepperidge Farm 100% whole wheat (PW); c = LC/MS-MRM peak area responses; d = Signal recovery for compound i in mixture A(%)=[Measured response of i in the mixture A/(averaged response of i in the two corresponding unmixed samples)]×100

The Maillard-derived compounds HMF, DDMP, Acortatarin A and C, PBA, and ARP were mainly found in the bread crust (**Fig. 2-4**). A similar concentration pattern of both HMF and DDMP among the five samples is easily observed; both compounds are generated in the initial stages of Amadori generation from 3-deoxyglucosone and 1-deoxyglucosone, respectively (Jiang & Peterson 2013). The concentration of ARP had a similar pattern to TRP, but with much higher distribution in bread crusts compared to crumbs, indicating the utilization of TRP to form ARP and further supporting the observed lower concentration of TRP in the crust versus the crumb (**Fig. 2-3**). Additional degradation products of deoxyglucosone (PBA, Acortatarin A, and Acortatarin C) (Jiang & Peterson 2013) were found only in crust, and are all higher in concentration in the AW crust than in the other samples.

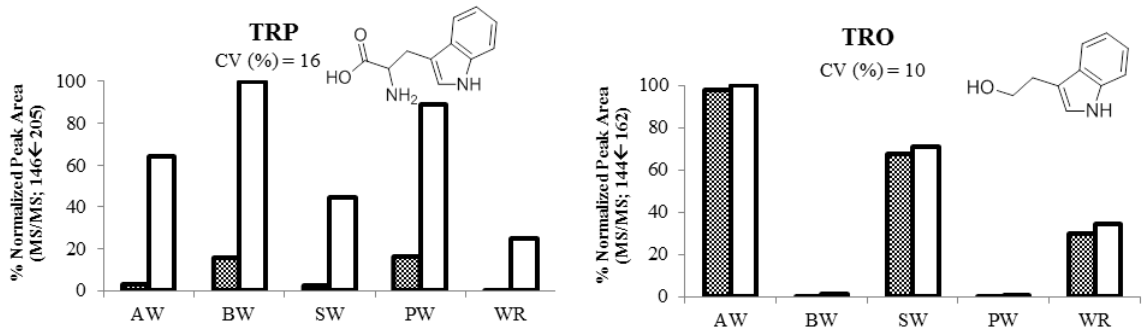

Figure 2-3. Mean relative concentrations of $_{L}$-tryptophan (TRP) and tryptophol (TRO) for the crust ■ and crumb □ of wheat bread; Arnold Soft Family 100% whole wheat (AW), Brownberry 100% whole wheat (BW), Sara Lee Soft & Smooth 100% whole wheat (SW), Pepperidge Farm 100% whole wheat (PW), and Wonder Classic refined wheat (WR).

Pearson correlation analysis of the perceived bitterness and relative concentration of these eight bitter compounds is shown in **Table 2-2**. In the crust, HMF and DDMP were found to be highly significantly correlated (p-value = 0.01); as well as both TRO and Acortatarin C (p-value = 0.05). In the crumb, the relative concentration of ARP and TRP were highly significantly correlated (p-value = 0.01) to bitterness intensity. Because it is anticipated multiple compounds contributed to the bitterness in wheat bread, compounds that did not significantly correlate to bitterness perception does not necessary mean they did not contribute to bitter taste. Correlation analysis simply suggests compounds that could be analyzed (concentration) to predict bitterness perception in wheat bread at a level of statistical confidence.

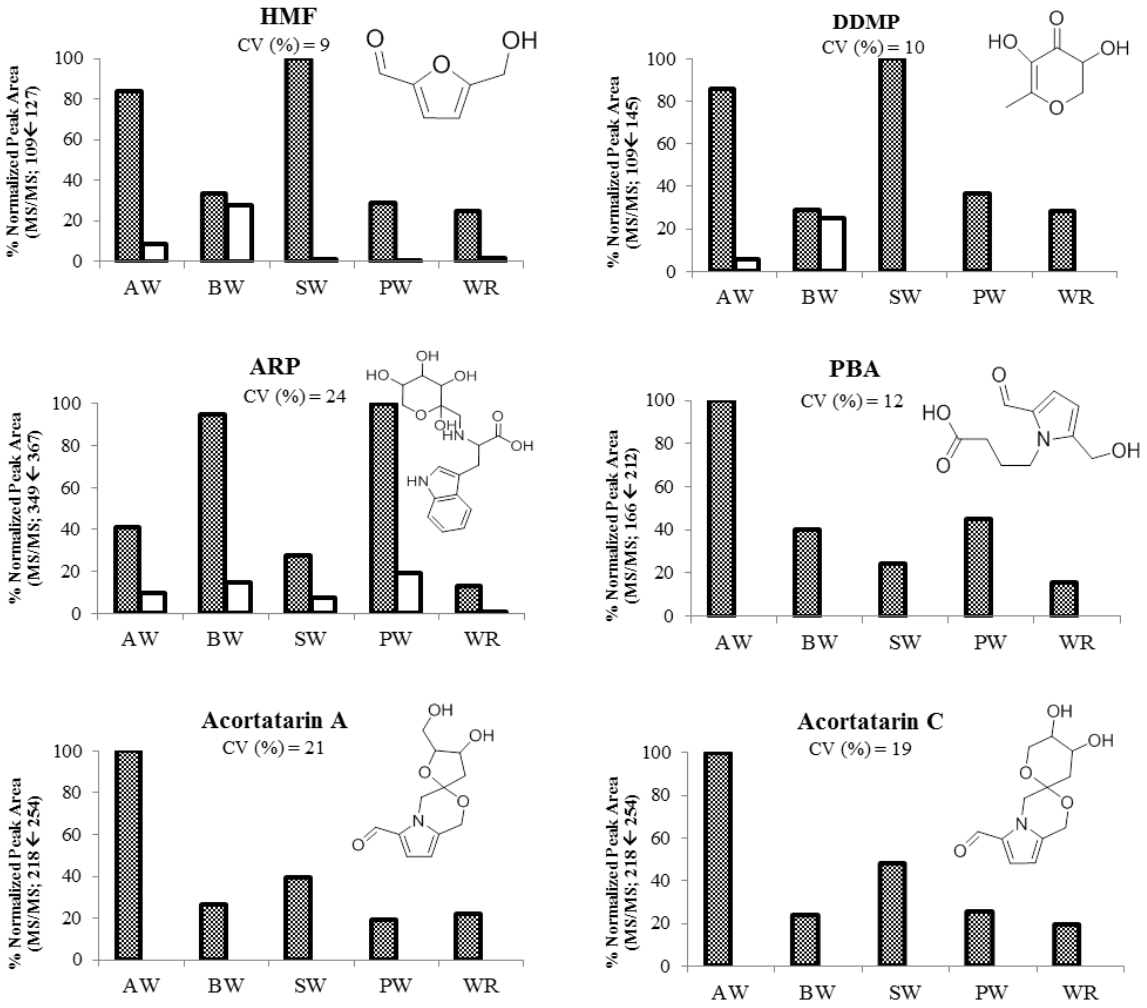

Figure 2-4. Mean relative concentrations of six bitter compounds 5-(hydroxymethyl)furfural (HMF), 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (DDMP), TRP Amadori rearrangement product (ARP), 4-(2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl)butanoic acid (PBA), Acortatarin A, and Acortatarin C for the crust ■ and crumb □ of wheat bread; Arnold Soft Family 100% whole wheat (AW), Brownberry 100% whole wheat (BW), Sara Lee Soft & Smooth 100% whole wheat (SW), Pepperidge Farm 100% whole wheat (PW),and Wonder Classic refined wheat (WR).

Table 2-2. Determination coefficients derived from the Pearson correlation analysis between the bitter compound concentration and the perceived bitterness in the crust and crumb.

Compound[a]	Correlation with Bitterness Intensity			
	Crust		Crumb	
	R^2	P value	R^2	P value
TRP	0.29	0.35	0.91	0.01
TRO	0.84	0.03	0.11	0.58
ARP	0.16	0.51	0.91	0.01
HMF	0.93	0.01	0.22	0.43
DDMP	0.95	0.00	0.24	0.40
PBA	0.31	0.33	-	-
Acortatarin A	0.64	0.10	-	-
Acortatarin C	0.76	0.05	-	-

a = $_L$-Tryptophan (TRP), tryptophol (TRO), TRP Amadori rearrangement product (ARP), 5-(hydroxymethyl)furfural (HMF), 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (DDMP), 4-(2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl)butanoic acid (PBA).

Further review of the data suggested two of the correlations for ARP and TRO may have been caused by indirect relationships. ARP was 3-7 fold higher in concentration in the crust than the crumb (**Fig. 2-4**), yet it was not correlated to the bitterness in the crust (p-value = 0.51) but was significantly correlated to bitterness in the crumb (p-value = 0.01). This suggested that ARP may have been indirectly related to the bitterness of the crumb and caused by the correlation to the concentration to TRP.

Additionally TRO also was significantly correlated to the bitterness in the crust (p-value = 0.03); however it is not correlated to the bitterness in crumb even though its concentration in the crumb is almost the same as that in the crust. Therefore, TRO is unlikely to be a bitter marker in the crust.

In conclusion, bitterness in commercial wheat bread made from whole wheat and refined wheat flour were significantly correlated to both fermentation (TRP) and Maillard reaction products (HMF, DDMP, Acortartin C). For the crumb, TRP was highly significantly correlated to bitterness; for the crust, HMF and DDMP were highly significantly correlated to bitterness (Acortartin C at a lower significance level). Overall, these findings provide a chemical bitter fingerprint to monitor bitterness generation in wheat bread and provide the food industry an improved basis to optimize palatability of whole wheat products to promote consumption and health impact.

Further work will aim at developing quantification methods to investigate the relative impact of each compound on bitterness intensity. Additionally related pathways of generation are being investigated to provide understanding how the whole grain composition (e.g., amino acid, polyphenol composition) impacts bitterness generation in wheat based food products.

Chapter 3

Characterization of Bitter Compounds in Whole Wheat Bread Crumb

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Synopsis

Consumer acceptability of whole wheat foods is challenged by negative bitter flavor attributes. In this study, bitter compounds in whole wheat bread crumb were isolated using sensory-guided multi-dimension fractionation techniques. The compounds with the highest bitterness intensity were subsequently identified by LC-MS and NMR techniques as L-tryptophan, Wessely-Moser isomers apigenin-6-C-galactoside-8-C-arabinoside & apigenin-6-C-arabinoside-8-C-galactoside, and 9,12,13-trihydroxy-trans-10-octadecenoic acid (pinellic acid). Sensory bitter recombination experiments were conducted at the reconstituted concentrations determined in saliva during bread mastication. Pinellic acid was reported to contribute the most to the bitterness perception. The origin of pinellic acid was generated during bread making; the content increased more than 30-fold once the flour was hydrated and produced into bread.

Introduction

A hurdle to the consumption of whole grain foods is related to production challenges limiting the development of products with high flavor quality. When cereal foods are manufactured with whole grain instead of refined grain flour, lower product

acceptability is observed (McMackin et al. 2013; Bakke & Vickers 2007; Burgess-Champoux et al. 2006). Whole-grain products are commonly associated with bitterness, dryness, astringency, and other negative sensory attributes(Bakke & Vickers 2007). The compounds responsible for these negative flavor attributes in whole grain foods have been poorly defined, limiting production strategies for product flavor improvement.

Among the limited number of studies focused on characterizing bitterness in whole grain foods(Heiniö et al. 2003; Heiniö et al. 2008; Biermann & Grosch 1979), Jiang and Peterson(Jiang & Peterson 2013) reported the Maillard reaction was a main mechanism of bitterness development in wheat bread crust. They identified eight bitter compounds in the bread crust; six were Maillard reaction products that included the TRP Amadori rearrangement product (ARP), 5-(hydroxymethyl)furfural (HMF), 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (DDMP), 4-(2-formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl)butanoic acid (PBA), Acortatarin A, and Acortatarin C. Two of the compounds were identified as the amino acid $_{L}$-tryptophan (TRP) and its metabolite tryptophol (TRO). In a follow-up study, the chemical profile of these bitter compounds was investigated in relation to the perceived bitterness of the crust of four commercial whole wheat breads and one refined wheat bread (Bin et al. 2012). Highly significant correlations ($\alpha < 0.05$) were observed between the perceived bitterness in the crust and the quantity of three Maillard reaction products, specifically 5-(hydroxymethyl)furfural (HMF), 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (DDMP) and Acortatarin C. These findings provided a chemical fingerprint to profile bitterness in whole wheat products.

The crumb portion of bread consists of more than 80% of the bread mass, has higher moisture content and reaches lower processing temperature than the crust during baking. Therefore, Maillard reaction products in the crumb would be distinct from the bread crust, in terms of the chemical species and generation pathways. Consequently, further investigation of the compounds and the origin of bitterness in whole wheat foods, specifically in the crumb portion, is warranted. Thus, the objective of the current study was to identify the main bitter compounds in the crumb of whole wheat bread.

This project investigates the origin and development pathways of bitterness in whole wheat foods from both the native cereal grains and the processing steps of bread production. The goal is to provide the food industry with more insights into practical improvement strategies for manufacturing whole-wheat products, or to greater extent whole grain products, with reduced bitterness to ultimately provide a basis for flavor improvement.

Materials and Methods

Chemicals. Ethanol (Absolute, HPLC Grade), methanol (HPLC grade), and formic acid (98-100%, puriss) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). [$13C_{18}$]Linoleic acid (99 atom % ^{13}C, 97%) was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Water was purified through Barnstead Nanopure Diamond water purification system (Thermo Scientific, Dubuque, IA, USA). All other chemicals were of analytical reagent grade from Fisher Scientific (Pittsburg, PA) and Sigma Chemical Co. (St. Louis, MO).

Food Samples. Bread was made using an modified sponge-dough method (AACC International 2010). Hard Red Spring (HRS) and Hard White Winter (HWW) flour was obtained from ConAgra Foods (Omaha, NE). In brevity, this method involves a two-step process. In the first step, sponge was made by mixing whole wheat flour (478 g), water (350 g), instant dry yeast (7.4 g), yeast food (7.4 g), vital wheat gluten (22 g), and ascorbic acid (20 ppm). The sponge dough was fermented for 3 hours at 86°F, and then the rest of the ingredient incorporated, including whole wheat flour (257 g), water (185 g), instant dry yeast (2.5 g), salt (17.6 g), sugar (74 g), sodium stearoyl lactylate (1.5 g), and soy oil (25.2 g), to make the dough. The dough was then subjected to sheeting, proofing (110/100° F), and finally baking at 400°F for 23 minutes.

Preparation of Bread Crumb Extract. Five hundred g of HRS bread-crumb was ground in a blender with liquid nitrogen and then extracted by 75% aqueous ethanol (1 L × 3) for 16 h. The extract was centrifuged, supernatant was freed from ethanol under vacuum, and freeze-dried. For further sample clean up, the powder from the freeze-dried sample was re-solubilized with 120 ml of ethanol: water (20:80 v/v) and two 60 ml aliquots was passed through a 12 g preconditioned C18 SPE cartridge (Supelco, Bellefonte, PA, USA) and eluted with 30 ml of ethanol, respectively. Both hydrophilic and hydrophobic eluents from C18 SPE were pooled, concentrated under vacuum to yield 40 ml of crumb extract and then filtered through 0.45 µm Nylon syringe filter (Millex, Billerica, CA, USA). Further fractionation was performed by RP-HPLC techniques.

Preparative HPLC Fractionation – First Dimension. Jiang and Peterson (2013) reported that in the bread crust portion, bitter substances were largely presented as

hydrophobic compounds with small molecular weight (MW < 1000). Therefore, for initial fractionation of the whole wheat bread crumb extract an RP-HPLC column was selected for analysis. A preparative HPLC system consisting of a binary pump system (Shimadzu LC-8A), a manual injector (Rheodyne 3725i), a UV/Vis detector (Shimadzu SPD-10Ai), and a fraction collector (Shimadzu FRC-10A) was used for fractionation. Five ml aliquots were separated using a preparative Agilent Pursuit® C18 column (21.2 × 150 mm, 5µm). The mobile phase was maintained at a flow rate of 15 ml/min using binary solvent system of 0.1% formic acid in water (A) and methanol (B). The elution gradient started at 10% B (0-5 min), linearly increased to 30% B (5-10 min), then to 50% B (10-20 min), then ramped 80-100% B (20-24 min), held at 100% B (24-26 min), and re-equilibrated at 10% B (26-40 min). The absorbance of the UV/Vis detector was set at 280 nm. The effluent was collected in 26 fractions. Fractions were collected from eight runs, individually combined and freeze-dried twice to completely remove the solvent. The resulting residues obtained were then dissolved in 15 ml water, and each fraction was evaluated by a trained sensory panel for bitterness intensity. The dose for evaluation was 1 ml, which was equivalent to extracts from 25 g of bread sample. The fractions with the highest bitter intensity were identified as #11, #16, and #21-25 by and are shown in **Figure 3-1**. Based on LC-MS analysis, Fraction #11 (**Fig. 3-1**) was easily identified as $_{L}$-tryptophan, a known bitter amino acid, which was further confirmed by ^{1}H NMR.

Semi-Preparative Purification of Bitter Fractions – Second Dimension. Targeted bitter fractions #16 and #21-25 (**Fig. 3-1**) from the first dimensional fractionation were subsequently further purified by a second HPLC separation, tasted and the primary bitter

regions analyzed by LC-MS, which identified the tentative structures in those fractions. Fraction #16 (**Fig. 3-1**) contained a mixture of flavonoid-C-glycoside isomers, which was further fractionated by preparative HPLC on a Agilent Zorbax® Bonus-RP column (21.2 × 100 mm, 5 µm) using a binary solvent system of 0.1% formic acid in water (A) and acetonitrile (B) at a flow rate of 10 ml/min. Separation of flavonoid-C-glycoside isomers that co-eluted from first dimension was successfully achieved by applying gradient elution from 10% B to 30% B (7-27 min). Purity of the flavonoid-C-glycoside isomers was > 96% as determined by NMR. Fractions #21-25 (**Fig. 3-1**) were determined by LC-MS to be hydroxylated fatty acids. In fractions #22 and #23 a major component (ESI^- *m/z* 329) was present in both fractions with co-elutions of its isomer (ESI^- *m/z* 329) and oxidized product (ESI^- *m/z* 327). Separation of hydroxylated fatty acids in fractions #22-23 was performed on a semi-preparative Restek Pinnacle C18 column (10.0 × 250 mm, 5 µm) using a binary solvent system of 0.1% formic acid in water (A) and acetonitrile (B) at a flow rate of 3.5 ml/min. Gradient elution was applied from 50% to 80% B (4-20 min). Since these hydroxylated fatty acids are non-active UV/Vis compounds, on-line mass directed fractionation was achieved by using a semi-preparative HPLC-MS system consisting of a Shimadzu® HPLC pumping system and a Waters® ZQ Mass Spectrometer. A minor portion of effluent was split into the MS detector and the remainder was sent to the fraction collector. Collection of major hydroxylated fatty acids by MS was obtained by triggering the collection of the *m/z* 329 and 327 under ESI^-. The purified fractions were freeze-dried twice to completely remove solvent. The isolates were dissolved in 5% aqueous ethanol solution, passed through a 5 g preconditioned C18

cartridge (Supelco, Bellefonte, PA, USA), eluted with ethanol, and then diluted with water to <5% ethanol and freeze dried twice.

Sensory Screening of Food Isolates. Three panelists were trained to evaluate bitter taste intensity using caffeine solutions as references (0.03, 0.08 and 0.15% of caffeine wt/wt in water corresponding to bitterness intensity ratings of 2, 5 and 10, respectively). For each fraction reconstituted as an aqueous sample, panelists were asked to pipette 1 ml to the tongue while wearing a nose clip, and evaluate the bitterness intensity of the sample according to the references. Water and unsalted crackers were used as palate cleansers. For each fraction, the bitterness intensity score was recorded as a result of the panel's consensus.

Precaution Taken for Sensory Analysis of Food Fractions and Taste Compounds. All fractions from food samples for taste analysis, prior to sensory testing, were liberated from solvent by rotary evaporation and were subsequently freeze-dried twice. GC/MS or 1H NMR revealed that fractions treated by the above protocol are free of solvents and suitable for sensory analysis. Approval for the sensory evaluation protocol was granted by the Ethics Committee, University of Minnesota (IRB #1505E70948).

Identification of $_L$-Tryptophan, Apigenin-6-C-arabinose-8-C-galactose, and 9,12,13-Trihydroxy-trans-10-octadecenoic Acid. *$_L$-Tryptophan*: LC/MS-Tof (ESI^-), *m/z* = 203.0815 (3.0 ppm, $[M-H]^-$). ^1H and ^{13}C NMR (700 MHz, D_2O) spectra were in agreement with the Spectral Database for Organic Compounds (SDBS)(National Institute of Advanced Industrial Science and Technology (AIST) n.d.).

Apigenin-6-C-arabinoside-8-C-galactoside: LC/MS-Tof (ESI⁻), *m/z* 563.1395 (2.0 ppm, $[M-H]^-$). ^{1}H NMR (900 MHz, DMSO-d_6) δ 8.30 [d, 2H, *J* = 8.7 Hz, H-C(2',6')], 6.89 [d, 2H, *J* = 8.7 Hz, H-C(3',5')], 6.86 [s, 1H, H-C(3)], 4.72 [d, 1H, *J* = 4.6 Hz, H-C(1'', Glc)], 4.71 [d, 1H, *J* = 4.6 Hz, H-C(1'', Ara)]; ^{13}C NMR (900 MHz, DMSO-d_6) δ 182.4 [C-4], 164.3 [C-2], 161.3 [C-7], 161.0 [C-4'], 158.3 [C-5], 155.3 [C-9], 129.5 [C-2'] 128.8 [C-6'], 121.1 [C-1'] 116.2 [C-3'], 116.2 [C-5'], 108.1 [C-6], 103.8 [C-8] , 102.6 [C-10], 102.2 [C-3], 80.7 [Gal, C-5], 76.6 [Ara, C-3], 75.8 [Ara, C-1], 75.0 [Gal, C-3], 74.3 [Gal, C-1], 74.0 [Ara, C-2], 73.9 [Ara, C-4], 70.2 [Ara, C-5], 69.7 [Gal, C-2], 69.3 [Gal, C-4], 61.0 [Gal, C-6].

Apigenin-6-C-galactoside-8-C-arabinoside: LC/MS-Tof (ESI⁻), *m/z* 563.1395 (2.0 ppm, $[M-H]^-$). ^{1}H NMR (900 MHz, DMSO-d_6) δ 7.96 [d, 2H, *J* = 8.7 Hz, H-C(2',6')], 6.94 [d, 2H, *J* = 8.7 Hz, H-C(3',5')], 6.81 [s, 1H, H-C(3)], 4.37 [d, 1H, *J* = 4.1 Hz, H-C(1'', Glc)], 4.36 [d, 1H, *J* = 4.1 Hz, H-C(1'', Ara)]; ^{13}C NMR (900 MHz, DMSO-d_6) δ 182.4 [C-4], 164.3 [C-2], 161.3 [C-7], 161.0 [C-4'], 158.3 [C-5], 155.3 [C-9], 129.2 [C-2'] 128.8 [C-6'], 121.4 [C-1'] 116.2 [C-3'], 116.2 [C-5'], 108.1 [C-6], 103.8 [C-8] , 102.6 [C-10], 102.2 [C-3], 80.0 [Gal, C-5], 76.9 [Ara, C-3], 75.1 [Ara, C-1], 74.7 [Gal, C-3], 74.0 [Gal, C-1], 73.3 [Ara, C-2], 70.5 [Ara, C-4], 70.2 [Ara, C-5], 68.6 [Gal, C-2], 68.4 [Gal, C-4], 61.2 [Gal, C-6].

9,12,13-trihydroxy-trans-10-octadecenoic acid (Pinellic acid). LC/MS-Tof (ESI⁻), *m/z* 329.2326 (0.6 ppm, $[M-H]^-$). ^{1}H NMR (700 MHz, DMSO-d_6) δ 5.69 [m, 2H, H-C(10,11)], 4.04 [q, 1H, *J* = 6.5 Hz, H-C(9)], 3.89 [t, 1H, *J* = 6.2 Hz, H-C(12)], 3.40 [m, 1H, H-C(13)], 2.14 [t, 2H, *J* = 7.7 Hz, H-C(2)], 0.91 [t, 3H, *J* = 6.5 Hz, H-C(18)].

Liquid Chromatography – Mass Spectrometry/tof (LC-MS-tof) Analysis. Samples were analyzed on a Waters ACQUITY UPLC system interfaced with Xevo™ G2 QTOF mass spectrometer (Waters, Milford, MA, USA). Two μL were injected on an ACQUITY UPLC BEH C18 1.7 μm column (2.1 mm × 50 mm) (Waters, Milford, MA, USA) at 25ºC. The mobile phase was maintained at a flow rate of 300 μL/min using a binary solvent system of 0.1% formic acid in water (A) and acetonitrile (B). The elution gradient started at 5% B (0-1 min), linearly increased to 50% B (1-6 min), then increased to 100% B (6-8 min) held at 100% B (8-10 min), and re-equilibrated at 5% B (10-15 min). The Xevo™ G2 QTOF mass spectrometer was equipped with a LockSpray™ dual electrospray ion source. The operational parameters were as follows: ESI+, source temperature 110°C; desolvation temperature 350°C; capillary voltage 1.5 kV. ESI-, source temperature 110°C; desolvation temperature 350°C; and capillary voltage 2.0 kV. Reserpine (100 mg/L) was used as the internal lock mass reference compound to obtain exact mass measurements. For CID MS/MS analysis, the CID energies are 20-25 eV. LC/MS data processing was performed using Waters® MassLynx™ 4.1 software.

Nuclear Magnetic Resonance Spectroscopy (NMR). The ^{1}H, ^{13}C, HMQC, and HMBC NMR experiments were performed on a Bruker 700 MHz spectrometer (Bruker, Billerica, MA, USA) with TXI cryoprobe. Data processing was performed using Bruker® TopSpin™ 2.1.

Quantitative Analysis of Bitter Compounds in Flour and Bread (LC/MS/MS). Five grams of flour, crumb or crust were extracted with 75% ethanol aqueous solution (50 ml) spiked with an internal standard butyl 4-hydroxybenzoate (100 gm/kg),

respectively. The extract was centrifuged, the supernatant was pooled, filtered, and the ethanol removed under vacuum and remaining aqueous extract was freeze-dried. The residue from the freeze-dried sample was re-extracted with 25 ml of methanol: water (50:50 v/v). Five ml of the extract was passed through a 500 mg preconditioned C18 cartridge (Supelco, Bellefonte, PA, USA) and eluted with 2 ml of methanol. The methanol from the pooled eluent was removed under vacuum and the concentrate was re-solubilized into 4 ml of 10% methanol aqueous solution and filtered through 0.20 µm Nylon syringe filter (Millex, Billerica, CA, USA) prior to LC-MS analysis. All samples were prepared in duplicate. Quantitative LC-MS/MS analysis of the bitter compounds was conducted using multiple reaction-monitoring (MRM) acquisition. A Waters ACQUITY UPLC system interfaced with a Quattro Premier XE^{TM} mass spectrometer (Waters, Milford, MA, USA) was used. Two µL of injection was separated on an ACQUITY UPLC BEH C18 1.7 µm column (2.1 mm × 50 mm) (Waters, Milford, MA, USA) held at 25ºC. The mobile phase was maintained at a flow rate of 300 µL/min using binary solvent system of 0.1% formic acid in water (A) and acetonitrile (B). The elution gradient started at 5% B (0-1 min), linearly increased to 50% B (1-6 min), then increased to 100% B (6-8 min) held at 100% B (8-10 min), and re-equilibrated at 5% B (10-15 min). The Quattro Premier XE^{TM} mass spectrometer was equipped with an electrospray ionization probe and the MS/MS ion transitions and collision energy for the bitter compounds were as follows: $_{L}$-tryptophan, ESI^{-} 203 →116 (12eV); flavone-C-glycoside, ESI^{-} 563 → 443 (25eV); 9,12,13-trihydroxytrans-10-octadecenoic acid, ESI^{-} 329 →211 (15eV). Quantification was conducted with a 5 point calibration curve that showed good

linearity; $r^2 > 0.99$. Butyl 4-hydroxybenzoate was used as an internal standard and monitored using the following transition: *m/z* 193 → 136 (15ev).

Quantitation of Bitter Compounds in Expectorated Saliva from Bread Mastication. Three panelists (two females and one male) were instructed to masticate a slice of HRS whole wheat bread (crumb only) in duplicate. During oral processing of the bread samples, participants expectorated their saliva containing the food residues into a 250 ml centrifuge bottle. The oral processing of the entire slice was kept at approximately 10 minutes. The collected saliva samples were centrifuged; then 5 ml of supernatant was sampled, loaded onto a 500 mg preconditioned C18 cartridge (Supelco, Bellefonte, PA, USA), and eluted with 2 ml of methanol. The methanol from the pooled eluent was removed under vacuum and the concentrate was re-solubilized into 4 ml of 10% methanol aqueous solution and filtered through 0.20 µm Nylon syringe filter (Millex, Billerica, CA, USA). The concentrations of the major bitter compounds in the saliva were measured by LC-MS/MS using the method described above (*Quantitative Analysis of Bitter Compounds in Flour and Bread*).

Pairwise Bitterness Ranking Test – Friedman's test. A trained sensory panel (aged 22–45 years, consisting of five males and six females) evaluated the bitterness contribution of the identified compounds. Treatments (samples) were prepared by reconstituting each bitter compound in an aqueous solution at the concentration reported in expectorated saliva. The samples were as follows: (A) $_L$-Trp (67 mg/L); (B) Apigenin-C-glycosides (59 mg/L); (C) pinellic acid (116 mg/L). Each panelist was presented with three pairs of samples coded with random 3-digit numbers. The presentation order of the

sample within pairs, between pairs and among panelists was randomized and balanced. Panelists were asked to pipette 1 ml sample onto the tongue while wearing a nose clip and select the sample perceived as more bitter within each pair. Evaluations were conducted with a three-minute break between the sample pairs. Water and unsalted crackers were used as palate cleansers. The results were evaluated by a Friedman-type statistical analysis. The rank sum of each sample for the attribute "more bitter" was computed.

Results and discussion

Identification of major bitter compounds in the whole wheat bread crumb

The first dimensional HPLC chromatogram with the fractionation scheme and corresponding relative bitterness intensity of each fraction for the HRS breadcrumb extract is illustrated in **Figure 3-1**. The fractions with the highest bitterness intensity are marked with the corresponding fraction number. Overall three main bitter regions were observed across the chromatogram: fraction #11, #16, and #21-25 (**Fig. 3-1**). The bitter fraction #16 and 21-25 were further purified through secondary HPLC fractionation using a modified revered phase column; the fractions were similarly profiled by sensory analysis to select the main bitter peaks. The bitter compounds were subsequently identified by LC/MS and NMR analysis.

For the bitter fraction #11 (**Fig. 3-1**) the bitter compound $_{L}$-tryptophan ($_{L}$-Trp, **Figure 3-2**) at > 99% purity was identified. Characterization of $_{L}$-Trp was easily

accomplished by ^{1}H NMR and confirmed by an authentic compound. $_{L}$-Trp has been reported as one of the bitterest amino acids(Mitteilungen 1965).

For fraction #16 (**Fig. 3-1**), the second HPLC separation is shown in **Figure 3-3A** and identified flavone-C-glycosides as the primary bitter compounds. Accurate mass measurement reported a pseudomolecular weight ion with an *m/z* 563.1395 (2.0 ppm,

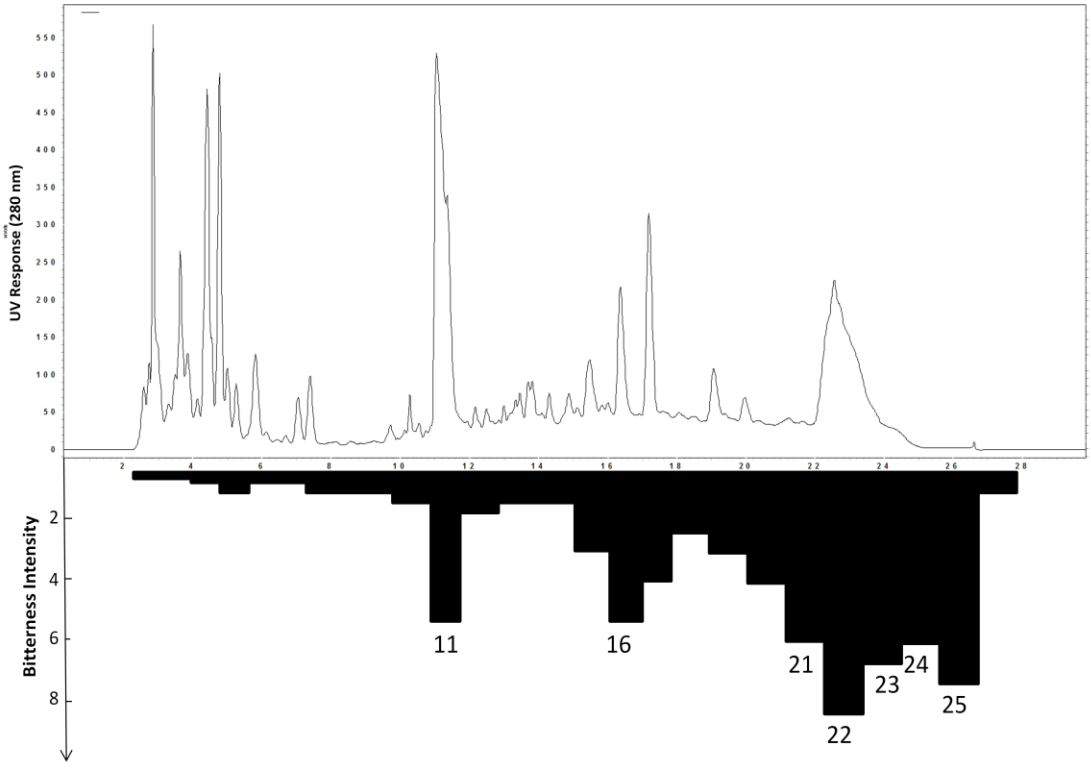
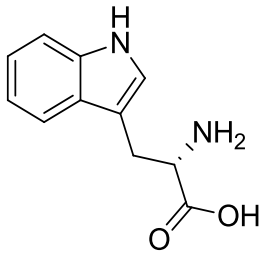


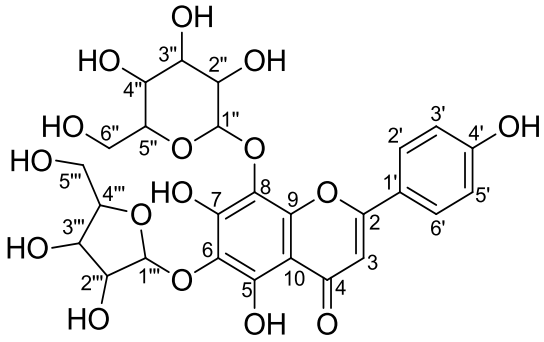
Figure 3-1. HPLC chromatogram and sensory-guided fractionation of HRS bread extract; bitterness levels of 2 and 5 were equivalent to 0.03% and 0.08% caffeine aqueous solution, respectively

$[M-H]^{-}$) with a predicted molecular formula of $C_{26}H_{29}O_{14}$. The ESI^{+} QTOF-MS-MS fragmentation pattern (**Figure 3-4**) is indicative of the structure of flavone-C-glycosides: *m/z* 547.2 E_1^{+} $[M+H-18]^{+}$, 529.2 E_2^{+} $[M+H-36]^{+}$, 511.2 E_3^{+} $[M+H-54]^{+}$, 427.1 [M+H-18-

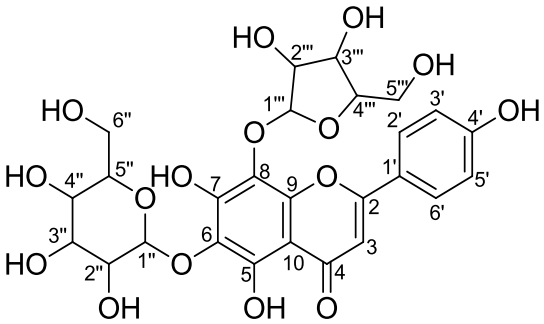
120]$^+$, 409.1 [M+H-36-120]$^+$, 379.1 [M+H-36-150]$^+$. The spectrum is annotated according to the previously reported tandem MS fragmentation principles of flavonoids(Waridel et al. 2001). The major bitter compounds of **Figure 3-3A** were characterized by ^{1}H and ^{13}C NMR to be Wessely–Moser isomers of apigenin-C-



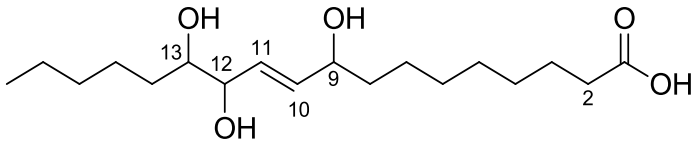
***L*-tryptophan**



apigenin-6-C-pentoside-8-C-hexoside



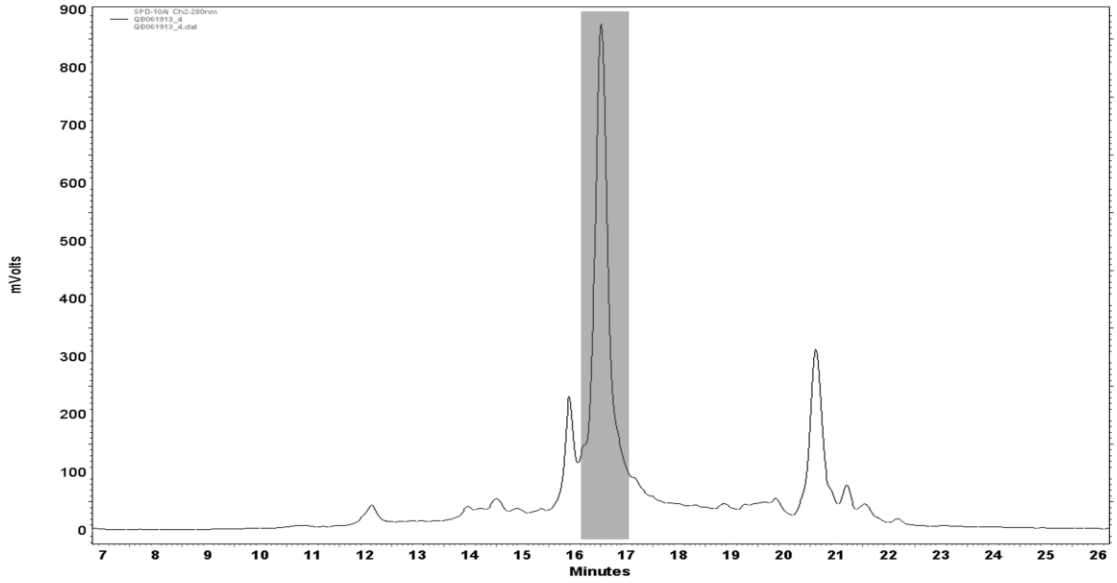
apigenin-8-C-pentoside-6-C-hexoside



(*E*)-9,12,13-trihydroxy-10-octadecenoic acid (pinellic acid)

Figure 3-2. Structures of the major bitter compounds in whole wheat bread crumb.

(A)



(B)

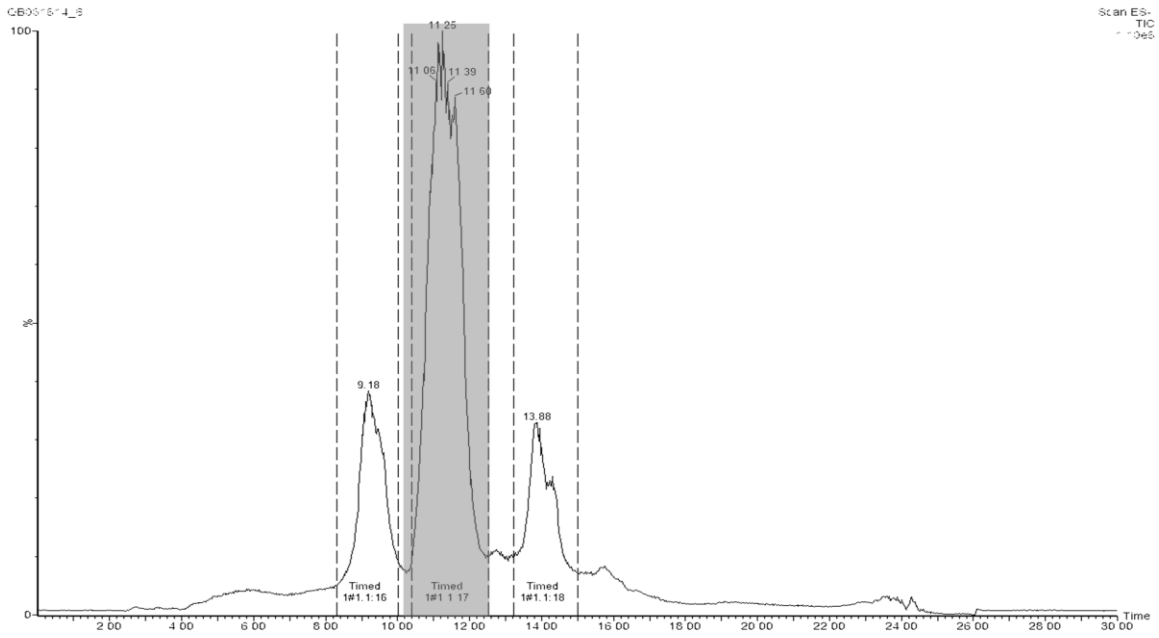


Figure 3-3. Second dimension HPLC-UV of fraction #16 (A) and #22 (B) from first dimension RPLC fractionation; highlighted regions were reported as main bitter region.

glycosides, consisting apigenin-6-C-galactoside-8-C-arabinoside and apigenin-6-C-arabinoside-8-C-galactoside (**Figure 3-2**) at a ratio of 3:2, with >90% purity. The ^{13}C NMR data was in agreement with the identification of apigenin-C-diglycosides by ^{13}C NMR in wheat flour(Asenstorfer et al. 2006). The carbons were assigned according to Besson *et al*.(Besson et al. 1985; Besson et al. 1984). These flavone-C-glycosides are native components from wheat bran that have been previously isolated from bran of hard red spring wheat(Feng et al. 1988).

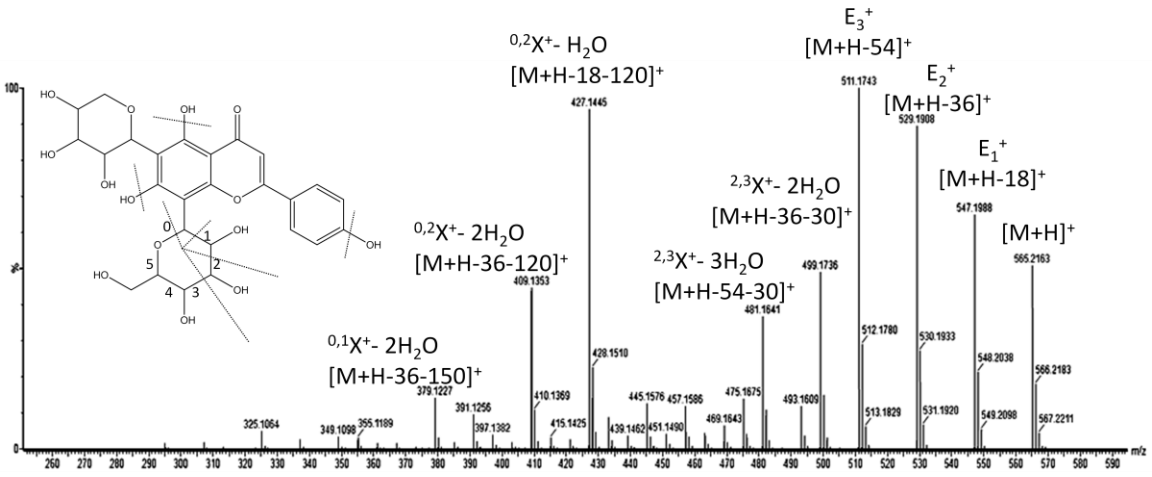


Figure 3-4. ESI$^+$ Q-TOF-MS–MS spectra of precursor ion (*m/z* 565.2) of the fraction #16.

The third bitter region (fractions #21-25, **Fig. 3-1**) with the highest perceived bitterness intensity were pooled, further purified under secondary HPLC separation (shown in **Figure 3-3B**) and the main bitter compound identified was characterized by LC-MS (**Figure 3-2**) and ^1H NMR as 9,12,13-trihydroxy-trans-10-octadecenoic acid

(pinellic acid) (Sabitha et al. 2009). This hydroxy fatty acid was the oxidation products of linoleic acid (Baur et al. 1977) and was further confirmed by adding [^{13}C]-labeled linoleic acid into the flour before water addition for dough formation, and [^{13}C]-labeled hydroxy fatty acid was detected at the corresponding retention time. Trihydroxy fatty acids have been previously reported as bitter substances(Baur et al. 1977), which can be formed from enzymatic oxidation of linoleic acid when incubating linoleic acid with a suspension of cereal (e.g. wheat, barley, rye, oats)(Graveland 1973) and leguminosae (e.g. peas)(Arens & Groseh 1974). LC-MS screening of fractions 21-25 revealed fraction #21-23 majorly consisted of a mixture of 9,12,13-trihydroxy-trans-10-octadecenoic acid and 9,10,13-trihydroxy-trans-10-octadecenoic acid in a ratio of 8:1. Fraction #24 (**Fig. 3-1**) consisted of mixtures of 9-hydroxy-octadecadienoic acid and 13-hydroxy-octadecadienoic acid, which have been reported to have higher bitterness threshold than the trihydroxy fatty acids(Baur et al. 1977; Biermann & Grosch 1979). Fraction #25 (**Fig. 3-1**) consisted mainly of free fatty acids, of which linoleic acid is the major one (data not shown). The purity of the pinellic acid was > 99% as determined by ^{1}H NMR. This hydroxy fatty acid was the oxidation product of linoleic acid (Baur et al. 1977) and was further confirmed by adding [^{13}C]-labeled linoleic acid into the flour before water addition for dough formation, and detection of the [^{13}C]-labeled hydroxy fatty acid at the corresponding retention time. Trihydroxy fatty acids have been previously reported as bitter substances (Baur et al. 1977), which can be formed from enzymatic oxidation of linoleic acid when incubated in a suspension of cereal (e.g. wheat, barley, rye, oats) (Graveland 1973) and leguminosae (e.g. peas) (Arens & Groseh 1974).

Profiling of Bitter Compounds in Saliva during Bread Mastication

A key component of flavor characterization involves the sensory validation of the compounds identified. Taste is a human response and consequently, compounds identified should be validated in the context of perception from food products. Traditional sensory approaches have involved measuring the taste activity value (TAV) of a compound. The TAV is calculated by the ratio between the concentration (in food) and the taste threshold concentration of a compound in water, in order to evaluate the contribution of individual tastants to the overall taste of foods (Hofmann, Ottinger, et al. 2004). However, the forces that govern the dissolution of tastants in saliva (delivery) during food mastication are complex (e.g. starch-flavor complexation) (Conde-Petit et al. 2006) and some tastants are more likely to be extracted by saliva and to be perceived than others.

To account for the influence of delivery on the tastants contribution to the food, the three identified bitter compounds (**Fig. 3-2**) were quantified in expectorated saliva after oral processing of whole wheat bread. Mean saliva concentrations (± SD) of targeted bitter compounds in expectorated saliva during bread mastication were determined by LC-MS-MRM as follows: $_{L}$-Trp, 67 ± 11 mg/L; Apigenin-C-glycosides, 59 ± 7.6 mg/L; Pinellic acid, 116 ± 25 mg/L. In comparison to the extraction yield of a 75% EtOH aqueous solution, in the extraction of the bitter compounds by saliva was 20-35%; $_{L}$-Trp was the highest (34.5%), followed by Apigenin-C-glycosides (25.4%), and pinellic acid was the lowest (20.7%). The concentrations of the three bitter compounds

determined in the saliva were consequently used for taste recombination sensory analysis in order to gain a more realistic understanding of their individual contribution in the perceived bitterness of whole-wheat products.

Sensory Ranking of the Bitter Compounds

All three bitter compounds at the concentration determined in saliva were reported by the sensory panel to have a recognizable bitter taste with a generally low intensity. To compare the relative contribution of these compounds to the bitterness intensity of the bread sample, the samples were evaluated by a pairwise ranking test (Meilgaard et al. 2006). The sensory data are shown in **Table 3-1** for the 11 panelists. Each "row" sample was selected as being "more bitter" than each "column" sample. For example, when **Pinellic acid** was presented with **$_{L}$-Trytophan**, it was perceived as "more bitter" by 7 of 11 panelists, and perceived "less bitter" by 4 of 11 panelists. Pinellic acid ranked as significantly more bitter than apigenin-C-glycosides (α= 0.05).

Table 3-1. Bitterness rank sums of $_{L}$-Trytophan, apigenin-C-glycosides, and pinellic acid[1].

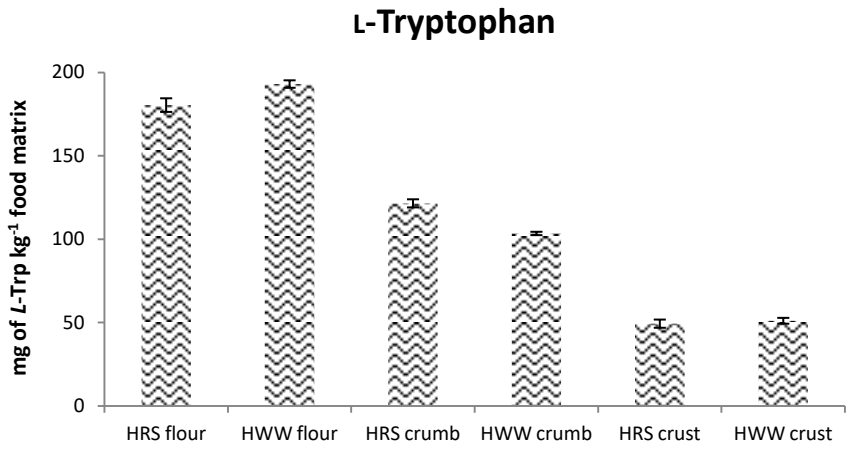
Row Samples (Less Bitter)	Column Samples (More Bitter)		
	$_{L}$-Trytophan	**Apigenin-C-glycosides**	**Pinellic acid**
$_{L}$-Trytophan	-	4	7
Apigenin-C-glycosides	7	-	8
Pinellic acid	4	3	-
Rank Sum	**33**[ab]	**28**[a]	**38**[b]

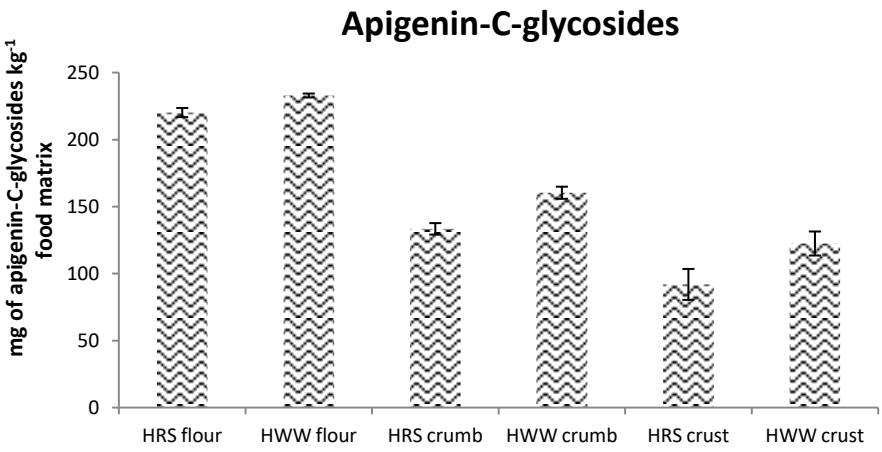
[1] = Sum of each sample was computed by adding the sum of row frequencies to twice the sum of column frequencies, ranging from 22 to 44; Friedman's *T* was 354 and greater than the critical *T* value (7.82) at significance level 0.05. Tukey's HSD is 9.5 (α= 0.05). Different letters a and b indicate significant difference at level 0.05.

Profiling of Bitter Compound Concentration from Flour to Bread

To evaluate the origin of the identified bitter compounds in whole wheat bread, the concentrations of the three major bitter compounds were measured in the flour and bread (crumb and crust) samples (**Figure 3-5**) provided by ConAgra Foods (Omaha, NE). Samples from different wheat classes, Hard Red Spring (HRS) and Hard White Winter (HWW) were examined. The concentration of $_{L}$-Trp was found to decrease from flour to bread, indicating its degradation during food processing and likely due to consumption by the Maillard reaction during baking (Yaylayan & Forage 1992). The apigenin-C-glycosides were also indicated to be native components in wheat flour and similarly degraded in bread. However the pinellic acid was found to be generated during bread making, with the concentration increasing more than 30-fold from the flour to the bread. The mechanism of enzymatic formation of trihydroxyoctadecenoic acids (e.g. pinellic acid) from linoleic acid has been previously proposed (Graveland 1970), and it has been suggested that trihydroxyoctadecenoic acids are products of decomposition of linoleic acid hydroperoxides (Gardner 1975), involving an epoxidation pathway. This pathway provides a model understanding of enzymatic oxidation of linoleic acid with the known starting material (free linoleic acid) and enzymes, however, the knowledge related to generation of bitter trihydroxyoctadecenoic acids from linoleic acid in whole wheat foods

is extremely limited due to a lack of understanding of the key pathway of trihydroxyoctadecenoic acid generation in the matrix of whole wheat foods. It





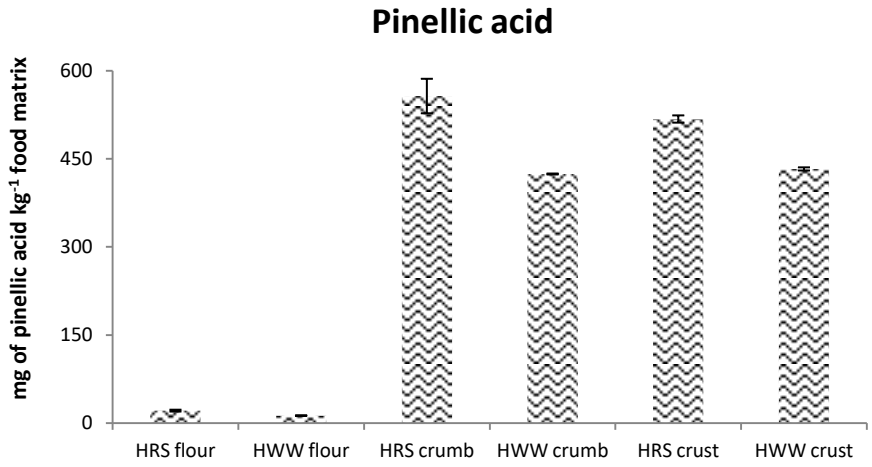


Figure 3-5. Relative concentrations of $_{L}$-Trp, apigenin-C-glycosides, and pinellic acid across HRS (hard red spring) and HWW (hard white winter) bread and flour samples.

is hypothesized that both enzymatic and non-enzymatic oxidation of linoleic could contribute to the increase in bitter trihydroxyoctadecenoic acid in the final food products. The next chapter (Chapter 4) investigates those hypotheses, including the key stages of formation of trihydroxyoctadecenoic acid from flour to bread and elucidating the mechanism for each stage of generation.

In summary, the oxidative changes of linoleic acid in whole wheat flour during food processing was reported to be the main contributor to the bitterness of the bread crumb, with a smaller contribution from endogenous bitter compounds. These findings provide a better understanding of the origin of bitterness in whole wheat products and additionally a basis to develop breeding and processing strategies to minimize bitterness in wheat bread, thus improving the product flavor quality, promoting consumption, and providing opportunities to simplify ingredient formulations (less sugar and salt).

Chapter 4

Mechanisms and Key Factors of Generation of Bitter Pinellic Acid during Whole Wheat Bread Processing

Synopsis

The origin and mechanism of generation of pinellic acid, a known bitter compound in wheat bread, were investigated using a stable isotope analytic technique using [$^{13}C_{18}$]linoleic acid. Results indicated pinellic acid was primarily generated during bread manufacturing and that free linoleic acid in flour is the major precursor. The most influential stages of bread making for generation of pinellic acid were during dough hydration, followed by the baking step. Factors that impact pinellic acid generation during whole wheat bread processing were also studied, including the influence of wheat class; hard red spring (HRS) and hard white winter (HWW) and flour storage temperature (-20°C, ambient, and 37°C). HRS wheat entries on average generate higher level of pinellic acid during dough kneading than HWW wheat varietals; however, the % yield of pinellic acid from linoleic acid is higher for HWW wheat varietals. Furthermore, generation of pinellic acid during thermal processing via non-enzymatic mechanisms influence both wheat classes. Storage temperature of flour was found to influence the free linoleic acid content and the subsequent generation of pinellic acid in dough. Ambient temperature storage significantly increased pinellic acid formation possibly due to the noted increase of free linoleic acid content, while maintaining lipoxygenase activity during storage. It is hypothesized that generation of linoleic acid hydroperoxide as a

precursor for pinellic acid occurred in flour during storage. In summary, the mechanisms of pinellic acid formation during bread-making were elucidated and provide an improved basis to develop guidelines to reduce bitterenss in whole wheat foods. This could ultimately lead to improvement of the flavor quality, and result in increased consumption of whole grain bread.

Introduction

Whole grain foods are considered to be a key component of a healthy diet as an increasing amount of studies support that whole grain consumption can reduce the risk of several chronic diseases, such as coronary heart disease (Liu et al. 1999), cardiovascular disease (Jacobs & Gallaher 2004), type 2 diabetes (McKeown et al. 2002; Liu et al. 2000), and cancer (Jacobs et al. 1998), as well as help with weight maintenance. According to the dietary guidelines for Americans, at least half of total grain intake should be whole grains. However, less than 5 percent of Americans consume the minimum recommended amount of whole grains (U.S. Department of Agriculture and U.S. Department of Health and Human Services 2010). Increasing the intake of whole grains for consumers has been a challenge and amongst the most influential factors is the perceived lower flavor quality of whole grain products when compared to refined (Kantor et al. 2001). Whole grain products are commonly associated with bitter, astringent, and other negative sensory attributes. Bitterness of bread crumb has been the focus of research as it comprises more than 80% of the bread, thus having a great sensorial impact during consumption. In addition, bitterness in crumb is expected to be developed through

very distinct pathways when compared to crust due to its higher moisture content and temperature exposure. Major bitterness compounds reported (Bin & Peterson 2016) in the bread crumb includes $_{L}$-tryptophan, Wessely-Moser isomers apigenin-6-C-galactoside-8-C-arabinoside & apigenin-6-C-arabinoside-8-C-galactoside, and 9,12,13-trihydroxy-trans-10-octadecenoic acid (pinellic acid), with the pinellic acid being the most influential tastants determined by sensory recombination experiment. $_{L}$-tryptophan and apigenin-C-glycosides are native in grain and found to be degraded during bread-making, whereas the pinellic acid was found generated during bread-making (Bin & Peterson 2016).

Trihydroxy fatty acids have been previously reported to form via enzymatic oxidation of linoleic acid when incubating linoleic acid with a suspension of cereal (e.g. wheat, barley, rye, oats)(Graveland 1973) and leguminosae (e.g. peas)(Arens & Groseh 1974) and a mechanism was proposed for their formation (**Figure 4-1**). It has been suggested that trihydroxyoctadecenoic acids are products of decomposition of linoleic acid hydroperoxides, formed under the enzymatic oxidation of linoleic acid by lipoxygenase. The linoleic acid hydroperoxides further undergoes epoxidation and hydrolysis to give trihydroxyoctadecenoic acids. This pathway is rather a model understanding of enzymatic oxidation of linoleic acid with known precursors and enzymes. Bitterness development during different bread-making stages (e.g. dough kneading, fermentation, and baking) requires further elucidation in order to gain better understanding of important precursor, influential factors and the stages of formation or degradation during bread making. Improved knowledge of bitterness development in whole wheat food would allow for the identification and potential control of key factors

that reduce the bitterness during whole wheat food manufacturing. Wheat class is considered an important factor that genetically affects the composition of wheat flour and baking quality. Storage of flour is also an important factor, which is accompanied by a cascade of enzymatic and chemical changes that lead to reduced bread sensory quality.

Wheat grown in the US is categorized into six classes: hard red winter (HRW), hard red spring (HRS), soft red winter (SRW), durum, hard white (HW), and soft white (SW). The “red” and “white” are representing the color of the kernel are typically used for bread making. Red wheat is commonly perceived in the industry as more bitter than the white wheat.

Another important factor that may influence the pinellic acid formation in wheat foods is the storage of flour. Freshly milled flours are commonly subjected to storage ranging from 3 to 9 months at ambient temperature. However, actual shelf life could be shorter or longer depending on environmental conditions such as temperature and humidity during storage. In addtion, shelf life of whole wheat flour is considerably shorter than white flour (Doblado-Maldonado et al. 2012). The most unstable components in whole wheat flour are considered to be the lipids, which are readily degraded via the enzymatic lipase activity resulting in loss of flour functionality (Doblado-Maldonado et al. 2012). Lipase (EC 3.1.1.3) hydrolyzes triacylglycerols to free fatty acids and diglycerides, which is also known as hydrolytic rancidity (O’Connor et al. 1992). From flavor perspective, lipase activity in whole wheat flour can lead to a decrease in sensory quality of whole grain flour (Hansen & Rose 1996) as acceptability of bread made from whole wheat flours is inversely related to their hydrolytic rancidity.

In this study, the influence of storage conditions on the linoleic acid content in flour and subsequently on the levels of bitter pinellic acid in bread was investigated, in order to provide insights for improving flour storage practices aiming to reduce bitterness in whole wheat foods.

The overall goal of this study was to better understand the mechanisms and key factors of formation of pinellic acid for the development of improvement strategies in manufacturing whole wheat products with reduced bitterness and better flavor quality. The influence of wheat class on the bitter pinellic acid generation from flour to bread was also investigated.

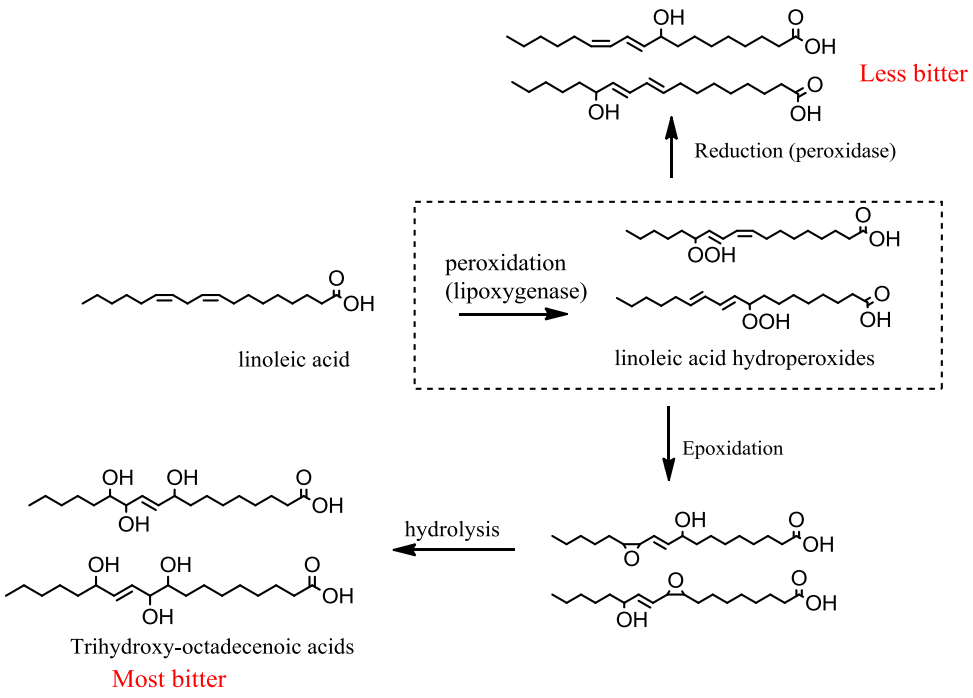


Figure 4-1. Chemical pathway of enzymatic generation of bitter trihydroxy-octadecenoic acid from linoleic acid(Graveland 1970).

Materials and Methods

Chemicals. [$^{13}C_{18}$]Linoleic acid (99 atom % ^{13}C) was purchased from Sigma-Aldrich (St. Louis, MO, USA)

HRS and HWW Wheat Varietals. HRS wheat varietals, Knudson, Marshall, Glenn, Elgin-ND, RB07, WB Mayville, Prosper, and Faller, were harvested from plots in Crookston, Lamberton, Morris, Roseau, St. Paul and Waseca, and on-farm sites near Benson, Fergus Falls, Hallock, LeCenter, Kimball, Oklee, Perley, Stephen and Strathcona, Minnesota in 2014. HWW wheat varietals, Snowmass, Nuplains, N11MD2129W, N11MD2157W, N11MD2166W, N13MD2589W, N13MD2517W, Alice, and NW07505 were harvested from plots in Sidney and Nebraska in 2014. Whole wheat flours were obtained via grinding using a UDY Cyclone Sample Mill (UDY Corporation, Fort Collins, CO, USA).

Bread Model-Pathway Study. Whole wheat flour (100 g), water (60 g), active dry yeast (5.3 g), and sodium chloride (1.5 g) were mixed with a mechanical kneader at room temperature for 8 min at low speed and then for 8 min at high speed. Immediately after dough kneading, two replicates of 3% dough mass (4.9 g each) were sampled for subsequent solvent extraction. Four replicates of 3% dough mass were sampled for fermentation. The dough was left to proof at 30 °C for 120 min. Loaves were allowed to rest for about 5 min before being shaped. The formed dough was then fermented at 28 °C for 60 min. After fermentation, two replicates were taken for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis to determine pinellic acid generation in

the matrix. The remaining two were baked at 190 °C for 15 min. The bread samples were further analyzed by LC-MS/MS to quantify pinellic acid in the sample.

Free and Hydroxylated Fatty Acid Extraction. Whole wheat flour (3 g), dough samples before fermentation, after fermentation, and bread samples were extracted with 15 ml × 2 of methanol-chloroform (50:50 v/v) mixture spiked with [$^{13}C_{18}$]linoleic acid (100 ng/ml) as internal standard. The mixture was homogenized at room temperature for 30 min and centrifuged. The supernatant was concentrated using Rotavap and the residue was re-solubilized with 4 ml of methanol, which was passed through a 500 mg preconditioned C18 cartridge (Supelco, Bellefonte, PA, USA) and eluted with 2 mL of methanol for further sample clean up. The methanol and eluent were pooled and filtered through 0.20 µm Nylon syringe filter (Millex, Billerica, CA, USA) prior to LC-MS/MS analysis (see section *Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS) Analysis below).*

[$^{13}C_{18}$]Linoleic Acid Isotope Labeling Study. 45 mg [$^{13}C_{18}$]linoleic acid was added into flour (12 g), along with additional bread model ingredients to a final ratio as described above (section *Bread Model-Pathway Study*), immediately before dough kneading. Equal samples (5 g of dough) were collected, before and after fermentation and after baking, and were subjected to LC-MS analysis to study the [^{13}C]-labeled reaction products (see section *Liquid Chromatography – Mass Spectrometry (LC-MS/ToF below) Analysis).* The [$^{13}C_{18}$]linoleic acid acted as free linoleic acid marker to characterize the key precursors of trihydroxy-octadecenoic acids, and quantitatively elucidate the

contribution of free linoleic acid, along with its reaction intermediates, to the generation of trihydroxy-octadecenoic acids, in the big picture of linoleic acid oxidation in food.

Liquid Chromatography – Mass Spectrometry (LC-MS/ToF) Analysis. LC-MS/Tof analysis was used to study ^{13}C-labeled reaction products in linoleic Acid-$^{13}C_{18}$ labeled flour and bread samples. Samples were analyzed on a Waters ACQUITY UPLC system interfaced with Xevo™ G2 QTOF mass spectrometer (Waters, Milford, MA, USA). Samples (2 μL) were injected on an ACQUITY UPLC BEH C18 1.7 μm column (2.1 mm × 50 mm) (Waters, Milford, MA, USA) at 25ºC. The mobile phase was maintained at a flow rate of 300 μL/min using binary solvent system of 0.1% formic acid in water (A) and acetonitrile (B). The elution gradient started at 5% B (0-1 min), linearly increased to 50% B (1-6 min), then increased to 100% B (6-8 min) held at 100% B (8-10 min), and was subsequently re-equilibrated at 5% B (10-15 min). A Xevo™ G2 QTOF mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization probe was utilized for analysis. Data was collected in positive and negative ionization mode and the operational parameters were as follows: ESI+, source temperature 110°C; desolvation temperature 350°C; capillary voltage 1.5 kV. ESI-, source temperature 110°C; desolvation temperature 350°C; capillary voltage 2.0 kV. Data processing was performed using Waters® MassLynx™ 4.1 software.

Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS) Analysis. Quantitative LC-MS/MS analysis was used to quantitatively monitor pinellic acid level in food matrices using multiple reaction-monitoring (MRM) acquisition. A Waters ACQUITY UPLC system interfaced with a Quattro Premier XE™ mass

spectrometer (Waters, Milford, MA, USA) was used. Samples (2 μL) were injected onto an ACQUITY UPLC BEH C18 1.7 μm column (2.1 mm × 50 mm) (Waters, Milford, MA, USA) held at 25ºC. The mobile phase was maintained at a flow rate of 300 μL/min using binary solvent system of 0.1% formic acid in water (A) and acetonitrile (B). The elution gradient started at 5% B (0-1 min), linearly increased to 50% B (1-6 min), then increased to 100% B (6-8 min) held at 100% B (8-10 min), and re-equilibrated at 5% B (10-15 min). The inlet condition was the same as in the method described in the LC-MS/ToF section above. A Quattro Premier XE^{TM} mass spectrometer (Waters, Milford, MA, USA) was equipped with an electrospray ionization probe and the MS/MS ion transitions and collision energy were as follows: pinellic acid ESI- 329 --> 211 (collision energy: 15eV; cone voltage 30V). Butyl 4-hydroxybenzoate was used as an internal standard and monitored using the following transition: *m/z* 193 -->136 (collision energy: 15eV; cone voltage 30V).

Results and Discussion

Generation of THOA during bread-making: Key Stages and Precursor

Isotope labeling analysis using [$^{13}C_{18}$]linoleic acid was conducted in order to elucidate the role of free linoleic acid in generation of pinellic acid including the key changes during each stage of bread-making: flour, dough initial (dough immediately after kneading), dough final (dough after fermentation), and bread. [$^{13}C_{18}$]Linoleic acid was added at a 1:1 ratio with the natural content of free linoleic acid in flour used (0.4%), along with other ingredients typical of bread manufacture, immediately before dough

kneading. The dough initial, dough final, and bread samples were analyzed for LC-QTOF-MS analysis. **Figure 4-2** shows the TIC chromatogram obtained using Masslynx^{TM} Strips function with Cluster Output (Δm=18.06, $^{13}C_{18}$) indicating the $^{13}C_{18}$-labeled products in the final bread sample. As notated on the chromatogram, the major $^{13}C_{18}$-labeled products include pinellic acid (9,12,13-trihydroxy-trans-10-octadecenoic acid) and its isomer 9,10,13-trihydroxy-trans-10-octadecenoic acid at 10:1 peak area ratio, hydroperoxide isomers, and 9-hydroxy-octadecadienoic acid.

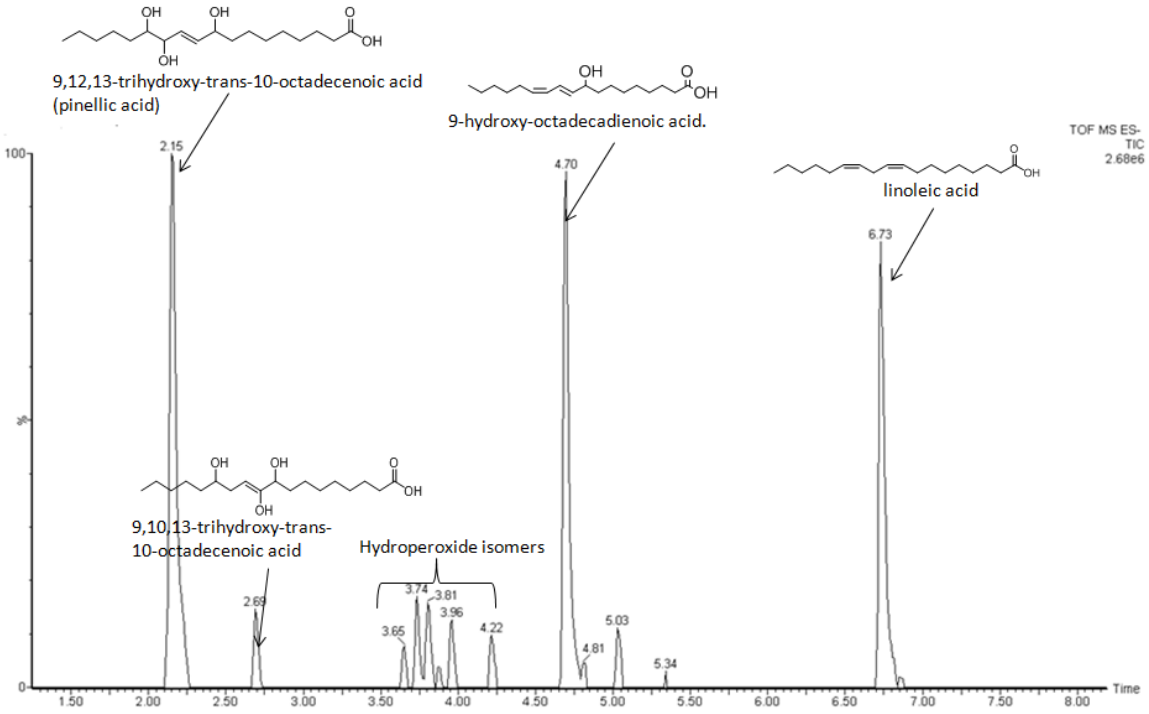
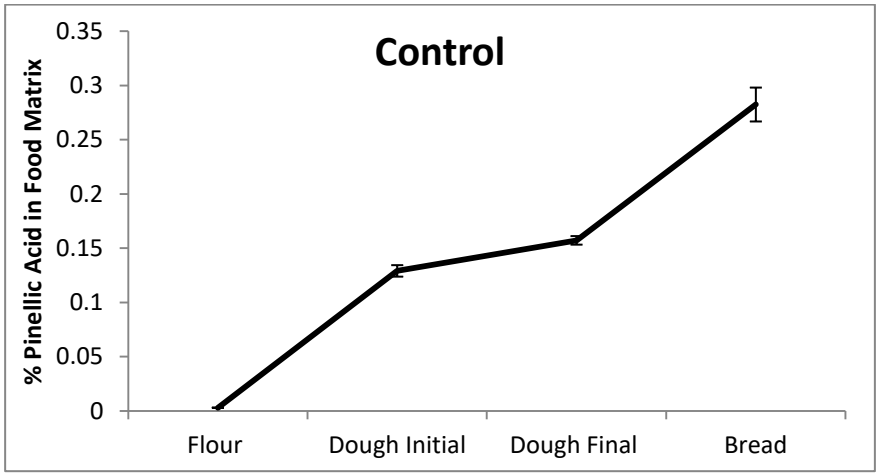


Figure 4-2. TIC chromatogram indicating the $^{13}C_{18}$-labeled products in the final bread sample. Identification of the compounds was based on ESI-MS/MS fragmentation spectra.

Quantitative analysis of unlabeled and [^{13}C]-labeled pinellic acid during bread-making was conducted by LC-MS/MS analysis. **Figure 4-3A** shows the results obtained

from the analysis of the control sample, and demonstrated the generation of pinellic acid throughout the bread-making process. Figure **4-3B** shows results obtained from the analysis of the 1:1 stable isotope labeled sample, and both the levels of unlabeled and [^{13}C]-labeled pinellic acid at each stage of bread-making. The flour stage was also included ([^{13}C]-labeled pinellic acid level is 0). The results indicated that the change of the [^{13}C]-labeled pinellic acid during the bread-making process followed closely the trend of the unlabeled pinellic acid. Thus, this finding suggested that free linoleic acid is an exclusive precursor of the trihydroxy octadecenoic acid during whole wheat bread processing. Results additionally indicated that the total level of pinellic acid increased more than 40 fold during the dough kneading process (dough initial), when the flour was mixed with water and the gluten network was formed. During fermentation (from dough initial to dough final) no significant change was observed while after baking a significant increase was additionally noted, and the labeled product also exhibited the same trend. These results suggest that multiple pathways, both enzymatic and non-enzymatic, are involved in the generation of pinellic acid. The mechanism of generation of pinellic acid during production of whole wheat bread was further investigated. The following sections discuss both enzymatic and non-enzymatic mechanistic pathways of generation of pinellic acid in whole wheat bread.

A.



B.

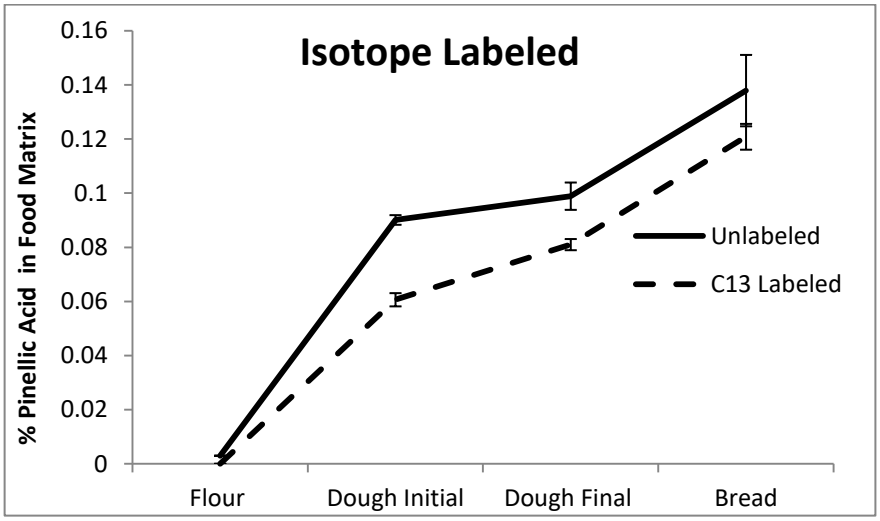


Figure 4-3. **A.** Generation of pinellic acid during bread making: flour, dough initial (dough sampled immediately after kneading), dough final (dough sampled after fermentation), and bread. **B.** Concentration of unlabeled vs. [^{13}C]-labeled pinellic acid in food matrix (normalized to the same flour mass, µmol/g) during stages of bread making:. (12.6 umol/g [$^{13}C_{18}$]linoleic acid was added into the flour.)

Enzymatic Generation of Pinellic Acid during Dough Kneading

Pinellic acid is a trihydroxyoctadecenoic acids (Tri-OH), which has been previously reported to be formed by the enzymatic oxidation of linoleic acid when

incubating linoleic acid with a suspension of cereal (e.g. wheat, barley, rye, oats) and leguminosae (e.g. peas) (Arens & Groseh 1974) and a mechanism has been previously proposed (Graveland 1973). In brevity, in the presence of oxygen, linoleic acid is oxidized by lipoxygenase and forms two isomeric linoleic acid hydroperoxides (LOOH): the 13-hydroperoxy-9,11-octadecadienoic acid and 9-hydroperoxy-10,12-octadecadienoic acid. Enzymatic epoxidation of the hydroperoxides by a water-insoluble factor that is localized in gluten leads to hydroxy-epoxy-octadecenoic acids. The hydroxy-epoxy-octadecenoic acids further undergoes hydrolysis to give Tri-OH. Decomposition of LOOH can also lead to 13-hydroxy-9,11-*octadecadienoic* acid and 9-hydroxy-10,12-octadecadienoic acid.

Tri-OH has also been reported to increase during storage of soybeans at 22ºC. Incubation of the components of soybean, glutathione, and horse radish peroxidase with LOOH showed that breakdown of LOOH to Tri-OH is facilitated by the protein fraction from soybean containing lipoxygenase and peroxidase activities. Thiol groups of protein/free thiol (glutathione) were also found to be involved in the formation of Tri-OH.

In order to understand whether the linoleic acid or enzyme (lipoxygenase/peroxidase) is a limiting factor in the enzymatic formation of pinellic acid during dough kneading, dependence of free linoleic acid concentration on the formation of pinellic acid was investigated. HRS whole wheat flour (with 0.4% original linoleic acid) was treated with added linoleic acid: 0% (control), 0.4%, and 1.0% respectively, and mixed into dough. Pinellic acid was measured in the dough and determined that there

is no significant difference between the treatments (**Figure 4-4**). Therefore, it was suggested that the lipoxygenase/peroxidase activity and not the concentration of linoleic acid, was limiting the formation of pinellic acid during dough mixing. Results are in agreements with previous studies reporting a self-destruction mechanism accounting for the losses in lipoxygenase during dough mixing (Delcros et al. 1998). These researchers reported the highest losses of lipoxygenase were observed at the beginning of the mixing period, whereas lipoxygenase presence rapidly decreased as mixing time increases. About 2/3 of the activity is lost during the first 5 minute of mixing. Our results (Fig. 4-4) indicated that enzyme activity is the rate-limiting factor of the formation of pinellic acid during dough mixing. Given the loss of lipoxygenase activity during mixing, this would explain why there was not a further increase in pinellic acid during dough fermentation.

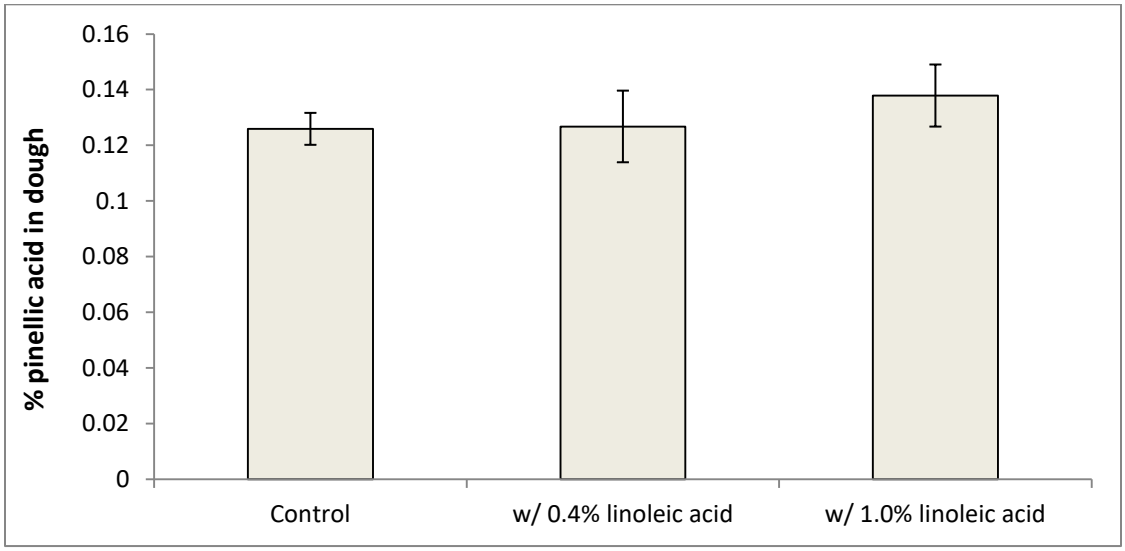


Figure 4-4. Dependence of linoleic acid concentration on the formation of pinellic acid in dough. Results indicates that formation of pinellic acid is not significantly different between the treatments ($\alpha = 0.05$).

Non-Enzymatic Generation of Pinellic Acid during Bread Baking

The results obtained from isotope labeling experiment (**Figure 4-3)** further indicated that the baking cycle is another step where generation of pinellic acid during was observed, as levels increased by ~40% when compared to dough stage. As discussed above, enzymatic activities during dough mixing lead to the generation of bitter Tri-OH from free linoleic acid. For the baking stage, enzymatic pathways are unlikely as most are denatured and deactivated at high temperature. Therefore non-enzymatic generation of Tri-OH acids was proposed.

As shown in **Figure 4-1**, the enzymatic pathway of bitter Tri-OH from linoleic acid, 9-hydroxyoctadecadienoic acid (HOA), the less bitter byproduct, was also formed during dough kneading **(Figure 4-2).** As monitored during baking, more pinellic acid was formed, however not for HOA (see **Table 4-1**). The TIC ratio of pinellic acid/HOA increased as baking time increased, which indicated that the thermal energy facilitated non-enzymatic formation of pinellic acid as its level continuously increased during baking; whereas the formation of HOA is mainly of enzymatic origin as baking does not change its level.

Table 4-1. Time course change of TIC ratio of Pinellic Acid/HOA during baking

Baking time (min)	TIC Ratio of Pinellic Acid/HOA
0	1.00
4	1.56
8	1.60
12	1.72
16	1.84

Previous work suggests that LOOH, generated enzymatically from linoleic acid, is a major precursor of pinellic acid. LOOH can decompose via non-enzymatic pathways shown in **Figure 4-5** (Gardner 1975), which involves free radical mechanism facilitated by Fe-cysteine redox cycle and forming hydroxyoctadecadienoic acid that further reacts with hydroxyl radical to form pinellic acid. As elevated temperature is commonly reported to increase the rate of hydroxyl radicals reactions (Christensen et al. 1982), this radical pathway provides a reasonable hypothesis for the mechanism by which heat (baking) facilitates non-enzymatic formation of pinellic acid.

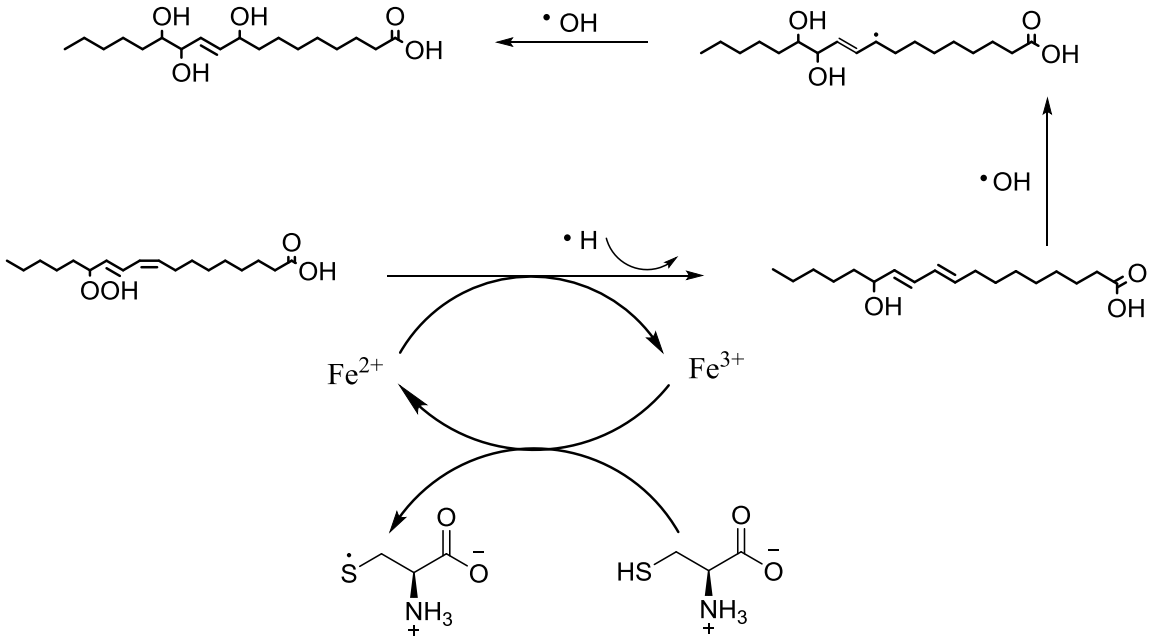


Figure 4-5. Non-enzymatic generation of pinellic acid from LOOH(Gardner 1975).

Influence of wheat classes on the profile of precursor (linoleic acid) and generation of pinellic acid during bread making.

The percent of free linoleic acid in flour for HRS and HWW wheat samples is reported in **Figure 4-6A**. Comparing 8 varietals of each HRS and HWW wheat classes, results indicated that HRS wheat samples have significantly higher linoleic acid content in the flour, and also generate higher pinellic acid during dough kneading. On average, HRS had a 97% higher content of free linoleic acid when compared to the HWW samples. Correspondingly, HRS flours generated 34% more pinellic acid during dough kneading than HW wheat entries on average (**Figure 4-6B**); however, the % yield of pinellic acid from linoleic acid is higher for HWW wheat (27.3%) as compared to HRS wheat (40.2%). As discussed earlier, generation of pinellic acid was found to be independent of linoleic acid concentration. Rather, enzymatic activity of lipoxygenase which converts free fatty acids into lipid hydroperoxides was hypothesized to be an important limiting factor. Results implied lipoxygenase activity is higher for HWW wheat. Decomposition of lipid hydroperoxides can be enzymatic, which is facilitated by enzymatic activity of peroxidase and lipoxygenase that occur during dough kneading. Other than enzymatic decomposition, chemical factors such as Fe-cysteine can also facilitate decomposition of lipid hydroperoxides into pinellic acid (Gardner 1975), which can impact formation during both dough-making and baking. During baking, there is 45% increase in pinellic acid from dough to flour for HRS wheat, and 49% increase for HWW wheat samples. These results suggested that the non-enzymatic generation of pinellic acid influence both HRS and HWW samples during baking (**Figure 4-7**).

In conclusion, by comparing 8 varietals of each HRS and HWW wheat classes, HRS wheat varietals, on average, have higher generation of pinellic acid during bread-making than HWW wheat varietals. As observed, HRS wheat generally have higher free linoleic acid content in the flour than HWW wheat, however, previous section has suggested that enzymatic activity of lipoxygense/peroxidase, rather than the free linoleic acid content, serve as a rate-limiting factor in generation of pinellic acid in dough. Non-enzymatic generation of pinellic acid influences both HRS and HWW samples during baking. This study of the influence of wheat class on the formation of pinellic acid serves as a practical example of understanding the mechanism of pinellic acid generation from ingredients to finished products, and provides insights into better breeding strategies and wheat varietal selections to reduce the bitterness and improve the flavor profile of whole wheat products.

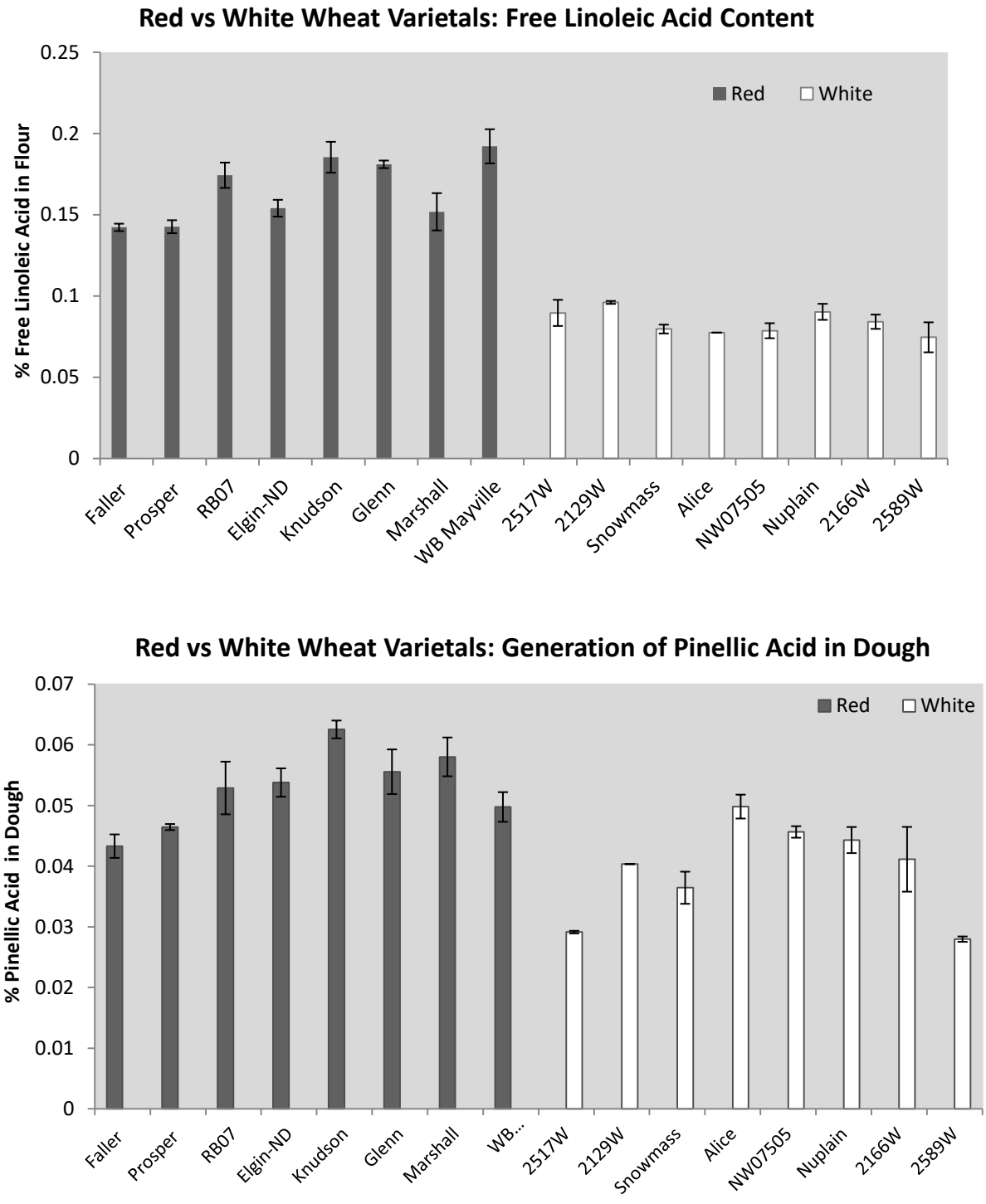


Figure 4-6. (A) % free linoleic acid content in flour for HRS and HWW wheat entries. (B) % pinellic acid content in dough for HRS and HWW wheat entries.

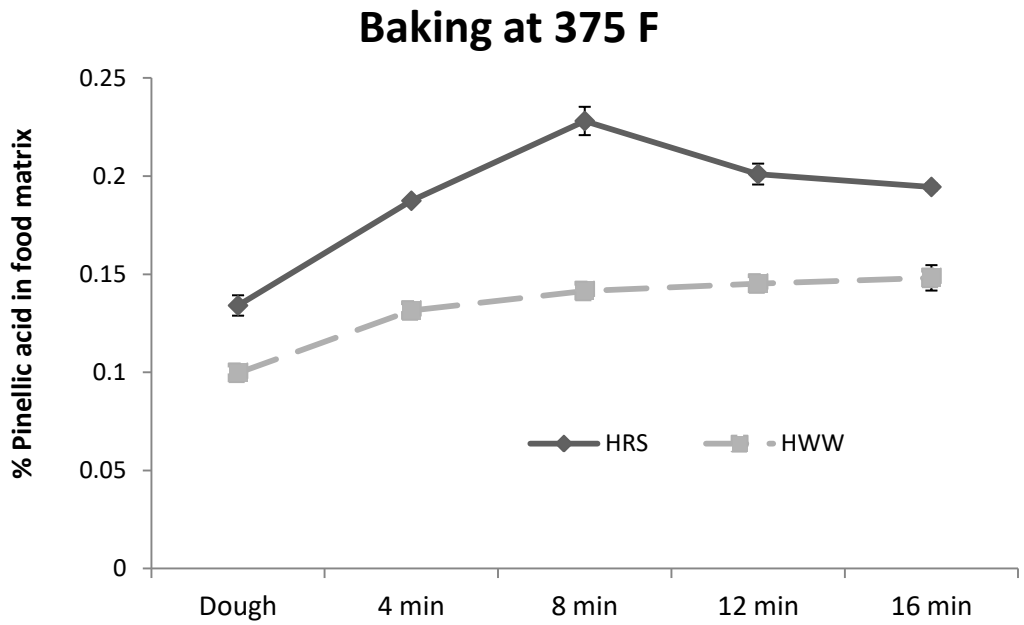


Figure 4-7. Time course change of % pinellic acid in HRS vs. HWW samples, respectively, during baking at 375 F.

Influence of storage temperature on the profile of precursor (linoleic acid) and generation of THOA during bread making.

Influence of flour storage temperature on the linoleic acid content of flour, and the generation of pinellic acid in dough was investigated. Results of free linoleic acid content in four types of flours (HRS whole wheat, HWW whole wheat, HRS refined wheat, HWW refined wheat) stored for 4 months at three difference temperatures, -20 °C, ambient temperature (~25 °C), and 37 ºC, are shown in **Figure 4-8A**. Both ambient temperature and 37ºC had a significant impact on the free linoleic acid content, which indicated that hydrolytic rancidity from the activity of lipase was higher at those

temperatures when compared to freezing at -20 °C. Higher contents of free linoleic content can affect overall bread quality. Polyunsaturated fatty acids reduce the lipid binding capacity of gluten, which reduces gas holding capacity of gluten and affects elasticity and final volume of the bread (Alvarez-Jubete et al. 2010).

The pinellic acid content in the dough samples prepared from flours stored at different temperatures revealed that the ambient temperature had the greatest influence increasing the generation of pinellic acid in dough likely reflecting the effect of storage temperature on the enzymatic activity lipoxygenase and subsequently the generation of pinellic acid. Lipid peroxidation can still occur through the action of lipoxygenase during storage, resulting in slow generation of precursors of pinellic acid (e.g. LOOH) when stored at suitable temperatures. Ambient temperature is suggested to be the optimal temperature for this enzymatic process. Moreover, results indicated (Figure **4-8B)** that the influence of storage temperature on pinellic acid level was greater in HWW whole wheat varietals when compared to HRS whole wheat varietals. This is in agreement with the above-mentioned hypothesis of lipoxygenase activity and generation of pinellic acid precursors as it has been previously reported that lipoxygenase exhibits lower activity during storage with higher level of antioxidants in wheat (Wang and Toledo, 1987). Since the antioxidant activities in red wheat are higher than the white wheat, lipoxygenase activity during storage is expected to be limited in HRS and therefore the impact of storage on pinellic acid formation is expected to be greater on HWW samples. The results also showed that influence of storage temperature on pinellic acid level was significant in

refined flours (both HRS and HWW), indicating the activity of the lipoxygenase in refined wheat flour samples resulted in significant impact during storage.

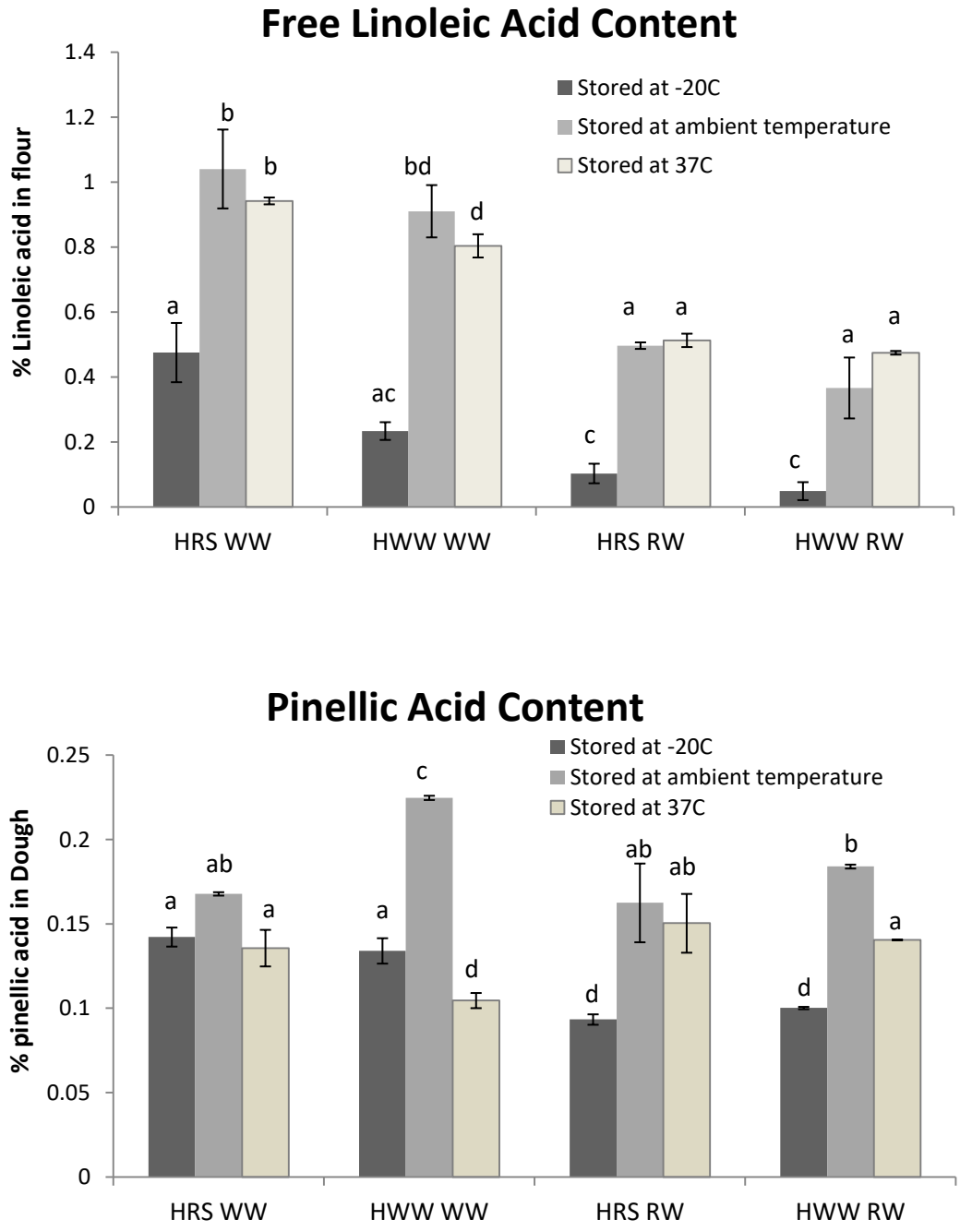


Figure 4-8. (A) Influence of storage temperature on the free linoleic acid content in four types of wheat flours. (B) Influence of storage temperature on the pinellic acid content in dough samples from four types of wheat flours. HRS = hard red spring, HWW = hard

white winter, WW = whole wheat, RW = refined wheat. Different letters mean significantly different (p-value < 0.05).

In conclusion, this chapter examined the mechanism of the formation of pinellic acid, a key bitter compound in whole wheat bread, during bread-making. Stable isotope labeling study indicated that free linoleic acid in flour was the major contributor to the formation of pinellic acid that occurred in dough kneading and baking. Enzymatic generation of LOOH, a precursor of pinellic acid, occurred during dough mixing with the lipoxygenase/peroxidase activities, is the rate-limiting factor (oxidation of free linoleic acid to LOOH). Decomposition of the LOOH into pinellic acid was found to occur both enzymatically during dough mixing, and non-enzymatically during baking. Influence of wheat class and flour storage on the generation of pinellic acid was further investigated. HRS wheat entries on average generated higher level of pinellic acid during dough kneading than HWW wheat entries, as influenced by both enzymatic and non-enzymatic pathways. Temperature of flour storage temperature also significantly influenced generation of pinellic acid in dough. When stored at ambient temperature, significantly higher pinellic acid formation was observed in the dough, which suggesed that ambient temperature facilitates the enzymatic generation of precursors of pinellic acid, free linoleic acid and linoleic acid hydroperoxides, by allowing both lipase and lipoxygenase activity during storage. Overall this work provided insight regarding the mechanistic pathway of generation of a key bitter marker in whole wheat products. In addition critical environmental and intrinsic factors were elucidated facilitating the development of guidelines for better manufacturing practices for high flavor quality in whole wheat foods.

Chapter 5

Conclusions and Future Work

5.1. Conclusions

In summary, this project has provided a better understanding of the chemical basis for bitterness development in whole wheat foods. Whole wheat bread crust and crumb were both studied. Chemical markers for bitterness in bread crust is generated from the Maillard reaction. Bread crumb bitterness is distinctive from the crust and the key bitter compounds consisted of $_{L}$-tryptophan, apigenin-C-glycosides, and 9,12,13-trihydroxy-trans-10-octadecenoic acid (pinellic acid), with the pinellic acid determined to be the most influential bitterant by sensory recombination model evaluation. $_{L}$-tryptophan and apigenin-C-glycosides were found to be native in wheat flour and reported to be degraded during the bread manufacture process whereas the pinellic acid was generated during bread manufacture. Isotope labelling study using [$^{13}C_{18}$]linoleic acid as a free linoleic acid marker indicated that the natural free linoleic acid in flour contributes majorly to the formation of pinellic acid that occurs in two bread-making stages: dough kneading and baking. The mechanism of generation for the pinellic acid was further investigated and suggested to involve a few parts. First is the enzymatic oxidation of linoleic acid by lipxygenase to linoleic acid hydroperoxides (LOOH), which is during the dough kneading when the gluten network was formed. Second includes the decomposition of the LOOH into pinellic acid, both enzymatically and non-enzymatically. The enzymatic pathway involves epoxidation of the LOOH followed by hydrolysis, which was suggested to be

major pathway during dough kneading; the non-enzymatic decomposition of LOOH involves free radical mechanism facilitated by transition metal and forming hydroxyoctadecadienoic acid that further reacts with hydroxyl radical to form pinellic acid, which was suggested to be major pathway during baking. In addition, study of the influence of wheat class and flour storage temperature on the enzymatic formation of pinellic acid in dough provide insights into wheat varietal selections and flour storage practice to reduce the bitterness in the products. Results showed that Hard Red Spring wheat entries generally have higher linoleic acid content in the flour and generates higher level of pinellic acid during dough kneading than Hard White wheat entries. Temperature of flour storage temperature also plays key role influencing the linoleic acid content in flour and generation of pinellic acid in dough. Ambient temperature has the greatest impact by significantly increasing pinellic acid formation through lipoxygenase activity which can generate precursors of pinellic acid during storage.

5.2. Translatable outcomes

With the knowledge of bitterness composition, origin, and development pathway in whole wheat foods, we have gained critical insights into improvement strategies for manufacturing whole wheat foods with better flavor quality. With effective strategies in reducing bitterness, sugar and salt content can also be reduced in many applications. In addition, the knowledge can be further extended to a broad range of whole grain products (e.g. barley, rye, and oat).

5.3. Future Work

This project has majorly focused on processed-derived bitterness in whole wheat foods; however, bitterness in food may undergo further development during storage. During food storage, biochemical changes of food components can be another source of bitterness, which results in loss of flavor quality of food. As mentioned above, research in beer has discovered that isomerization of hop acids (e.g. cohumulone, humulone, and adhumulone) into iso-α-acids causes increased bitterness in beer during storage. Future work can focus on the bitterness changes during whole wheat food storage to address: 1) whether some bitter compounds, such as pinellic acid, can be further generated during storage; 2) degradation of pinellic acid (e.g. oxidation, isomerization, cross-linking, etc.) in storage conditions and how the degradation pathway influence bitterness and overall flavor quality.

References

AACC International, 2010. Baking Quality of Bread Flour - Sponge-Dough, Pound-Loaf Method 10-11.01.

Alvarez-Jubete, L. et al., 2010. Baking properties and microstructure of pseudocereal flours in gluten-free bread formulations. *European Food Research and Technology*, 230(3), pp.437–445. Available at: http://link.springer.com/10.1007/s00217-009-1184-z [Accessed September 28, 2016].

Arens, D. & Groseh, W., 1974. Non-volatile Reaction Products from Linoleic Acid . Comparison of a Ground Pea Suspension with a Purified Pea Lipoxygenase. *Z. Lebensm. Unters.-Forsch*, 156, pp.292–299.

Asenstorfer, R.E., Wang, Y. & Mares, D.J., 2006. Chemical structure of flavonoid compounds in wheat (Triticum aestivum L.) flour that contribute to the yellow colour of Asian alkaline noodles. *Journal of Cereal Science*, 43(1), pp.108–119. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0733521005001074 [Accessed November 14, 2013].

Bakke, A. & Vickers, Z., 2007. Consumer liking of refined and whole wheat breads. *J. Food Sci.*, 72(7), pp.S473-480. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17995660 [Accessed December 5, 2011].

Ballesteros, J.A. & Weinstein, H., 1995. [19] Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods in Neurosciences*, 25, pp.366–428.

Baur, C. et al., 1977. Enzymatic Oxydation of Linoleic Acid : Formation of Bittertasting

Fatty Acids. *Z. Lebensm. Unters.-Forsch*, 164, pp.171–176.

Besson, E. et al., 1985. C-glycosylflavones from Oryza Sativa. *Phytochemistry*, 24(5), pp.1061–1064.

Besson, E. et al., 1984. Identification of neoschaftoside as 6-C-β-d-glucopyranosyl-8-C-β-l-arabinopyranosylapigenin. *Phytochemistry*, 23(1), pp.159–161.

Biermann, U. & Grosch, W., 1979. Bitter-Tasting Monoglycerides from Stored Oat Flour. *Z. Lebensm. Unters.-Forsch*, 169, pp.22–26.

Bin, Q. et al., 2012. Chemical markers for bitterness in wheat bread. *Flavour and Fragrance Journal*, 27(6), pp.454–458. Available at: http://doi.wiley.com/10.1002/ffj.3124 [Accessed July 13, 2013].

Bin, Q. & Peterson, D.G., 2016. Identification of bitter compounds in whole wheat bread crumb. *Food Chemistry*, 203, pp.8–15. Available at: http://dx.doi.org/10.1016/j.foodchem.2016.01.116.

Van Boekel, M.A.J.S., 1998. Effect of heating on Maillard reactions in milk. *Food Chemistry*, 62(4), pp.403–414.

Burgess-Champoux, T. et al., 2006. Perceptions of children, parents, and teachers regarding whole-grain foods, and implications for a school-based intervention. *Journal of nutrition education and behavior*, 38(4), pp.230–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16785092 [Accessed June 29, 2015].

Burgess-champoux, T. et al., 2006. Perceptions of Children , Parents , and Teachers Regarding Whole-Grain Foods , and Implications for a School-Based Intervention. *Journal of nutrition education and behavior*, 38, pp.230–237.

Christensen, H., Sehested, K. & Corfitzen, H., 1982. Reactions of hydroxyl radicals with hydrogen peroxide at ambient and elevated temperatures. *The Journal of Physical Chemistry*, 86(9), pp.1588–1590. Available at: http://pubs.acs.org/doi/abs/10.1021/j100206a023 [Accessed August 16, 2016].

Conde-Petit, B., Escher, F. & Nuessli, J., 2006. Structural features of starch-flavor complexation in food model systems. *Trends in Food Science & Technology*, 17(5), pp.227–235. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0924224405003420 [Accessed November 16, 2013].

Cutzach, I. et al., 1997. Identification of volatile compounds with a "toasty" aroma in heated oak used in barrelmaking. *J. Agric. Food Chem.*, 45(6), pp.2217–2224. Available at: http://pubs.acs.org/doi/abs/10.1021/jf960947d.

Delcros, J.F. et al., 1998. Effect of mixing conditions on the behavior of lipoxygenase, peroxidase, and catalase in wheat flour doughs. *Cereal Chemistry*, 75(1), pp.85–93.

Doblado-Maldonado, A.F. et al., 2012. Key issues and challenges in whole wheat flour milling and storage. *Journal of Cereal Science*, 56(2), pp.119–126. Available at: http://dx.doi.org/10.1016/j.jcs.2012.02.015.

Drake, M.A., Gerard, P.D. & Chen, X.Q., 2001. Effects of sweetener, sweetener concentrate, and fruit flavor on sensory properties of soy fortified yogurt. *Journal of Sensory Studies*, 16(4), pp.393–405. Available at: http://doi.wiley.com/10.1111/j.1745-459X.2001.tb00309.x [Accessed May 11, 2016].

Drewnowski, A., 2001. The Science and Complexity of Bitter Taste. *Nutrition Reviews*, 59(6), pp.163–169.

Drewnowski, A. & Gomez-carneros, C., 2000. Bitter taste , phytonutrients , and the consumer : a review 1 – 3. *The American Journal of Clinical Nutrition*, (22), pp.1424–1435.

Engel, W., Bahr, W. & Schieberle, P., 1999. Solvent assisted flavour evaporation - a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. *European Food Research and Technology*, 209(3–4), pp.237–241.

Feng, Y., McDonald, C.E. & Vick, B.A., 1988. C-Glycosylflavones from Hard Red Spring Wheat Bran. *Cereal Chemistry*, 65, pp.452–456.

Frank, O., Jezussek, M. & Hofmann, T.H.H., 2003. Sensory Activity , Chemical Structure , and Synthesis of Maillard Generated Bitter-Tasting 1-Oxo-2,3-dihydro-1H-indolizinium-6-olates. *Journal of Agricultural and Food Chemistry*, 51, pp.2693–2699.

Frank, O., Ottinger, H. & Hofmann, T., 2001a. Characterization of an intense bitter-tasting 1H,4H-quinolizinium-7-olate by application of the taste dilution analysis, a novel bioassay for the screening and identification of taste-active compounds in foods. *J. Agric. Food. Chem.*, 49(1), pp.231–238. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11170582.

Frank, O., Ottinger, H. & Hofmann, T., 2001b. Characterization of an intense bitter-

tasting 1H,4H-quinolizinium-7-olate by application of the taste dilution analysis, a novel bioassay for the screening and identification of taste-active compounds in foods. *Journal of agricultural and food chemistry*, 49(1), pp.231–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11170582.

Gardner, H.W., 1975. Decomposition of linoleic acid hydroperoxides. Enzymic reactions compared with nonenzymic. *Journal of agricultural and food chemistry*, 23(2), pp.129–36. Available at: http://www.ncbi.nlm.nih.gov/pubmed/805802.

Ghiron, A.F. et al., 1988. Studies on the Role of 3-Deoxy-D-erythr-gol ucosulose (3-Deoxyglucosone) in Nonenzymatic Browning. Evidence for Involvement in a Strecker Degradation. *J. Agric. Food Chem.*, 36, pp.677–680.

Graveland, A., 1973. Analysis of lipoxygenase nonvolatile reaction products of linoleic acid in aqueous cereal suspensions by urea extraction and gas chromatography. *Lipids*, 8(3), pp.599–605.

Graveland, A., 1970. Enzymatic Oxidations of Linoleic Acid and Glycerol-1-Monolinoleate in Doughs and Flour-Water Suspensions. *Journal of the American Oil Chemists' Society*, 47, pp.352–361.

Grosch, W., 1994. Determination of Potent Odourants in Foods by Aroma Extract Dilution Analysis (AEDA) and Calculation of Odour Activity Values (OAVs). *Flavour and Fragrance Journal*, 9, pp.147–158.

Grosch, W., 2001. Evaluation of the Key Odorants of Foods by Dilution Experiments, Aroma Models and Omission. *Chemical Senses*, 26(5), pp.533–545. Available at: http://chemse.oxfordjournals.org/content/26/5/533.abstract\nhttp://chemse.oxfordjou

rnals.org/content/26/5/533.full.pdf.

Hansen, L. & Rose, M.S., 1996. Sensory acceptability is inversely related to development of fat rancidity in bread made from stored flour. *Journal of the American Dietetic Association*, 96(8), pp.792–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8683011 [Accessed March 24, 2016].

Harnack, L., Walters, S.-A.H. & Jacobs, D.R., 2003. Dietary intake and food sources of whole grains among US children and adolescents: data from the 1994-1996 Continuing Survey of Food Intakes by Individuals. *Journal of the American Dietetic Association*, 103(8), pp.1015–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12891150 [Accessed April 27, 2016].

Hayase, F., 2000. Recent Development of 3-Deoxyosone Related Maillard Reaction Products. *Food Sci. Technol. Res.*, 6(2), pp.79–86.

Heiniö, R.-L. et al., 2003. Milling fractionation of rye produces different sensory profiles of both flour and bread. *LWT - Food Science and Technology*, 36(6), pp.577–583. Available at: http://linkinghub.elsevier.com/retrieve/pii/S002364380300063X [Accessed July 4, 2013].

Heiniö, R.-L. et al., 2008. Quantities of phenolic compounds and their impacts on the perceived flavour attributes of rye grain. *Journal of Cereal Science*, 47(3), pp.566–575. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0733521007001324 [Accessed June 12, 2013].

Hidalgo, F.J., Leon, M.M. & Zamora, R., 2006. Antioxidative activity of amino phospholipids and phospholipid/amino acid mixtures in edible oils as determined by

the rancimat method. *Biochemistry*, pp.5461–5467.

Hodge, J.E., 1953. Browning reactions in model systems. *Journal of Agricultural and Food Chemistry*, 1, pp.928–943.

Hodge, J.E., 1955. The Amadori Rearrangement. *Advan. Carb. Chem.*, 10, pp.169–205.

Hofmann, T., Ho, C. & Pickenhagen, W., 2004. Challenges in Taste Research : Present Knowledge and Future Implications. In *ACS Symposium Series*. p. 1.

Hofmann, T., Ottinger, H. & Frank, O., 2004. The Taste Activity Concept : A Powerful Tool to Trace the Key Tastants in Foods. In *ACS Symposium Series*. pp. 104–124.

Institute of Medicine, 2009. *School Meals: Building Blocks for Healthy Children*,

Jacobs, D.R. et al., 1998. Whole grain intake and cancer : An expanded review and meta-analysis. *Nutr. Cancer*, 30(2), pp.85–96.

Jacobs, D.R. & Gallaher, D.D., 2004. Whole grain intake and cardiovascular disease: A review. *Curr. Atheroscler. Rep.*, 6(6), pp.415–423.

Jeleń, H.H., Majcher, M. & Dziadas, M., 2012. Microextraction techniques in the analysis of food flavor compounds: A review. *Analytica Chimica Acta*, 738, pp.13–26.

Jiang, D. et al., 2009. Identification of hydroxycinnamic acid-maillard reaction products in low-moisture baking model systems. *Journal of agricultural and food chemistry*, 57(21), pp.9932–43. Available at: http://dx.doi.org/10.1021/jf900932h [Accessed March 24, 2016].

Jiang, D. & Peterson, D.G., 2013. Identification of bitter compounds in whole wheat bread. *Food chemistry*, 141(2), pp.1345–53. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/23790923 [Accessed July 13, 2013].

Jiang, D. & Peterson, D.G., 2010. Role of hydroxycinnamic acids in food flavor: A brief overview. *Phytochemistry Reviews*, 9(1), pp.187–193.

Kantor, L.S. et al., 2001. Choose a Variety of Grains Daily , Especially Whole Grains : A Challenge for Consumers. *J. Nutr.*, 131(3), pp.473–486.

Kokosa, J.M., Przyjazny, A. & Jeannot, M., 2009. *Solvent Microextraction: Theory and Practice*, John Wiley & Sons. Available at: https://books.google.com/books?hl=en&lr=&id=U4G9GbiHfFMC&pgis=1 [Accessed May 5, 2016].

Liu, R.H. & Adom, K.W., 2007. *Whole Grain Phytochemicals and Antioxidant Activity*, Blackwell Publishing.

Liu, S. et al., 2000. A prospective study of whole-grain intake and risk of type 2 diabetes mellitus in US women. *Am. J. Public Health*, 90(9), pp.1409–1415. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1447620&tool=pmcentrez&rendertype=abstract.

Liu, S. et al., 1999. Whole-grain consumption and risk of coronary heart disease: results from the Nurses' Health Study. *Am. J. Clin. Nutr.*, 70(3), pp.412–419. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10479204.

Liukkonen, K.-H. et al., 2003. Process-induced changes on bioactive compounds in whole grain rye. *The Proceedings of the Nutrition Society*, 62(1), pp.117–122.

Margolskee, R.F., 2002. Molecular mechanisms of bitter and sweet taste transduction. *The Journal of biological chemistry*, 277(1), pp.1–4. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/11696554 [Accessed September 28, 2016].

Martins, S.I.F.S., Jongen, W.M.F. & Van Boekel, M.A.J.S., 2000. A review of Maillard reaction in food and implications to kinetic modelling. *Trends in Food Science and Technology*, 11(9–10), pp.364–373.

Matsunami, H., Montmayeur, J.P. & Buck, L.B., 2000. A family of candidate taste receptors in human and mouse. *Nature*, 404(6778), pp.601–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10766242.

McKeown, N.M. et al., 2002. Whole-grain intake is favorably associated with metabolic risk factors for type 2 diabetes and cardiovascular disease in the Framingham Offspring Study. *Am. J. Clin. Nutr.*, 76(2), pp.390–398. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12145012.

McMackin, E. et al., 2013. Whole grains and health: attitudes to whole grains against a prevailing background of increased marketing and promotion. *Public health nutrition*, 16(4), pp.743–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23102407 [Accessed June 29, 2015].

Meilgaard, M., Civille, G.V. & Carr, B.T., 1997. *Sensory Evaluation Techniques*, CRC Press.

Meilgaard, M.C., Carr, B.T. & Civille, G.V., 2006. Pairwise Ranking Test: Friedman Analysis - Comparing Several Samples in All Possible Pairs. In *Sensory Evaluation Techniques*. CRC, p. 109.

Mintel Oxygen, 2013. *Bread - US*,

Mitteilungen, K., 1965. The Taste of L- and D-Amino Acids. *Cell. Mol. Life Sci.*, 21(12),

pp.692–694.

Moskowitz, M.R. et al., 2009. Identification of hydroxycinnamic acid-maillard reaction products in low-moisture baking model systems. D. S. Mottram & A. J. Taylor, eds. *Journal of agricultural and food chemistry*, 57(21), pp.9932–43.

Moskowitz, M.R. et al., 2012. Influence of endogenous ferulic acid in whole wheat flour on bread crust aroma. *Journal of agricultural and food chemistry*, 60(45), pp.11245–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23106092.

Moskowitz, M.R. & Peterson, D.G., 2010. *Hydroxycinnamic Acid – Maillard Reactions in Simple Aqueous Model Systems* D. S. Mottram & A. J. Taylor, eds., Washington, DC: American Chemical Society. Available at: http://dx.doi.org/10.1021/bk-2010-1042.ch006 [Accessed March 24, 2016].

Mottram, D.S. & Edwards, R.A., 1983. The role of triglycerides and phospholipids in the aroma of cooked beef. *Journal of the Science of Food and Agriculture*, 34(5), pp.517–522. Available at: http://doi.wiley.com/10.1002/jsfa.2740340513 [Accessed May 4, 2016].

Mottram, D.S. & Elmore, J.S., 2010. *Controlling Maillard Pathways To Generate Flavors* D. S. Mottram & A. J. Taylor, eds., Washington, DC: American Chemical Society. Available at: http://dx.doi.org/10.1021/bk-2010-1042.ch014 [Accessed May 2, 2016].

Mottram, D.S., Wedzicha, B.L. & Dodson, A.T., 2002. Acrylamide is formed in the Maillard reaction. *Nature*, 419(6906), pp.448–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12368844 [Accessed August 14, 2016].

Namiki, M., 1988. Chemistry of Maillard reactions: Recent studies on the browning reaction mechanism and the development of antioxidants and mutagens. *Advances in Food Research*, 32, pp.115–184.

National Institute of Advanced Industrial Science and Technology (AIST), Spectral Database for Organic Compounds SDBS. Available at: http://sdbs.db.aist.go.jp/sdbs/cgi-bin/cre_index.cgi.

Nursten, H.E., 2005. *The Maillard reaction: chemistry, biochemistry, and implications*, Cambridge, UK: Royal Society of Chemistry.

O'Connor, J., Perry, H.J. & Harwood, J.L., 1992. A comparison of lipase activity in various cereal grains. *Journal of Cereal Science*, 16(2), pp.153–163. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0733521009801471.

Plutowska, B. & Wardencki, W., 2008. Application of gas chromatography-olfactometry (GC-O) in analysis and quality assessment of alcoholic beverages - A review. *Food Chemistry*, 107(1), pp.449–463.

Reineccius, G., 2005. *Flavor Chemistry and Technology* second., CRC Press. Available at: https://books.google.com/books?id=lWQHFi0ioD8C&pgis=1.

Van Ruth, S.M., 2001. Methods for gas chromatography-olfactometry: A review. *Biomolecular Engineering*, 17(4–5), pp.121–128.

Sabitha, G. et al., 2009. Synthesis of (-)-Pinellic Acid and Its (9R, 12S, 13S)-Diastereoisomer. , 92, pp.2052–2057.

Sapis, J.-C. & Ribéreau-Gayon, P., 1969. No Title. *Annales de Technologie Agricole*, 18, p.207.

Sapis, J.-C. & Ribéreau-Gayon, P., 1965. On the presence in wine of tyrosol, tryptophol, phenylethyl alcohol and gamma-butyrolactone, secondary products of alcoholic fermentation. *C. R. Acad. Sci. Hebd. Séances Acad. Sci.*, 261(8), pp.1915–1916.

Scharbert, S., Holzmann, N. & Hofmann, T., 2004. Identification of the astringent taste compounds in black tea infusions by combining instrumental analysis and human bioresponse. *Journal of Agricultural and Food Chemistry*, 52(11), pp.3498–3508.

Slavin, J., Jacobs, D. & Marquart, L., 2011. Whole grain consumption and chronic disease : Protective mechanisms Whole-Grain Consumption and Chronic Disease : Protective Mechanisms. *Nutr. Cancer*, (December), pp.37–41.

Sosulski, F., Krygier, K. & Hogge, L., 1982. Free, esterified, and insoluble-bound phenolic acids. 3. Composition of phenolic acids in cereal and potato flours. *Journal of Agricultural and Food Chemistry*, 30(2), pp.337–340. Available at: http://dx.doi.org/10.1021/jf00110a030 [Accessed March 24, 2016].

de Souza, M.D.C.A. et al., 2006. Characterization of cachaça and rum aroma. *Journal of agricultural and food chemistry*, 54(2), pp.485–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16417309 [Accessed May 6, 2016].

Steinhart, H., 2005. The Maillard Reaction. Chemistry, Biochemistry and Implications. By Harry Nursten. *Angewandte Chemie International Edition*, 44(46), pp.7503–7504. Available at: http://doi.wiley.com/10.1002/anie.200585332 [Accessed August 14, 2016].

Tressl, R., Kersten, E. & Rewicki, D., 1993. Formation of 4-Aminobutyric Acid Specific Maillard Products from [1-13C]-D-Glucose, [1-13C]-D-Arabinose, and [1-13C]-

D-Fructose. *J. Agric. Food Chem.*, 41, pp.2278–2285.

U.S. Department of Agriculture, 2012. Federal Register. , 77(17).

U.S. Department of Agriculture and U.S. Department of Health and Human Services, 2010. *Dietary Guidelines for Americans*, U.S. Government Printing Office.

U.S. Department of Agriculture & U.S. Department of Health and Human Services, 2010. *Dietary Guidelines for Americans, 2010* 7th ed. T. Vilsack & K. Sebelius, eds., Washington, DC: U.S. Government Printing Office.

Waridel, P. et al., 2001. Evaluation of quadrupole time-of-flight tandem mass spectrometry and ion-trap multiple-stage mass spectrometry for the differentiation of C-glycosidic flavonoid isomers. *Journal of chromatography. A*, 926(1), pp.29–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11554416.

Yaylayan, V.A. & Forage, N.G., 1992. A kinetic model for the reaction of tryptophan with glucose and mannose—the role of diglycation in the Maillard reaction. *Food Chem.*, 44(3), pp.201–208. Available at: http://linkinghub.elsevier.com/retrieve/pii/0308814692901888.

Yaylayana, V.A. & Huyghues-Despointes, A., 1994. Chemistry of Amadori rearrangement products: Analysis, synthesis, kinetics, reactions, and spectroscopic properties. *Crit. Rev. Food Sci. Nutr.*, 34(4), pp.321–369.

Yucel, U. & Peterson, D.G., 2015. Effect of Protein–Lipid–Salt Interactions on Sodium Availability in the Mouth and Consequent Perception of Saltiness: In Solutions. *Journal of Agricultural and Food Chemistry*, 63(34), pp.7487–7493. Available at: http://pubs.acs.org/doi/10.1021/acs.jafc.5b02311 [Accessed August 15, 2016].

Zamora, R. & Hidalgo, F.J., 2005. Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Critical Reviews in Food Science and Nutrition*, 45(1), pp.49–59. Available at: <Go to ISI>://WOS:000226137000003\nhttp://www.tandfonline.com/doi/pdf/10.1080/10408690590900117.

Zhang, Q. et al., 2008. A perspective on the Maillard reaction and the analysis of protein glycation by mass spectrometry: probing the pathogenesis of chronic disease. *Journal of proteome research*, 8(2), pp.754–769.

Zhang, Y. et al., 2003. Coding of Sweet, Bitter, and Umami Tastes: Different Receptor Cells Sharing Similar Signaling Pathways. *Cell*, 112(3), pp.293–301.